NEOTROPICAL FROGS AS A STUDY SYSTEM FOR GLUCOCORTICOID REGULATION AND A FOUNDATION FOR K-12 OUTREACH

BY

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THESIS

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ABSTRACT

Neotropical frogs have attracted the attention of scientists, naturalists, and the general public for decades. These creatures have a rich, interdisciplinary research history, spanning behavior, physiology, development, evolution, and ecology, and serve as a captivating foundation for public engagement. In my master's work, I took advantage of these unique animals for research and outreach. Here, I summarize my work leveraging frogs to investigate steroid hormone regulation across development and engage K-12 students in hands-on learning.

Answering questions from a hormonal perspective allows biologists to conduct research that integrates multi-level biological phenomena. Glucocorticoids (GCs) are steroid hormones that are well studied for their involvement in many biological processes, especially the stress response. There are two main GCs produced by vertebrates – cortisol and corticosterone. Animals are assumed to have a primary GC that stays consistent across life stages and sampling methods, but growing evidence suggests that GC regulation is not as straightforward as we once thought. To connect previous work focused on GC abundance to specific developmental contexts, I quantified both cortisol and corticosterone across seven distinct life stages of northern glassfrogs (*Hyalinobatrachium fleischmanni*) using water and body sampling methods. I found that corticosterone was more abundant than cortisol, as predicted in amphibians, but the magnitude of this difference varied across life stages and depended on sampling method. Further, I found that cortisol and corticosterone and water and body hormone levels were not correlated in most life stages of *H. fleischmanni*. My findings address inconsistencies in our assumptions about GCs, provide interesting avenues for future research on the dynamic regulation of cortisol

and corticosterone across development, and call for careful preliminary investigation of GCs across contexts in future work.

The unique biology of neotropical frogs is the inspiration from which my K–12 outreach initiative “Frogs in Class” was created. Outreach in the K–12 setting is particularly important in bridging the gap between science and society. Frogs in Class addresses this gap by using poison frogs as a phenomenon through which to teach K–12 life science learning standards. The program educates participants about poison frogs and how to care for them, provides necessary supplies and support for classrooms to adopt tadpoles from the Fischer Laboratory frog colony, and presents six modules that capitalize on the current developmental stage of each classroom’s frog to engage students in learning diverse biological concepts. Each module is presented by biologists, aligned with Next Generation Science Standards (NGSS), and connects the developmental stage of each classroom’s frog to a broader biological theme. Frogs in Class allows students to experience the fascinating life-history of frogs in real time, and simultaneously leverages students’ curiosity about their frogs to engage them in broader learning objectives, including how we can utilize model systems to help us answer central questions in biology, how we can mitigate climate change, and what it means to be a scientist. Here, I share my insights following the piloting of Frogs in Class during the 2023–2024 school year. Taken together, my work demonstrates how neotropical frog development is a fascinating backdrop for investigations of GC regulation and for meaningful community outreach.

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To my mom, for your unwavering love and support

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Abstract

Answering questions from a hormonal perspective can allow biologists to conduct research that integrates multi-level biological phenomena. Glucocorticoids (GCs) are steroid hormones that are particularly well-studied because of their involvement in many physiological processes. There are two main glucocorticoids produced by vertebrates – cortisol and corticosterone. Species and sometimes classes of animals are assumed to have a primary GC that is consistent across life stages and sampling methods, but growing evidence suggests that GC regulation is not as straightforward as we once thought. To connect studies that have highlighted discrepancies in our assumptions about GC abundance across time and taxa, we investigated both cortisol and corticosterone levels across life stages and sampling methods. We measured cortisol and corticosterone abundance in egg clutches, early-stage tadpoles, mid-stage tadpoles, late-stage tadpoles, froglets, juveniles, and adults of northern glassfrogs (*Hyalinobatrachium fleischmanni*) using water and body sampling. We found that corticosterone was more abundant than cortisol, as predicted in amphibians, but the magnitude of this difference varied across life stages and depended on the sampling method. When we looked at the correlation between both GCs across life stages, we found that there was no significant correlation between cortisol and corticosterone levels within most life stages. Counter to expectations that water hormone levels reflect those in the body, we found that water and body samples were not correlated in most life stages. These findings address inconsistencies in our assumptions of GCs, and we advocate for careful

preliminary investigation of GCs across contexts in future work. We also encourage further exploration of the dynamic regulation of cortisol and corticosterone across development.

Introduction

Biological phenomena occur across many levels of organization (i.e., population, organism, organ, cell, chromosome, gene, etc.) and timescales (i.e., immediate, developmental, and evolutionary), across which hormones have long been accepted as physiological coordinators (Brück 1983; McGlothlin and Ketterson 2008; Finch and Rose 1995). For example, hormones integrate genetic, neurological, and tissue-level processes to respond acutely to environment changes but also to coordinate life stage transitions, such as metamorphosis and puberty, and to influence evolutionary variation in complex traits (Blaustein 2012; Lipshutz et al. 2019; Cox, McGlothlin, and Bonier 2016). Consequently, answering questions from a hormonal perspective allows biologists to conduct research that integrates multi-level biological phenomena.

Glucocorticoids (GCs) are a class of steroid hormones that are particularly well-studied because of their involvement in diverse physiological processes including the immune response, behavior, metabolism, development, and maintaining homeostasis in the face of environmental fluctuations (reviewed in Buckingham 2006). GCs are particularly well known as key players in the physiological stress response which has been of keen interest across biological disciplines, from clinical settings – where GCs are fundamental in the treatment of some diseases (inflammatory and auto-immune diseases) and the source of others – to ecological studies where they are often used as a proxy for stress levels in captive and wild populations of vertebrates and therefore directly inform conservation decisions (Sheriff et al. 2011; Reeder and Kramer 2005; Cain and Cidlowski 2017; Narayan 2013; Buckingham 2006). The implications GCs have for

diverse biological fields calls for careful evaluation of our fundamental understating of their regulation and functionality across time, contexts, and taxa.

There are two main glucocorticoids produced by vertebrates — cortisol and corticosterone (Koren et al. 2012; Buckingham 2006). Most vertebrates produce a combination of the two GCs which are often considered physiologically interchangeable because they act on the same receptors (Ovejero et al. 2013; Romero et al. 2008). Even so, one is usually considered primary based on dominance and/or abundance. Dominance is defined by measuring which GC is most responsive to a physiological or environmental stressor, and abundance refers to the relative amount of each GC (Aerts 2018). The dominant GC, often the more abundant as well, is generalized as the primary GC for groups of animals based on phylogenetic relationships (Hancock 2010; Reeder and Kramer 2005). Cortisol is typically investigated as the primary GC in fish (Sadoul and Geffroy 2019) and mammals (Koren et al. 2012), and corticosterone is typically investigated as the primary GC in birds (Blas 2015), reptiles (Meylan, Haussy, and Voituron 2010), and amphibians (Moore and Jessop 2003). However, these generalizations are sometimes based on studies in a limited number of species that have often focused on a specific life stage.

Despite general patterns, there are exceptions to class- and even species-level GC-dominance. For example, most mammals are considered cortisol-dominant (Sheriff et al. 2011) yet rodents — the most common mammalian model systems — are corticosterone-dominant (Katsu and Iguchi 2016), and bats show interspecific variation in GC-dominance (Reeder et al. 2004; Widmaier and Kunz 1993). There is also evidence that challenges the assumption of a species having a primary GC. For instance, in zebra finches (T*aeniopygia guttata*) corticosterone was more abundant in plasma, cortisol was more abundant in immune tissues, and there was no

difference in abundance in the brain, demonstrating that GCs vary across tissue type (K. L. Schmidt and Soma 2008). In addition to variation across tissue type, the abundance of cortisol versus corticosterone has been shown to vary in response to stressor type (natural versus physiological) (Gong et al. 2015) and across sexes (Reeder et al. 2004), seasons (Vera, Antenucci, and Zenuto 2011; Romero 2002), time of day (Widmaier and Kunz 1993), developmental stages (Dalle and Delost 1974), and sampling methods (Millikin et al. 2019). These studies highlight the striking variation present in GC abundance both across and within species and highlight the importance of additional work exploring the dynamics of GCs across contexts and timescales in more species/clades.

Amphibians are an interesting focal group in which to explore glucocorticoids across contexts because there is variation in GC abundance within the group, they have a unique biphasic life history in which GCs play a critical role, and non-invasive waterborne sampling methods are possible and hold importance for research and conservation efforts. At the class-level, amphibians are generally considered corticosterone-dominant (Carr 2011) but there are exceptions. Cortisol is the dominant GC in adult hellbender salamanders (*Cryptobranchus alleganiensis*), having a higher abundance than corticosterone following restraint and a physiological stressor (Hopkins et al. 2020). Westrick and colleagues (2023) measured GC abundance across five species of Dendrobatid poison frogs and found that cortisol was more abundant in water samples for three out the five species and there was no difference in abundance between GCs for the other two species (Westrick, Paitz, and Fischer 2023). These studies highlight the variation and complexity of GCs within amphibians.

The variation that we see across species is especially interesting in amphibians because of their unique developmental trajectory. Anurans (frogs and toads) undergo profound

morphological and physiological changes during metamorphosis and corticosterone plays an essential role in mediating these changes (Shewade et al. 2020; Sachs and Buchholz 2019; Jaffe 1981; Kulkarni and Buchholz 2014; Krug et al. 1983; Jolivet Jaudet and Leloup Hatey 1984). Typically, corticosterone levels increase during tadpole development and peak at metamorphic climax (Kulkarni and Buchholz 2014; Wright et al. 2003; Jaffe 1981). Additionally, corticosterone has been well-studied in adult amphibians, as this is typically the targeted life stage for studies looking into GC abundance and dominance. However, we are lacking studies that bridge these two life stages to explore GC variation across these drastically different developmental contexts — from egg clutch through adulthood.

Amphibians are also an interesting focal group because their GCs can be sampled non-invasively using waterborne hormone collection methods (Gabor et al. 2013). In fish and amphibians, excreted levels of GCs are generally interpreted as an integrated measure of internal GCs across the sample collection period (Baugh et al. 2018; Sadoul and Geffroy 2019). The simplicity and non-invasiveness of this method make it a powerful tool for collecting repeated measures in small-bodied animals, especially valuable in behavioral studies, and for conservation efforts for amphibians whose populations are declining worldwide (Ruthsatz, Rico-Millan, et al. 2023; Kiesecker 2011). In general, water-borne sampling works well and correlates with whole-body or plasma GC levels in various anuran species (Baugh et al. 2018; Forsburg et al. 2019; Gabor et al. 2013). However, there is also evidence that correlations between excreted and internal GCs may be life stage and/or context dependent. Water and body samples were found to be correlated in some, but not all, metamorphic life stages in Northern Leopard frogs (*Lithobates pipiens*) (McClelland and Woodley 2021) and European common frogs (*Rana temporaria*) (Ruthsatz, Rico-Millan, et al. 2023). In spotted salamanders (*Ambystoma maculatum)*, plasma

and water corticosterone levels were not correlated in larvae, metamorphs, or adults (Millikin et al. 2019). These examples document the variation in GC abundance captured by water versus body sampling methods, but we lack an understanding of the biological significance of these patterns. In summary, the literature on GCs in amphibians, specifically anurans, emphasizes the complexity of GCs and provides challenges and opportunities for further exploration of GC abundance across contexts and timescales.

Within amphibians, we were particularly interested in exploring GC regulation in glassfrogs. Northern glassfrogs (*Hyalinobatrachium fleischmanni*) are arboreal frogs that range from Mexico to northern parts of South America and are named for their transparent ventral skin and muscles as adults (Cruz, Urgiles, and Sánchez-Nivicela 2017; Villa 1984; Cisneros-Heredia and Mcdiarmid 2007). *H. fleischmanni* are small-bodied frogs who are of conservation concern ("Consideration of Proposals for Amendment of Appendices I and II" 2019), making them a useful species in which to compare body and water sampling methods. Further, it was recently discovered that *H. fleischmanni* shunt approximately 89% of their red blood cells to their liver while sleeping which could have interesting effects on GC regulation since GCs are transported through the circulatory system (Taboada et al. 2022). We were intrigued by *H. fleischmanni* and their unique biology specifically, as well as the broader amphibian literature.

We aimed to understand whether the relationship between cortisol and corticosterone changes across development in a single species. To explore this idea, we investigated glucocorticoid abundance across seven distinct life stages (i.e., egg clutches, early-stage tadpoles, mid-stage tadpoles, late-stage tadpoles, froglets, juveniles, and adults) in northern glassfrogs (*H. fleischmanni*) using water and body sampling techniques. We asked whether and how (1) corticosterone and cortisol abundance, (2) the relationship between GCs, and (3) the

relationship between sampling methods vary across life stages from egg clutches to adults. This study provides a comprehensive overview of GC dynamics across development in glassfrogs, contributing a novel case study to the growing GC literature.

Methods

Animal husbandry

We bred and housed all glassfrogs in the Fischer Laboratory frog colony at the University of Illinois Urbana-Champaign (UIUC) under IACUC protocols #20147 and #23099. We kept frogs in the same temperature- and humidity-controlled room on a 12-hour on, 12-hour off light cycle that was shifted to facilitate sampling during the frogs' wake cycle. Terraria consisted of a drainage layer, soil, sphagnum moss, and an abundance of tropical plants. Each terrarium housed 5–8 frogs and received automatic misting so that humidity always remained above 60% RH and temperature ranged from 20-25°C. We fed frogs a diet of gut-loaded crickets and vitamin-dusted flightless fruit flies three times weekly on an alternating basis. We cleaned tanks weekly and performed health checks daily. Tadpoles were housed in aquatic tanks containing approximately 20 liters of reverse osmosis (RO) water heated to 25±1°C. Each aquatic tank contained two clusters of live plants, a sponge filter, and 30–50 tadpoles. We fed tadpoles three times weekly with Repashy Solient Green and Community Plus (Repashy Specialty Pet Products). We performed health checks daily and partial water changes weekly.

Staging

We staged glassfrogs using the Gosner staging system which classifies frogs into 46 stages from fertilized embryo through metamorphosis (Gosner 1960). Stages based on morphological characteristics rather than age are commonly used in amphibians because developmental rate varies based on intrinsic factors and environmental conditions. We classified individuals into seven life stages: egg clutches (5–9 days old), early tadpoles (no external limb development; Gosner stages 25–30), mid tadpoles (two visible limbs; Gosner stages 31–35), late tadpoles (four visible limbs; Gosner stages 36–40), froglets (0–6 months post-metamorphosis), juveniles (6–12 months post-metamorphosis), and adults (sexually mature — egg producing or calling) (Gosner 1960). We staged tadpoles using a stereo microscope (Leica S9E).

Water-borne hormone collection

To collect water-borne hormone samples, we placed each tadpole/frog in a 4x4x5cm hexagonal glass jar filled with 50mL of RO water and left them to soak for one hour. We sampled all frogs 2–3 hours into their 12-hour wake cycle to control for diel fluctuations in GCs (Wright et al. 2003). For terrestrial individuals (i.e., froglets, juveniles, and adults), we rotated the jars every 15 minutes to ensure each frog remained in the water for the duration of the sampling period. After sampling, we recorded body length, and returned individuals to their home tank or euthanized them immediately for whole-body (froglets, juveniles) or plasma (adults) sampling. We filtered all water samples through Whatman™ Grade 1 Qualitative Filter Paper to remove any solid particles, such as dirt or fecal matter. We stored all samples at -20ºC until hormone extraction. In total, we collected 144 water-samples between February 2023 and

February 2024 (n = 15 early-stage tadpoles, n = 9 mid-stage tadpoles, n = 22 late-stage tadpoles, n = 30 froglets, n = 13 juveniles, and n = 55 adults).

Whole-body and plasma hormone collection

We collected plasma from adults only and whole bodies for all other life stages because only adults were large enough to provide an adequate amount of plasma for GC quantification. We refer to both plasma and whole-body samples as body samples for the rest of the manuscript for ease. To compare water-borne and whole-body glucocorticoid levels, we euthanized a subset of individuals immediately following water-borne hormone collection (n = 13 egg clutches, n = 15 early tadpoles, n = 9 mid tadpoles, n = 7 late tadpoles, and n = 15 froglets). We euthanized tadpoles using sodium bicarbonate buffered tricaine methane sulfonate (MS-222), a commonly used anesthetic for fish and amphibians, and froglets using benzocaine (Orajel™), as recommended by the American Veterinary Medical Association (Topic Popovic et al. 2012; Ramlochansingh et al. 2014; Leary et al. n.d.). Following euthanasia, we rinsed all individuals with RO water and recorded their weight and length. We stored bodies in microcentrifuge tubes at -80ºC until hormone extraction. To prepare for hormone extraction, we cut the bodies into small pieces and pulverized them into a fine powder using a mortar, pestle, and liquid nitrogen. We recorded the mass of powder recovered and then placed the powder in a centrifuge tube with 5 mL of methanol overnight. The next day, we centrifuged the samples for 15 minutes at 2000 rpm and 4ºC. We added the supernatant of each sample to a 50 mL conical tube and added approximately 45 mL of RO water to each sample to bring it to 50 mL total.

We collected egg clutches at 5–9 days old and froze them at -20ºC until hormone extraction. Similar to whole-body samples, egg clutches were pulverized using a mortar, pestle,

and liquid nitrogen. We weighed the homogenized powder of each clutch and prepared them for hormone extraction as in Paitz & Dugas (2021).

We anesthetized adults (n =12) with benzocaine and then euthanized them via rapid decapitation. We immediately collected blood from the trunk of the body using heparinized microhematocrit capillary tubes and placed the blood in heparinized microcentrifuge tubes. We separated plasma from whole blood by centrifuging for 5 minutes at 5,000 rpm. We stored plasma supernatant at -20ºC until quantification.

Hormone extraction

We extracted steroid hormones from water and whole-body samples via solid-phase extraction using Sep-Pak® C18 cartridges (Waters Corporation) on a vacuum manifold. We activated the cartridges with 6 mL of 100% methanol, rinsed them with 6 mL of RO water, and then ran our samples through the cartridges. We eluted total steroid hormones from the columns with 4 mL of 100% methanol. We desiccated the samples in a dry heating block at 37 ºC under nitrogen gas and stored them at -20 ºC until quantification via enzyme linked immunosorbent assay (ELISA) analysis.

Enzyme linked immunosorbent assays

We used DetectX® cortisol and corticosterone ELISA kits from Arbor Assays™ to quantify GC levels in our samples. We followed the manufacturer's protocol except for the use of the same assay buffer (X065) for both kits. Reconstituting samples with assay buffer X065 allowed us to run each sample on both cortisol and corticosterone plates, per manufacturer's recommendation. We reconstituted water and whole-body samples using 500 µL of assay buffer.

Before plating, we added 5–23 µL plasma samples to the respective dissociation reagent included in each kit in a 1:1 ratio. Per protocol, we incubated the plasma and dissociation reagent mixture for at least 5 minutes at room temperature before we added assay buffer. We added 220 µL of assay buffer to each sample being prepared for corticosterone quantification and 110 µL of assay buffer to each sample being prepared for cortisol quantification. We used the 100 µL curve for the corticosterone kit to capture the full range of corticosterone present in our samples. We read samples at an optical density of 450 nm. Arbor Assays™ reports the cross reactivity for their cortisol ELISA plates with corticosterone as 1.2% and for their corticosterone ELISA plates with cortisol as 0.38%. We used the MyAssay software (www.myassays.com) recommended by Arbor Assays™ to fit the standard curve for each plate and calculate the concentration of cortisol and corticosterone for each sample. Since samples were run in duplicate, we used the average concentration of the two wells per sample for body samples and used this value to calculate the release rate (pg/hr) for water samples. We removed samples with a coefficient of variance (CV) greater than 20%, which is the standard cutoff for ELISA assay analysis (Kinn Rød et al. 2017; Kifude Carolyne M. et al. 2008), and those that fell outside of the standard curve. 17% of corticosterone samples and 41% of cortisol samples were excluded and the average CV of all samples was 17.59.

Statistical analysis

We ran all statistical analyses using R (version 4.4.0) in R Studio (version 2024.04.0+735; R Core Team 2024; Posit team 2024). We log-transformed all hormone data to achieve normality. We were broadly interested in how GC abundance varies by life stage, GC type, and sampling method in glassfrogs. Therefore, we ran a linear mixed model with GC

abundance predicted by GC type, sampling method, and life stage, and their interactions. Because there was a significant three-way interaction, we ran subsequent models to look at both cortisol and corticosterone within each sample type and life stage. We excluded sex from our models because only adults could be confidently sexed and in a reduced model with adults only, sex had no effect on GC level ($F_{1,34.424}$=0.267, p=0.609). We calculated effect sizes (η2) for all models using the F_to_eta2 function in the effectsize package in R. We interpreted η2 > 0.01 as a small effect, η2 > 0.06 as a medium effect, and η2 > 0.14 as a large effect.

To ask how GC abundance varied by life stage, we fit two linear mixed models, one for water samples and one for body samples, using log-transformed hormone level as the independent variable with GC type (cortisol vs corticosterone), life stage, and their interaction as main effects and individual ID as a random effect. We ran type III ANOVAs on each model to assess significance ($p<0.05$) and post-hoc pairwise comparisons using the emmeans package (Lenth R 2024) to compare GCs within each life stage as appropriate. We were primarily interested in within-life-stage differences in the relationship between cortisol and corticosterone rather than between-life-stage differences because we expected fundamental differences between life stages resulting from developmental variation in morphology, body size, metabolic rate, et cetera. Because of marked differences in GC abundance between life stages, we ran subsequent models within each life stage.

Since we found different patterns of GC abundance in water versus body samples, we wanted to understand the extent to which the relationship between GC type and abundance depended on sampling method. To do this, we ran a separate linear mixed model for each life stage with hormone level predicted by main effects of GC type, sample type (water vs body), and their interaction as main effects and individual ID as a random effect. We ran type III ANOVAs

on each model to assess significance. In life stages where there was a significant effect of sampling type on the relationship between hormone level and type, we tested for correlation between cortisol and corticosterone in each life stage in both water and body samples using the cor.test function (Pearson method).

To ask whether water release rate was predicted by body concentration within each life stage, we ran a linear model with water predicted by body, GC type, and the interaction between the two as main effects, and individual ID as a random effect. We removed egg clutches and juveniles from this model due to only having one sample type for these groups. Lastly, in life stages where there was a significant interaction between body concentration and GC type, we tested for correlation between water release rate and body concentration by GC type using the cor.test function (Pearson method).

Results

Does relative glucocorticoid abundance vary by life stage?

Corticosterone is generally considered dominant in amphibians and is known to vary throughout metamorphosis but since cortisol has been found to be more abundant in some amphibians than previously thought, we were curious how both corticosterone and cortisol might vary across life stages in *H. fleischmanni*. Overall, we found that GC concentration is predicted by the interaction between GC type, sampling method, and life stage ($F_{4,215.16} = 10.80$, $p < 0.0001$, $\eta 2 = 0.17$). In body samples, we found that the relationship between GC concentration and GC type depends on life stage ($F_{5, 57.115} = 27.218$, $p < 0.0001$, $\eta 2 = 0.70$) (Figure 1a). Post hoc comparisons showed that corticosterone is significantly higher than cortisol in egg clutches, mid-stage tadpoles, late-stage tadpoles, froglets, and adults, but there was no significant

difference between corticosterone and cortisol in early-stage tadpoles (Table 1a). In water samples, we also found that the relationship between GC level and GC type depends on life stage ($F_{5, 109.49} = 4.16$, $p = 0.0017$, $\eta 2 = 0.16$) (Figure 1b). Specifically, corticosterone was significantly higher than cortisol in early- and late-stage tadpoles, but there was no significant difference between corticosterone and cortisol in mid-stage tadpoles, froglets, juveniles, or adults (Table 1b).

What is the relationship between corticosterone and cortisol within each life stage?

Between life stages, our previous models showed distinct statistical differences in GC abundance, and this makes sense biologically since there are fundamental differences between life stages (e.g., body size). Therefore, we were interested in the relative abundance of cortisol versus corticosterone within each life stage and how that relationship might differ between life stages and by sampling method.

When we compared relative abundance of cortisol versus corticosterone in body versus water samples within each life stage, we found that the magnitude difference between cortisol and corticosterone depended on sampling method for all life stages (early-stage tadpoles: $F_{1, 40.521} = 7.884$, $p = 0.008$, $\eta 2 = 0.16$; mid-stage tadpoles: $F_{1, 20.978} = 22.869$, $p = 0.0001$, $\eta 2 = 0.52$; late-stage tadpoles: $F_{1,42} = 18.999$, $p < 0.0001$, $\eta 2 = 0.31$; froglets: $F_{1,45.715} = 6.500$, $p = 0.014$, $\eta 2 = 0.12$; and adults: $F_{1,43.804} = 24.610$, $p < 0.0001$, $\eta 2 = 0.36$). Therefore, we looked at the correlation between corticosterone and cortisol within each life stage and sampling method. In body samples, we found a significant positive correlation between cortisol and corticosterone in eggs and early-stage tads only (Table 2a, Figure 2). In water samples, we found a significant

negative correlation between cortisol and corticosterone in late-stage tadpoles only (Table 2b, Figure 2).

Is water release rate predicted by body concentration in each life stage?

Given that the above analyses suggested that different sampling methods show different patterns in abundance, we were next interested in how the relationship between sampling methods varied within life stages and by GC type. We did not include egg clutches and juveniles in this analysis as we only had one sample type for these life stages. We found that the relationship between water release rate and body concentration varied between cortisol and corticosterone in early-stage tadpoles ($F_{1, 17.506} = 20.371$, $p < 0.0001$, $\eta 2 = 0.54$) only. When we looked at the correlation between water release rate and body concentration in early-stage tadpoles, we found a significant, negative correlation for corticosterone ($t_{13} = -4.229$, $r = -0.761$, $p = 0.0001$) but not cortisol (Table 3). For all other life stages, the correlation between water and body was not statistically different between the two GCs. Combining GCs for other life stages, we found that water release rate and body concentration were significantly and positively correlated in late-stage tadpoles ($t_{10} = 6.736$, $r = 0.905$, $p < 0.0001$) and froglets ($t_{22} = 2.366$, $r = 0.450$, $p = 0.027$), and not significantly correlated in mid-stage tadpoles, froglets, or adults (Table 3).

Discussion

Recent findings and long-standing discrepancies have called attention to the interesting variation present in GC regulation that has previously been smoothed over by the assumption that each taxon has a primary GC, and that it remains the primary GC across life stages and

sampling methods (Vera, Zenuto, and Antenucci 2017; Koren et al. 2012; McClelland and Woodley 2021). Here, we explored this variation by measuring cortisol and corticosterone abundance across seven life stages in glassfrogs using both water- and body-sampling methods. We found variation in *H. fleischmanni* GCs based on life stage, GC type, and sampling method. We discuss interpretations, limitations, and implications of our findings below.

Glucocorticoids across development

Previous work exploring glucocorticoids across ontogeny has primarily focused on early development in mammals and fishes (Wada 2008). In amphibians, studies have demonstrated the importance of corticosterone in mediating metamorphosis (e.g., Chambers et al. 2011; Jolivet Jaudet and Leloup Hatey 1984; Shewade et al. 2020; Kulkarni and Buchholz 2014), and other studies have surveyed corticosterone levels in adults as a biomarker for stress (Narayan 2013; Barsotti et al. 2019; Gavel et al. 2021; Tennessen et al. 2018). However, we are lacking information about GC abundance in the period between metamorphosis and adulthood, as well as a comprehensive overview of cortisol *and* corticosterone at all stages of development within an amphibian species. We addressed this knowledge gap by exploring both GCs within seven distinct life stages in glassfrogs.

We found measurable levels of both cortisol and corticosterone in every life stage of northern glassfrogs. Interestingly, while we found that corticosterone is overall more abundant than cortisol, the magnitude of this difference varies across life stages and depends on sampling method. To our knowledge, there is no previous work documenting differences in the balance between corticosterone and cortisol across development. Because GCs are involved in many processes (e.g., immune response, metabolism, development, behavior, stress response) and the

context in which these processes are taking place change across the life span, it is possible that the changing balance between corticosterone and cortisol across development reflects changes in the expression of these processes. For example, cortisol was found to be the predominant GC in immune tissues of zebra finches (T*aeniopygia guttata*) at hatching but its abundance decreased with age while corticosterone was the predominant GC in plasma at hatching and its abundance increased with age — suggesting that cortisol may play a distinct role in early development of the immune system in song birds (K. L. Schmidt and Soma 2008). While additional work is needed to determine the mechanistic basis and functional consequences of the GC variation that we found, our results set the stage for future studies to explore why the balance between corticosterone and cortisol varies across development.

Overall, we found that the difference between GCs lessens with age and when corticosterone is significantly higher than cortisol, that difference is more pronounced in body samples than it is in water samples. These results support the growing evidence that GC regulation across development is more complex than is sometimes assumed – not only did the extent to which corticosterone was more abundant vary by life stage, but the data also displayed different patterns in abundance between body and water sampling methods. Our findings influence our fundamental understanding of GCs which carries important implications for studies that use GCs as a proxy for stress in amphibians. This is particularly influential for the field of conservation physiology, where reliability of hormone data is necessary for accurately informing conservation decisions (Narayan 2013).

Exploring the relationship between cortisol and corticosterone

There are few studies that measure both cortisol and corticosterone abundance from the same samples, as the two GCs are generally assumed to be functionally interchangeable (Koren et al. 2012). Of the studies that report both values, some suggest that cortisol and corticosterone may play distinct physiological roles. This is because the two GCs show variation by context — such as time, where cortisol showed seasonal variation but corticosterone did not in tuco-tucos (*Ctenomys talarum*) (Vera, Antenucci, and Zenuto 2011), or in adult poison frogs where corticosterone and cortisol were equally abundant but only cortisol responded to a physiological stressor (Westrick, Paitz, and Fischer 2023). When we looked at the correlation between both GCs across life stages, we found that there was no significant correlation between cortisol and corticosterone levels within most life stages, suggesting physiological independence. Possible mechanisms that could decouple cortisol and corticosterone include differences in free versus bound GCs to corticosteroid binding globulins (CBGs) or GC receptors, which could be mediated by differing binding affinities between cortisol and corticosterone (Breuner and Orchinik 2002). Moreover, GCs are products of complex metabolic pathways composed of many precursors (e.g. adrenocorticotropic hormone (ACTH)) and conversion enzymes which provide numerous opportunities for modulation of GC production (Koren et al. 2012; Vera, Antenucci, and Zenuto 2011). For example, cortisol is the only stress-responsive GC in tuco-tucos despite detectable baseline levels of both GCs, suggesting that cortisol has a greater sensitivity to adrenocorticotropic hormone (ACTH) — a precursor for GC production (Vera, Antenucci, and Zenuto 2011). Further exploration of these pathways is needed.

We found a few exceptions to the general trend of no correlation between cortisol and corticosterone, however. In water samples of late-stage tadpoles, as corticosterone increased,

cortisol decreased which could reflect the importance of corticosterone during metamorphic climax. In body samples, we found a significantly positive correlation between GCs for egg clutches and early-stage tadpoles. Higher sample sizes for these groups compared to others could be driving some of these differences. Yet, it is interesting to note that for these life stages that showed significant correlations between GCs, the correlations were not the same across sampling method. We explore differences between water and body samples in depth below.

Taken together, cortisol and corticosterone are present in all life stages but not correlated in most for both body and water sampling methods, and therefore we would not suggest using one GC as a proxy for the other in most contexts in *H. fleischmanni*. Instead, our results emphasize the context-dependent nature of GCs and how generalized assumptions about their abundance can cause serious bias in applied studies.

Exploring the relationship between water and body sampling methods

While GCs have traditionally been measured in plasma, the validation of water-borne hormone sampling in amphibians has expanded research questions and applications. This non-invasive method allows for repeated sampling, even in small-bodied species, opening doors to physiological studies in basic and conservation research (Gabor et al. 2013). Yet, some studies have found that GC abundance in water samples does not always reflect that in body samples (Millikin et al. 2019; Ruthsatz, Rico-Millan, et al. 2023). To explore variation in GC abundance between sampling methods, we collected corticosterone and cortisol using both sampling methods in early-, mid-, and late-stage tadpoles, froglets, and adults.

As expected, we found that the relative abundance of GCs in body were higher than in water. This is unsurprising because body samples capture the level of actively circulating

hormones while water samples capture the level of excreted hormones, which should always be less than what is in the body. We were more interested, however, in whether the relationship between excreted and internal levels might vary depending on developmental stage. We found that the relationship between water and body levels did not vary by GC type (cortisol versus corticosterone) in any life stage except early-stage tadpoles. Further, internal and excreted GC levels were significantly correlated in late-stage tadpoles and froglets only. Taken together, these findings suggest that water levels only predict body levels under limited circumstances in *H. fleischmanni*. Though body and water hormone levels are often assumed to be correlated, another study demonstrated that this is not always the case. McClelland and Woodley (2021) found that water and body corticosterone samples were only correlated in one life stage — in their case, prometamorphic tadpoles (comparable to mid-stage tadpoles in this study).

In our results and those of McClelland and Woodley (2021), the differences in the relationship between water and body samples across life stages could indicate variation in GC secretion throughout development. Previous work in mammals, cultured fish, and birds, have shown intricate relationships between GCs, life-stage transitions, and GC secretion, where the trade-off between the costs and benefits of GCs are critical to survival and reproduction (Wada 2008). That is, GCs are necessary for metabolism, growth, and coping with various stressors but also impair immune function and cognition and can cause high mortality rates (Sapolsky, Romero, and Munck 2000; Saino et al. 2005; Eriksen et al. 2006; Morici, Elsey, and Lance 1997). These trade-offs are exacerbated under certain circumstances, such as a seabird chick needing high levels of corticosterone to induce begging and increase food intake at the cost of low growth efficiency and cognitive abilities (Kitaysky et al. 2003). In the context of anurans, it would not be surprising if our results reflect changes in excretion of both GCs throughout

development given the dramatic physiological and morphological changes that tadpoles undergo during metamorphosis. Perhaps variation in the excretion of GCs indicates changing internal levels across development and this dynamic is shaped by the trade-offs associated with GCs, such that an organism excretes more to reduce the costs of high circulating GC levels. The differences between water and body levels of GCs could also be driven by morphological changes across development that impact the secretion of GCs, such as skin keratinization and gill degeneration during late metamorphosis (Burggren and West 1982; Nishikawa, Kaiho, and Yoshizato 1989).

Overall, we argue against using waterborne hormone collection as a predictor for body GC concentrations for most life stages in this species. Rather, we recommend further investigation into the mechanisms that could be driving differences between water and body levels of GCs throughout development so that we can understand and accurately interpret the information that we receive from water samples. Moreover, these differences across development in our own work as well as others suggest that there are dynamic biological processes underpinning the regulation of GCs that we have yet to fully understand.

Additional considerations

There are some caveats to our work resulting from our study design and the difficulty of making quantitative comparisons across vastly different life stages. First, we elected to use waterborne hormone sampling for all life stages to make data most comparable and because of current interest in this non-invasive sampling technique (e.g., Nowicki et al. 2024; Ruthsatz, Eterovick, et al. 2023; Bókony et al. 2024). While tadpoles are aquatic adults are terrestrial, and this difference could affect the level of stress individuals experience during water sampling — it

may not be as stressful for a tadpole to be transferred from one container of water to another compared to an adult that is moved from a terrestrial habitat to a container of water. A further consequence of this difference is that terrestrial individuals (froglets, juveniles, and adults) were rotated every 15 minutes during the sampling period to ensure that they remained in water, but tadpoles did not require rotation. If waterborne sampling is indeed more stressful for terrestrial life stages, we might have expected relatively larger differences between the two GCs resulting from a greater increase in corticosterone following an acute stressor, however this is opposite of the pattern we found. Future studies in captive frogs could use multi-day habituation procedures to test for and reduce the impacts of sampling procedures, however, we note this approach is unrealistic in wild species of conservation concern.

Second there are various challenges and considerations for comparing body and water samples. Differences in body versus water samples are likely in part related to the fact that body samples provide a more proximate measurement of GC abundance than water samples which integrate GC abundance across the entire sampling period (Gabor et al. 2013). Additionally, our 'body' samples differed between age classes as only adults were large enough for plasma collection and we therefore collected whole bodies for younger life stages. Finally, humane collection of plasma and body samples required euthanasia prior to sample collection and the anesthetic used for this procedure may themselves induce stress. Tadpoles were euthanized using MS-222 and froglets and adults were euthanized using benzocaine. We are unaware of any studies in anurans, but previous work has shown that MS-222 can induce an increase in cortisol in fish (Barton and Peter 1982). Wedemeyer (1970) measured cortisol in fish 12 minutes after application of either MS-222 or benzocaine and found no increase in cortisol, suggesting that this time frame was not long enough to detect changes in cortisol levels. We prioritized quick (< 3

minute) euthanasia for all individuals in our study to minimize effects of handling stress (Romero and Reed 2005) and the study above suggests this timeframe is also short enough to minimize any potential stress effects directly from anesthetics. While the above impose some limitations on interpretation, they also provide avenues for future studies to further distinguish important technical considerations from interesting biological phenomena.

Conclusions

Our investigation of glucocorticoids across life stages and sampling methods adds to the expanding literature emphasizing GC variance across contexts. We explored cortisol and corticosterone abundance across seven distinct life stages using both water and body sampling methods, connecting the dots between valuable previous work that focused on early anuran development by providing the first overview of GCs across the entire lifespan of an amphibian. While we expected between-life-stage differences in overall GC abundance due to body size differences, we were intrigued by the variation in the balance between corticosterone and cortisol between life stages and by the complexities of the within-life-stage differences that we found. While corticosterone tended to be more abundant than cortisol, the magnitude of this difference varied by life stage and sampling method, where differences were more pronounced in body samples. Cortisol and corticosterone were mostly uncorrelated across life stages, supporting growing evidence that suggests physiological independence of these GCs. Lastly, whether water hormone levels predicted body hormone levels varied across life stages, with no relationship between the two sampling methods in most stages of development. Taken together, these data emphasize the within-life-stage diversity and complexity of GCs and call for further exploration

of GCs across contexts. We suggest careful investigation of GC abundance by GC type, life stage, and sampling method, as a preliminary step for future work.

Tables

Table 1: Difference between corticosterone and cortisol abundance within each life stage in (a) body and (b) water samples as visualized in Figure 1. Estimates are from post hoc comparisons using emmeans. Body samples were not collected for juveniles and water samples were not collected for eggs. Sample size (n) is reported per GC type.

Life stage	**t**	**n**		**df**	**p**
		corticosterone	**cortisol**		
(a) body					
Eggs	5.408	13	11	57.2	**< 0.0001**
Early-stage tadpoles	-0.654	15	14	55.6	0.516
Mid-stage tadpoles	9.851	9	7	58.6	**< 0.0001**
Late-stage tadpoles	11.867	7	6	57.0	**< 0.0001**
Froglets	6.798	14	11	62.2	**< 0.0001**
Juveniles	-	-	-	-	-
Adults	6.931	12	12	54.4	**< 0.0001**
(b) water					
Eggs	-	-	-	-	-
Early-stage tadpoles	3.809	15	14	95.5	**0.0002**
Mid-stage tadpoles	1.814	9	6	114.5	0.072
Late-stage tadpoles	3.458	20	13	134.3	**0.0007**
Froglets	-0.206	22	23	135.8	0.837
Juveniles	-0.360	12	11	106.6	0.720
Adults	0.227	52	36	121.8	0.821

Table 2: Correlation between cortisol and corticosterone within each life stage in (a) body samples and (b) water samples. Body samples were not collected for juveniles and water samples were not collected for eggs. Sample size (n) is reported per individual animal with paired cortisol and corticosterone samples for most individuals.

Life stage	r	n	df	p
(a) body				
Eggs	0.643	11	9	**0.033**
Early-stage tadpoles	0.943	14	12	**< 0.001**
Mid-stage tadpoles	0.227	7	5	0.624
Late-stage tadpoles	0.708	6	4	0.115
Froglets	0.555	10	8	0.096
Juveniles	-	-	-	-
Adults	-0.437	12	10	0.155
(b) water				
Eggs	-	-	-	-
Early-stage tadpoles	0.299	14	12	0.299
Mid-stage tadpoles	0.701	6	4	0.121
Late-stage tadpoles	-0.650	11	9	**0.030**
Froglets	0.085	15	13	0.764
Juveniles	0.470	10	8	0.170
Adults	0.285	33	31	0.108

Table 3: Correlation between body and water GC levels within each life stage. Body samples were not collected for juveniles and water samples were not collected for eggs, so those life stages are excluded. Since GC type (cortisol versus corticosterone) affected the correlation between body and water in early-stage tadpoles only, all other life stages reflect the correlation between water and body with combined GCs. Sample size (n) is reported per individual animal with paired body and water samples for most individuals.

Life stage	r	n	df	p
Early-stage tadpoles *(cortisol)*	-0.169	14	12	0.564
Early-stage tadpoles *(corticosterone)*	-0.761	15	13	**< 0.001**
Mid-stage tadpoles	0.300	12	10	0.343
Late-stage tadpoles	0.905	12	10	**<0.001**
Froglets	0.450	24	22	**0.027**
Adults	0.375	19	17	0.113

Figures

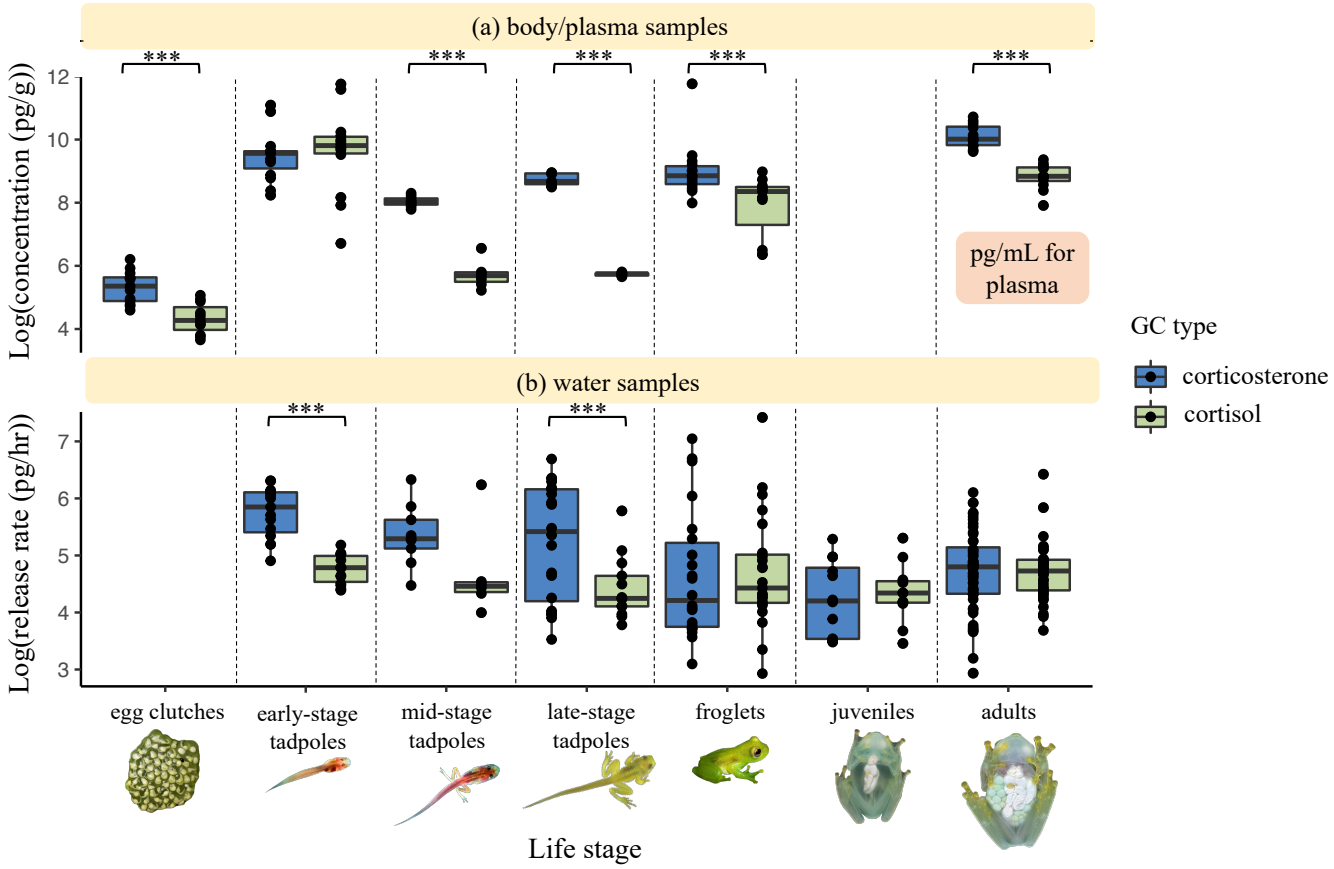

Figure 1: Glucocorticoid abundance varies across life stages and by sample type. This figure displays corticosterone (in blue) and cortisol (in green) abundance across seven distinct life stages within body (a) and water (b) samples. Panel (a) shows the concentration of whole-body samples in pg/g of mass recovered and the concentration of plasma samples for adults in pg/mL. Panel (b) shows release rate in pg/hr for water samples. Boxplots show the median, the first and third quartiles, and whiskers as 1.5x the interquartile range. Black dots show individual data points.

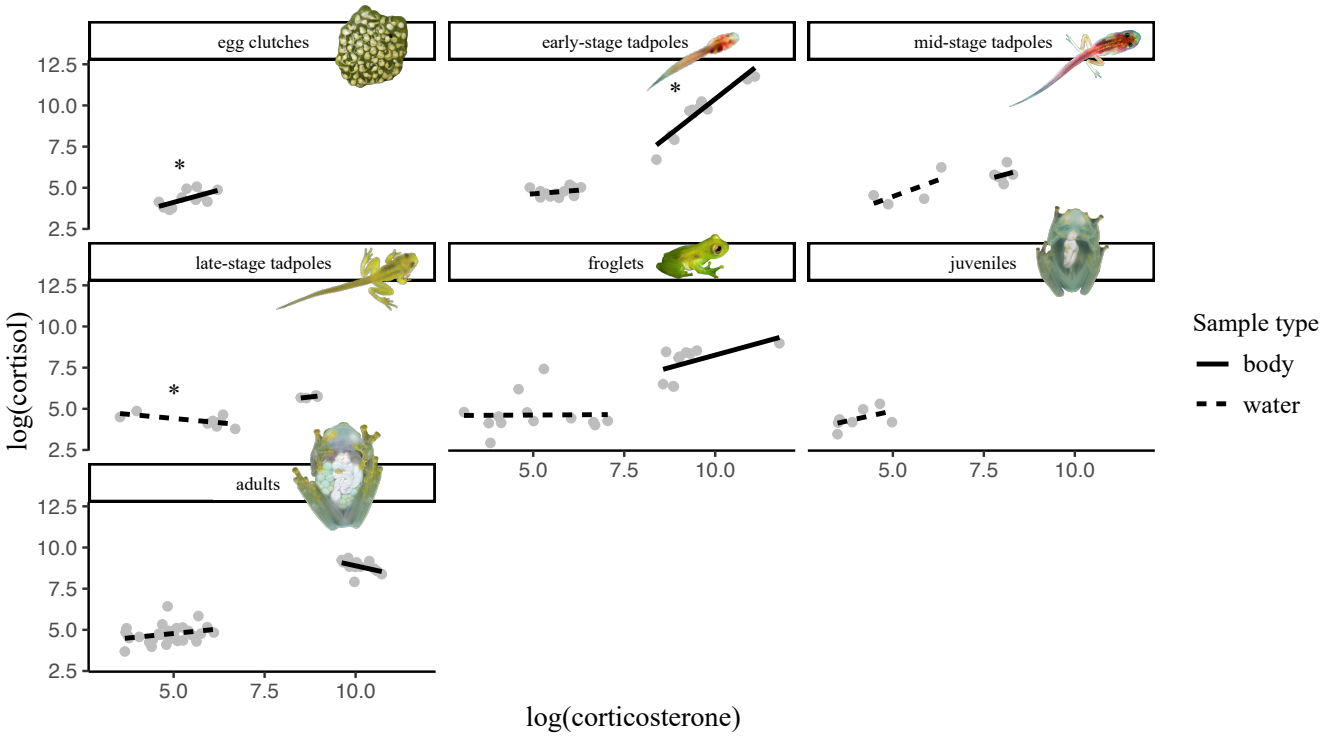

Figure 2: The correlation between cortisol and corticosterone abundance varies by life stage and sample type. Solid lines show the correlation between GCs in body samples (pg/g dry mass for whole-body samples and pg/mL for plasma) and dashed lines show the correlation between GCs in water samples (pg/hr). Asterisks denote significant correlations between cortisol and corticosterone levels. Egg clutches only show the correlation between GCs in body samples since water samples were not collected. Similarly, the correlation between GCs is shown only in water for juveniles since body samples were not collected for this life stage.

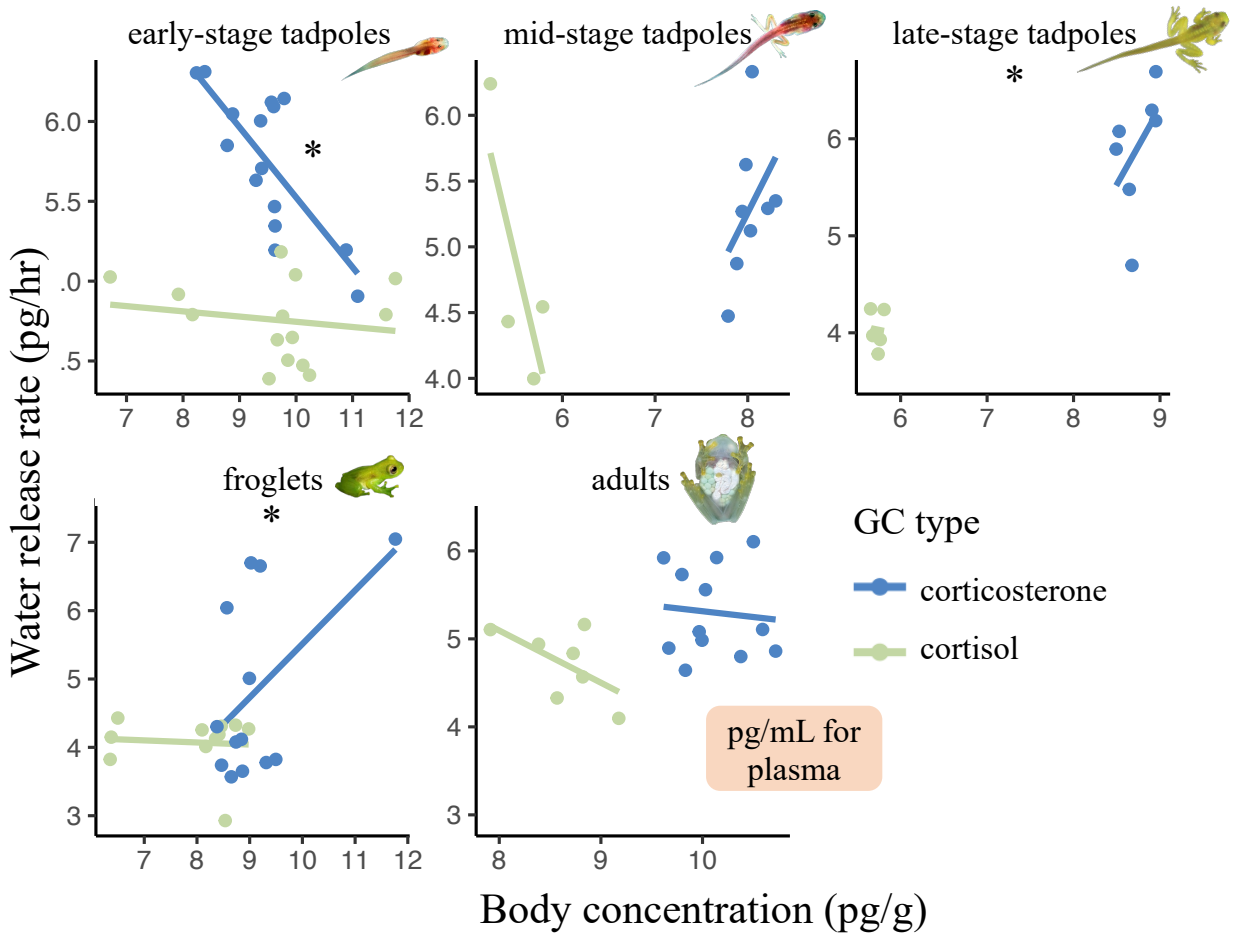

Figure 3: Correlation between water and body corticosterone (in blue) and cortisol (in green) levels across life stages. Water and body samples are positively correlated in late-stage tadpoles and froglets but not in other life stages. Egg and juvenile life stages are excluded since egg clutches were not water-sampled and juveniles were not body-sampled. Asterisks denote significant correlations between body and water GC levels (specifically corticosterone levels in early-stage tadpoles).

CHAPTER 2: FROGS IN CLASS: LEVERAGING NEOTROPICAL FROGS AS A FOUNDATION FOR K–12 OUTREACH

Introduction

Science outreach serves as a two-way bridge connecting scientists and society – scientists benefit by developing effective communication skills, increasing support and awareness of science, gaining broader insight from those outside of academia, and investing in the future of scientific research (Clark et al. 2016; Varner 2014). Society can gain understanding of and trust in the process of science and can use their scientific knowledge to make informed decisions (Laursen et al. 2007; Stieben, Halpin, and Matyas 2017). Science outreach or communication can take many forms, such as podcasts, community events, blogs, social media accounts, or television shows. One area in which outreach is considered especially important for increasing scientific literacy and preparing a diverse STEM workforce is in K–12 education, a structure that the majority of the U.S. population shuffles through (Golle et al. 2022).

Research has shown that interest in STEM and STEM test scores steadily decline as early as the 6–8th grades, and many students opt out of higher-level science courses by high school (Moskal and Skokan 2011). Synergistically, science educators and/or elementary teachers willing to teach science are limited in quantity, and this can lead to students being taught by long-term substitute teachers or other educators whose expertise lies elsewhere (Marco-Bujosa et al. 2024). Ultimately, these challenges result in less diverse and fewer numbers of people pursuing STEM careers despite an all-time-high demand for STEM professionals, and a growing disconnect between science and society (Veenstra, Padró, and Furst-Bowe 2012; Benish 2018; Ufnar, Bolger, and Shepherd 2017). It is because of these barriers, however, that K–12 science

education is a powerful setting in which to foster understanding of and support for the sciences. In fact, research shows that early exposure to science, a sense of belonging in the STEM environment, and awareness of STEM career paths are critical factors in whether a person pursues a STEM profession (Alvarez, Edwards, and Harris 2010; Laursen et al. 2007; Merritt et al. 2021).

It is essential that organizations outside of the K−12 realm contribute their expertise to enrich science education and alleviate pressure on educators, leading to the cultivation of trust between science and society and increased interest in STEM career paths by diverse people (Veenstra, Padró, and Furst-Bowe 2012). To contribute to this need, I designed and implemented Frogs in Class, a program aimed at providing hands-on biological lessons through the lens of poison frogs and their developmental stages. These lessons were led by biologists, providing repeated exposure to a scientist in the classroom, and grounded in Next Generation Science Standards (NGSS) for third through fifth grade classrooms. Here, I describe my experience piloting Frogs in Class and aspirations for the future of the program.

Frogs in Class

Overview

Frogs in Class is a collaboration between the Fischer Laboratory, an animal behavior laboratory at the University of Illinois Urbana-Champaign, and local third grade classrooms. The program educates participants about poison frogs and how to care for them, provides necessary supplies and support for classrooms to adopt tadpoles from the Fischer Laboratory frog colony, and presents six modules that capitalize on the current developmental stage of each classroom's tadpole/frog to engage students in diverse biological concepts. These modules are used across

eight to ten months, following the school year and the approximate time it takes for tadpoles to reach adulthood. The six modules are: (1) Fantastic Frogs: an overview of frog life history and husbandry; (2) Terrific Tadpoles: the impact of environmental cues on development; (3) Fragile Froglets: a fragile life stage in a changing climate; (4) Jumping Juveniles: special features of a frog's survival toolbox; (5) Adaptive Adults: the diverse and unique behaviors of frogs; (6) Life of a Scientist: a tour of the Fischer Lab. Each module overviews the frog's life stage, connects that developmental stage to a bigger biological theme, and cultivates STEM skills through emphasis on the scientific process.

Alignment with Next Generation Science Standards

I created the modules from a foundation of Next Generation Science Standards (NGSS). NGSS are science content standards rigorously developed and published by scientists and science education experts in 2013 (Bybee 2014). The standards emphasize three dimensions of scientific learning: disciplinary core ideas, scientific and engineering practices, and cross cutting concepts. Parts of each of these three dimensions combine to form one performance expectation (PE) and these PEs build cohesively from K–12th grade (States 2013). I focused specifically on the 3–5th grade-band performance expectations when developing Frogs in Class. While my 60-minute visits surely did not address a performance expectation thoroughly, it provided a starting point from which educators could go further in depth. Or, potentially, my visit provided the only exposure students may have to this scientific idea in their classroom, as the level of NGSS-aligned or any science instruction varies widely from teacher to teacher and school to school across the elementary grades. Nonetheless, by aligning my outreach efforts with the standards by which science educators teach, I intended to expand upon the concepts that students were

learning about in their classrooms, ensuring that the time I took from their school day was worthwhile and mutually beneficial for the biologists, teachers, and students.

Frogs as an anchoring phenomenon

Students gain scientific skills and literacy by "doing science", in other words, by asking questions and creatively and rigorously answering those questions (Manz 2019). Science teachers use a strategy referred to as an "anchoring phenomenon routine" to engage students with an intriguing circumstance that motivates curiosity and raises questions as a way of introducing a topic (Weizman, Shwartz, and Fortus 2008). The questions generated from interacting with or observing the anchoring phenomenon can be used to initiate and structure a storyline unit - a coherent series of investigations or units driven by student interest (Reiser et al. 2021). Frogs in Class leverages these instructional routines by providing each classroom with a tank of poison frogs as an anchoring phenomenon. Arriving as drab-colored tadpoles and developing into bright, charismatic frogs with distinct behaviors, poison frogs are an excellent anchoring phenomenon. The name "poison frog" itself generates many questions in an elementary classroom. Not only do the frogs inspire wonder when they are first adopted into the classroom, but they continue to motivate question-asking as they progress through different life stages. As adults, poison frogs display parental care, where males will guard and hydrate their eggs and then transport their tadpoles on their backs to pools of water. The themes for the six modules are designed to address anticipated questions that may be generated from student observations– such as why the frogs are so colorful or why they tap their toes when they eat. In this way, Frogs in Class provides a cohesive learning experience for students that is driven by student interest and based on best science education practices.

A scientist in the classroom

Importantly, the modules were presented by Fischer Lab members which helps demystify misconceptions about scientists, facilitate conversation about the various identities of scientists and different paths to a STEM career, and the challenges that arise on that journey. Having scientists in the classroom is an important component of Frogs in Class because they provide necessary representation of diverse identities (e.g., ethnicity, gender, ability, socioeconomic status, sexual orientation) and, relatedly, support a sense of belonging in STEM environments which is essential for motivation to pursue a STEM career and to be successful in doing so (Ufnar, Bolger, and Shepherd 2017; Merritt et al. 2021). I designed Frogs in Class to be a long-term program providing repeated exposure to scientists with the intent of maximizing the opportunities available for students to reap the benefits of a scientist in their classroom.

School demographics

I piloted Frogs in Class in four third-grade classrooms at a rural-area school and six third-grade classrooms at an urban-area school, reaching approximately 180 students. Due to time constraints at the urban school, only the first two modules were piloted at this location while all six modules were piloted at the rural school. Over 80% of students at the urban school were members of racial and ethnic minorities and the school actively receives the highest level of Title I assistance, available only to schools with high percentages of children from low-income families. This school was chosen for its racial/ethnic and socioeconomic statuses. There is a critical need to make science more accessible for underrepresented groups that have been systematically excluded from STEM, and inclusive, empowering science outreach provides a pathway for beginning to break down the various barriers faced by underserved groups (Golle et

al. 2022). In another context, the small, rural elementary school also receives the highest level of Title I assistance, reflecting a high population of students from low-income families. Exposure to STEM opportunities are typically scarce for children in rural schools due to several constraints, including reduced access to technology, advanced courses, extracurricular activities, and university-led outreach efforts (Hendrickson et al. 2020). As an alumna of this rural school, my curiosity about biology was hindered by the lack of opportunities to explore my interests, and a lack of exposure to diverse people in STEM and their careers. It is because of my lived experiences that I am passionate about filling this gap between K−12 schools and biological research by providing opportunities for underrepresented groups to explore STEM concepts, people, and careers.

(Module 1) Fantastic Frogs: an overview of frog life history and husbandry

Fantastic Frogs is the kick-off to Frogs in Class and touches on NGSS Performance Expectation 3-LS4-3 (construct an argument with evidence that in a particular habitat some organisms can survive well, some survive less well, and some cannot survive at all). In this unit, students were introduced to the research happening in the Fischer Lab, learned about poison frog life history, constructed a terrarium, adopted tadpoles, and became a "frog friend" (i.e., received training on how to care for their frogs). To begin, I introduced myself and the Fischer lab by sharing about our study system, the poison frogs, and giving a brief overview of the research we conduct in the Fischer Laboratory. Next, we constructed each terrarium as a classroom, simultaneously discussing why each component is important for the health of the frogs and, conversely, how the frogs contribute to the health of their ecosystem (Figure 1A). For example, when students added a drainage layer, we discussed how proper water drainage minimizes

disease that could be caused by stagnant water on the surface, or how frog droppings are an excellent fertilizer for the plants in the terrarium. To make the construction of the terrarium as hands-on as possible, I divided tasks so that each student was able to contribute in some way. Once the terrarium was complete, the students became "frog friends" by learning how to properly care for their adopted tadpoles. Here, I emphasized that every biologist that joins the Fischer Lab undergoes similar training because animal care is a critical part of studying captive animals. Each classroom received a weekly checklist and the rotation of student responsibility for frog care tasks was decided by teachers.

During Fantastic Frogs, I was pleasantly surprised by the expertise some students had about frogs and the overall enthusiasm of all students. Looking forward, I will allot more time with each classroom for this module, as one hour of time was simply not enough to thoroughly cover each of the components. A possible alternative would be to split Fantastic Frogs into two modules, with the first module being an introduction to poison frogs and an opportunity for the biologist and students to become familiar, and the second module being a training on how to care for frogs and construct their terraria. In summary, Fantastic Frogs introduced poison frogs, their life history, and husbandry, and provided students with exposure to a biologist and their laboratory.

(Module 2) Terrific Tadpoles: the impact of environmental cues on development

My second module, Terrific Tadpoles, was inspired by NGSS performance expectations 4-LS1-2 (use a model to describe that animals receive different types of information through their senses, process the information in their brain, and respond to the information in different ways) and 3-LS3-2 (use evidence to support the explanation that traits can be influenced by the

environment). Here, I used a long-term tadpole experiment to demonstrate the significance and usefulness of the scientific method. After a discussion about the unique developmental context of tadpoles and the various aspects of their environment, students learned about the interesting questions Fischer Lab biologists ask and seek to answer in tadpoles. Using a recent Fischer Lab study that investigated the impact of environmental cues on development as a springboard, students asked whether tadpoles in heated or room-temperature water would metamorphose quicker. To answer this question, we put two tadpoles in a tub of water with an aquarium heater and two tadpoles in a tub of water without a heater. As a group, we determined the importance of keeping all other variables, such as feeding frequency and light cycle, consistent. I gave each student a blank notebook and they recorded their research question, hypothesis, and made a table in which to record their data regarding the size and morphology of their tadpoles over time. I also encouraged the students to use their notebooks as a space to record any observations about their frogs throughout the school year. Once their experiment was started and their notes were taken, I ended the visit with a Frog Friend training specific to supporting tadpoles through and after metamorphosis.

The significance of using the scientific process as a guide during Terrific Tadpoles was only realized once I was already in the classroom and exploring students' STEM skillset. When I asked each classroom of children to raise their hand if they had heard of the scientific method or process, less than 10% of students raised their hand. While the predefined "steps" of the scientific process are not the only way of approaching science inquiry, they provide a guide for engaging in meaningful, comprehensive science experiences which are pivotal in early science education (Gerde, Schachter, and Wasik 2013).

A challenge we experienced with Terrific Tadpoles was the fluctuating temperatures of school buildings. The heating system of both schools was powered off at the end of the school day which led to fatally low temperatures for some of the tadpoles throughout the night. This resulted in unexpected tadpole fatalities during the experiment. Once the teachers and I were able to pinpoint the issue, we began experimenting with alternative ways of keeping heat within the tanks, including heating mats, location changes within the classroom, and insulating the tanks. Ultimately, we found that covering tanks with towels or blankets overnight kept the temperature of each terrarium at a preferable level. While this unanticipated challenge impacted the original goal of Terrific Tadpoles, it also provided a wonderful opportunity to discuss with students the adaptive nature of "doing science". We were able to compare their data to that of a published scientific study and discuss why the two might differ and what changes we would make if we were to run the experiment again. In the future, I will be sure to address temperature fluctuations before tadpoles are adopted into classrooms. Nonetheless, Terrific Tadpoles provided students with a realistic experience with the scientific process while building observational and note-taking skills.

(Module 3) Fragile Froglets: a fragile life stage in a changing climate

Fragile Froglets addresses NGSS performance expectation 5-ESS3-1 (obtain and combine information about ways individual communities use science ideas to protect the Earth's resources and environment) and capitalizes on the delicateness of a recently metamorphosed froglet to emphasize how a changing climate is challenging for all animals, especially those in sensitive life stages. I began by polling the students about whether they had heard of "climate change" before. Surprisingly, zero students raised their hand in response to my question. My

experience with the students reflects research that shows that climate change education is not yet a priority among science educators due to outdated standards and frameworks of science education (Sharma 2012). To address this gap in K−12 education, we began a discussion about what climate change is, what contributes to it, and how it impacts animals, especially amphibians who are indicator species. The students brainstormed small changes that they could make in their daily routines to help mitigate their contribution to climate change and took action to "save the frogs" by creating posters demonstrating these changes (Figure 1B).

The process of brainstorming and then sharing their ideas and how they relate to climate change prompted a lot of inquiry and conversation between students and with me. In contrast to Terrific Tadpoles, which was more structured, the free time dedicated to creating posters during Fragile Froglets provided an informal time for the students and I to interact and get to know each other. Their posters were hung in the school hallways which hopefully encouraged further discussion about climate change amongst their peers. In all, the value of Fragile Froglets was at least two-fold: (1) students with no prior knowledge of climate change reached a level of understanding that allowed them to propose a change they could make to help lighten their contribution to climate change, and (2) less-structured time built into the visit allowed for deeper connection between the scientist in the classroom and the students. While Fragile Froglets went over well, I would like to expand this module in future years to give students the opportunity to present their posters to students outside of Frogs in Class.

(Module 4) Jumping Juveniles: special features of a frog's survival toolbox

Jumping Juveniles touches on NGSS Performance Expectation 4-LS1-1 (construct an argument that plants and animals have internal and external structures that function to support

survival, growth, behavior, and reproduction). We leveraged the juvenile life stage to dive deeper into the many adaptations of poison frogs that often attract attention and motivate questions, such as their bright coloration, toxicity, and parental care strategies. Using videos of native Illinois and poison frog species in their natural habitats, students compared visible adaptations of the two groups of frogs, such as differences in coloration and behavior, as well as differences in their environments. After individually recording their observations, students shared their ideas to make a classroom list of native versus poison frog adaptions, and temperate versus tropical forest characteristics. I then gave each student a blank, foldable box and encouraged them to draw either a temperate or tropical forest on the outside using markers. Then, I gave each student air-dry clay that they used to make a model of either a poison frog or a native frog fitted to their environment, showcasing the adaptations unique to each (Figure 1C). I gave students who chose to model poison frogs brightly colored clay and glitter to symbolize their toxins and I gave dull green and brown clay to students who chose to model native frogs. In addition to coloration and toxicity, students also modeled the parental care strategy of their frog by adding tadpoles on the backs of poison frogs to symbolize their care for their young in the form of tadpole transportation, and by adding large amounts of eggs in a separate part of the box for native frogs to symbolize their lack of care.

Jumping Juveniles was inspired by research showing that making and revising models of scientific ideas deepens student understanding of scientific concepts (Merritt et al. 2021). Indeed, I observed many students smooshing together their clay to revise their model of a frog and its adaptions after conversing with their peers and/or comparing their models amongst each other. A challenge I experienced in piloting Jumping Juveniles was the desire of students to create unrealistic models. For example, many students modeled dyeing poison frogs with groups of

tadpoles on their backs when dyeing poison frogs carry only one tadpole at a time for very important biological reasons (i.e., their tadpoles are aggressive and cannibalistic). While I didn't discourage student creativity by having them correct their models, I did use this as an opportunity to have interesting conversations with the students about various features of poison frogs or frogs in general. This led to authentic inquiry in every classroom. Overall, Jumping Juveniles provided an opportunity for students to creatively model their knowledge of adaptions in the context of frog coloration, toxicity, and behavior.

(Module 5) Adaptive Adults: the diverse and unique behaviors of frogs

Adaptive Adults addresses NGSS performance expectation 3-LS2-1 (construct an argument that some animals form groups that help members survive) by immersing students in the field of animal behavior. Starting with a game of charades, students acted out different animals for their classmates. I connected their experience playing charades with animal behavior – highlighting how different animals behave in unique ways and how this allowed the students to act like a specific animal and to determine the animals being displayed by their peers. I then shared videos of various poison frog behaviors with each classroom, such as toe tapping, calling, and parental care in the form of tadpole transportation, before introducing the more abstract idea of "bold" behavior. I explained that boldness can be considered an aspect of an animal's personality and can be measured by putting an animal in a new environment and recording how much they move around this new space. I introduced three species of poison frogs who vary in their natural toxicity levels and asked students to hypothesize which one might be the boldest. Then, using video data I collected in the Fischer Laboratory, students recorded the amount of time each frog spent moving. I emphasized that these videos were collected for an experiment I

conducted as a scientist in a research laboratory to try to demystify what it looks like to be a scientist. The students observed three frogs of each of the three species before concluding that the most toxic species was in fact the boldest.

Compared to Terrific Tadpoles, which was an example of a long-term experiment, the students seemed to really enjoy asking a question and answering it in one classroom visit. The students were very enthused when proposing their hypotheses and even more so when interpreting their results. This activity also provided an opportunity to reinforce comprehensive science inquiry using the scientific method. Perhaps most importantly, Adaptive Adults demystified what it means to be a professional scientist by having students complete a mini version of an experiment that was conducted in the Fischer Laboratory. Looking forward, I aim to design a behavioral experiment conducive to a classroom environment so that students can experience the study of animal behavior in a more hands-on, authentic way.

(Module 6) Life of a Scientist: a tour of the Fischer Lab

My last module was devoted to demystifying who scientists are, what scientists do, and how to become a scientist. To do this, I invited our collaborating classrooms on a tour of the Fischer Lab. However, due to financial and time constraints classrooms were not able to physically tour the lab. Instead, I created a virtual tour of the Fischer Lab spaces and incorporated interviews of my fellow lab members. I collected questions from the students in advance to be sure that their curiosities and uncertainties were addressed. I curated interviews to have Fischer Lab members share about their personal interests, such as their hobbies and favorite frog species, as well as their research interests. After getting to know a few lab members through interviews, students were then taken on a virtual tour of the Fischer Laboratory bench and office

spaces, fly room, and three frog rooms. In each of these areas, members of the lab explained the purpose of any special equipment and the reasoning behind various aspects of our frog facilities, such as the automatic misting systems or careful labelling of our frog tanks. In the video, we compared our large-scale frog colony to an individual classroom tank to highlight the ways in which care strategies change to accommodate the large number of frogs needed for experimentation. Life of a Scientist provided, for most students, a first exposure to a laboratory space and the diverse scientists that work within it. Through personal interviews, students were able to connect with biologists through similar interests outside of science – an important aspect of demystifying scientists and fostering a perspective that science is for everyone (Merritt et al. 2021). In future years, I would like to incorporate an opportunity for students to ask questions to a panel of scientists in real time.

Conclusions

Outreach efforts targeting K−12 students break down barriers between science and society, tackle inequities in science accessibility for underserved groups, as well as alleviate pressure on a burdened education system (Veenstra, Padró, and Furst-Bowe 2012; Golle et al. 2022). In response to a need for K−12 outreach opportunities, I piloted Frogs in Class - a long-term outreach collaboration between the Fischer Laboratory and local third grade classrooms. Through six modules connecting the current life stage of the students' adopted frog to broad biological concepts, approximately 180 students were able to engage in hands-on, NGSS-aligned science activities. Importantly, students experienced repeated exposure to a scientist in their classroom (Figure 1D), a necessary experience for changing ideas about what science is and who can do it (Figure 1E) (Laursen et al. 2007).

Moving forward, I plan to continue Frogs in Class with revised materials and ultimately assess its efficacy through student and teacher surveys. In revising the modules, I aim to create learning experiences that are more closely aligned to NGSS by providing opportunities for students to be active generators of knowledge rather than passive recipients. I hope to achieve this by structuring the modules in a way that encourages students to make sense of biological concepts through scientific practices (e.g., planning investigations and engaging in evidence-based arguments) and embracing the inherent uncertainty that comes with those practices. I would also like to expand upon the current modules to create more NGSS-based curricula for teachers to implement in addition to the modules led by scientists. That is, I aim to develop detailed, frog-inspired lesson plans that teachers can access and lead independently. Altogether, Frogs in Class provides an example of creating a meaningful K–12 outreach experience that prioritizes student learning and exposure to scientists and is steeped in best education practices.

Figure

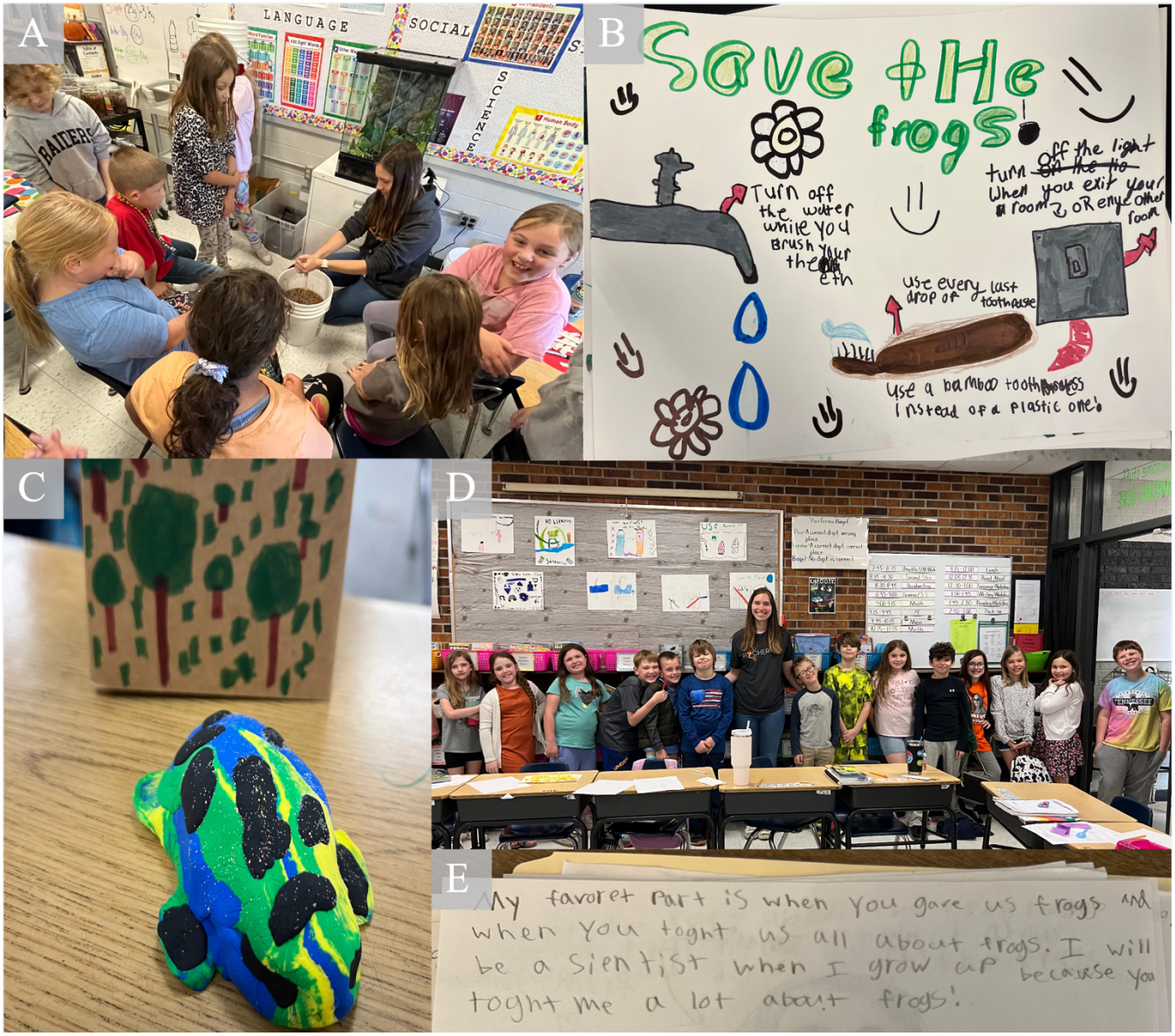

Figure 4: Photos of (A) constructing a tank with students during Fantastic Frogs, (B) student poster from Fragile Froglets, (C) student model of a poison frog and its habitat from Jumping Juveniles, (D) end of the year photo with a classroom, and (E) student note that writes, “My favorite part is when you gave us frogs and when you taught us all about frogs. I will be a scientist when I grow up because you taught me a lot about frogs!”

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