Why not both? A case study measuring cortisol and corticosterone in poison frogs

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Abstract

A general tenet in stress physiology is that the hypothalamic-pituitary-adrenal (HPA) axis predominantly produces one glucocorticoid (GC) in response to stressors. Researchers typically measure only the GC believed to be dominant in their species of interest; however, the two main glucocorticoids produced by vertebrates– cortisol and corticosterone — show variation across species in absolute levels, relative proportions, and stress responsivity. Therefore, generalizing assumptions and focusing on a single GC may not tell the whole story. In this study, we measured both cortisol and corticosterone in water samples collected from five species of Dendrobatid poison frogs. Understanding the full picture of the physiological stress response in poison frogs is of special interest due to their susceptibility to environmental stressors and conservation status. Counter to the dogma that corticosterone is dominant and more abundant in amphibians, we found cortisol was more abundant than corticosterone in four of the five species. *Dendrobates tinctorius* was unique in releasing equal amounts of cortisol and corticosterone. When we stimulated the HPA axis in *D. tinctorius*, we found corticosterone was responsive to adrenocorticotropic hormone (ACTH), while cortisol did not change. Comparing plasma and water samples, we found water-borne cortisol was positively correlated with cortisol in plasma and the relationship between plasma and water-borne corticosterone was positive but not statistically significant. These results challenge the broadly accepted assumption that corticosterone is 'dominant' in amphibians and add to the growing literature highlighting the importance of measuring both GCs to understand (stress) physiology.

Keywords: ACTH challenge, anurans, corticosterone, cortisol, Dendrobatids, glucocorticoids

1. Introduction

Researchers across disciplines are interested in understanding stress. The ability to measure stress physiology non-invasively in the lab and in the wild is key for behavioral ecologist and conservation biologists seeking to understand animal behavior in a changing world. In studying stress physiology, we often measure glucocorticoids (GCs), most commonly cortisol and corticosterone, produced by the adrenal cortex in response to stress (Sapolsky et al., 2000). Glucocorticoids are of keen interest not only as a readout of stress, but also because they elicit a variety of physiological, morphological, and behavioral responses (MacDougall-Shackleton et al., 2019). For example, we know elevated levels of corticosterone can have negative impacts on tadpoles through reducing growth rate, altering morphology, and shortening time to metamorphosis which affects growth and survivorship later in life (Glennemeier and Denver, 2002; Kulkarni and Gramapurohit, 2017).

Most studies measure only a single GC, the one considered to be "dominant". The dominant GC is formally defined as the one released in response to adrenocorticotropin hormone (ACTH), though dominance is often assumed based on higher abundance. Which GC is dominant varies across species, with generalizations assumed based on taxon. Yet a key thing that is often overlooked in stress physiology is the fact that other glucocorticoids are present, often in substantial amounts, and can be produced independently of the HPA axis being activated during times of stress (Bornstein et al. 2016). Thus, to fully understand the role glucocorticoids play in population health, we need to move beyond simply studying the glucocorticoid we assume to be dominant.

1.1 Cortisol vs corticosterone –

Glucocorticoids are not only involved in the "stress response" (MacDougall-Shackleton et al., 2019). In fact, glucocorticoids are involved in many physiological processes including, but not limited to, energy metabolism (Vegiopoulos and Herzig, 2007), immune response (Cruz-Topete and Cidlowski, 2015), growth and development (Seckl and Meaney, 2004), reproduction (Whirledge and Cidlowski, 2017), and cardiovascular function (e.g., Cruz-Topete et al., 2016). Because of their widespread influences, changes in GCs and/or their receptors can be immensely impactful on survival and fitness. While acutely elevated GC levels often have adaptive

functions, in many species chronically elevated GCs can disrupt normal physiology and decrease fitness (reviewed in Bonier et al., 2009).

Cortisol and corticosterone are often considered interchangeable in their physiological function (Sutanto and De Kloet, 1987). Typically, either cortisol or corticosterone is considered the dominant GC produced by a species because it is more abundant at baseline and increases in response to stressors or ACTH. ACTH challenges are often used to measure the responsiveness of the adrenals, to identify the dominant GC, and to validate that the method of hormone measurement is detecting an increase in HPA axis activity (Palme, 2019; Touma and Palme, 2005). However, the GC that is not responsive or less responsive to ACTH can also play an important physiological role. For instance, in humans, cortisol is considered the primary effector hormone of the HPA (Brück, 1983). However, corticosterone has been studied in humans for its role in the central nervous system (Raubenheimer et al., 2006) and pre-natal responses to stressors (Wynne-Edwards et al., 2013). In other cortisol-dominant species, the rate of conversion from corticosterone to aldosterone may vary resulting in more or less circulating corticosterone in cortisol-dominant species (Koren et al., 2012).

Despite large interspecific variation in GC levels and relative proportions of cortisol and corticosterone (Koren et al., 2012), we commonly make default assumptions about which GC is more abundant (and therefore often assumed to be dominant) based on taxa. The consequence of this generalization is that studies typically measure only the GC that is generally considered dominant, such as measuring only cortisol in fish and only corticosterone in birds. Yet, these assumptions and generalizations can be misleading. For example, we find GC dominance varies across closely and more distantly related mammals. In rodents, corticosterone is the considered the dominant GC in rats and mice (Crisler et al., 2020), while cortisol is dominant in North American red squirrels (*Tamiasciurus hudsonicus*) and Arctic ground squirrels (*Spermophilus parryii*) (Boonstra and Mccoll, 2000). Yet another species in Rodentia, the golden hamster (*Mesocricetus auratus*), secretes approximately equal amounts of both cortisol and corticosterone (Albers et al., 1985; Kollack-Walker et al., 1997; Ottenweller et al., 1985). Interestingly, a recent study of GC concentrations in hair found wide among-individual variation in the ratio of cortisol to corticosterone across three otariid species (Stellar sea lions [*Eumetopias jubatus*]; California sea lions [*Zalophus californianus*], and Northern fur seals [*Callorhinus ursinus*]), though cortisol and corticosterone were correlated within-individuals suggesting either

cortisol or corticosterone could be measured to assess HPA axis activity (Keogh et al., 2020). Similarly, there are also population differences in the ratio of cortisol to corticosterone in wild sea otters (*Enhydra lutris*) across the species range with fecal corticosterone concentrations ranging from around 10-85% of cortisol concentrations (Larson et al., 2009). These highly variable findings within and across species suggest that we should use caution when following dogmatic assumptions about which GC to measure based on, order, family, or even population within the same species (Hancock, 2010).

As for variation in (relative) abundance, more studies are showing the importance of testing interspecific variation in GC responses even among closely related species. For instance, across mammals, we see all combinations of which GC responds to acute stress. Some species respond with an increase in cortisol but not corticosterone (mice [*Mus musculus*] Gong et al., 2015; Javan rusa deer [*Rusa timorensis*] van Mourik et al., 1985; tuco-tucos [*Ctenomys talarum*] Vera et al., 2012, 2011), while in others corticosterone responds but not cortisol (clouded leopard [*Neofelis nebulosa*] Young et al. 2004), and still in others *both* GCs respond (ferrets [*Mustela putorius*] Rosenthal et al., 1993; red deer [*Cervus elaphus*] Smith and Bubenik, 1990). Sea otters are an interesting case where some studies have found an increase in cortisol in response to ACTH (Murray et al., 2020; Wasser et al., 2000), yet others find an acute stressor (catching and handling) causes an increase in corticosterone suggesting a non-ACTH-driven regulation of corticosterone (Larson et al., 2009). Across cortisol-dominant mammals, cortisol and corticosterone are often, but not always, positively correlated with one another (reviewed in Koren et al., 2012). Baleen whales are a great example of how species vary in their correlation between the two GCs with a positive correlation in the North Atlantic right (*Eubalaena glacialis*) and blue (*Balaenoptera musculus*) whales but not in the bowhead whale (*Balaena mysticetus*) (Hunt et al., 2018). In baleen whales, the two GCs may synchronize when under severe stress or in an energetically demanding physiological state such as reproduction (Hunt et al., 2018, 2017). While in some cases there may be financial or logistical limitations preventing the measurement of both GCs, whenever possible we should explore both to discover potentially interesting and informative patterns.

1.2 Glucocorticoids in amphibians –

Non-invasively studying the stress physiology of amphibians can be instrumental in understanding how species respond to multifactor environmental stressors, such as habitat modification, climate variability, and disease, and can inform conservation strategies for at-risk species (Kumar and Umapathy, 2019; Narayan, 2013; Palme, 2019; Walls and Gabor, 2019). Corticosteroids also play critical roles in metamorphosis (Denver, 2009; Kulkarni and Buchholz, 2014). Corticosterone is typically referenced as the dominant and more abundant glucocorticoid in amphibians (Moore and Jessop, 2003; Norris and Carr, 2013; Wingfield and Romero, 2011), and occasionally listed as amphibians' only glucocorticoid (Katsu and Iguchi, 2016). Consequently, the vast majority of studies on stress physiology in amphibians only measure corticosterone. This dogma prevails despite a history of cortisol being detected in several species of anurans (frogs and toads) (e.g., Dale, 1962; Krug et al., 1983) and a growing number of studies demonstrating that the assumption of corticosterone dominance and/or greater abundance does not hold for all species of amphibians. For example, hellbenders (*Cryptobranchus alleganiensis*) mainly produce cortisol in response to a stressor (Hopkins et al., 2020). Additionally, cortisol has been measured in multiple species of amphibians (terrestrial and aquatic) using dermal swabs (Santymire et al., 2018), and cortisol increases in response to ACTH in green treefrogs (*Hyla cinerea*), American toads (*Anaxyrus americanus*), and red-spotted newts (*Notophthalamus viridescens*) (Santymire et al., 2018). Previous studies have suggested aquatic amphibia rely on cortisol as their primary GC, as many fishes do, whereas semi-terrestrial or terrestrial amphibia typically use corticosterone (Jungreis et al., 1970), yet the vast majority of studies on a popular aquatic model species, the clawed frog (*Xenopus* sp.), measure corticosterone (e.g., Jaudet and Hatey, 1984; Kulkarni and Buchholz, 2012; Shewade et al., 2020). Measuring GCs in more amphibians will help us discern the role of different GCs across different life histories and inform our general understanding of amphibian physiology. The myriad of contradictory studies about the primary GC in amphibians brings into question whether corticosterone can indeed be assumed to be the primary GC as we expand our study of endocrinology to more non-traditional species.

1.3 Stress physiology in Neotropical poison frogs –

Many Neotropical poison frog (Dendrobatidae) species are threatened due to risks including agrochemicals (Angulo et al., 2019), climate change (Whitfield et al., 2007), smuggling (Auliya et al., 2016), and habitat destruction or fragmentation (Becker et al., 2007; Cushman, 2006). Due to their susceptibility to the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) (Courtois et al., 2015) and conservation status (Guillory et al., 2019), the development of non-invasive measurements of GCs is important for studying poison frog physiology and informing conservation efforts. Neotropical poison frogs are primarily diurnal and terrestrial, and have been gaining attention in behavioral ecology and neuroethology due to their interesting parental behavior (Lötters et al., 2007; Roland and O'Connell, 2015; Westrick et al., 2023). In addition, poison frogs vary in size but generally are relatively small, with an average snout-vent length (SVL) of ~17-60 mm. Thus, repeated plasma sampling is not feasible, and we need to further explore non-invasive methods of studying the endocrinology of poison frogs.

Our goal for this study was to investigate which glucocorticoid is the primary hormone of the HPA axis in Dendrobatid poison frogs. Both cortisol (Fischer and O'Connell, 2020) and corticosterone (Nowicki et al., 2022) have been measured in poison frogs, albeit in different species and sample types (plasma from *Dendrobates tinctorius* and water from *Ranitomeya imitator*, respectively). We tested interspecific variation in the relationship between cortisol and corticosterone and focused on the response of both glucocorticoids to a physiological stressor in one species. We used non-invasive water-borne hormone sampling to allow for repeated measures from the same individuals (Baugh et al., 2018; Gabor et al., 2013; Rodríguez et al., 2022). To examine species variation in glucocorticoid dominance among poison frogs, we took "baseline" water samples from five species of Dendrobatids: the golden poison frog (*Phyllobates terribilis*), mimic poison frog (*Ranitomeya imitator*), Zimmermann's poison frog (*Ranitomeya variabilis*), dyeing poison frog (*Dendrobates tinctorius*), and Anthony's poison frog (*Epipedobates anthonyi*). These species share many behaviors, such as parental care, and are endemic to tropical regions of Central and South America, but vary in body size and toxicity in the wild (Wells, 2007). *P. terribilis* and *D. tinctorius* are the largest of the group (~37-60 mm SVL), *E. anthonyi* are intermediate (~19 to 25 mm SVL), and both *Ranitomeya* are among the smallest poison frogs (~17-22 mm SVL). To confirm whether the more abundant GC is also dominant, i.e. elevates in response to an acute stress signal, we additionally performed an ACTH

challenge in our primary focal species (*D. tinctorius*). In *D. tinctorius*, we then compared glucocorticoid concentrations between water and plasma samples from the same individuals to assess whether water samples are representative of circulating hormone levels in blood. All together this study informs our understanding of the 'stress' physiology of this particularly vulnerable clade of frogs and contributes important data to the puzzle of GC dominance in amphibians.

2. Materials and methods

2.1 Animal husbandry –

We housed all frogs in our breeding colony at University of Illinois Urbana-Champaign in glass terraria (18x18x18", 12x12x18", or 36x18x18" Exo Terra®) with soil, sphagnum moss, live tropical plants, and coconut shelters. We kept frogs on a 12:12 h light cycle and fed them flightless *Drosophila* fruit flies (*D. melanogaster* or *D. hydei*) dusted with vitamin supplements three times weekly. Our automated misting system kept humidity >75% and the room was kept at 70-76ºF. We housed adult frogs in groups of two to three individuals of the same species, either one or two males with one female (*E. anthonyi*, *D. tinctorius*, *R. variabilis*, *R. imitator*) or as a group of two to three juveniles that were too young to be sexed at the time of sampling (*P. terribilis*). We weighed frogs prior to sampling by placing them in a plastic container on a scale and subtracting the weight of the container. All procedures were approved by UIUC Institutional Animal Care and Use Committee (protocol #20147).

2.2 Hormone sampling –

To collect water-borne hormones, we placed individual frogs in glass containers with RO water based on size. For the smaller poison frogs (*E. anthonyi*, *R, variabilis*, and *R. imitator*), we used 4cm x 4cm x 5cm hexagonal glass jars with lids filled with 15 mL of RO water. For the larger frogs (*D. tinctorius* and *P. terribilis*), we used 6.5cm x 6.5cm x 6cm glass tubs with lids and 40 mL of RO water. Our goal was to use enough water to cover frogs' highly vascularized pelvic skin in a small enough container that the frogs would stay in the water and not climb the walls. We left frogs in the water for one hour before returning them to their home terraria or a new water tub for additional sampling. We collected all samples 4.5-8 hours into the frog's 12 h

light cycle. We filtered water samples with Whatman® 1 filter papers to remove any large particulates, such as fecal matter, prior to steroid hormone extraction (see Section 2.4). To compare across species, we collected n=84 water samples (*D. tinctorius* n = 6, *E. anthonyi* n = 20, *P. terribilis* n = 20, *R. imitator* n = 20, *R. variabilis* n = 18). With n=32 samples, we subsampled half of the water for additional projects and stored each subsample separately. We stored all water samples at -20ºC until extraction. For the plasma and water-borne hormone comparison, we collected water samples as described above for *D. tinctorius* (n = 10) immediately prior to collecting plasma. For n=5 samples, we split water samples in half for additional projects. To collect plasma samples, we anesthetized frogs with benzocaine (Orajel™) and euthanized via rapid decapitation. We used heparinized microhematocrit capillary tubes to collect trunk blood from the body. We combined all the collected blood from an individual in a microcentrifuge tube and centrifuged for 5 min at 5,000 rpm to separate the plasma. Using a micropipette, we collected the plasma supernatant and stored all plasma samples at -20°C.

2.3 ACTH hormone challenge –

For the ACTH hormone challenges, we used a within-individual repeated measures design with 18 adult *D. tinctorius* (9 male, 9 female). Each frog experienced an injection of 0.5 ug ACTH per g body weight (Sigma-Aldrich®, product no. A7075) diluted in 0.9% saline and a vehicle injection of only 0.9% saline with 6 days between injections. The order of injections was counterbalanced across individuals such that half of the frogs were first injected with vehicle, and half were first injected with ACTH. Prior to the start of trials, we dissolved the ACTH in saline at a concentration of 0.125 ug/µL and aliquoted the solution into insulin syringes which were frozen at -20°C until the day of injection, along with saline vehicle aliquots. On the day of the trial, we thawed the aliquots of saline and ACTH and calculated the volume needed to inject each frog with a dosage of 0.5 ug ACTH per g body weight, based on previous work using ACTH challenges in Neotropical frogs (Baugh et al., 2018). Since the precision of the insulin syringes was limited, we rounded to the nearest 10 µL to get the volume for injection with both ACTH and saline. For each trial, we collected a water-borne hormone sample, as described above, for one hour prior to injection as our “pre-injection” sample. We then used insulin syringes to inject frogs intraperitoneally. After injecting, we immediately placed the frogs in a new water bath for one hour to collect the “post-injection” sample. After the post-injection

248 sample, we returned frogs to their home terraria. We repeated this process the following week
249 with the alternate treatment (ACTH or saline). We performed all trials between five and seven
250 hours after the lights turned on. We split each water sample (four samples per 18 frogs = 72
251 samples total) in half for additional projects and stored subsamples separately.
252
253 *2.4 Steroid hormone extraction –*
254 For all water samples, we used C18 cartridges (Waters™ Corporation, Milford, MA) for
255 solid-phase extraction to collect steroid hormones. With cartridges on a vacuum manifold, we
256 used a vacuum pump to prime the filters with 6 mL 100% methanol. We then rinsed the
257 cartridges with 6 mL RO water before passing the sample through the cartridge. We eluted the
258 hormones from the cartridges with 4 mL of 100% methanol into 13x100mm borosilicate glass
259 vials. We dried samples under a stream of nitrogen gas in a 37°C dry heating block until
260 completely dry. We stored desiccated samples at -20°C until reconstituted with assay buffer for
261 enzyme linked immunosorbent assay (ELISA) analysis (see Section 2.6).
262 To measure glucocorticoids from plasma samples, we used the dissociation reagent included
263 in our ELISA kits (see Section 2.6). We combined 5 µL of dissociation reagent and 5 µL of
264 plasma, vortexed, and incubated for a minimum of 5 min at room temperature. We then added
265 450 µL of assay buffer to make a 1:100 dilution before running samples on ELISA plates.
266
267 *2.6 Enzyme linked immunosorbent assays –*
268 We used DetectX® ELISA kits from Arbor Assays™ (Ann Arbor, MI) to measure total
269 cortisol and corticosterone concentration in plasma and water samples. We used each kit
270 according to the manufacturer's instructions except for using the same assay buffer (catalog no.
271 X065) for both kits to allow us to split samples across cortisol and corticosterone plates,
272 following manufacturer recommendations. For the corticosterone kit, we followed instructions
273 for the 100 µL format because the standard curve for the 100 µL format can measure smaller
274 concentrations than the 50 µL format. The cortisol ELISA plate required 50 µL of sample for
275 each well, or 100 µL to run samples in duplicate. Initially, we reconstituted samples with 225 µL
276 of assay buffer to ensure an even split of the original sample across both cortisol and
277 corticosterone plates. Starting with the cortisol ELISA plate, we pipet 50 µL of samples and
278 standards into wells in duplicate (100 µL in total). We followed the rest of the manufacturer's

instructions for adding the conjugate and antibody to the cortisol plate. We then added 105 µL of assay buffer to further increase the volume of the remaining 125 µL of each sample so we could use the 100 µL assay format for corticosterone to allow for measurement of lower concentrations. We pipet 100 µL of each sample into wells in duplicate (200 µL in total) on the corticosterone ELISA plate and proceeded according to the manufacturer's instructions. For both cortisol and corticosterone, we measured the optical density at 450 nm. After removing samples with a coefficient of variation (CV) > 20% (n = 110 out of 352 total measurements), the average intra-assay CV across all samples was 8.4% for cortisol and 8.2% for corticosterone. The cross reactivity for the cortisol ELISA plate with corticosterone is reported by the manufacturer as 1.2% and for the corticosterone ELISA plate cross reactivity with cortisol is reported as 0.38%.

We used a four-parameter logistic curve (4PLC) regression to fit the respective standard curve on each plate using MyAssay software recommended by the manufacturer (www.MyAssay.com). Based on this curve, we calculated the concentration of cortisol and corticosterone (pg/mL of assay buffer) for each sample and averaged across duplicates. Due to low concentrations of both GCs in some species, some samples were too low to fall on the standard curve (n=30 out of 352 total measurements), though some had concentrations of cortisol that were too high (n=12). To be conservative, for samples that were off the standard curve in either direction (too low or too high), we censored the data at the minimum or maximum detection limits of the assay, respectively. To confirm the censored data was not skewing our findings, we ran additional analyses with only samples measuring within the assay detection limits and CV < 20%, and the overall pattern of results remained the same (Supplemental Materials, Figure S1). To calculate the release rate from the concentration, we multiplied the concentration by the volume of assay buffer used for initially reconstituting the sample (0.225 mL) to get mass (pg) of the hormone in the sample and divided by collection time (one hour) to get the release rate. For samples that were split in half prior to extraction, we multiplied the rate (pg/hr) by two to allow comparison with samples that were not split.

2.7 Statistical analysis –

We used R v4.2.2 (R Core Team, 2022) in RStudio v3.1.446 (Posit Team, 2023) for all statistical analyses and ran all analyses on log transformed hormone release rates to normalize residuals. We first tested each assay for parallelism by combining 24 additional water samples

310 from adult *D. tinctorius* to create a highly concentrated pooled sample and then serial diluting by
311 1/2 of the reconstituted extracts for a dilution curve with seven samples in total. This serial
312 dilution sample was split four ways to test parallelism across four different ELISA plates for
313 steroid hormones, including cortisol and corticosterone. We confirmed parallelism by comparing
314 the slope of the serially diluted curve to the slope of the standard curve using an interaction term
315 in a linear model. We found the dilution curves were not significantly different from the standard
316 curve for their respective hormone (cortisol: ß=-0.34, SE = 1.08, p = 0.76; corticosterone: ß=-
317 5.09, SE = 3.06, p = 0.12).
318 Across species, we were primarily interested in whether the release rates of the two GCs
319 differed within species, rather than absolute GC differences between species. To this end, we fit
320 a linear mixed model for log transformed hormone release rate with a fixed effect of the
321 interaction between species and glucocorticoid type (corticosterone vs cortisol) with sample ID
322 as a random effect. We ran a Type III ANOVA on the linear mixed model to assess the overall
323 effect of the interaction term. We then ran post-hoc simultaneous pairwise comparisons using the
324 multcomp package (Hothorn et al., 2008) to specifically look at the difference in release rate
325 between cortisol and corticosterone within species (e.g., *D. tinctorius* cortisol vs. *D. tinctorius*
326 corticosterone). We did not include sex as a covariate since not all individuals could be
327 confidently sexed at time of sampling. However, in a reduced model with only individuals that
328 we could confidently sex, we found no overall effect of sex on GC production (Table S1). To
329 increase our sample size for *D. tinctorius* in the species comparison, we included samples on the
330 first day of trials before the injection during ACTH challenges in the analysis (n=22 out of 29
331 total measurements of *D. tinctorius* samples). Our final species comparison dataset included 29
332 measurements from *D. tinctorius* (15 corticosterone, 14 cortisol), 25 measurements from *E.*
333 *anthonyi* (12 corticosterone, 13 cortisol), 23 measurements from *P. terribilis* (8 corticosterone,
334 15 cortisol), 22 measurements from *R. imitator* (8 corticosterone, 14 cortisol), and 19
335 measurements from *R. variabilis* (3 corticosterone, 16 cortisol).
336 For the ACTH challenges, we fit a linear mixed effects model to ask if there was an effect of
337 treatment (ACTH or control) on either cortisol or corticosterone. The model had the following
338 main effects: GC type (cortisol or corticosterone), time point (pre- or post-injection), and
339 injection type (ACTH or saline). We also included a three-way interaction between GC type,
340 time point, and injection type. This interaction indicated if the difference between pre- and post-

injection varied by injection type and whether this relationship varied by GC type. In other words, is there a change in cortisol from pre- to post-injection based on injection type and is this change (or lack thereof) the same or different in corticosterone? We included trial ID as a nested random effect within frog ID to account for repeated measures across trials and individuals. We used post-hoc pairwise comparisons using the multcomp package to interpret interaction effects. After filtering for CV, our sample size was 26 pre- and 26 post-injection cortisol measurements and 27 pre- and 30 post-injection corticosterone measurements. Because our main interest was how the within-individual response to the injection varied across injection types, all four samples for each individual were run on the same plate to reduce possible confounding effects of inter-plate variation within individuals and trials.

We calculated the original plasma sample concentration by multiplying the concentration measured in the assay by the dilution factor. To examine the relationship between water-borne glucocorticoids and circulating glucocorticoids in plasma, we fit linear models (R packages lme4 (Bates et al., 2015) and lmerTest (Kuznetsova et al., 2016)) for both cortisol and corticosterone with plasma concentrations (log transformed pg/mL) as the fixed effect predicting water hormone release rate (log transformed pg/hr) for the matched plasma-water samples.

3. Results

3.1 Species variation in relative glucocorticoid abundance –

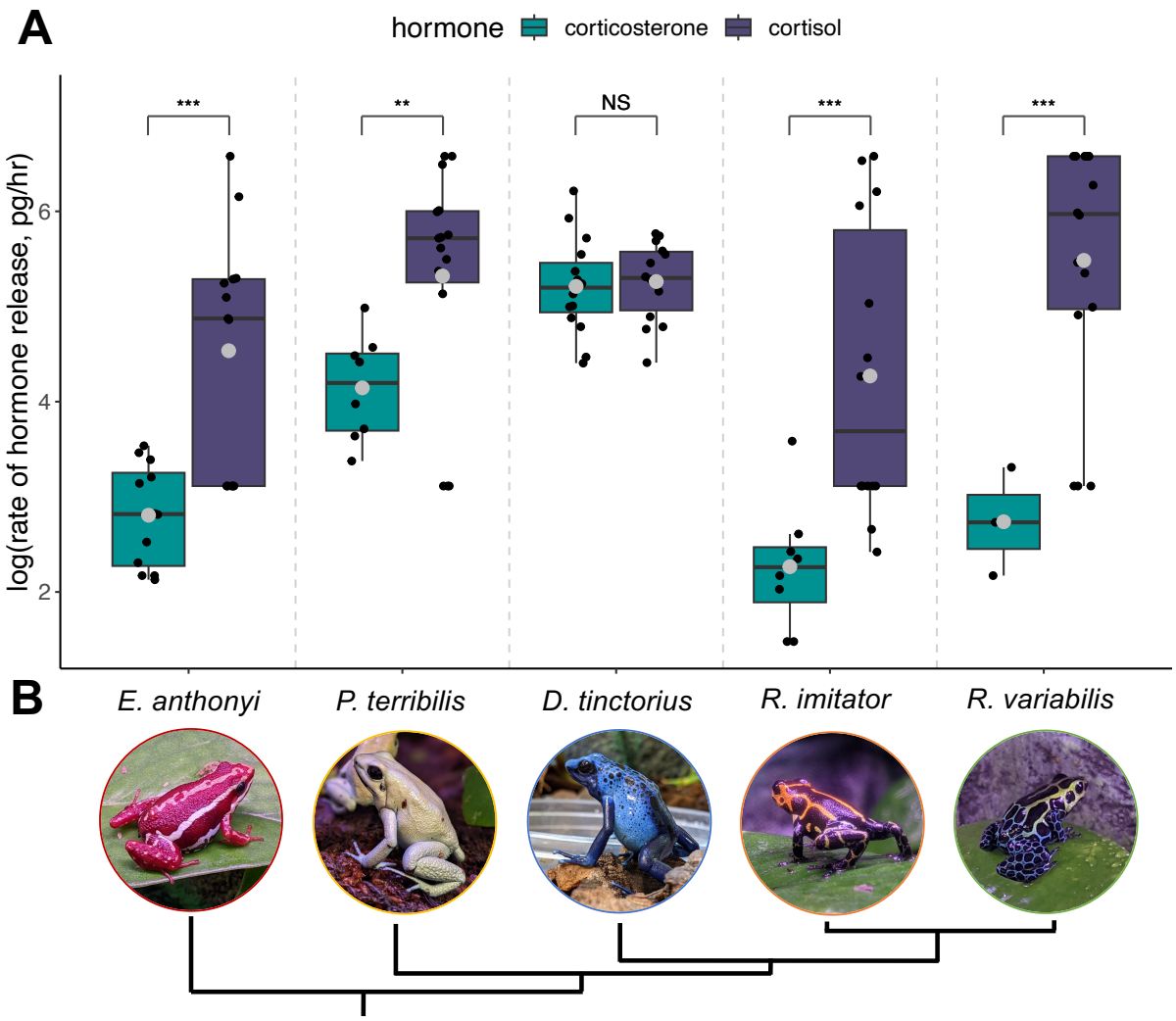
Overall, we found species differed in which glucocorticoid was more prevalently released in the water ($F_{9,49.44}$=14.49, $p < 0.0001$; Fig 2). Specifically, pairwise comparisons within species revealed *D. tinctorius* was unique in releasing almost equal amounts of corticosterone and cortisol, while the other four species released more cortisol (Table 1; Fig 2). This pattern remained the same if we exclude all samples that did not fall within the range of the assays (Table S2; Fig S1).

371 ***Table 1.*** Results from simultaneous multiple pairwise comparisons of means for hormone release
372 rate across species. P-values < 0.05 are in bold.

	log(release rate, pg/hr): cortisol - corticosterone			
species	*estimates*	*std. error*	*z-value*	*p-value*
D. tinctorius	0.036	0.33	0.11	1.00
E. anthonyi	1.68	0.36	4.73	**< 0.0001**
P. terribilis	1.27	0.39	3.22	**0.006**
R. imitator	2.07	0.40	5.13	**< 0.0001**
R. variabilis	2.89	0.60	4.78	**< 0.0001**

373

374 ***Figure 1.*** Species variation in relative glucocorticoid abundance. (A) For four out of the five
375 species tested, the release rate of corticosterone was lower than the release rate of cortisol,
376 suggesting cortisol is the dominant glucocorticoid in these species. In *D. tinctorius*, we found
377 cortisol and corticosterone were released at the same rate. Boxplots for corticosterone are teal
378 (left) and for cortisol are purple (right). Grey dots indicate the mean for each group. Black dots
379 represent individual samples. (NS = not significant, ** = $p < 0.001$, *** = $p < 0.0001$) (B) The
380 species names correspond to the respective photos beneath the x-axis labels. The tree below the
381 photos indicates the phylogenetic relationships between species (Guillory et al., 2019).



3.2 ACTH challenges in D. tinctorius –

The effect of an injection (ACTH vs vehicle) varied by GC and also injection type (type of GC x injection type x time point: ß= -0.97, p = 0.024; Table 2; Fig 2). We found decreased GC release rates post-injection (ß=-0.59, p = 0.007; Table 2) and this effect was driven by a decrease in corticosterone in saline injected frogs (pairwise comparison of corticosterone between pre- and post-injection saline: ß = 0.59, z = 0.21, p = 0.031; Fig 2). After an injection, frogs had higher corticosterone release rates when injected with ACTH compared to saline alone (pairwise comparison of corticosterone between ACTH and saline post-injection: ß = 1.36, z = 5.86, p <0.001; Fig 2). Cortisol release did not vary pre- and post-injection for either type of injection

(pairwise comparisons of cortisol between pre- and post-injection: saline ß = -0.02, z = -0.09, p = 1.00, ACTH ß = -0.03, z = -0.03, p = 1.00; Fig 2).

Table 2. Results from linear model for hormone release rate across injection type and time points. The reference group is corticosterone, control (saline) injection, and pre-injection time point. P values < 0.05 are listed in bold.

	log(release rate, pg/hr)		
Predictors	*Estimates*	*CI*	*p*
intercept	4.69	4.34 – 5.04	**<0.001**
glucocorticoid type (cortisol)	0.17	-0.26 – 0.60	0.429
injection type (ACTH)	0.40	-0.08 – 0.89	0.098
time point (post)	-0.59	-1.02 – -0.17	**0.007**
cortisol x ACTH	0.06	-0.54 – 0.66	0.842
cortisol x post-injection	0.61	0.00 – 1.22	**0.048**
ACTH x post-injection	0.95	0.37 – 1.53	**0.002**
cortisol x ACTH x post-injection	-0.97	-1.80 – -0.13	**0.024**
Random Effects			
σ^2	0.29		
$\tau_{00\ trial:frogID}$	0.12		
$\tau_{00\ frogID}$	0.02		
ICC	0.32		
N_{trial}	35		
N_{frogID}	18		
Observations	109		
Marginal R^2 / Conditional R^2	0.298 / 0.525		

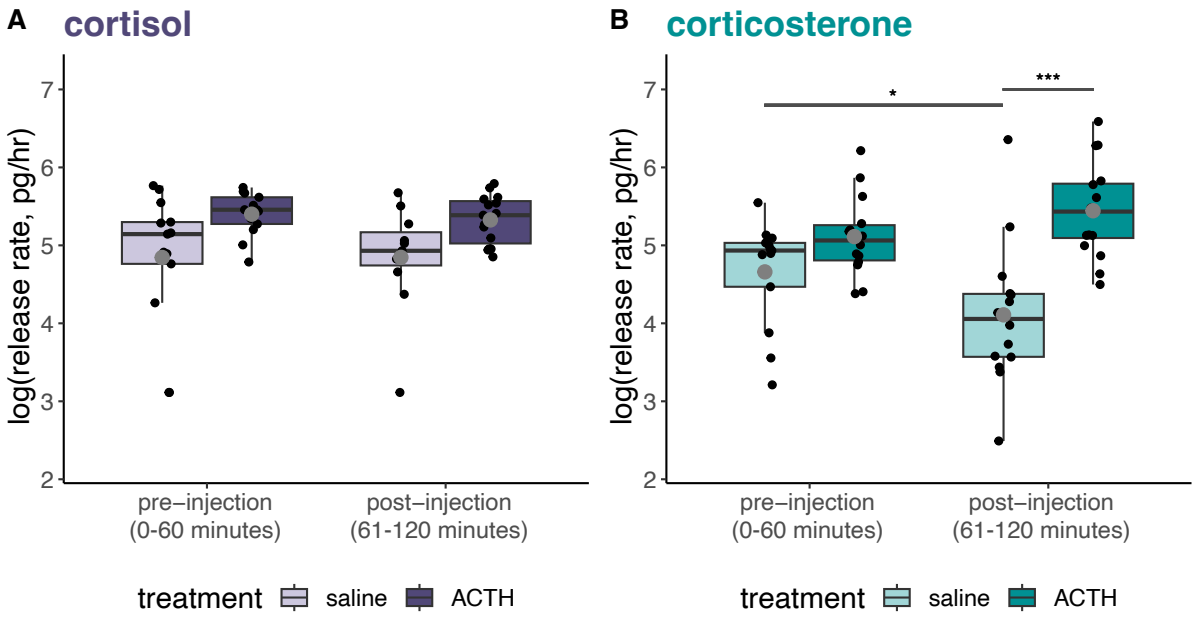


Figure 2. Glucocorticoid response to an ACTH injection compared to a saline control injection. (A) We found no difference in cortisol release rate between samples taken before and after injection of either saline or ACTH. (B) In contrast, corticosterone release rate was higher after an ACTH injection than after a saline injection. Purple boxplots indicate cortisol data. Boxplots for corticosterone data are in teal. Lighter colors (on the left within injection groups) indicate the control/saline injection for the respective glucocorticoid. Grey dots indicate the mean for each treatment group. Black dots are individual water samples. (* = $p < 0.05$, *** = $p < 0.001$)

3.3 Plasma vs. water glucocorticoids –

When we compared water glucocorticoids with plasma glucocorticoids in *D. tinctorius*, the concentration of cortisol in plasma was positively correlated with the release rate of cortisol in water (ß=1.10, CI=0.51 – 1.70, R^2/R^2 adjusted = 0.818/0.782 p = 0.005; Fig 3). We found a slightly positive but statistically non-significant relationship between corticosterone concentration in plasma and corticosterone release rate in water (ß=0.20, CI = -0.31 – 0.72, R^2/R^2 adjusted = 0.108/-0.019, p = 0.387; Fig 3).

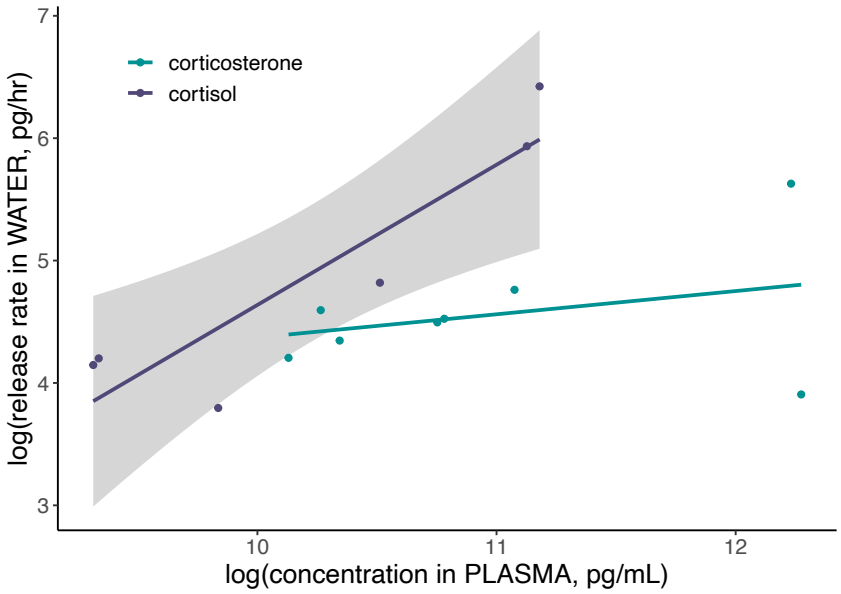


Figure 3. Correlations between glucocorticoids in plasma and water. The concentration of cortisol, but not corticosterone, in plasma is positively correlated with the concentration in water samples from the same individuals. Grey shaded area is the confidence interval around the statistically significant linear regression for cortisol.

4. Discussion

Glucocorticoids are involved in many physiological processes that are essential for survival and fitness (MacDougall-Shackleton et al., 2019; Nicolaides et al., 2015); therefore, understanding glucocorticoids and HPA axis dynamics can inform conservation efforts and improve our understanding of how animals are being impacted by environmental changes and threats (Busch and Hayward, 2009; Millspaugh and Washburn, 2004). Often the GC that responds most to an ACTH challenge (the dominant GC) is also the more abundant GC (e.g. Flament et al., 2012). To better understand the stress physiology of Dendrobatids, we measured water-borne glucocorticoids in multiple poison frogs and performed an ACTH hormone challenge and plasma-water correlations in our focal species, *D. tinctorius*. Our findings challenge the common assumption that corticosterone is both more abundant and the dominant GC in frogs and – more broadly – the assumptions that relative abundance predicts dominance.

We demonstrate that focusing only on the ACTH-responsive GC can overlook potentially important ACTH-independent regulation of other abundant GCs.

4.1 Species variation in glucocorticoid dominance –

Results from all five poison frog species across the *Dendrobatidae* family contradict the common assumption that corticosterone is both the dominant and most abundant GC in amphibians (Moore and Jessop, 2003; Norris and Carr, 2013; Wingfield and Romero, 2011), and in particular in terrestrial amphibians (Jungreis et al., 1970). In water-borne hormone samples from five poison frog species, we found that most species released more cortisol than corticosterone. On average, both *Ranitomeya* species released more than twice as much cortisol as corticosterone (Fig 1). The difference between cortisol and corticosterone was less dramatic for *P. terribilis* and *E. anthonyi* but cortisol was still released at more than 1.25x the rate of corticosterone (Fig 1). In contrast, in *D. tinctorius* we detected approximately equal amounts of both glucocorticoids (Fig 1). In our study, we opportunistically chose these five species of poison frogs, and it would be interesting to know if this pattern extends to all Dendrobatids.

Most studies comparing cortisol and corticosterone within-species are done in mammals with few systematic studies in other taxa (Vera et al., 2017). Despite the generally accepted rule that corticosterone dominant and therefore more abundant in amphibians, the few studies that have looked at both cortisol and corticosterone in amphibians report a range of empirical results with no consistent pattern. In some species, corticosterone is the dominant glucocorticoid, as canonically expected. For example, Rio Grande Leopard tadpoles released more corticosterone than cortisol overall and increased corticosterone release rates in response to an ACTH challenge, but decreased cortisol release rates (Forsburg et al., 2019). Similarly, in American bullfrogs (*Rana catesbeiana*), corticosterone in plasma responded to ACTH whereas cortisol was undetectable (Johnston et al., 1967). In contrast, other studies in amphibians find cortisol is the more abundant glucocorticoid, as we find here for multiple species of poison frogs, and responsive to ACTH. For instance, in fecal samples from two species of Panamanian harlequin frogs (*Atelopus certus* and *Atelopus glyphus*) both cortisol and corticosterone were responsive to ACTH, but cortisol metabolites were detected at much higher concentrations overall (Cikanek et al., 2014). Yet in other taxa, both cortisol and corticosterone are detectable at relatively equal concentrations, similar to our finding for *D. tinctorius*. In the common water frog (*Rana*

esculenta), cortisol and corticosterone are detectable in plasma and both are elevated in ovulatory and postovulatory phases compared to the preovulatory phase (Gobbetti and Zerani, 1993). Further equivocal results about cortisol and corticosterone in amphibians, including contradictory results in the same species (such as *Xenopus laevis* and *Rana pipens*), are nicely summarized in Table 2 in Hopkins et al. (2020). With empirical support for dominance of either GC in amphibians, as well as variation in the relationship between dominance and abundance, we caution against making a general assumption about which GC is responsive to stress, even across closely related species.

These inconsistent findings on GC dominance in amphibians and other taxa beg the question of what could explain this variation. It has been suggested that cortisol and corticosterone may in fact have some different physiological functions and be differentially regulated within a species. Focusing only on the ACTH-responsive or "dominant" GC ignores ACTH-independent regulation of the other GC, which may be just as important (Bornstein et al., 2008; Bornstein and Chrousos, 1999). The two GCs could be playing different roles across age classes and metabolic functions. For instance, in a study on zebra finches (*Taeniopygia guttata*), the more abundant GC varied by tissue type with more corticosterone (the dogmatic primary GC of birds) detected in plasma, but more cortisol detected in immune tissues (Schmidt and Soma, 2008). Additionally, corticosterone and cortisol may differentially bind to species-specific corticosteroid-binding globulins resulting in differing amounts of bound and unbound corticosteroid available in circulation (Breuner and Orchinik, 2002; Martin and Ozon, 1975; Westphal, 1986). Pharmacological studies also suggest differential glucocorticoid receptor-mediated gene regulation based on which type of corticosteroid is available (e.g. Nixon et al., 2016; Pariante et al., 2003) and differential transport of each corticosteroid across the blood-brain barrier (e.g. Karssen et al., 2001).

Notably, there is some evidence in frogs that the dominant glucocorticoid relative glucocorticoid abundance may shift throughout development from tadpole to adult (Dale, 1962; Jungreis et al., 1970; Krug et al., 1983; McClelland and Woodley, 2021). Previous studies have found cortisol to be dominant in aquatic larval tadpoles (Dale, 1962), but corticosterone to be dominant in terrestrial adults of the same species (Jungreis et al., 1970). This developmental shift is not unique to amphibians. For instance, in rabbits (*Oryctolagus cuniculus*), the ratio of cortisol to corticosterone shifts from cortisol-dominant to corticosterone-dominant as they develop from

504 fetus to adult (Mulay et al., 1973). The functional reason for this potential ontogenetic shift in
505 glucocorticoid dominance across development is unclear and warrants further study across
506 species and developmental stages. At a mechanistic level, the enzymes necessary for cortisol
507 production may be regulated by different stimuli than the enzymes for corticosterone production,
508 and these stimuli may be restricted to specific developmental stages. In particular, researchers
509 should explore non-ACTH routes of GC release (reviewed in Bornstein et al., 2008; Bornstein
510 and Chrousos, 1999; Lesouhaitier et al., 1995). Release of glucocorticoids by extrapituitary
511 mechanisms, including neurotransmitters, neuropeptides, and cytokines, is far less studied and
512 less understood than activation of the adrenals by ACTH (Bornstein et al., 2008; Bornstein and
513 Chrousos, 1999; Lesouhaitier et al., 1995). Amphibians are especially promising for studies
514 across developmental stages given their biphasic life histories that lead to dramatically different
515 physiological challenges at different life stages.
516
517 *4.2 Glucocorticoid response to adrenal activation in D. tinctorius –*
518 Our second goal in this study was to identify which glucocorticoid was responsive to
519 activation of the adrenal glands via injection of ACTH, i.e. the dominant GC by the standard
520 definition. In *D. tinctorius*, we found the release rate of corticosterone decreased following a
521 saline injection, indicating frogs were habituating to the sampling process and no longer
522 mounting a stress response, however an ACTH injection prevented this decrease in
523 corticosterone, suggesting a stress response relative to controls (Fig 2). Interestingly, cortisol did
524 not respond to either an injection of ACTH or saline (Fig 2) suggesting cortisol is not responsive
525 to acute adrenal activation and does not fluctuate across short time periods (i.e., does not show
526 an increased stress response or habituation). In combination, these results suggest corticosterone
527 is more responsive to acute adrenal activation than cortisol, despite approximately equal amounts
528 of cortisol and corticosterone prior to injections. Typically, the GC that responds most to adrenal
529 activation through an ACTH challenge is also the more abundant GC, but this was not the case in
530 *D. tinctorius*. Golden hamsters are another example where the more abundant GC is not the GC
531 that responds to acute adrenal activation. A previous study found corticosterone was higher than
532 cortisol at baseline in golden hamsters, yet cortisol but not corticosterone was elevated in
533 response to chronic stress (Ottenweller et al., 1985). Our finding that the GC that responded to an

534 ACTH challenge is not the most abundant GC in *D. tinctorius* further muddies our understanding
535 of which GC plays a primary role in stress physiology in poison frogs.
536 Our current study shows corticosterone responds to ACTH, however a previous study in *D.*
537 *tinctorius* found an increase in cortisol in plasma during an energetically costly behavior, tadpole
538 transport (Fischer and O'Connell, 2020). Taken together these results suggest corticosterone and
539 cortisol may serve distinct functions and can be released via distinct mechanisms depending on
540 context. As discussed above, there are many non-ACTH routes of GC production, including
541 neurotransmitters and neuropeptides involved in parental care (Bornstein and Chrousos, 1999).
542 The parental state involves a myriad of physiological changes (Numan, 2020; Rosenblatt and
543 Snowdon, 1996) and perhaps the unique demands of parenting rely on the production of
544 glucocorticoids through non-ACTH routes. Though speculative, we suggest that distinct ACTH
545 and ACTH-independent mechanisms could provide modular regulation of parental care versus
546 other stressors (McGlothlin and Ketterson, 2008).
547 In addition, the functionality of cortisol and corticosterone within a species may be more
548 distinct than we typically assume (Schmidt and Soma, 2008; Vera et al., 2017), leading to
549 preferential expression of one GC over the other in some contexts and synchronized release in
550 other contexts. In some species, cortisol is more responsive to acute stress than corticosterone
551 (Gong et al., 2015; Koren et al., 2012; Venkataseshu and Estergreen, 1970; Vera et al., 2012,
552 2011), however the two GCs may also simultaneously elevate in more chronic states with high
553 energetic burdens. For instance, both cortisol and corticosterone increase during pregnancy in
554 humans (Wintour et al., 1978), little brown bats (*Myotis lucifugus,* Reeder et al., 2004), North
555 Atlantic right whales (Hunt et al., 2017), and Asian elephants (*Elephas maximus*, Kajaysri and
556 Nokkaew, 2014). In essence, the ratio of the two GCs in circulation may be dependent on
557 physiological state (Gong et al., 2015). In the context of increased cortisol during parenting in *D.*
558 *tinctorius* (Fischer and O'Connell, 2020), an increase in corticosterone, but not cortisol,
559 following an ACTH injection suggests non-ACTH routes of GC production may be important in
560 upregulating cortisol during behavioral states with higher energetic demand. Such modular
561 regulation could occur through changes in the regulation of the enzyme required to produce
562 cortisol (17α-hydroxylase). Further studies are needed to explore these intriguing possibilities.
563 We note our current study and Fischer and O'Connell (2020) measured hormones in different
564 sample types (plasma vs. water); however, we did find a positive correlation between plasma

concentration and water release rate for cortisol. Additionally, water-borne hormones are an integrative measure of hormone release rate over time, rather than measuring one moment in time as in plasma. The collection of water-borne hormones and plasma is inherently separated in time and therefore, perhaps our null expectation should not be a tight relationship between plasma and water-borne hormones. Moreover, the relationship between plasma and water-borne hormones could be life stage-dependent in amphibians, as previously demonstrated in Northern Leopard frogs (*Lithobates pipiens*; McClelland and Woodley, 2021). Finally, even when plasma and water hormone levels are correlated, GCs may differ in their release rates such that relative abundances differ between plasma and water. For example, in túngara frogs (*Physalaemus pustulosus*), cortisol was present in higher concentrations than corticosterone in water-borne samples, but corticosterone was higher in plasma samples (Baugh et al., 2018). Thus, while most studies of water-borne hormones in amphibians find a positive relationship between plasma and water measurements as we did here, the complexities of this relationship require further study.

4.3 Conclusions

Altogether, these findings highlight the importance of measuring both cortisol and corticosterone when investigating the complex, multivariate roles of GCs in physiology, development, and behavior. Even in well-studies species, measuring both GCs could bring to light new information about the differential regulation and functionality of cortisol and corticosterone, which may not be as interchangeable as we assume (Hancock, 2010). Our results suggest poison frogs go against the textbook dogma of corticosterone being dominant and therefore more abundant in terrestrial amphibians. We found cortisol released at higher rates than corticosterone in multiple species. *D. tinctorius* were distinctive in releasing nearly equal amounts of corticosterone and cortisol yet follow the general assumption of corticosterone dominance by responding to ACTH with an increase in corticosterone. These findings inspire more questions than they answer. For instance, why does *D. tinctorius* show different relative GC abundance than other poison frogs? Are cortisol and corticosterone independently regulated through ACTH and non-ACTH routes? Are poison frogs unique among terrestrial frogs in producing more cortisol than corticosterone? We clearly have a lot left to learn about the stress physiology of this unique clade of frogs and amphibians in general. We can say with confidence that there is interesting, under-appreciated, and unexplored variation in HPA activity and

glucocorticoids, and that simplifying assumptions about the relationship between GC abundance and dominance do not hold.

Ultimately, we need more studies measuring both GCs whenever possible. We encourage more researchers to investigate both GCs in their own focal species, regardless of the dogma, as the "non-dominant" GC may in fact be more abundant. With data across more species, we can look for broader patterns in GC dominance and abundance to investigate whether there are adaptive and/or ecological explanations for the diverse patterns emerging across species, populations, and life stages. We can also test hypotheses about potential unique physiological roles for cortisol versus corticosterone, which may have previously been obscured due to measurement of only a single GC. Additionally, measuring both cortisol and corticosterone may better inform conservation practices through improving our understanding of stress physiology across species and environments.

Declarations of Competing Interest none

CRediT authorship contribution statement

SEW: conceptualization, methodology, validation, formal analysis, investigation, data curation, writing – original draft, writing – review & editing, visualization, project administration, funding acquisition. **RTP:** conceptualization, methodology, writing – review & editing, supervision. **EKF:** conceptualization, methodology, writing – review & editing, supervision, funding acquisition.

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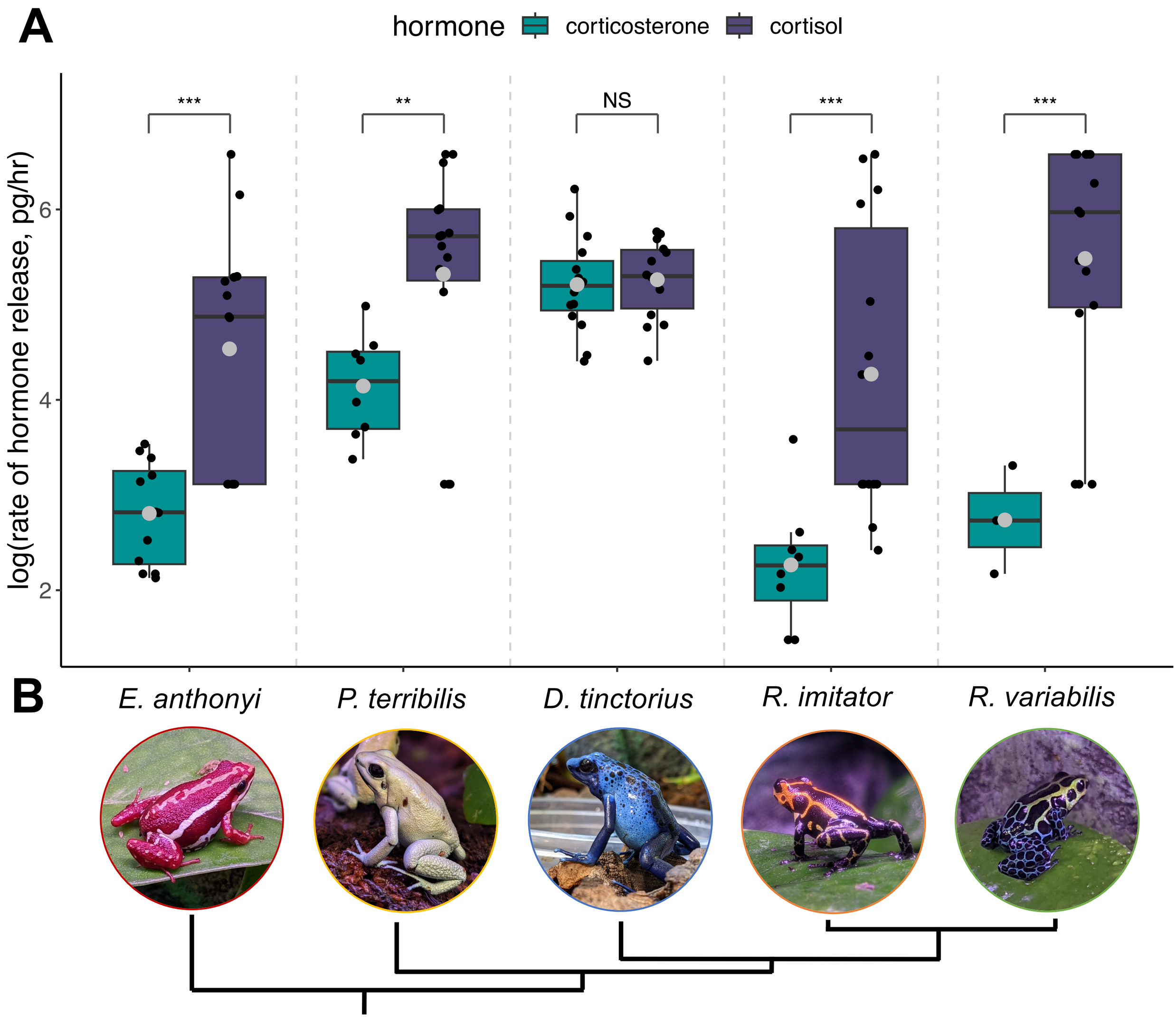
A
hormone
corticosterone
cortisol
log(rate of hormone release, pg/hr)
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B
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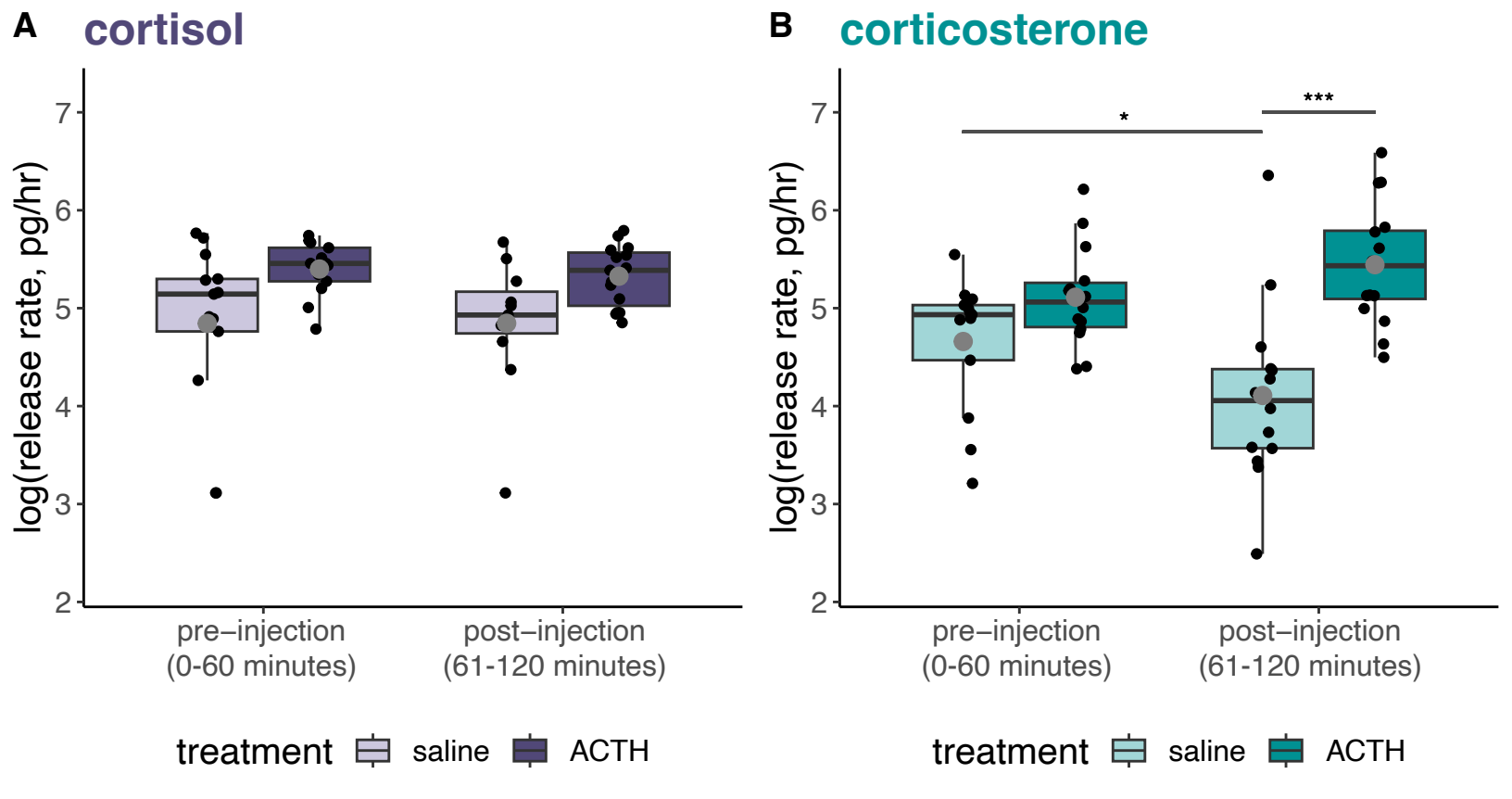
P. terribilis
D. tinctorius
R. imitator
R. variabilis


A
cortisol
log(release rate, pg/hr)
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pre–injection
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treatment
saline
ACTH
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treatment
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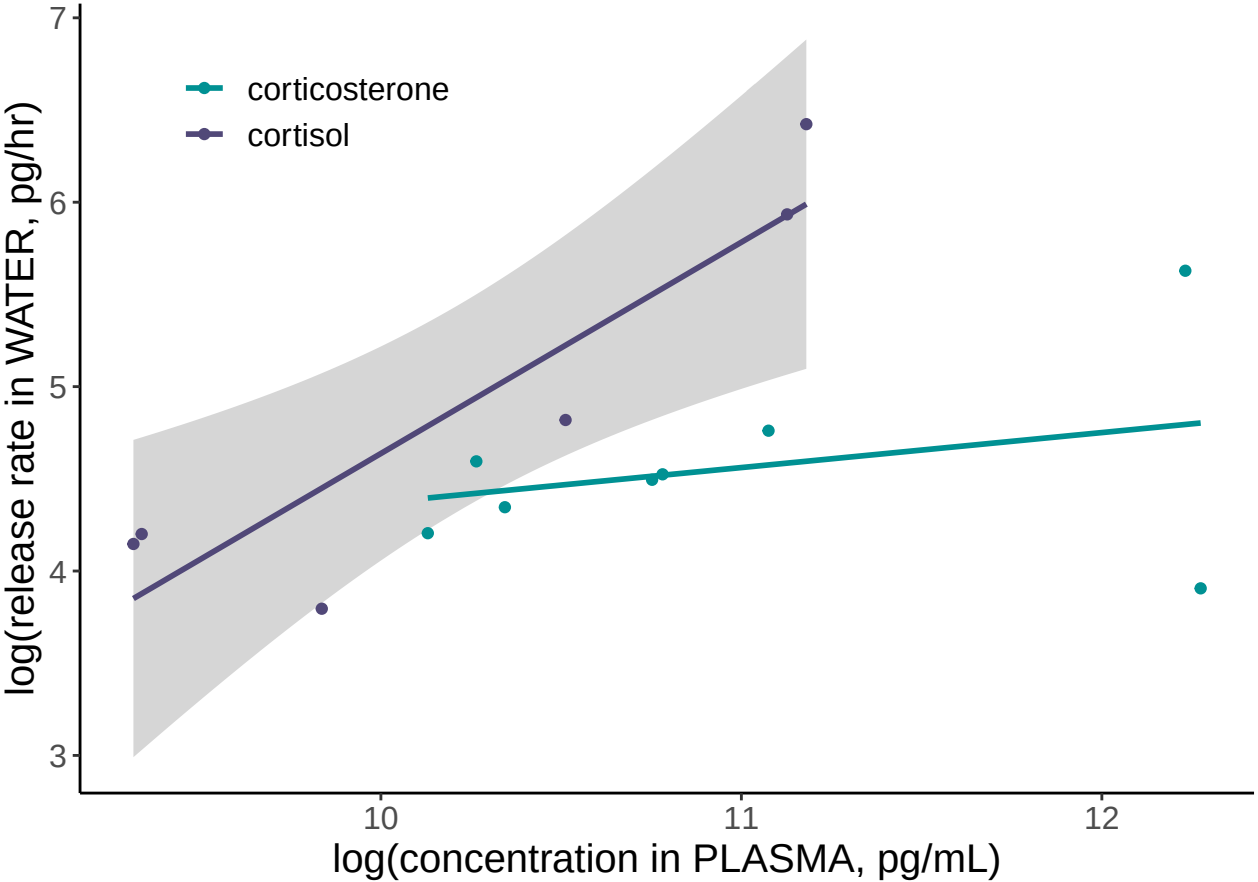
corticosterone
cortisol
log(release rate in WATER, pg/hr)
7
6
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log(concentration in PLASMA, pg/mL)