Research article

Dermal glucocorticoids are uncoupled from stress physiology and infection

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Ongoing amphibian population declines are caused by factors such as climate change, habitat destruction, pollution and infectious diseases not limited to chytridiomycosis. Unfortunately, action is taken against these factors once population collapses are underway. To avoid these *post hoc* responses, wildlife endocrinology aims to analyse physiological mediators that predict future population declines to inform wildlife management. Mediators typically investigated are stress hormones known as glucocorticoids, which are produced by the Hypothalamus—Pituitary—Interrenal axis (HPI axis). The HPI axis is the part of the endocrine system that helps amphibians cope with stress. Chronic increases in glucocorticoids due to stress can lead to immune dysfunction, which makes amphibians more susceptible to infectious diseases. Despite this predictive potential of glucocorticoids, interpretation of glucocorticoid data is confounded by sampling design and type. Glucocorticoid monitoring classically involves blood sampling, which is not widely applicable in amphibians as some are too small or delicate to sample, and repeated samples are often valued. To address this, we tried to validate skin swabbing via corticosterone (CORT) and adrenocorticotropin hormone (ACTH) injections in adults of two amphibian species: Eastern red-spotted newts, *Notophthalmus viridescens viridescens,* with natural skin infections with *Batrachochytrium dendrobatidis* (*Bd*) upon collection in the field, and Northern leopard frogs, *Rana (Lithobates) pipiens,* raised in captivity and naïve to *Bd* exposure. Further, we determined the predictive potential of skin glucocorticoids on *Bd* load in the field via correlations in Eastern red-spotted newts. We found that hormones present in the skin are not related to the HPI axis and poorly predict infection load; however, skin hormone levels strongly predicted survival in captivity. Although skin swabbing is not a valid method to monitor HPI axis function in these species, the hormones present in the skin still play important roles in organismal physiology under stressful conditions relevant to wildlife managers.

Lay Summary

Conservation biologists are interested in finding new methods to monitor amphibian populations. Therefore, we evaluated a 'stress' hormone sampling technique, which involved swabbing amphibian skin. We found that although the technique does not adequately monitor stress hormones, these hormones still predicted survival in captivity.
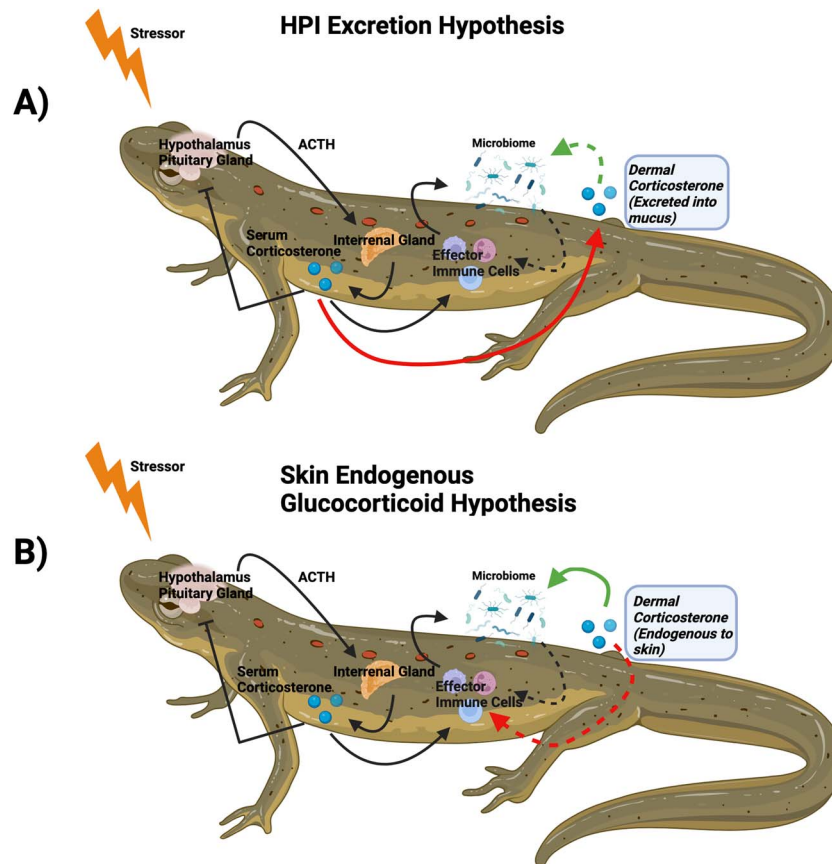
Graphical Abstract

Introduction

Along with declining global biodiversity trends, amphibian extinction rates continue to climb in the 21st century, resulting in an ongoing biodiversity crisis (Cowie et al., 2022; Luedtke et al., 2023; McKee et al., 2004). Although all groups of vertebrates are under pressure, amphibians are particularly susceptible to the climactic and anthropogenic disturbances that are driving the biodiversity crisis, such as habitat degradation, pollution, atmospheric warming and infectious diseases (Luedtke et al., 2023). As infectious agents continue to spread amongst the herpetofauna (Bosch et al., 2006; Fisher & Garner, 2020), conservation physiologists need to take both responsive and proactive actions in order to protect amphibian biodiversity (Assis et al., 2023; McEwen & Wingfield, 2003; Narayan et al., 2019).

Proactive actions rely on predicting population collapses before they occur and understanding causal connections between biological indicators and future declines, with multi-sector readiness (Wikelski & Cooke, 2006). One class of biological indicators that have known physiological connections to organismal health are compounds known as glucocorti-

coids (Romero, 2004). Glucocorticoids are steroid hormones produced by the Hypothalamus–Pituitary–Adrenal/Interrenal axis (HPA/I) (Botía et al., 2023; Ledón-Rettig et al., 2009; Romero, 2004; Wingfield & Romero, 2011). The HPA/I axis is classically associated with the vertebrate 'stress response' to social, trophic, pathogenic or environmental stressors (Wingfield & Romero, 2011). Amphibians are no exception, since all vertebrate taxa release corticosterone (CORT) or cortisol into the serum upon exposure to noxious stimuli or when injected with adrenocorticotropin hormone (ACTH): the hormone that causes the production of glucocorticoids by the interrenal glands (Leboulenger et al., 1986; Selye & Collip, 1936). Glucocorticoids have many downstream effects, such as altering immune system function, increasing blood glucose via shifts in metabolism and changes in behaviour or reproductive activity (Cannon, 1915; Selye & Collip, 1936). As a consequence, there are cases where glucocorticoids were used to predict population declines in certain taxa, such as the impact of an oil spill on marine iguanas, *Amblyrhynchus cristatus* (Wikelski et al., 2001). Despite this predictive potential, the effects these hormones have depended on the amounts produced, their frequency, the species or population being investigated and the timing of hormone release

(Bryant *et al.*, 2022; Chambers, 2011; Denver, 2021; Jackson *et al.*, 2021; Kirschman *et al.*, 2018; van Kesteren *et al.*, 2019).

In addition to physiological cofactors that can influence the effect glucocorticoids have on an organism; hormone sampling methods are incredibly diverse and can further impact the measurements. The classic method for measuring and monitoring glucocorticoid levels involves obtaining serum samples from multiple individuals at varying time points following capture restraint (Romero & Wingfield, 2015). Although used across a wide range of taxa, this method is only applicable to organisms that are large enough to obtain serum samples without euthanasia and organisms that can be handled safely (Narayan, 2013; Narayan *et al.*, 2019). To circumvent this, researchers have been able to obtain glucocorticoid samples from faeces (Hadinger *et al.*, 2015), hair (Sheriff *et al.*, 2011), nails (Fokidis *et al.*, 2023), feathers (Freeman & Newman, 2018; Gormally *et al.*, 2020) whale spout and baleen (Thompson *et al.*, 2014; Trumble *et al.*, 2018), aquarium water (McClelland & Woodley, 2021; Tornabene *et al.*, 2021a, 2021b), urine (Narayan & Hero, 2011; Sadoul & Geffroy, 2019) and saliva (Garde & Hansen, 2005; Hammond *et al.*, 2018). This broad range of sample types falls under the category of non-invasive methods (Narayan *et al.*, 2019; Romero & Wingfield, 2015). Non-invasive methods are important for monitoring endangered or at-risk species of amphibians, as many species that are susceptible to infectious diseases are too small to draw serum samples from (Narayan, 2013; Narayan *et al.*, 2019). Despite the potential convenience for researchers and amphibians alike, the physiological interpretation of non-invasively sampled glucocorticoids is a challenge, as the biologically active compound is present only in the serum (Romero, 2004; Smith & Vale, 2006). As more non-invasive methods arise, rigorous physiological validation of each method is required to better understand the causal connection between non-invasively sampled glucocorticoids and future population losses.

Prior investigation has showcased the potential to obtain glucocorticoid samples via dorsoventral swabbing of amphibian skin and mucus; however, interpretation of the data is limited by the use of too few biological replicates (Santymire *et al.*, 2018). Later work attempted to further validate this technique, though with mixed results (Santymire *et al.*, 2021; Scheun *et al.*, 2019), perhaps indicating species-specific physiology. Dermal swabbing aims to monitor HPI function; however, clinical research in humans has demonstrated that epidermal cells synthesize their own Pro-opiomelanocortin (POMC), ACTH and Cortisol (Hannen *et al.*, 2017; Jozic *et al.*, 2014; Phan *et al.*, 2021; Vukelic *et al.*, 2011). If this is occurring in amphibians, then dermal swabbing would not be appropriate for monitoring HPI function, or at least have substantial amounts of signal masked by the skin's endocrine activity.

Glucocorticoids are known to affect disease processes in complex ways in various taxa, but the relationship between HPI function and chytridiomycosis outcome is under ongoing investigation; though research is limited by hormone sampling techniques available for each taxon (Fonner *et al.*, 2017; Gabor *et al.*, 2015, 2018; Pereira *et al.*, 2023; Peterson *et al.*, 2013; Searle *et al.*, 2014). Many amphibians are susceptible to chytridiomycosis, which is caused by *Batrachochytrium dendrobatidis* (*Bd*) and *Batrachochytrium salamandrivorans* (*Bsal*; (Fisher & Garner, 2020). A variety of factors can influence species or population specific outcomes, such as immune repertoire (Assis *et al.*, 2023; Grogan *et al.*, 2018; Murone *et al.*, 2016; Pereira *et al.*, 2023), skin microbiome composition (Colombo *et al.*, 2015; Knutie *et al.*, 2018, 2018; Woodhams *et al.*, 2014), pollutant exposure (Bosch *et al.*, 2021; Buck *et al.*, 2015; Jiménez *et al.*, 2021), climate change (Bosch *et al.*, 2006; Bradley *et al.*, 2019) and timing of infection (Bosch *et al.*, 2021; Kirschman *et al.*, 2018).

Since monitoring *Bd* and *Bsal* involves obtaining dermal swabs to obtain DNA to quantify through quantitative PCR (qPCR) (Boyle *et al.*, 2004), monitoring skin glucocorticoids concomitantly would be simple to implement in the field. Before dermal swabbing can be implemented in the field, we need to determine if skin glucocorticoids picked up by swabs are related to HPI function. Even if dermal glucocorticoids are unrelated to HPI function, if they still can predict survival or infection load, then the swabbing technique could still have a role in predicting outcomes in amphibians threatened by disease. Therefore, the questions addressed by this study are threefold:

1) Do skin swabs adequately uptake glucocorticoids?
2) Are dermal glucocorticoids related to HPI axis activity? Do they respond to acute stress?
3) Can dermal glucocorticoids predict infection load or survival?

To test whether skin glucocorticoids are related to HPI function, Eastern red-spotted newts, *Notophthalmus viridescens viridescens* (hereafter, Eastern newts), and Northern leopard frogs, *Rana* (*Lithobates*) *pipiens* (hereafter, Northern leopard frogs), were selected as models due to their small size, high abundance, and sensitivity to disease and anthropogenic disturbances (Bouchard *et al.*, 2009). Adults of both species were sampled. The Eastern newts had natural skin infections with *Bd* upon collection in the field, and the frogs were raised in outdoor mesocosms from frog eggs, thus naïve to *Bd* exposure. The swabs used on both amphibians were evaluated for potential matrix effects by spiking solutions with known amounts of CORT and swabbing them. CORT was the primary hormone studied due to the lack of detection of cortisol in the skin of both amphibian species even during the injection challenge. Groups of Eastern newts and Northern leopard frogs were injected with saline solution, ACTH and CORT, and then swabbed repeatedly over a 24-h period to determine if CORT in the skin is coupled to HPI function. Finally, *Bd* load and survival were monitored in Eastern newts and statistically tested for correlation with dermal CORT.

Materials and Methods

Swab matrix validation, hormone extraction and enzyme-linked immunosorbent assays

To determine if rayon swabs can effectively adsorb steroid hormones, we conducted a study using three different types of swabbing medium samples. This included mucus collected from 12 captive Eastern newts, water with the same parameters as the newts' housing and enzyme-linked immunosorbent assay (ELISA) buffer (Cayman Chemical Company). ELISA buffer served as a positive control, as the spiked CORT is known to be chemically stable whilst dissolved in the ELISA buffer. Housing water and mucus were chosen as experimental groups because both medium types would end up on the swabs when swabbing any amphibian. Newt mucus was collected in a 50-ml conical tube using a cell scraper to gently rub the mucus off the newt skin. To do this, we restrained newts by their tails with their heads facing towards the inside of the tube. After holding the conical at an angle to allow the newts to rest their stomachs at the top, we gently scraped their dorsal and ventral surfaces. Once mucus coated the scraper, the newt was removed and placed back into its respective holding tub and the mucus was smeared onto the bottom of the conical. Since we house aquatic Eastern newts, some housing water got into the sample, although this reflects what would be obtained through dermal swabbing if an adult newt were immediately swabbed after capture from an aquatic habitat in the field. ELISA buffer (1X) was made from a 10X stock diluted into MiliQ water according to instructions from the CORT Cayman Chemical ELISA kit. Water used to house Eastern newts was obtained from a reservoir tank in the animal care facility containing reverse osmosis water reconstituted with dilute salts and sodium hydroxide to a pH range of 7.4–7.6 and conductivity of 300 μs/cm. In total, 800 μl of each medium type was collected (Fig. 1).

We also included three swabs that were not used to swab anything ('neat') as a control to measure the background produced by the swab itself. Each swab type (excluding 'neat' swabs) was further split into two 400-μl samples: one spiked with 600 pg/ml of CORT, and one without any spike. We placed 400 μl of each sample type into sterile Petri dishes and used three sterile rayon CLASSICQSwabs to rub the surface of the dish. The medium was streaked across the dish surface three times before flipping the swabs over and swabbing it three more times. Next, we placed the swabs into 1.5-ml microcentrifuge tubes containing 500 μl of 70% ethanol and stored them at 4°C overnight for hormone extraction (Fig. 1).

The next day, we removed the swabs from the ethanol solution and split the 'neat' swab samples into two 250-μl samples: one spiked with 2500 pg/ml of CORT into the ethanol and one without a spike to determine extraction efficiency. We then placed all tubes into a SpeedVac and allowed the ethanol to evaporate under vacuum for 3 h at 60°C. We reconstituted the samples in 200 μl of 1X ELISA buffer (Cayman Chemical Company), vortexed them
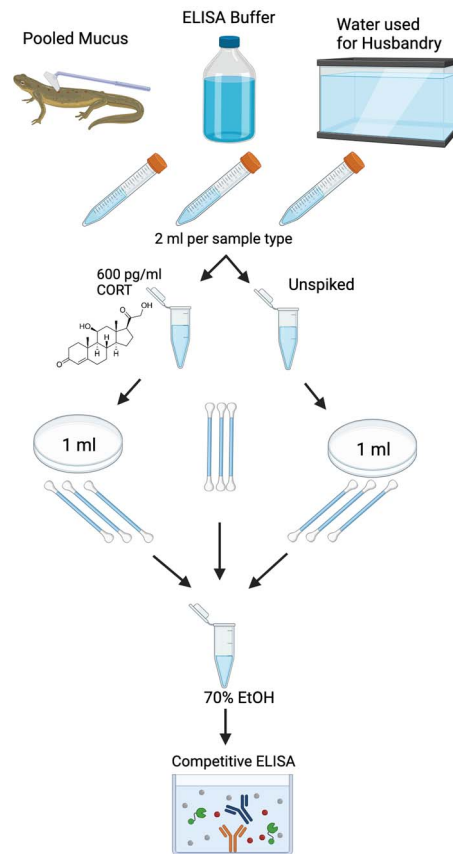
Swab Validation Experiment

Figure 1: Visualization of the swab matrix validation protocol utilized in the current study. This figure was made using Biorender.

for 1 min, sonicated them for 20 min and left them to shake at 150 rpm on an orbital shaker (Carolina Orbital Shaker, Item # 216230) for 30 min to reconstitute the hormone. Finally, we stored the samples at 4°C for future use.

Once all samples were reconstituted, we ran each sample on a CORT ELISA from Cayman Chemical Company. We loaded 50 μl of each sample into each well and ran them in triplicate. We read the plates on a POLARstar Omega plate reader at 412 nm according to the manufacturer's instructions. We recorded the resulting absorbance values on a spreadsheet. All samples were run 'neat'.

Field swabs, sampling and husbandry

To measure the natural variation in CORT obtained through dermal swabbing, we collected 35 adult Eastern red-spotted newts, *N. viridescens viridescens*, from the George D. Aiken Wilderness in Woodford, Vermont, in May 2023. The site consisted of a high-altitude, low-pH sphagnum bog that is situated next to a trail with little tree cover. Adult Eastern

newts were captured by dip-netting and each handled with a new pair of latex gloves and promptly swabbed for baseline CORT. We recorded their snout-vent length (SVL) (mean = 40.5 mm, standard deviation = 0.3 mm), weights (mean = 1.98 g, standard deviation = 0.29 g) and sex (most were in breeding condition), then placed them into sterile Whirlpack bags containing 30 ml of sterile artificial pond water for the collection of dermal mucus, which was used for a separate project. After 30 min, we re-swabbed the newts for CORT and swabbed for both *Bd* and the microbiome. We then placed them into Whirlpack bags containing water from the surrounding bog and transported them back to the lab in a cooler. After 6 h, we swabbed the newts for CORT and drip-acclimated them onto a flow-through aquarium (Aquaneering) system, housing each newt individually. The *Bd*/microbiome swabs were obtained using sterile Dryswabs from medicalwire (MW 113) and CORT swabs were obtained using CLASSIQSwabs from Copan Diagnostics.

On the Aquaneering system, we housed the Eastern newts at 18°C and changed the water twice a week. The newts were kept in 0.8-l holding tanks with 3 × 3 cm styrofoam floats and PVC tube hides. After each water change, we fed them bloodworms thawed in system water and allowed them to acclimate for 3 weeks before conducting experiments. Dermal swabs were obtained each week to gauge changes in dermal CORT over time whilst individual survival was monitored for 2.5 months after capture. After initial mortality plateaued during acclimation, only non-diseased newts were used in experiments.

Northern leopard frog, *R.* (*Lithobates*) *pipiens*, eggs were collected from Shelburne Bay, Shelburne, Vermont, in April of 2023 for a separate project that was occurring concomitantly. We reared the eggs in a 15°C Conviron environmental chamber (Controlled Environments, Pembina, North Dakota) with a 12-h light cycle to replicate natural developmental conditions. Whilst the eggs were developing, we prepared outdoor mesocosms by filling 1136-l tanks with 950 l of local water and 22.5 kg of natural play sand (Pavestone, Atlanta Georgia), and covering them with 40% shade cloth. After 2 weeks, we introduced 500 ml of concentrated zooplankton from a local pond to each tank. Once tadpoles reached the free-swimming stage, each mesocosm was stocked with 20 tadpoles, for a density of 0.021 individuals per litre. Mesocosms were supplemented with 6.5 g of Aquatic Gel Diet Herbivorous Fish Formula (Mazuri Exotic Animal Nutrition, St. Louis, Missouri) every other week. As tadpoles metamorphosed throughout the spring and summer, the resulting froglets were transferred to a separate 1136-l mesocosm. This tank was held at an angle to create aquatic and terrestrial habitats with a planted margin to allow the frogs to hide. We fed frogs calcium-dusted crickets three times per week, *ad libitum*. Once all frogs reached an SVL of 1 cm, they were used for experimentation, with 27 Northern leopard frogs that were not used in any experimental manipulations being selected for the current study. All frogs were naïve to *Bd* exposure, which was confirmed with qPCR of both the zooplankton concentrate and swabs from individual frogs (see DNA extraction and qPCR section below).

The physiological validation experiments occurred in June 2023 for newts (Fig. 2), and in October 2023 for frogs upon completion of metamorphosis and growth to minimum size.

Newt wash

To determine if the mucus washes performed in the field had an impact on dermal CORT readings, 12 Eastern newts that had been living on the Aquaneering system for more than a year were selected for testing. One group was placed in Whirlpack bags filled with sterile artificial pond water (Wyngaard & Chinnappa, 1982), whilst the other group was returned to the holding tank they were taken from after their baseline CORT swab was obtained. After 30 min, the newts were swabbed again and put back in their holding tanks. Once the experiment was finished, the newts were given water changes and fed. All swabs were extracted as mentioned in the previous section, spiked to examine extraction efficiency, and immediately analysed using an ELISA.

Physiological validation

Before experimentation, all newts and frogs received a 100% water change to eliminate any waterborne or faecal glucocorticoids that may have been present. The experiments began at 1000 h and finished at 1000 h the following day. The newts and frogs were not fed on the day of the experiments. Each amphibian was randomly assigned to one of three treatment groups. Roughly equal numbers of male and female Eastern newts were assigned to each treatment group; however, the sex of the Northern leopard frogs was unknown as they were too small to be reliably sexed. In the newt experiment, samples size for the control group ($n = 7$), and experimental groups CORT ($n = 6$) and ACTH injected ($n = 6$; Fig. 2) were robust for detecting significant differences in skin CORT expected based on other studies (e.g. Scheun *et al.* 2019). During the frog experiment, the ACTH group had seven frogs, whilst the other two groups had six frogs. Newts were randomly assigned to Aquaneering containers, whilst frogs were randomly assigned to different mesocosms arranged in a Latin-squares matrix, with nine bins in total. Although these sample sizes were small, we expected to see differences in CORT on the scale of 2–10 orders of magnitude between the control group and both experimental groups (Hammond *et al.*, 2018; Scheun *et al.*, 2019; Wingfield & Romero, 2011).

The first group was injected with 100 µl of 1 IU/kg synthetic ACTH (Prospec Bio HOR-279) in 0.9% saline vehicle; the second group received an injection of 100 µl of 0.446 ug/g CORT (501 320, Cayman Chemical Company) in a 0.9% saline vehicle, whilst the third group received only 0.9% saline of equal volume (Fig. 2). These concentrations were chosen according to previous work performed on both Eastern newts and Northern leopard frogs (Hammond *et al.*, 2018; Santymire *et al.*, 2018; Scheun *et al.*, 2019). Before the
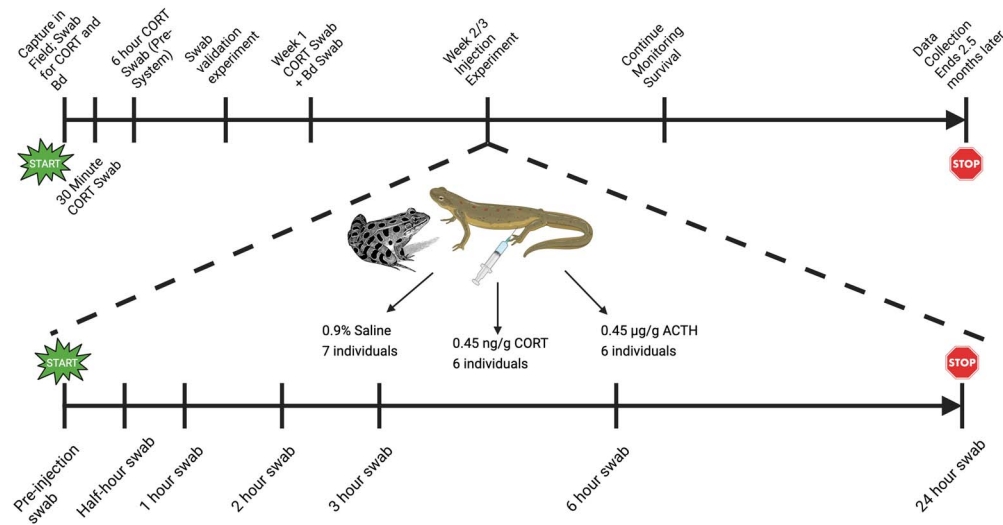
Experiment Timing

Figure 2: The experimental timeline of experiments with Eastern newts, *N. viridescens viridescens*, or with Northern leopard frogs, *R. (Lithobates) pipiens,* carried out over the summer of 2023. This figure was made using Biorender.

injections, each amphibian was swabbed with both a fine-tipped rayon swab for microbiome and Bd sample collection along with a rayon swab for a baseline measure of glucocorticoids. The corresponding glucocorticoid swabs were collected at Hours 0.5, 1, 2, 3, 4, 6 and 24 after the injection (Fig. 2). The glucocorticoid swabs were placed into 500 µl of 70% ethanol inside a microcentrifuge tube and were stored overnight at 4°C for reconstitution the next day. The microbiome swabs were stored in sterile microcentrifuge tubes and stored at −20°C until DNA extractions could be performed.

Once the swabs were reconstituted following the methods described above, they were analysed for both CORT and cortisol using the Cayman Chemical CORT ELISA kit (501320) and Cayman Chemical cortisol ELISA kit (500360). Fifty microlitres of the sample was loaded into triplicate wells. The resulting DNA from the microbiome swabs was used for Bd qPCRs to monitor disease load throughout the experiment.

DNA extraction and qPCR

DNA was extracted from *Bd*/microbiome swabs using the gMax Mini extraction Kit (IBI Scientific). Swabs were suspended in an extraction buffer containing lysozyme followed by an incubation at 37°C for an hour. Afterward, the extraction supernatants were loaded onto spin columns, washed twice and then eluted to obtain purified DNA extracts according to the manufacturer's instructions. All extracts were stored at −20°C for long-term storage.

To determine infection load, qPCR was performed following Boyle *et al.* (2004): 5 µl of sample was used for 25 µl

total reactions using the ITS forward and reverse primers in Taqman Master Mix. To detect DNA amplification, FAM was used as the fluorescent reporter. The plates were run on a C1000 Touch Thermal Cycler (BIO-RAD).

Data analysis and curation

All absorbance data obtained via hormone ELISAs were analysed on a spreadsheet that Cayman Chemical provides for generating standard curves to determine the concentrations of analytes. Once these concentrations were calculated, all the resulting data was placed into CSV files for analysis in R. The Cq values obtained directly from the thermocycler were converted to log ITS starting copy numbers by generating a standard curve in Microsoft Excel. Additionally, all qPCR data, field metrics and compiled hormone titres were placed into a single Excel file with multiple spreadsheets (available in Supplementary Materials: Compiled_Newt_Experimental_Data.xlxs).

For the injection series, linear mixed models made through the glmmTMB package (Brooks *et al.*, 2017) were constructed using newt ID as a random effect. Variables such as treatment, SVL, weight and sex were implemented as predictors of dermal CORT/cortisol (Supplemental Materials Section 3: Statistical Models, Supplementary Tables S1 and S2). Models with the lowest Akaike information criterion (AIC) values were selected for *post hoc* statistical testing, which consisted of 95% confidence interval (CI) generation using the emmeans package (Lenth *et al.*, 2024) to examine treatment effects. For the matrix validation assay, a suite of linear models generated using the base R linear model function were compared using

the AIC, with the model generating the lowest AIC value without violating assumptions selected for further statistical analysis. Sample types, along with spiked and unspiked groups, were compared using Welch Two-Sample *t*-tests to determine if there are statistical differences associated with each sample type. All model assumptions were checked using the performance package (Lüdecke *et al.*, 2021) and all plots were generated using ggplot2 functions (Wickham *et al.*, 2019). Using the dplyr package, cleaning and rearrangement of datasets was performed (Wickham *et al.*, 2019).

The relationship between dermal CORT, disease load and survival in captivity was assessed using a mix of both linear models and Cox regressions using the ggsurvfit (Sjoberg *et al.*, 2024) and gtsummary packages (Sjoberg *et al.*, 2021) for analysis. Cox regressions were plotted according to both dermal CORT and disease load using the contsurvplot package (Denz & Timmesfeld, 2023) to eliminate any statistical manipulations that would have arisen due to the discretizing of continuous predictors. Cox regression assumptions were checked using functions from the survminer package (Kassambara *et al.*, 2024). All code and data used in this study are available on github (https://github.com/VictQuad/Dermal_CORT_Code_Data).

Results

Rayon swabs as a matrix

In total, 21 swabs and their corresponding extracts were collected. Across media types, spiked CORT was recovered through swabbing (Fig. 3a). Swab spike recovery was 66.7% on average, meaning that 66.7% of the 600 pg/ml spike was detected on the ELISA post-extraction across all medium types (Fig. 3b). Spiked swabs had higher detected CORT on average compared to their unspiked counterparts ($n = 18$, t = 14.426, df = 9.4638, $P = 9.3*10^{-8}$, Upper CI = 357.2473 pg/ml, Lower CI = 261.0176 pg/ml; Welch Two-Sample *t*-test). Extraction efficiency was 97% (Supplemental Materials: Fig. S2).

Primary hormone, capture-restraint and washing

CORT was found in the skin of both Eastern red-spotted newts and Northern leopard frogs, whilst cortisol was not able to be detected using an ELISA (Supplemental Materials: Figs. S5 and S6). Unpublished preliminary results indicate cortisol was present below the detection limits of the ELISA based on LC–MS, and thus not present at physiologically meaningful quantities in these species (Jason Evans, *pers. comm.*).

Average dermal CORT decreased 30 min after capture in Eastern newts but returned to near-field levels after 6 h. Newts that were washed before the 30-min swab did not have any differences in dermal CORT compared to newts that were left unwashed before the 30-min swab ($n = 11$, $P > 0.05$; Supplemental Materials: Figs. S1, S3, S4).

Dermal CORT poorly predicts infection load, yet predicts survival in captivity

All Eastern newts captured from the field ($n = 35$) were infected with *Bd*. *Bd* load in the field was 1×10^6 copies on average, whilst in captivity, the average increased to 1.5×10^6 copies. This 50% increase was not correlated with dermal CORT (Fig. 4a, slope $= -0.255 \pm 0.761$ with 95% confidence, adjusted-$r^2 = 0.06$; Supplemental Materials: Model Outputs 1-6). Despite this, dermal CORT on the first week in captivity strongly predicted survival after 2 months (Fig. 4b $P = 0.033$, Score (logrank) test $= 4.68$, corrected regression coefficient $= 1.002–1.006$, standard error $= 0.002$, $n = 35$; Supplemental Materials: Model Outputs 1-6). *Bd* load was not as strongly predictive of survival as dermal CORT was (Fig. 4c; $P = 0.3$, Score (logrank) test $= 0.03$, corrected regression coefficient $= 1.18–0.8478$, standard error $= 0.1612$, $n = 35$).

Dermal CORT is uncoupled from HPI function

The model with the lowest score ($\Delta AIC = 0.0$, DF = 11; Supplemental Materials: Table S1) was the linear mixed-effects model for Eastern newts and a simple linear model for Northern leopard frogs ($\Delta AIC = 0.0$, DF = 9; Supplemental Materials: Table S2). During the duration of the injection series, two ACTH-injected and one CORT-injected newts perished after the third hour. Both models for newts and frogs resulted in no statistically significant differences between ACTH-, Saline- and CORT-injected amphibians (Fig. 5, $P > 0.05$ for all comparisons).

The error bars in Fig. 5a–b have substantial overlap at most time points, with the only major difference being the larger CORT detected in the saline group compared to the CORT- and ACTH-injected group at 24 h in newts. The 95% CIs estimated using treatment contrasts all overlapped with a contrast estimate of zero (Fig. 5c, d), which indicates no measured effects of injection type on dermal CORT in both Eastern newts and Northern leopard frogs.

Discussion

Swabs are a suitable matrix

Rayon swabs were able to recover 66.7% of the spiked CORT on average, differing from swabs that were unspiked. All medium types contributed some background on the ELISA; however, swabs on their own also contribute to this background, which can limit the sensitivity of this method at detecting dermal CORT. Despite this, if dermal CORT was responsive to HPI stimulation, then any differences in concentration >100 pg/ml would be detected. Considering
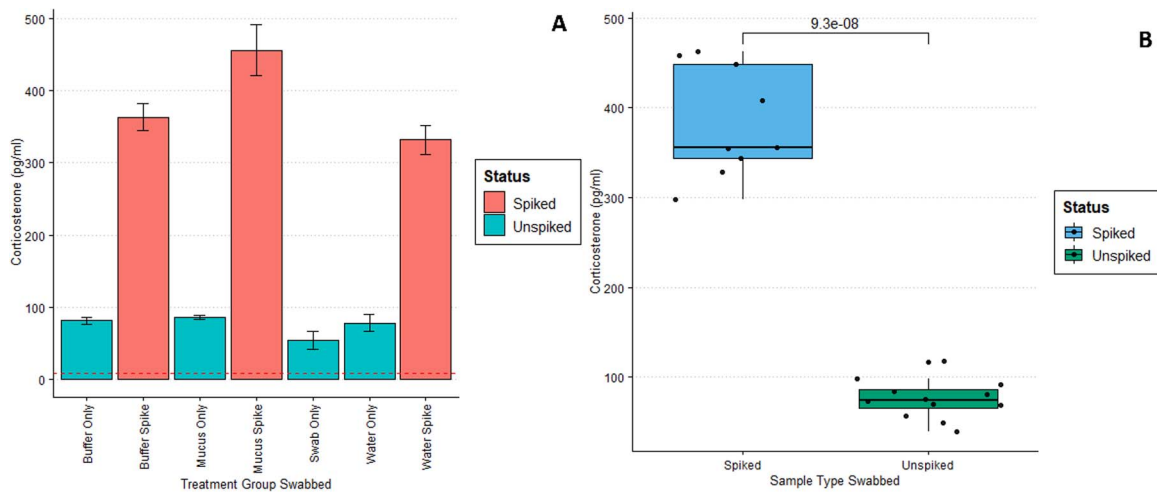
Figure 3: Corticosterone on rayon swabs. (a) Mean corticosterone detected according to each medium type and spike status. Vertical bars represent standard error across triplicates. All mucus was obtained from captive Eastern newts, *N. viridiescens viridescens*. The line close to the bottom of the figure indicates the lower limit of detection for the ELISA (8.8 pg/ml). (b) CORT detected according to spike status. Since all mediums were spiked with 600 pg/ml, this results in an extraction efficiency of 66.7% averaged across media. The number above the bar is the *P*-value obtained through a Welch Two-Sample *t*-test. The dots represent individual data points.
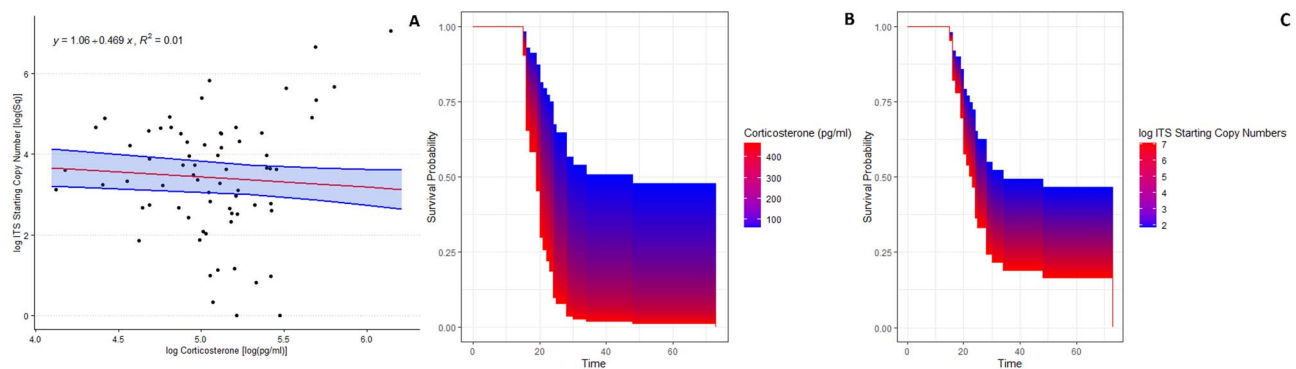

Figure 4: *Batrachochytrium dendrobatidis* (*Bd*) infection, dermal CORT and survival. (a) Relationship between the log of both CORT and *Bd* load indicated by ITS gene copy number. The model with the lowest AIC is fitted to the data, with the shaded region being the 95% CI around the estimated model parameters. (b) Survival curves obtained through Cox regression depicting survival probabilities associated with varying levels of dermal CORT on *N. viridescens viridescens* monitored over 2 months in captivity. (c) Survival curves obtained through the same regression as in (b), however, *Bd* infection load [log(ITS copy numbers)] was used as a predictor instead of dermal CORT.

that ACTH injection in amphibians can cause serum CORT or cortisol levels to increase to the nanogramme/millilitre range, we expected to detect dermal glucocorticoids within that range after a certain period if the skin mucus is linked to the HPI axis via excretion or excitation. Therefore, rayon swabs are a suitable method for collecting dermal glucocorticoids from amphibians.

Dermal CORT and stress physiology

Our study was limited by both smaller than expected sample sizes and an inability to obtain plasma samples to directly show CORT in the serum. The smaller sample sizes came from the loss of individual animals in captivity, which ended

up being a feature of the study, as survival analysis was performed, but nonetheless limited which individuals were experimented on during the physiological validation. Plasma samples would have yielded more concrete results but were impossible to obtain without lethal sampling in both species used in this study.

Despite these limitations, the CORT-injected group served as a means of avoiding lethal plasma sampling, since the measured hormone was directly placed into the serum in amounts that are beyond the natural ranges in both species (Hammond *et al.*, 2018; Pereira *et al.*, 2023). Since the bolus of CORT placed into the serum was never seen on the skin in both species, this suggests that little, if any, plasma CORT
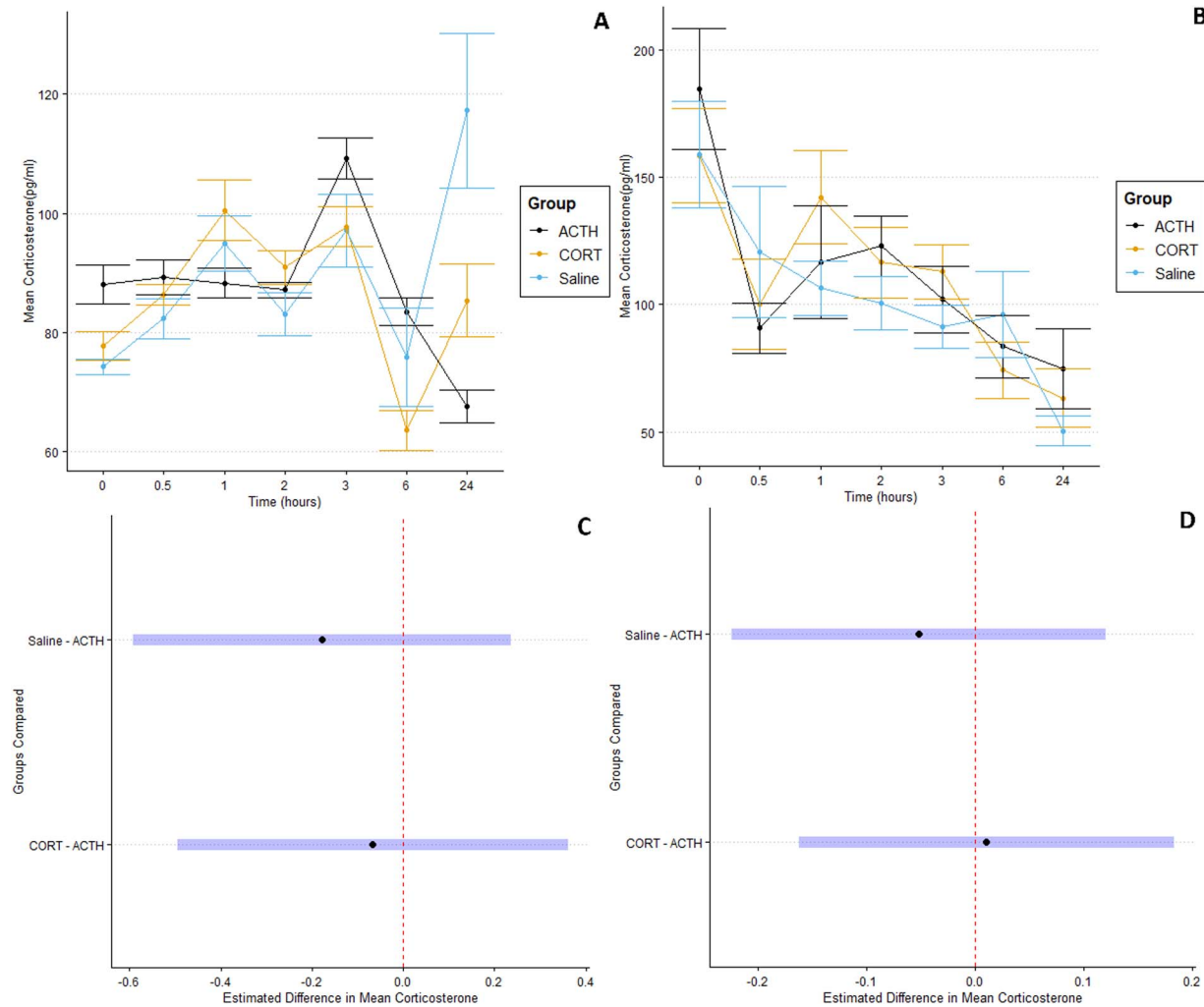
Figure 5: Experimental tests of HPI-axis stimulations and dermal CORT quantification. Mean estimates of Eastern newt (a) and Leopard frog (b) dermal CORT detected post-injection according to each time point sampled. Vertical bars indicate the standard error of the mean and median for each graph, respectively. Mean and median estimates were obtained from biological replicates within each injection group. (c) The 95% CIs obtained from the model with the lowest AIC examining the effects of each injection treatment on the estimated mean CORT obtained overall in the Eastern newt models. Overlap with the dotted line indicates confidence in an estimate for no contrasting effect on measured CORT when compared to another treatment group. (d) The 95% CIs obtained in the same manner as (c), except the data used to generate the above intervals was derived from Northern leopard frogs.

was excreted onto the skin during the injection challenge. Furthermore, ACTH injected into the serum elicited no response in dermal CORT. All of this suggests that the CORT present in the skin of both Eastern newts and Northern leopard frogs is uncoupled from CORT present in the serum. Therefore, HPI function cannot adequately be monitored using dermal swabs in these two amphibians as was previously suggested (Santymire *et al.*, 2018; Santymire *et al.*, 2021), although validated in an African amphibian, the edible bullfrog, *Pyxicephalus edulis* (Scheun *et al.*, 2019).

One caveat is that the Eastern newts were near their physiological limits due to ongoing *Bd* infections. Indeed,

three newts expired during the 24-h injection experiment. In the field, most Eastern newt populations are infected with widely ranging *Bd* infection prevalence and loads (Raffel *et al.*, 2010). Thus, our results may be representative of field conditions for this species. In addition, a pilot study with healthy *Bd*-infected newts collected from the same population in summer 2022 but conducted in outdoor mesocosms also demonstrated a lack of significant impacts on dermal CORT of rough handling and filtered *Bd* metabolites when compared to control treatments (Supplemental Materials: Figs. S7–S12). Conversely, Northern leopard frogs are often not infected in the field or can recover from *Bd* infections (Wilber *et al.*, 2022). Plus, the Northern leopard frogs were raised and fully

acclimatized to the environmental conditions present during experimentation, so it is highly likely that the frogs were not at their maximum hormone release rates. Despite this, there was still no signal detected in the Northern leopard frogs, suggesting uncoupling.

If dermal CORT is unrelated to the HPI axis, what explains the signal obtained from the mucus? Over the last two decades, work in humans has shown that the skin acts as its own endocrine organ, producing a wide variety of steroidal compounds or their precursors endogenously (Hannen *et al.*, 2017; Jozic *et al.*, 2014; Phan *et al.*, 2021; Vukelic *et al.*, 2011). Some of these compounds, namely POMC, ACTH and CORT, are synthesized by keratinocytes, which function independently of the HPA axis (Phan *et al.*, 2021). The uncoupling of dermal CORT from CORT present in the serum as we have shown in two amphibians here provides preliminary evidence that epidermal cells may endogenously produce CORT. To further evaluate this idea, transcriptomic data paired with immunohistochemical validation could demonstrate the endogenous production of HPI elements in the epidermis or granular glands of amphibians. In addition to immunohistochemistry, amphibian epidermal cell cultures could be used to determine if the skin is responsible for dermal glucocorticoid signals observed via skin swabbing.

CORT, Bd infection load and survival

Dermal CORT was poorly predictive of *Bd* infection load (Fig. 4a). *Bd* infection load was weakly predictive of survival (Fig. 4b). Infection load often correlates with survival in amphibians susceptible to *Bd* chytridiomycosis (Grogan *et al.*, 2018). Since all the Eastern newts used in the experiment were infected, there were no uninfected and infected comparisons to be made, which could explain the lack of a statistical association (Fig. 4b, c; Supplemental Materials Section 3: Statistical Models) between survival and infection load. The rapid increase in infection load when newts were brought into captivity may have been the result of a release from the environmental pH constraint on *Bd* growth (Piotrowski *et al.*, 2004, Woodhams *et al.*, 2018) upon moving newts from an acidic bog to a slightly alkaline captive environment. This perhaps precipitated chytridiomycosis-associated mortality.

The relationship between dermal CORT and survival was not related to HPI stimulation. Assuming the relationship is not spurious, it could also hint at a novel role that dermal CORT plays in the physiology of amphibian skin. The Eastern newts were collected from a bog with a low pH and low conductivity but were housed in water with a pH of ~7.4 and higher conductivity. Given the shift in water parameters, it could be possible that dermal CORT participates in some form of osmoregulatory mechanism (Chambers, 2011; Smith & Vale, 2006). Recent work has demonstrated that skin glucocorticoids also influence the microbes that live on the skin in *Boana faber* (Neely *et al.*, 2023). Therefore, it is likely that microbiome assemblage shifted in concert with, and potentially in response to, dermal CORT, which can

influence survival against Bd (Woodhams *et al.*, 2018). Further investigation is required to understand the physiological mechanisms underlying the observed survival data.

Conservation implications

The urgency of developing methods to monitor and assess at-risk amphibian populations will only increase as the climate changes and as anthropogenic activity extends further into wetland habitats. Although initially promising as a non-invasive method, dermal swabs cannot adequately monitor HPI activity in two North American amphibian species. Instead, the signal obtained from dermal swabbing appears to be a consequence of the endogenous production of glucocorticoids rather than hormones excreted from the serum into the mucus. Therefore, conservationists should exercise caution when applying the dermal swabbing technique to field populations, as the technique likely monitors CORT that is not related to acute HPI-axis function. Further, this data calls attention to a need for more rigorous testing of methods as imprecise methods could lead to incorrect conclusions about wild populations. The data presented serve as both a cautionary tale and a source of further investigation, as the function of glucocorticoids in the skin across vertebrates remains a mystery.

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Author contributions

V.Q.: Conceptualization (equal); investigation (lead); animal husbandry (equal); formal analysis (lead); methodology (equal); data curation (equal); writing—original draft (lead); writing—review and editing (equal).

B.I.: investigation (supporting); animal husbandry (supporting); writing—review and editing (supporting).

N.M.D.: animal husbandry (equal); writing—original draft (supporting); writing—review and editing (supporting).

K.W.: investigation (supporting); writing—review and editing (supporting).

L.M.R.: Conceptualization (equal); methodology (equal); writing—review and editing (equal); project administration (supporting); funding acquisition (supporting).

D.C.W.: Conceptualization (equal); methodology (equal); data curation (equal); writing—review and editing (equal);

project administration (lead); funding acquisition (lead); resources (lead).

Conflicts of Interest

The authors declare no competing interests.

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Data availability

Additional information regarding statistics and prior experiments can be found in Supplemental Materials. In addition, all of the Eastern newt data are provided in a Supplementary Materials file: Compiled_Newt_Experimental_Data.xlxs, and all analyses used during the experiment along with all of the collected data are available on github (https://github.com/VictQuad/Dermal_CORT_Code_Data).

Supplementary Material

Supplementary Material is available at *Conservation Physiology* online.

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