Physiology & Behavior 297 (2025) 114951

Contents lists available at ScienceDirect

Physiology & Behavior

journal homepage: www.elsevier.com/locate/physbeh

Assessing the interconnected behavioral and physiological underpinnings of amphibian responses to fungal infection

Samantha A. Shablin *, Sofia Valencia Osorio, Carl N. Keiser

Department of Biology, University of Florida, Gainesville, FL 32611, United States

ARTICLE INFO

Keywords:
Activity level
Batrachochytrium dendrobatidis
Cuban tree frog
Corticosterone
Disease ecology
Immune response
Stress physiology

ABSTRACT

Pathogens inflict various costs onto their hosts from sublethal changes in physiology and behavior to intense pathology and mortality. The timing of host immune responses and concomitant changes in behavior may be jointly underpinned by an increase in glucocorticoid hormones. The temporal and causal links between these interrelated responses to infection remain equivocal in many host-pathogen systems. Here, using a fungal pathogen implicated in global amphibian declines, *Batrachochytrium dendrobatidis* (*Bd*), we examined sublethal consequences of infection in the Cuban tree frog (*Osteopilus septentrionalis*). Specifically, we tracked changes in the neuroendocrine stress response by measuring urinary corticosterone (CORT) metabolites as well as immunological activation via neutrophil/lymphocyte (N/L) ratios over three consecutive rounds of *Bd* exposure. Additionally, we measured both exploratory behavior and activity level in open field arenas to evaluate whether infection-mediated changes in behavior and stress physiology coincided. Pathogen-exposed individuals began testing positive for *Bd* after two rounds of exposure. After the third exposure, *Bd*-positive frogs had higher CORT levels compared to control frogs. While infection load was not associated with N/L ratio, CORT concentrations showed a negative correlation with N/L ratio, suggesting a link between endocrine activity and immune regulation. Furthermore, there were no differences in exploratory behaviors or activity levels between control and *Bd*-exposed frogs. The lack of a relationship between N/L ratios and infection load may indicate a lag between neuroendocrine and immunological responses in this host-parasite system. This is further supported by the delayed increase in CORT levels only after three rounds of pathogen exposure. Alternatively, the lack of a relationship may be due to the immunosuppressive capability of *Bd*. The varied impacts of infection on physiological biomarkers indicates a greater need for researchers to consider simultaneous changes to behavior, neuroendocrine, and immunological measures of stress in future host-pathogen studies.

1. Introduction

Infectious diseases are important from a conservation perspective because they can be a significant source of host mortality and therefore play a role in regulating populations. Sublethal responses to infection can also arise through a combination of physiological (e.g. mounting immune responses, changes in hormone concentrations) and behavioral mechanisms (e.g. changes in activity and antipredator behaviors) [1–4]. Concurrent changes to hormone profiles [5] and activation of immune defenses [6] can cause physiological stress to hosts, yet they continue to engage in ecological interactions throughout the course of infection [7]. As such, understanding the multifaceted, interconnected effects that parasites have on host physiology and behavior is imperative to build a more complete picture of emerging infectious diseases.

Changes in host behavior are common in host-parasite systems, from highly specific manipulations of behavior by tightly coevolved parasites to patterns like lethargy or diet shifts after infection by bacteria and viruses [8]. Although behavioral changes after infection are underpinned by diverse physiological mechanisms, the hypothalamus-pituitary-adrenal (HPA/HPI; interrenal in amphibians and fishes) axis is likely a strong mediator of the covariation between physiological and behavioral changes in infected animals. Corticosterone (CORT), a primary glucocorticoid (GC) hormone produced by the HPI axis, is involved in the stress response and is hypothesized to promote delayed glucose release that supports long-term energy recovery [9–11]. Though short-term increases in CORT are crucial for maintaining homeostasis during exposure to stressors and protect against infection [12–14], long-term elevations can lead to dampened immune

* Corresponding author at: Mississippi State University, Department of Biological Sciences, Mississippi State MS 39762, United States.
E-mail addresses: ss4856@msstate.edu (S.A. Shablin), ckeiser@ufl.edu (C.N. Keiser).

https://doi.org/10.1016/j.physbeh.2025.114951
Received 10 February 2025; Received in revised form 25 April 2025; Accepted 8 May 2025
Available online 8 May 2025
0031-9384/Published by Elsevier Inc.

responses and behavioral changes such as lethargy that increase overall mortality risk [15–18]. The immune system is upregulated with short-term HPI activity and increases in CORT levels during infection can also change immune parameters such as neutrophil, lymphocyte, and eosinophil counts [19–21]. Further, GCs can become unresponsive to stress over extended periods, which makes them imperfect measures of immune stress when used alone [22]. Studies focusing on stress responses to infection should collect GC data concurrently with other stress biomarkers to better understand behavior-physiology crosstalk during infection.

Circulating cellular components of the immune system represent important defenses against pathogens and also serve as stress bioindicators [23]. The ratio of two immune cells, neutrophils and lymphocytes, is a common measure of immune stress that typically increases alongside GCs in vertebrates, including amphibians [22,24–26]. Neutrophils perform phagocytic functions while lymphocytes regulate and redistribute immune defenses during infection [23,27,28]. Combining components of the innate and adaptive immune systems [29], neutrophil/lymphocyte (N/L) ratios communicate information on immune function and can be used alongside GCs to measure multiple facets of organismal stress responses [23]. Indeed, in amphibians, increases in CORT and elevated N/L ratios are commonly observed during stressful events, though some have suggested N/L ratios as a more reliable indicator of long-term stress when CORT may begin to attenuate and N/L ratios remain elevated [19,22,26,30–33]. Supporting this, Titon et al. [34] demonstrated that restraint, an immune challenge, and exogenous CORT application enhanced select immune responses in *Rhinella icterica* such as N/L ratios, though effects varied by dose and immune parameter, underscoring the complexity of CORT-induced immunomodulation [34]. Given the numerous environmental stressors and emerging infectious diseases leaving amphibian populations some of the most imperiled groups globally [35], conservation efforts require a better understanding of multifaceted amphibian stress responses to disease.

The amphibian skin disease chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), targets many species and causes diverse effects from mass mortality events to sublethal effects on physiology and changes to behavior [36,37]. *Bd*-infected amphibians often exhibit increased GCs [12,38–42], but variable results have been recorded across host species varying in disease susceptibility [43–46]. Even in the absence of disease, elevated GCs can affect behavior, development, and immunity [12,39–42]. After infection, increased baseline CORT levels are accompanied by decreased locomotory behaviors and impaired righting reflexes [39,47]. However, whether *Bd* jointly mediates changes in neuroendocrine, immune, and behavioral responses simultaneously remains relatively unexplored and studies combining these approaches will help us understand how seemingly resistant hosts cope with pathogens.

To disentangle the physiological mechanisms underlying behavioral changes after *Bd* infection, we focused on the Cuban tree frog (*Osteopilus septentrionalis*), an invasive frog in the southern United States. Cuban tree frogs can experience mortality from chytridiomycosis but often clear infections even after several rounds of exposure, and can survive chronic infections without showing clinical signs of disease [48]. Cuban tree frog tadpoles have shown a positive correlation between CORT release rates and *Bd* infection [43], but similar patterns in adult frogs remain untested. Although the Cuban tree frog is not of conservation concern, this study is beneficial to conservation efforts in two ways: (1) understanding the physiological underpinnings of disease can elucidate how some invasive species become so successful and (2) our methods can serve as a proof of concept with applications to ecophysiology studies in endangered amphibians. Here, we subjected *O. septentrionalis* to three rounds of *Bd* exposure while repeatedly measuring changes in stress physiology and behavior. After the final round of exposure, we also measured immune activity via circulating N/L ratio. Our objectives were to: (1) determine whether Cuban tree frogs undergo changes in activity and exploratory behaviors after *Bd* infection, (2) investigate how physiological stress biomarkers (CORT and N/L ratio) covary with *Bd* infection, and (3) determine whether changes in CORT covary with changes in behaviors.

2. Materials and methods

2.1. Ethical declaration

The collection, care, and use of experimental animals complied with guidelines of The University of Florida Institutional Animal Care and Use Committee (IACUC permit Number: 201810502) as well as the Florida Fish and Wildlife Conservation Commission (LSSC-17-00031B).

2.2. Frog collection and husbandry

From August to September 2020, we collected 41 adult *O. septentrionalis* using stationary, upright PVC pipes at the Natural Area Teaching Laboratory located at the University of Florida in Gainesville, FL. Average nightly temperature during collection was 23.3°C. Treefrogs naturally seek shelter inside of hollow PVC pipes, making this an effective method for collecting individuals. Upon capture, frogs were immediately placed into behavioral arenas in the field (see full description in "Behavioral Trials" section). Frogs were then weighed with a digital scale, snout-vent-length recorded with a digital caliper, sexed, and swabbed to detect any existing *Bd* infections. Individuals that were initially difficult to sex in the field were later identified via dissection of sex organs post-euthanasia. After initial measurements and behavioral assays, frogs were transported to the lab at the University of Florida (Fig. 1), each housed in its own plastic container (~19 cm x 33 cm x 23 cm) with PVC pipe for shelter, a shallow dish of water prepared using Holtfreter's solution, and a moist paper towel [49]. *Bd*-exposed and control frogs were housed in separate Percival environmental chambers with water dish and paper towels replaced twice weekly and full tank changes once weekly. During full changes, each frog was removed from its enclosure and placed into a new clean husbandry container. We kept frogs under an automated 12:12 hr light:dark schedule at 21°C during the day and 17°C during the night, with a relative humidity of 80%. Frogs were misted with water daily, inspected for signs of disease daily, and fed domestic crickets ad libitum.

2.3. Behavioral trials

We focused on activity and exploratory behavior because both are implicated in pathogen exposure risk and transmission potential [50] and activity level (e.g., lethargy) is often used as a behavioral proxy for disease in captive and wild animals. Behavioral trials were performed three times in total: at the field site immediately after capture, 24 hours later in the lab prior to *Bd* or control exposure, and in the lab after the third round of *Bd* of control exposure (~56 days later). All behavioral trials were filmed using a Nikon DSLR camera at night between 21:00-00:00. Cameras were stationed ~25 cm away from arenas with red light projected on top of the enclosures to view the frog in a dark setting (in field and lab). Prior to each assay, frogs were powdered with non-toxic, fluorescent powder manufactured by DayGlo (Cleveland, OH, USA) to track movement and make frogs more visible on camera. This powder has been previously used on amphibians with no adverse effects on health or behavior [51,52]. Each individual was then placed into a small, inverted plastic cup in the center of the behavioral arena for a 5-minute acclimation period before being released, after which frog behavior in the arena was filmed for 20 minutes. Each rectangular arena (21cm x 61cm x 18cm) contained six artificial plants constructed from wooden dowels and artificial leaves placed ~6 cm apart and adhered to the base using modeling clay. In the laboratory, assays were filmed similarly to those in the field, though were performed inside of Percival environmental chambers to maintain constant environmental conditions. From the videos, we measured activity level (total time the frog's

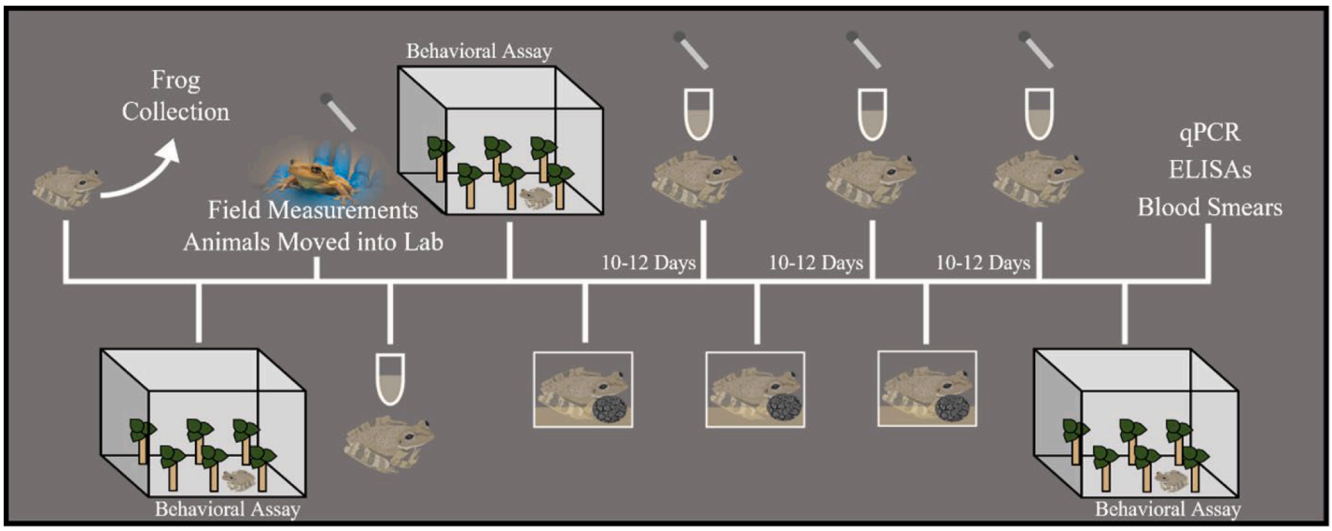

Fig. 1. Experimental timeline. Frog activity level and exploratory behavior were assayed once in the field upon capture and then twice in the laboratory, once prior to and once following experimental infection with *Bd*. All individuals underwent three rounds of exposure to either *Bd* zoospores or a control/sham treatment. Urinary CORT metabolite levels were measured once before exposure and then immediately prior to each subsequent round of exposure for a total of four sampling time points. Lastly, immune responses (N/L ratios) were assessed at the fourth time point before euthanasia. Pathogen exposure represented by zoospores in fluid, urine sampling represented by vial of liquid, skin swabs represented by cotton swab, behavioral trials represented by gray arenas with trees.

center of gravity spent moving) and exploration (the number of unique trees climbed). Upon completion of each trial, we rinsed frogs with dechlorinated water and returned them to their housing containers. Any behavioral videos where the frog disappeared outside of camera view for >30 seconds were removed from this analysis (total removed from pre-exposure assays = 14; total removed from post-exposure assays = 12; final sample sizes utilized in Table S1).

2.4. Bd detection and exposure

Frogs were swabbed prior to transport into the lab to ensure experimental animals were not already infected with *Bd*. To quantify *Bd* infection load, genomic DNA was first isolated from each swab using PrepMan Ultra (Applied Biosystems) and processed following methods from Boyle et al. [53], Hyatt et al. [54], and Longo et al. [55]. Once DNA was extracted, *Bd* loads were quantified via real-time qPCR using ITS gene fragments as a standard curve. Samples consisted of 2 uL 1:10 diluted DNA: deionized water and 8 uL Master Mix. All samples were run in duplicate against serially diluted *Bd* concentrations of 10^6-10 ITS gene fragments [53,54]. Ultrapure deionized water was also used as a negative control to monitor potential contamination. No animals had detectable *Bd* loads upon collection from the field.

We randomly assigned frogs to either a control (n = 14) or *Bd*-exposed (n = 27) treatment group. More individuals were assigned to the *Bd*-exposed group to account for anticipated mortalities due to chytridiomycosis. Frogs in the *Bd*-exposed group were submerged in a 5 mL liquid Holtfreter's solution containing 10^6 *Bd* zoospores for a 24-hour period during inoculations, while frogs in the control group were submerged in 5 mL Holftreter's solution. Frogs were exposed three times over the course of the experiment, 10-12 days apart, to assess whether measures of physiological stress would coincide with the development of immune responses [48]. One day prior to exposure, frogs were swabbed to determine infection status/load. Frogs were swabbed again 10 days after the final round of exposure. We utilized a standardized swabbing protocol that consisted of swabbing the dorsal surface, ventral surface, sides, and thighs a total of 10 times each and feet a total of 5 times each in a downwards motion [56].

2.5. Urinary CORT collection and validation

To limit animal distress and the effects of short-term handling on CORT levels, we measured non-invasive urinary CORT metabolites [57,58]. Glucocorticoid hormone metabolites can be reliably extracted from urine samples and such methods have been validated with enzyme-immunoassays (EIAs), which maintain detection sensitivity in numerous amphibian species that produce small sample volumes [58–60]. We collected a total of four urine samples from each frog, the first urine sample collected 24 hours after transport into the lab. Urine samples were not collected in the field because many individuals tended to either urinate during handling prior to capture or did not produce urine within five minutes of stimulation. A five-minute window was selected for urine collection based on evidence from Narayan et al. [61], which demonstrated that manual restraint of this duration does not significantly influence urinary corticosterone metabolite levels in cane toads, in contrast to extended restraint periods of 15 or 30 minutes. The next three urine samples were taken 10-12 days after each *Bd* exposure (Fig. 1), immediately before the next round of exposure to ensure that the 24-hour containment associated with exposure did not interfere with stress measurements. This timeframe was chosen because detectable *Bd* loads begin to appear eight days after exposure in *O. septentrionalis* [62]. On each sampling day between 15:00-20:00h, to minimize any variation in the stress response that may occur via diel rhythms [63], each frog was held over a sterile cup and gently massaged on the ventral side until a urine sample was collected. If no urine sample was collected within five minutes of stimulation (n = 44), the frog was returned to its housing container. The volume of urine samples varied from ~.025-1.5mL. Following collection, samples were frozen at -20°C until further processing.

Once thawed, samples were dried using a Speedvac and re-suspended in a 1:2 dilution with prepared assay buffer (Arbor Assays). Hormone metabolite concentrations were measured using an Arbor Assays DetectX Corticosterone ELISA kit (K014-H5, Ann Arbor, MI, USA). Assays were conducted following the manufacturer's provided protocol. For 50uL of sample input, manufacturer-reported assay sensitivity was 20.9 pg/mL. All urine samples were run in triplicate and standardized to creatinine (Cr) levels to control for varied water content across samples and for normalization of CORT [64]. If a triplicate produced a coefficient of variation > 20%, the respective sample was run

again in triplicate. Additionally, if not enough sample was available to run on creatinine and corticosterone assays, the sample was not used. Plates were processed at 450nm on a Synergy LX Plate Reader with final metabolite concentrations calculated as $\log_{10}$(pg CORT/μg Cr).

To accurately determine CORT metabolite concentrations, we performed a series of validation steps [65–67]. Tests of parallelism, linearity, and spike and recovery are commonly performed in assay validation [59,66], and were applied here by pooling six random urine samples. Two groups of pooled urine samples were collected and serially diluted 2:1, 1:1, 1:2, 1:4, 1:8, and 1:16 and assayed following the manufacturer's protocol to assess linearity and parallelism (Arbor Assays). Linearity and spike and recovery were tested until values fell between 80-120%. To determine spike and recovery, a known value of CORT (1250 pg/mL) was added to pooled samples and assayed at three serial dilutions [66]. Resulting concentrations were compared to control (assay buffer) spiked samples and un-spiked samples from the same pool. Intra- and inter-assay variation were determined by running known standards of 2500 and 78.125 pg/mL in triplicate. The intra- and inter- assay coefficients of variation (CV) were 6.2% and 6.4%, respectively.

2.6. White blood cell counts

At the end of the experiment, we collected a single measurement of immune stress via white blood cell counts. We collected blood smears from a total of 18 frogs from which blood collection was sufficient (n = 9 *Bd*-exposed, n = 9 control) from the facial maxillary/musculo-cutaneous vein with heparinized tubes immediately after euthanasia with MS-222. To mitigate acute stress-induced alterations in N:L ratios during the euthanasia process, individuals were placed into an immersive bath of MS-222 within 30 seconds of handling, using small containers positioned inside of their husbandry tanks to maintain environmental familiarity. Approximately 5uL of blood was pipetted onto the middle of a microscope slide and swiped horizontally with the edge of another slide before air drying for 10 minutes. Once dried, blood smears were stained using the Hema 3 Stain Set.

Slides were viewed under the 100x oil immersion objective of a DMi8 Leica microscope and approximately 200 frames were captured per slide. Frames were individually analyzed and each white blood cell was identified using methods described by [68] and [69]. White blood cells with ambiguous characteristics or on the edge of the frame were not counted. We attempted to identify all white blood cells (neutrophils, lymphocytes, monocytes, eosinophils, and basophils; Supplementary Fig. 1) though we used N/L ratios as a measure of immune function and stress. The N/L ratio often increases following exposure to stressors or infection [70], driven by an increase in neutrophils and concurrent decrease in lymphocytes [23]. However, this pattern is not consistently observed across all taxa or conditions [71].

2.7. Statistical analyses

Statistical analyses were performed in RStudio Version 4.1.0 [72]. Missing data from a specific time point were removed from analyses if, for example, urine samples were not produced (n = 44 removed in total), behavioral videos were rendered unusable (n = 36 in total), or if individuals died during the experiment (n = 8). We log10-transformed both CORT metabolite levels and *Bd* loads to meet normality assumptions. Moreover, we scaled frog mass values using the scale() function in models where this variable is specified as a fixed effect. All models and associated residuals were assessed for dispersion and zero inflation using DHARMa diagnostic tools [73].

To determine whether there were differences in frog behavior from the field and pre-exposure laboratory settings [74], we used generalized linear mixed models (GLMMs) with the glmmTMB package in R [74]. We used two models, one for the number of trees climbed and one for total time spent moving and assigned the following variables: time point (field vs. pre-exposure laboratory setting), treatment group (control vs. exposed group), and mass were used as fixed effects and individual ID as a random effect. To test whether behavior changed from pre- to post-exposure time points in the lab, we used two GLMMs (one for each behavioral response variable) for the effects of time point (pre- to post-exposure), treatment group (control vs. exposed group), mass, CORT level measured in the first and last assays, and an interaction between treatment group and time point with individual ID assigned as a random effect. We specified a negative binomial distribution and log link function for models utilizing the number of trees climbed as a response variable and a gamma distribution and log link function for models utilizing the total time spent moving as a response variable.

To assess CORT levels, we used linear models with the following fixed effects: treatment group (exposed vs. control), mass, assay number (1-4), and an interaction between treatment and assay number. In a separate model to assess the effect of *Bd* load on CORT, we used a linear model with mass, time point, *Bd* load, and a time point x *Bd* load interaction term. Individual ID was used as a random effect in these linear models. N/L ratios were analyzed using a GLMM with a zero-augmented gamma distribution, implemented in the R package glmmTMB. Fixed effects included body mass, CORT level, and *Bd* load.

Lastly, we used a Cox proportional hazards model to determine whether mortality was related to *Bd* exposure and CORT levels. Survival analysis was performed with the coxph() function within the coxme R package [75].

3. Results

3.1. Relationships between Bd exposure, CORT, and behavior

We found no differences in behavior (activity: estimate = 0.436, z = 1.354, df = 47, p = 0.176; exploration: estimate = 1.193, z = 1.675, df = 47, p = 0.094) between assays conducted in the field and pre-exposure laboratory settings. We found a positive trend between body mass and activity (estimate = 0.321, z = 1.896, df = 47, p = 0.058), though no relationship between body mass and exploratory behavior (estimate = 0.158, z = 0.555, df = 47, p = 0.579).

When comparing frog behavior before and after experimental treatments in the lab, we found that all individuals became less exploratory over time (Fig. 2a; estimate = -2.032, z = -2.300, df = 35, p = 0.021) but showed no change in activity level (Fig. 2b; estimate = 0.491, z = 1.469, df = 35, p = 0.142). Exposure treatment had no effect on frogs' change in activity (estimate = -0.144, z = -0.333, df = 35, p = 0.739) or exploration (estimate = 1.958, z = 1.435, df = 35, p = 0.151) across the two time points in the lab. We found no effect of mass on activity (estimate = 0.016, z = 0.130, df = 35, p = 0.896) or exploration (estimate = -0.241, z = -0.486, df = 35, p = 0.627). We found no relationship between CORT level and exploratory behavior (estimate = -0.004, z = -0.533, df = 35, p = 0.594), though we detected a negative correlation between CORT and activity level (estimate = -0.002, z = -2.008, df = 35, p = 0.045).

3.2. Relationships between Bd exposure and physiological measures of stress

We found no detectable signs of *Bd* infection for any frog sampled in the field or the first post-exposure time point. A total of 25% (6/24) of individuals in the *Bd*-exposed group remained *Bd*- negative after the second round of exposure (Figure S2). Nearly all *Bd*-exposed frogs (18/19) tested positive for *Bd* after the third round of exposure, while the control frogs remained *Bd*-negative through the entirety of the experiment (Figure S2). After the final round of infection, *Bd*-exposed frogs had 51% higher CORT levels compared to frogs in the control group (Fig. 3; estimate = 0.710, t = 2.373, df = 78, p = 0.020). We also detected a positive relationship between CORT and *Bd* load (Fig. 3; estimate = 0.107, t = 3.198, df = 100, p = 0.002). Mass did not show a

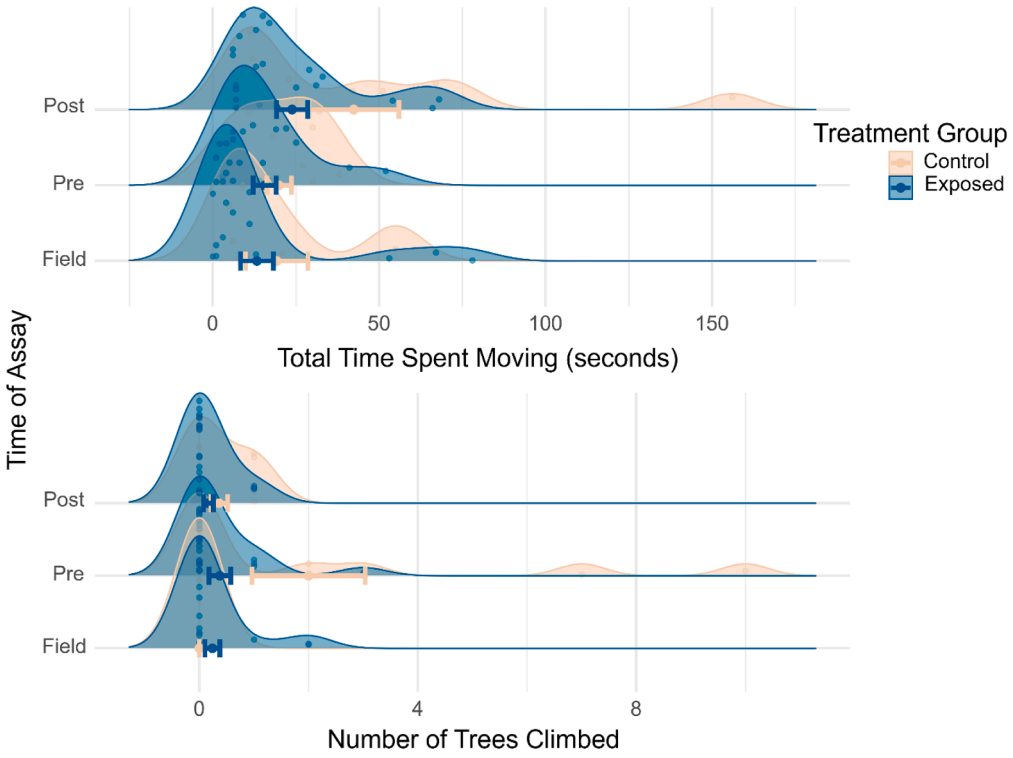

Fig. 2. Activity levels and exploratory behavior in Cuban treefrogs during 20-minute assays in the field and the lab. (a) Activity levels did not change from field to pre-exposure or post-exposure time points while (b) exploratory behaviors did not change from field to pre-exposure time points but decreased from pre-exposure to post-exposure time points. Treatment had no effect on either behavioral measurement, suggesting that the change in exploratory behavior was driven by acclimation to the lab setting. Curves represent density distributions of measurements; points on the x axis represent means and errors bars represent standard errors.

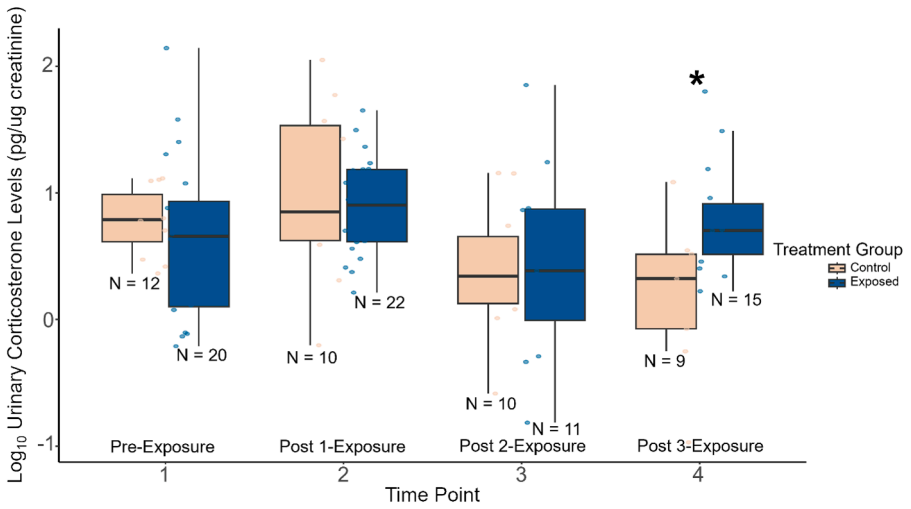

Fig. 3. Urinary CORT metabolite concentrations in control and *Bd*-exposed frogs across four sampled time points. *Bd*-exposed frogs had higher urinary CORT metabolite levels compared to control frogs at the 4th time point. The asterisk denotes a significant difference.

relationship with CORT (all $p > 0.31$). *Bd*-exposed frogs died more rapidly compared to control frogs (coefficient = 13.089, $p = 0.0003$; Supplementary Fig. 3), though CORT levels did not vary with survival (coefficient = 0.467, $p = 0.494$) (Fig. 4).

Although sample sizes for the zero-augmented gamma distribution model of N/L ratio were low (control $n = 9$, exposed $n = 9$) due to challenges in obtaining sufficient blood volume from smaller individuals, we report the following results given their potential biological relevance. A significant negative relationship was observed between CORT level and N/L ratio (estimate = -2.845, $z = -2.731$, $df = 9$, $p = 0.006$), while no relationship was found between N/L ratio and body mass (estimate = 0.111, $z = 0.418$, $df = 9$, $p = 0.676$) or Bd load (estimate = 0.179, $z = 1.447$, $df = 9$, $p = 0.148$).

4. Discussion

A hallmark of infectious disease ecology is that parasites can exert sublethal effects on host behavior and physiology throughout the course of infection. Physiological defenses can be costly and, coupled with behavioral changes after infection, can hinder an organism's ability to

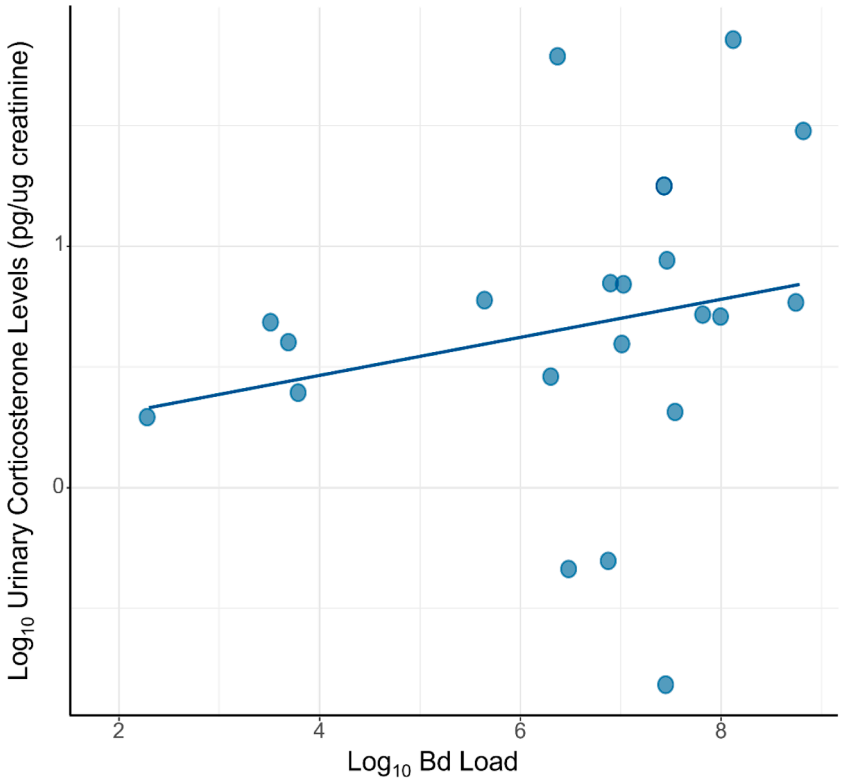

Fig. 4. Relationship between log10-transformed *Bd* load and log10-transformed urinary CORT metabolite concentrations in *Bd*+ individuals.

cope with additional environmental challenges. However, the causal linkages between physiological and behavioral changes during infection are often complex and diagnostic measurements of disease stress via one trait may not be as informative as more comprehensive approaches. Here, using Cuban tree frogs experimentally infected with the fungal pathogen *Bd*, we assessed host behavior alongside neuroendocrine and immunological measures of stress over time. We found that *Bd* exposure was associated with an increase in CORT levels, and CORT showed a negative relationship with the immune measure N/L ratio. Lastly, we found no effects of *Bd* or CORT on host activity and exploratory behavior.

4.1. *Bd* exposure and physiological measures of stress

The relationship between host immunity and neuroendocrine stress is a growing focus of ecoimmunology [24,25,34,76] and discoveries in this field could prove helpful in amphibian disease ecology given the multiple stressors associated with amphibian population declines globally. Species like the Cuban tree frog, which are susceptible to *Bd* infection yet populations appear to be highly resilient, represent important systems in which to test sublethal effects of disease on behavior and physiology [48]. We found that *Bd*-exposed Cuban tree frogs had higher CORT levels compared to control individuals, but only after three rounds of pathogen exposure. CORT levels were not associated with disease outcomes such as morbidity, suggesting that while CORT may reflect a physiological response to infection, it is not necessarily predictive of survival in this species. Additionally, a subset of individuals showed evidence of infection after two rounds of exposure, yet there was no concomitant increase in CORT until our next measurement 10 days later. This may indicate that changes in the stress response begin only after a critical period in the course of infection. Other amphibians have shown increased CORT levels two weeks after *Bd* infection [40]. However, the period between the onset of infection and the delayed increase in CORT is likely characterized by immune activation and immune stress.

We did not find a relationship between N/L ratio and infection load. Fernandez-Loras et al. [77] similarly found no differences in N/L ratios between *Bd*-infected and uninfected *Alytes obstetricans*, though the timeframe was not specified [77]. Other innate immune defenses that we did not measure, such as components of the amphibian skin mucus or antimicrobial peptides, were also likely defending hosts against *Bd* infection [78–81]. Alternatively, given the documented immunosuppressive properties of *Bd*, it is possible that infection inhibited the expected elevation in N/L ratio [77,82]. Additionally, N/L ratios may still increase after *Bd* infection, though not necessarily in an intensity-dependent manner as evaluated in this study. One limitation to this study was that *Bd* load was quantified solely through skin swabbing, which may yield imprecise assessments of fungal burden [83]. However, this approach was selected to facilitate repeated, nonlethal sampling of individuals for longitudinal monitoring. Although the neuroendocrine stress response measured via CORT levels was delayed in animals after infection, we found no concomitant increase in immune stress at the same time point. In another study, *Bd*-infected southern leopard frogs had increased N/L ratios only after showing symptoms of chytridiomycosis compared to pre-symptomatic frogs [84]. We were only able to sample white blood cells at the end of the experiment, but future studies would benefit from jointly measuring N/L ratios and CORT repeatedly before and after infection as these measures may not necessarily increase simultaneously [22].

A negative relationship between CORT and N/L ratio was detected, though the small sample sizes available at the end of the experiment may have contributed to the observed pattern. In the absence of infectious disease, De Assis et al. [19] described increased CORT levels in restrained *Rhinella icterica* toads without an increase in N/L ratio [19]. In CORT-treated *Litoria caerulea*, supraphysiological levels of CORT were not related to N/L ratios, but were related to decreases in other important immune cells, such as circulating eosinophils [85]. Narayan and Hero [32] found a positive relationship between CORT and N/L ratios in *R. marina* toads during transportation stress [57]. Though we provide evidence for a negative relationship between CORT and N/L ratios, this

observation could be explained by a migration of neutrophils from blood into tissues such as the skin where chytrid infection occurs. Indeed, neutrophils and macrophages are some of the first cells to respond to *Bd*-infection as they can engulf the fungal cells and may be more readily transported from circulation into the skin during infection [82]. Rollins-Smith [21] refers to this constant migration of leukocytes as a reason to maintain multiple measures of physiological stress and why leukocyte counts on their own may be imperfect [21]. We also found broad variation in N/L ratios among uninfected individuals (0 - 2.07). This could be explained by unidentified infections with other parasites such as ranaviruses, Perkinsea, and endoparasites [86–89]. Alternatively, individual variation in baseline immune activity has also been described in other vertebrates (e.g., in free-living female great tits) [90].

4.2. *Bd* exposure, stress, and behavior

Although changes to behavior during infection are a hallmark of many host-parasite systems, we found no differences in activity or exploratory behavior between *Bd*-exposed and control individuals. Rather, between the pre-infection behavioral assays and post-infection assays, we found that all frogs became less exploratory. This decrease in exploratory behavior may be due to individuals being released from the need to search for food as regular *ad libitum* feedings occurred [91].

We found a negative relationship between CORT and activity level, though we found no relationship between CORT and exploratory behavior. Previous studies have detailed various relationships between GCs and behavioral changes in amphibians [39,50]. For example, the salamander *Desmognathus ochophaeus* showed no relationships between experimentally elevated CORT levels and exploratory behaviors [92] and similar results have been shown with *Rana pipiens* tadpoles [93]. However, Belliure and Clobert [94] found that *Podacris muralis* lizards with elevated plasma CORT levels increased their time spent moving, potentially due to related increases in glucose utilization and energy increases [94].

Overall, our study emphasizes the importance of not only using multiple stress biomarkers in studying host responses to disease, but also repeated hormone and behavioral measurements. We identified multiple relationships between behavior, physiological stress, and immune response, though not all patterns were intuitive. Studying physiological and behavioral covariation across disease states will provide an integrative approach for understanding the multifaceted coping strategies in wildlife infectious diseases [95]. Although we focused on an invasive frog as a model system, our methods can be applied to other amphibians of conservation concern.

Funding

This work was supported by startup funds from C.N. Keiser and A.V. Longo at the University of Florida and a National Science Foundation Graduate Research Fellowship to S.A.S. [Project Number: 00129881].

CRediT authorship contribution statement

Samantha A. Shablin: Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Sofia Valencia Osorio:** Visualization, Methodology, Investigation. **Carl N. Keiser:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization.

Declaration of competing interest

The authors have no conflicts of interest to declare.

Acknowledgments

We would like to thank E. Richardson, D. Buttrick, M. Arena, G. Glen, M. Torres-Sánchez, and S. McGrath-Blaser for their assistance in field work and behavioral trials, J. Villate and A. Julian for help with frog husbandry, and S. Johnson for frog collection equipment. We would also like to thank the staff at the Natural Area Teaching Laboratory for allowing animal collection at their site. All animals were ethically handled under permits from the Institutional Animal Care and Use Committee (Permit Number: 201810502) and Florida Fish and Wildlife Conservation Commission (Permit Number: LSSC-17-00031B). Experiments were conducted in lab space and with resources provided by A.V. Longo.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.physbeh.2025.114951.

Data availability

All data generated and analyzed during this work and the associated R code are available on Figshare (DOI: 10.6084/m9.figshare.28381700).

References

[1] J.S. Adelman, C. Mayer, D.M. Hawley, Infection reduces anti-predator behaviors in house finches, J. Avian Biol. 48 (2017) 519–528.
[2] J. Agugliaro, C.M. Lind, J.M. Lorch, T.M. Farrell, An emerging fungal pathogen is associated with increased resting metabolic rate and total evaporative water loss rate in a winter-active snake, Funct. Ecol. 34 (2020) 486–496.
[3] A.I. Al-Afaleq, Biochemical and hormonal changes associated with experimental infection of chicks with infectious bursal disease virus, J. Vet. Med. Ser. B-Infect. Dis. Vet. Public Health 45 (1998) 513–517.
[4] M.J. Parris, E. Reese, A. Storfer, Antipredator behavior of chytridiomycosis-infected northern leopard frog (Rana pipiens) tadpoles, Can. J. Zool. 84 (2006) 58–65.
[5] J.H. Rakotoniaina, P.M. Kappeler, E. Kaesler, A.M. Hamalainen, C. Kirschbaum, C. Kraus, Hair cortisol concentrations correlate negatively with survival in a wild primate population, BMC. Ecol. (2017) 17.
[6] K. Schneeberger, A. Courtiol, G.A. Czirjak, C.C. Voigt, Immune profile predicts survival and reflects senescence in a small, long-lived mammal, the greater sac-winged bat (Saccopteryx bilineata), PLoS. One 9 (2014) e108268.
[7] T.T. Hammond, M. Vo, C.T. Burton, L.L. Surber, E.A. Lacey, J.E. Smith, Physiological and behavioral responses to anthropogenic stressors in a human-tolerant mammal, J. Mammal. 100 (2019) 1928–1940.
[8] Moore J (2002) Parasites and the behavior of animals. Oxford University Press on Demand.
[9] S.A. MacDougall-Shackleton, F. Bonier, L.M. Romero, I.T. Moore, Glucocorticoids and "stress" are not synonymous, Integr. Organismal Biol. 1 (2019).
[10] L.M. Romero, Seasonal changes in plasma glucocorticoid concentrations in free-living vertebrates, Gen. Comp. Endocrinol. 128 (2002) 1–24.
[11] R.W. Warne, E.J. Crespi, J.L. Brunner, Escape from the pond: stress and developmental responses to ranavirus infection in wood frog tadpoles, Funct. Ecol. 25 (2011) 139–146.
[12] C. Kindermann, E. Narayan, J. Hero, Urinary corticosterone metabolites and chytridiomycosis disease prevalence in a free-living population of male Stony Creek frogs (Litoria wilcoxii), Comparat. Biochem. Physiol.-Mol. Integr. Physiol. 162 (2012) 171–176.
[13] J.C. Owen, A. Nakamura, C.A.C. Coon, L.B. Martin, The effect of exogenous corticosterone on West Nile virus infection in Northern Cardinals (Cardinalis cardinalis), Vet. Res. (2012) 43.
[14] J.C. Wingfield, A.S. Kitaysky, Endocrine responses to unpredictable environmental events: stress or anti-stress hormones? Integr. Comp. Biol. 42 (2002) 600–609.
[15] I.J. Elenkov, G.P. Chrousos, Stress hormones, Th1/Th2 patterns, pro/anti-inflammatory cytokines and susceptibility to disease, Trends Endocrinol. Metab. 10 (1999) 359–368.
[16] L.M. Romero, M. Wikelski, Corticosterone levels predict survival probabilities of Galapagos marine iguanas during El Niño events, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 7366–7370.
[17] L.M. Romero, J.M. Reed, Repeatability of baseline corticosterone concentrations, Gen. Comp. Endocrinol. 156 (2008) 27–33.
[18] R.M. Sapolsky, L.M. Romero, A.U. Munck, How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions, Endocr. Rev. 21 (2000) 55–89.
[19] V.R. de Assis, S.C.M. Titon, A.M.G. Barsotti, B. Titon, F.R. Gomes, Effects of acute restraint stress, prolonged captivity stress and transdermal corticosterone

application on immunocompetence and plasma levels of corticosterone on the Cururu toad (Rhinella icterica), PLoS. One 10 (2015) e0121005.
[20] P.G. Falso, C.A. Noble, J.M. Diaz, T.B. Hayes, The effect of long-term corticosterone treatment on blood cell differentials and function in laboratory and wild-caught amphibian models, Gen. Comp. Endocrinol. 212 (2015) 73–83.
[21] Rollins-Smith LA, Amphibian immunity-stress, disease, and climate change, Dev. Comp. Immunol. 66 (2017) 111–119.
[22] A.K. Davis, D.L. Maney, The use of glucocorticoid hormones or leucocyte profiles to measure stress in vertebrates: What's the difference? Methods Ecol. Evol. 9 (2018) 1556–1568.
[23] A.K. Davis, D.L. Maney, J.C. Maerz, The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists, Funct. Ecol. 22 (2008) 760–772.
[24] V.R. Assis, S.C.M. Titon, Junior B Titon, F.R. Gomes, The impacts of transdermal application of corticosterone on toad (Rhinella icterica) immunity, Integr. Comp. Biol. 62 (2022) 1640–1653.
[25] P.G. Garcia Neto, S.C.M. Titon, S.M. Muxel, B. Titon Junior, A.C. de Figueiredo, F. R. Floreste, A.S. Lima, V.R. Assis, F.R. Gomes, Immune and endocrine alterations at the early stage of inflammatory assemblage in toads after stimulation with heat-killed bacteria (Aeromonas hydrophila), Comparat. Biochem. Physiol. Part A: Mol. Integr. Physiol. 291 (2024) 111606.
[26] M.J. Gavel, S.D. Young, R.L. Dalton, C. Soos, L. McPhee, M.R. Forbes, S. A. Robinson, Effects of two pesticides on northern leopard frog (Lithobates pipiens) stress metrics: Blood cell profiles and corticosterone concentrations, Aquat. Toxicol. (2021) 235.
[27] F.S. Dhabhar, A hassle a day may keep the doctor away: stress and the augmentation of immune function, Integr. Comp. Biol. 42 (2002) 556–564.
[28] B.G. Harmon, Avian heterophils in inflammation and disease resistance, Poult. Sci. 77 (1998) 972–977.
[29] J.Q. Richmond, A.E. Savage, K.R. Zamudio, E.B. Rosenblum, Toward immunogenetic studies of amphibian chytridiomycosis: linking innate and acquired immunity, Bioscience 59 (2009) 311–320.
[30] S.E. DuRant, W.A. Hopkins, A.K. Davis, L.M. Romero, Evidence of ectoparasite-induced endocrine disruption in an imperiled giant salamander, the Eastern hellbender (Cryptobranchus alleganiensis), J. Exp. Biol. 218 (2015) 2297–2304.
[31] M.J. Gavel, S.D. Richardson, R.L. Dalton, C. Soos, B. Ashby, L. McPhee, M. R. Forbes, S.A. Robinson, Effects of 2 neonicotinoid insecticides on blood cell profiles and corticosterone concentrations of wood frogs (Lithobates sylvaticus), Environ. Toxicol. Chem. 38 (2019) 1273–1284.
[32] E. Narayan, J.M. Hero, Urinary corticosterone responses and haematological stress indicators in the endangered Fijian ground frog (Platymantis vitiana) during transportation and captivity, Aust. J. Zool. 59 (2011 a) 79–85.
[33] R. Vasconcelos-Teixeira, S.C.M. Titon, B. Titon, M.L.M. Pompeo, F.R. Gomes, V. R. Assis, Stress response, immunity, and organ mass in toads (Rhinella diptycha) living in metal-contaminated areas, Biol. Trace Elem. Res. 200 (2022) 800–811.
[34] S.C.M. Titon, Junior B Titon, F.R. Gomes, V.R. Assis, Short-term stressors and corticosterone effects on immunity in male toads (Rhinella icterica): A neuroimmune-endocrine approach, Brain Behav. Immun. Health 13 (2021) 100230.
[35] S.N. Stuart, J.S. Chanson, N.A. Cox, B.E. Young, A.S.L. Rodrigues, D.L. Fischman, R. W. Waller, Status and trends of amphibian declines and extinctions worldwide, Science (1979) 306 (2004) 1783–1786.
[36] L.A. Brannelly, M.W.H. Chatfield, J. Sonn, M. Robak, C.L. Richards-Zawacki, Fungal infection has sublethal effects in a lowland subtropical amphibian population, BMC. Ecol. 18 (2018) 34.
[37] A.M. Kilpatrick, C.J. Briggs, P. Daszak, The ecology and impact of chytridiomycosis: an emerging disease of amphibians, Trends. Ecol. Evol. 25 (2010) 109–118.
[38] C.R. Gabor, M.C. Fisher, J. Bosch, A non-invasive stress assay shows that tadpole populations infected with Batrachochytrium dendrobatidis have elevated corticosterone levels, PLoS. One 8 (2013) e56054.
[39] C.R. Gabor, M.C. Fisher, J. Bosch, Elevated corticosterone levels and changes in amphibian behavior are associated with Batrachochytrium dendrobatidis (Bd) infection and Bd Lineage, PLoS. One 10 (2015) e0122685.
[40] C. Gabor, Z. Forsburg, J. Voros, C. Serrano-Laguna, J. Bosch, Differences in chytridiomycosis infection costs between two amphibian species from Central Europe, Amphibia-Reptilia 38 (2017) 250–256.
[41] C. Kindermann, E. Narayan, J. Hero, Does physiological response to disease incur cost to reproductive ecology in a sexually dichromatic amphibian species? Comparat. Biochem. Physiol. Part A -Mol. Integr. Physiol. 203 (2017) 220–226.
[42] J.D. Peterson, J.E. Steffen, L.K. Reinert, P.A. Cobine, A. Appel, L. Rollins-Smith, M. T. Mendonca, Host stress response is important for the pathogenesis of the deadly amphibian disease, chytridiomycosis, Litoria caerulea. Plos One 8 (2013) e62146.
[43] C. Gabor, S. Knutie, E. Roznik, J. Rhor, Are the adverse effects of stressors on amphibians mediated by their effects on stress hormones? Oecologia 186 (2018) 393–404.
[44] T. Hammond, P. Blackwood, S. Shablin, C. Richards-Zawacki, Relationships between glucocorticoids and infection with Batrachochytrium dendrobatidis in three amphibian species, Gen. Comp. Endocrinol. 285 (2020).
[45] J. Murone, J.A. DeMarchi, M.D. Venesky, Exposure to corticosterone affects host resistance, but not tolerance, to an emerging fungal pathogen, PLoS. One 11 (2016) e0163736.
[46] C.L. Searle, L.K. Belden, P. Du, A.R. Blaustein, Stress and chytridiomycosis: exogenous exposure to corticosterone does not alter amphibian susceptibility to a fungal pathogen, J. Exp. Zool. Part A-Ecol. Integr. Physiol. 321 (2014) 243–253.
[47] C.W. Fonner, S.A. Patel, S.M. Boord, M.D. Venesky, S.K. Woodley, Effects of corticosterone on infection and disease in salamanders exposed to the amphibian fungal pathogen Batrachochytrium dendrobatidis, Dis. Aquat. Organ. 123 (2017) 159–171.
[48] T.A. McMahon, B.F. Sears, M.D. Venesky, S.M. Bessler, J.M. Brown, K. Deutsch, N. T. Halstead, G. Lentz, N. Tenouri, S. Young, et al., Amphibians acquire resistance to live and dead fungus overcoming fungal immunosuppression, Nature 511 (2014) 224–227.
[49] D. DeNardo, Amphibians as laboratory animals, ILAR. J. 37 (1995) 173–181.
[50] J. Bliley, S. Woodley, The effects of repeated handling and corticosterone treatment on behavior in an amphibian (Ocoee salamander: Desmognathus ocoee), Physiol. Behav. 105 (2012) 1132–1139.
[51] A.W. Roe, K.L. Grayson, Repeated exposure to fluorescent powder does not affect survival or mass in Eastern red-spotted newts, *Notophthalmus viridescens*, Appl. Herpetol. 6 (2009) 295–299.
[52] P.A Ramírez, B.D. Bell, J.M. Germano, P.J. Bishop, N.J. Nelson, Tracking a small cryptic amphibian with fluorescent powders, New Zealand Jour. of Ecol. 41 (2017) 134–138.
[53] D.G. Boyle, D.B. Boyle, V. Olsen, J.A.T. Morgan, A.D. Hyatt, Rapid quantitative detection of chytridiomycosis (Batrachochytrium dendrobatidis) in amphibian samples using real-time Taqman PCR assay, Dis. Aquat. Organ. 60 (2004) 141–148.
[54] A.D. Hyatt, et al., Diagnostic assays and sampling protocols for the detection of Batrachochytrium dendrobatidis, Dis. Aquat. Organ. 73 (2007) 175–192.
[55] A.V. Longo, D. Rodriguez, D.D. Leite, L.F. Toledo, C.M. Almeralla, P.A. Burrowes, K.R. Zamudio, ITS1 copy number varies among Batrachochytrium dendrobatidis strains: implications for qPCR estimates of infection intensity from field-collected amphibian skin swabs, PLoS. One 8 (2013) e59499.
[56] K.M. Kriger, J.M. Hero, K.J. Ashton, Cost efficiency in the detection of chytridiomycosis using PCR assay, Dis. Aquat. Organ. 71 (2006) 149–154.
[57] E.J. Narayan, F.C. Molinia, J.F. Cockrem, J.M. Hero, Changes in urinary testosterone and corticosterone metabolites during short-term confinement with repeated handling in wild male cane toads (Rhinella marina), Aust. J. Zool. 59 (2011 b) 264–269.
[58] E. Narayan, J. Cockrem, J. Hero, Effects of temperature on urinary corticosterone metabolite responses to short-term capture and handling stress in the cane toad (Rhinella marina), Gen. Comp. Endocrinol. 178 (2012 a) 301–305.
[59] E. Narayan, F. Molinia, K. Christi, C. Morley, J. Cockrem, Urinary corticosterone metabolite responses to capture, and annual patterns of urinary corticosterone in wild and captive endangered Fijian ground frogs (Platymantis vitiana), Aust. J. Zool. 58 (2010 a) 189–197.
[60] E.J. Narayan, F.C. Molinia, C. Kindermann, J.F. Cockrem, J.M. Hero, Urinary corticosterone responses to capture and toe-clipping in the cane toad (Rhinella marina) indicate that toe-clipping is a stressor for amphibians, Gen. Comp. Endocrinol. 174 (2011 c) 238–245.
[61] E.J. Narayan, J.M. Hero, J.F. Cockrem, Inverse urinary corticosterone and testosterone metabolite responses to different durations of restraint in the cane toad (Rhinella marina), Gen. Comp. Endocrinol. 179 (2012 b) 345–349.
[62] C. Ramsay, J.R. Rohr, The application of community ecology theory to co-infections in wildlife hosts, Ecology. 102 (2021).
[63] C.W. Breuner, J.C. Wingfield, L.M. Romero, Diel rhythms of basal and stress-induced corticosterone in a wild, seasonal vertebrate, Gambel's white-crowned sparrow, J. Exp. Zool. 284 (1999) 334–342.
[64] E.J. Narayan, F.C. Molinia, K.S. Christi, C.G. Morley, J.F. Cockrem, Annual cycles of urinary reproductive steroid concentrations in wild and captive endangered Fijian ground frogs (Platymantis vitiana), Gen. Comp. Endocrinol. 166 (2010 b) 172–179.
[65] Z.R. Forsburg, C.B. Goff, H.R. Perkins, J.A. Robicheaux, G.F. Almond, C.R. Gabor, Validation of water-borne cortisol and corticosterone in tadpoles: recovery rate from an acute stressor, repeatability, and evaluating rearing methods, Gen. Comp. Endocrinol. 281 (2019) 145–152.
[66] T.T. Hammond, Z.A. Au, A.C. Hartman, C.L. Richards-Zawacki, Assay validation and interspecific comparison of salivary glucocorticoids in three amphibian species, Conserv. Physiol. 6 (2018).
[67] E.J. Narayan, Non-invasive reproductive and stress endocrinology in amphibian conservation physiology, Conserv. Physiol. 1 (2013).
[68] N.K. Bricker, R.E. Raskin, C.L. Densmore, Cytochemical and immunocytochemical characterization of blood cells and immunohistochemical analysis of spleen cells from 2 species of frog, Rana (Aquarana) catesbeiana and Xenopus laevis, Vet. Clin. Pathol. 41 (2012) 353–361.
[69] M.J. Forzan, T.G. Smith, R.V. Vanderstichel, N.S. Hogan, C.V. Gilroy, Hematologic reference intervals for Rana sylvatica (Lithobates sylvaticus) and effect of infection with Frog Virus 3 (Ranavirus sp., Iridoviridae), Vet. Clin. Pathol. 45 (2016) 430–443.
[70] S.S. Gervasi, E.G. Hunt, M. Lowry, A.R. Blaustein, Temporal patterns in immunity, infection load and disease susceptibility: understanding the drivers of host responses in the amphibian-chytrid fungus system, Funct. Ecol. 28 (2014) 569–578.
[71] K.E. Pereira, M.C. Bletz, J.A. McCartney, D.C. Woodhams, S.K. Woodley, Effects of exogenous elevation of corticosterone on immunity and the skin microbiome of eastern newts (Notophthalmus viridescens), Philos. Trans. R. Soc. B 378 (2023) 20220120.
[72] R Development Core Team (2021) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
[73] Hartig F (2021) DHARMa: residual diagnostics for hierarchical (multi-level /mixed) regression models. R package version 0.4.4.
[74] M. Brooks, K.K. Kristensen, K.J. van Benthem, A. Magnusson, C.W.B. Berg, A. Nielsen, H.J. Skaug, M. Maechler, B.M. Bolker, {glmmTMB} balances speed and

flexibility among packages for zero-inflated generalized linear mixed modeling, R. J. (2017) 378–400.
[75] Therneau TeM (2022) coxme: mixed effects Cox models.
[76] G.E. Demas, E.D. Carlton, Ecoimmunology for psychoneuroimmunologists: Considering context in neuroendocrine-immune-behavior interactions, Brain Behav. Immun. 44 (2015) 9–16.
[77] A. Fernandez-Loras, S. Fernandez-Beaskoetxea, E. Arriero, M.C. Fisher, J. Bosch, Early exposure to Batrachochytrium dendrobatidis causes profound immunosuppression in amphibians, Eur. J. Wildl. Res. 63 (2017).
[78] R.N. Harris, R.M. Brucker, J.B. Walke, M.H. Becker, C.R. Schwantes, D.C. Flaherty, B.A. Lam, D.C. Woodhams, C.J. Briggs, V.T. Vredenburg, et al., Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus, ISMe J. 3 (2009) 818–824.
[79] W.M. Holden, L.K. Reinert, S.M. Hanlon, M.J. Parris, L.A. Rollins-Smith, Development of antimicrobial peptide defenses of southern leopard frogs, Rana sphenocephala, against the pathogenic chytrid fungus, Batrachochytrium dendrobatidis, Dev. Comparat. Immunol. 48 (2015) 65–75.
[80] Rollins-Smith LA, The importance of antimicrobial peptides (AMPs) in amphibian skin defense, Dev. Comparat. Immunol. 142 (2023) 104657.
[81] D.C. Woodhams, V.T. Vredenburg, M.A. Simon, D. Billheimer, B. Shakhtour, Y. Shyr, C.J. Briggs, L.A. Rollins-Smith, R.N. Harris, Symbiotic bacteria contribute to innate immune defenses of the threatened mountain yellow-legged frog, Rana muscosa, Biol. Conserv. 138 (2007) 390–398.
[82] J.S. Fites, J.P. Ramsey, W.M. Holden, S.P. Collier, D.M. Sutherland, L.K. Reinert, A. S. Gayek, T.S. Dermody, T.M. Aune, K. Oswald-Richter, et al., The invasive chytrid fungus of amphibians paralyzes lymphocyte responses, Science (1979) 342 (2013) 366–369.
[83] F. Clare, O. Daniel, T. Garner, M. Fisher, Assessing the ability of swab data to determine the true burden of infection for the amphibian pathogen Batrachochytrium dendrobatidis, EcoHealth 13 (2016) 360–367.
[84] A.E. Savage, K.A. Terrell, B. Gratwicke, N.M. Mattheus, L. Augustine, R. C. Fleischer, Reduced immune function predicts disease susceptibility in frogs infected with a deadly fungal pathogen, Conserv. Physiol. 4 (2016).
[85] K. Kaiser, J. Devito, C.G. Jones, A. Marentes, R. Perez, L. Umeh, R.M. Weickum, K. E. McGovern, E.H. Wilson, W. Saltzman, Reproductive and immune effects of chronic corticosterone treatment in male White's treefrogs, Litoria caerulea, Conserv. Physiol. 3 (2015).
[86] J.M. Andrews, J.N. Childress, T.J. Iakovidis, G.J. Langford, Elucidating the life history and ecological aspects of Allodero hylae (Annelida: Clitellata: Naididae), a parasitic oligochaete of invasive Cuban tree frogs in Florida, J. Parasitol. 101 (2015) 275–281.
[87] R. Brenes, D.L. Miller, T.B. Waltzek, R.P. Wilkes, J.L. Tucker, J.C. Chaney, R. H. Hardman, M.D. Brand, R.R. Huether, M.J. Gray, Susceptibility of fish and turtles to three ranaviruses isolated from different ectothermic vertebrate classes, J. Aquat. Anim. Health 26 (2014) 118–126.
[88] A. Chambouvet, D.J. Gower, M. Jirku, M.J. Yabsley, A.K. Davis, G. Leonard, F. Maguire, T.M. Doherty-Bone, G.B. Bittencourt-Silva, M. Wilkinson, et al., Cryptic infection of a broad taxonomic and geographic diversity of tadpoles by Perkinsea protists, Proc. Natl. Acad. Sci. u S. a 112 (2015) E4743–E4751.
[89] N. Galt, M.S. Atkinson, B.M. Glorioso, J.H. Waddle, M. Litton, A.E. Savage, Widespread Ranavirus and Perkinsea infections in Cuban treefrogs (Osteopilus septentrionalis) invading New Orleans, USA, Herpetol. Conserv. Biol. 16 (2021) 17–29.
[90] A. Vermeulen, M. Eens, E. Zaid, W. Müller, Baseline innate immunity does not affect the response to an immune challenge in female great tits (Parus major), Behav. Ecol. Sociobiol. 70 (2016) 585–592.
[91] Wells KD (2010) The ecology and behavior of amphibians. University of Chicago press.
[92] J.L. Lewis, A.M. Sullivan, Salamander stress and duress: the relationship between CORT, autotomy and regeneration, and exploratory behaviour, Zoology 139 (2020).
[93] T.J. Hossie, B. Ferland-Raymond, G. Burness, D.L. Murray, Morphological and behavioural responses of frog tadpoles to perceived predation risk: a possible role for corticosterone mediation? Ecoscience 17 (2010) 100–108.
[94] J. Belliure, J. Clobert, Behavioral sensitivity to corticosterone in juveniles of the wall lizard, Podarcis muralis, Physiol. Behav. 81 (2004) 121–127.
[95] J.A. Marino, M.P. Holland, J.M. Maher, Predators and trematode parasites jointly affect larval anuran functional traits and corticosterone levels, Oikos. 123 (2014) 451–460.