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Relationships between glucocorticoids and infection with *Batrachochytrium dendrobatidis* in three amphibian species

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ABSTRACT

It is often hypothesized that organisms exposed to environmental change may experience physiological stress, which could reduce individual quality and make them more susceptible to disease. Amphibians are amongst the most threatened taxa, particularly in the context of disease, but relatively few studies explore links between stress and disease in amphibian species. Here, we use the fungal pathogen *Batrachochytrium dendrobatidis* (Bd) and amphibians as an example to explore relationships between disease and glucocorticoids (GCs), metabolic hormones that comprise one important component of the stress response. While previous work is limited, it has largely identified positive relationships between GCs and Bd-infection. However, the causality remains unclear and few studies have integrated both baseline (GC release that is related to standard, physiological functioning) and stress-induced (GC release in response to an acute stressor) measures of GCs. Here, we examine salivary corticosterone before and after exposure to a stressor, in both field and captive settings. We present results for Bd-infected and uninfected individuals of three amphibian species with differential susceptibilities to this pathogen (*Rana catesbeiana*, *R. clamitans*, and *R. sylvatica*). We hypothesized that prior to stress, baseline GCs would be higher in Bd-infected animals, particularly in more Bd-susceptible species. We also expected that after exposure to a stressor, stress-induced GCs would be lower in Bd-infected animals. These species exhibited significant interspecific differences in baseline and stress induced corticosterone, though other variables like sex, body size, and day of year were usually not predictive of corticosterone. In contrast to most previous work, we found no relationships between Bd and corticosterone for two species (*R. catesbeiana* and *R. clamitans*), and in the least Bd-tolerant species (*R. sylvatica*) animals exhibited context-dependent differences in relationships between Bd infection and corticosterone: Bd-positive *R. sylvatica* had significantly lower baseline and stress-induced corticosterone, with this pattern being stronger in the field than in captivity. These results were surprising, as past work in other species has more often found elevated GCs in Bd-positive animals, a pattern that aligns with well-documented relationships between chronically high GCs, reduced individual quality, and immunosuppression. This work highlights the potential relevance of GCs to disease susceptibility in the context of amphibian declines, while underscoring the importance of characterizing these relationships in diverse contexts.

1. Introduction

Wild organisms face multiple, interacting environmental challenges that have resulted in population declines for many species (Blaustein et al., 2011; Brook et al., 2008; Hayes et al., 2010). In some taxa, declines have been exacerbated by emerging infectious diseases, which have contributed to global losses of biodiversity (Hatcher et al., 2012; Skerratt et al., 2007; Thogmartin et al., 2013). One hypothesis that explains the recent onslaught of disease-related declines is that exposure to multiple environmental stressors may reduce individual quality and increase disease susceptibility (Aguirre and Tabor, 2008; Blaustein et al., 2011; Carey et al., 1999; Hing et al., 2016; Kiesecker, 2011; Rollins-Smith, 2017; Tompkins et al., 2015). This hypothesis has substantial, theoretical support. Glucocorticoids (GCs), for example, are metabolic hormones that increase when organisms experience energetic costs and threats to homeostasis and are one of the more commonly employed measure of "stress" (Sapolsky et al., 2000).

GCs increase in response to numerous types of anthropogenic environmental change (Busch and Hayward, 2009) and there are strong links between GCs and the immune system. Chronically elevated GCs

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are usually thought to be immunosuppressive (McEwen, 1997; Padgett and Glaser, 2003), while acute elevations in GCs (increases in circulating GCs in response to certain transient, stressful events) can sometimes temporarily enhance immune function (Dhabhar, 2000, 2009). For these reasons, it makes sense to develop separate hypotheses about how baseline (GC release that is related to standard, physiological functioning, Bonier et al., 2009) vs. stress-induced (elevated in response to an acute stressor) GCs may influence or respond to infection. Relationships between GCs and infection are complicated because, in addition to modulating susceptibility to disease, GCs can themselves be stimulated by inflammatory and immune responses (Chrousos, 1995; Sternberg, 2006). Moreover, because the hypothalamic-pituitary-adrenal/interrenal axis functions as a negative feedback loop in which GCs downregulate the hypothalamus and pituitary, in some situations animals experiencing chronic stress may have inhibited acute stress responses (Busch et al., 2008; Müller et al., 2009), and can also exhibit lower baseline GCs (Barton et al., 1987; Cyr and Romero, 2007; Rich and Romero, 2005; Torres-Medina et al., 2018).

A large body of literature experimentally characterizes relationships between GCs and immune function, but fewer studies explicitly test whether these relationships hold true in the context of wildlife diseases and declines (Hing et al., 2016). Because they are one of the most threatened taxonomic groups and are also particularly vulnerable to both environmental change and emerging infectious diseases, amphibians provide a valuable system for exploring these relationships (Skerratt et al., 2007; Vredenburg et al., 2010). *Batrachochytrium dendrobatidis* (Bd) is a fungal pathogen that threatens hundreds of amphibian species (Scheele et al., 2019). To date, a relatively small number of studies have explicitly characterized relationships between infection with Bd and GCs in amphibians (Table 1). Bd-positive amphibians tend to exhibit increased GCs, but results are variable, likely due to the diversity of species and life stages that have been examined. Studies that explore correlations between Bd infection and GCs or that experimentally infect animals with Bd and measure GCs largely identify positive relationships between Bd infection and GCs (Table 1). The few studies that experimentally manipulate GCs and then measure Bd susceptibility have inconsistent findings (Table 1). Altogether, previous work provides more evidence for Bd inducing increased GCs than for increased GCs making animals more susceptible or less tolerant to Bd. Previous work on Ranavirus, another amphibian pathogen, has also documented mixed results regarding the relationships between GCs and infection (Crespi et al., 2015; Kirschman et al., 2018; Reeve et al., 2013; Warne et al., 2011).

Here, we add to the body of literature exploring links between amphibian disease and GCs in amphibians by characterizing both baseline and stress-induced corticosterone (the primary GC hormone in amphibians) in both free-living and captive, Bd-positive and Bd-negative adults of three ranid frogs: bullfrogs (*Rana catesbeiana*), green frogs (*R. clamitans*), and wood frogs (*R. sylvatica*). These congeners exhibit differential tolerances to Bd infection, with *R. catesbeiana* showing low mortality, *R. clamitans* showing strain-dependent intermediate levels of Bd-induced mortality, and *R. sylvatica* showing the most consistent and greatest Bd-associated mortality of these three species (Bradley et al., 2015; Gahl et al., 2012; Searle et al., 2011). While Bd tolerance can vary intra-specifically across populations (Bradley et al., 2015), *R. catesbeiana* is generally thought to be a tolerant, reservoir species (Schloegel et al., 2010), and *R. sylvatica* has been used as a model susceptible species (Bradley et al., 2015; Eskew et al., 2018). The susceptibility/tolerance of *R. clamitans* to Bd is less well-studied, though it appears to be tolerant to at least one Bd strain from the northeast, and susceptible to a Panamanian strain (Gahl et al., 2012). We are not aware of any Bd-induced mass mortality events in the focal species, or of any declines in the wild that have been attributed to Bd in the focal species. Thus, one additional goal of this study was to characterize potential subtle, physiological changes associated with sublethal Bd-infection, even when the disease is asymptomatic.

Because chronic infection is known to increase baseline GCs, chronically increased GCs are known to be immunosuppressive, and high baseline GCs inhibit the hypothalamic pituitary axis, we hypothesized that Bd-positive individuals would exhibit elevated baseline corticosterone and an inhibited acute stress response. We also predicted that these patterns would be stronger in the least Bd-tolerant species, *R. sylvatica*, and weaker in the most Bd-tolerant species, *R. catesbeiana*. We also expected that corticosterone would vary with species, sex, and animal size, and that patterns might differ depending on context (field vs. captive).

2. Methods

2.1. Study species & sites

Adult frogs were captured by hand at night (~2100–0100 h) between March-August 2018 from 14 ponds near the Pymatuning Laboratory of Ecology, the University of Pittsburgh's biological field station, in Linesville, PA. Average distance between field sites was 8.07 ± 0.99 km (mean ± S.E.) and sites were very similar in their

Table 1
Summary of published studies examining relationships between Bd and GCs in amphibians.

Citation	Study Type*	GC Type	Species	Age Class	Result**
Fonner et al. (2017)	E_{GC}	Plasma	*Plethodon shermanii*	adults	+(n = 1)
Gabor et al. (2013)	C	Water-borne	*Alytes muletensis, A. obstetricans*	tadpoles	+(n = 2)
Gabor et al. (2015)	E_{Bd}, C	Water-borne	*A. muletensis, A. obstetricans*	tadpoles, juveniles	+(E_{Bd}, n = 1) +(C, n = 1)
Gabor et al. (2017)	E_{Bd}	Water-borne	*Bombina variegata, Hyla arborea*	tadpoles	+(n = 2)
Gabor et al. (2018)	E_{Bd}, E_{GC}	Water-borne	*Osteopilus septentrionalis*	tadpoles, juveniles	+(E_{Bd}, n = 1) −(E_{GC}, n = 1) NR (E_{GC}, n = 1)
Kindermann et al. (2012)	C	Urinary	*Litoria wilcoxii*	adults	+(n = 1)
Kindermann et al. (2017)	C	Urinary	*L. wilcoxii*	adults	+(n = 1)
Murone et al. (2016)	E_{GC}	Water-borne	*Anaxyrus americanus*	juveniles	−(n = 1)
Peterson et al. (2013)	C	Plasma	*L. caerulea*	adults	+(n = 1)
Searle et al. (2014)	E_{Bd}, E_{GC}	Whole Body	*A. boreas,* *Rana cascadae,* *R. catesbeiana*	tadpoles, juveniles	+(E_{Bd}, n = 3) −(E_{Bd}, n = 1) NR (E_{GC}, n = 4)

* C: correlational; E_{Bd}: Experimental – Bd manipulated; E_{GC}: Experimental – GCs manipulated

** +: Bd and GCs positively related; –: Bd and GCs negatively related; NR: no relationship. For papers with more than one study type, results are presented as the number of experiments finding a given result (+/–/NR) for each study type. Numbers larger than one indicate that more than one species or life stage had the same result for a given study type.

proximity to human activity and other environmental parameters; 12 of the 14 sites are ponds that originate from the same lake (Lake Pymatuning). Adult animals of both sexes were used in this study, and individuals of each species were captured primarily at the height of their breeding season (*R. sylvatica*: March-April; *R. catesbeiana* and *R. clamitans*: June-August). In general, males were calling at the time of capture; because individuals often stopped calling or fled as soon as human observers approached it was not possible to collect data on whether a given individual was or was not calling. It was not known whether female individuals had recently laid eggs or not. Bd prevalence for *R. catesbeiana* and *R. clamitans* at these study sites is highest (~50–80%) during the spring, but is still prevalent (~30–50%) during the summer (unpublished data). For *R. sylvatica* at the study sites, Bd prevalence is ~20% in the spring; prevalence in the summer is less certain, as it can be difficult to find this species outside of their spring breeding season at the study sites (unpublished data). Prevalence during the winter, when these species are hibernating, has not been measured. A fresh pair of nitrile gloves was used to handle each captured individual, and established methods were followed to avoid contamination between individuals (Phillott et al., 2010).

In captivity, animals were housed individually in plastic tanks (14 1/2″ L × 8 3/4″ W × 9 3/4″ H Large Rectangle, Critter Keeper). Room temperature was adjusted to stay near the range of local ambient temperatures, however, the facility tended to be warmer than average outdoor temperatures in spring and cooler than outdoor temperatures in summer given limitations of our shared-use facility. Specifically, in April outdoor temperatures (average low – average high) were 2–19 °C while the facility was 13–16 °C; in May outdoor temperatures were 8–19 °C while the facility was 16–21 °C, during June outdoor temperatures were 13–24 °C while the facility was 17–21 °C, and during July and August outdoor temperatures were 16–27 °C while the facility was 20–23 °C. Water was changed weekly and individuals were fed crickets biweekly. Animals were not fed on days of saliva sample collection. All applicable institutional and national guidelines for the care and use of animals were followed (IACUC protocol #s 17081291, 18052950; Pennsylvania Fish and Boat Commission Scientific Collector's Permit #2018-01-0078).

2.2. Bd testing

Animals were swabbed for *Bd* upon capture in the field using a fine-tipped swab (MW113; Medical Wire & Equipment Co.), which was rubbed five times down the dorsal, ventral and lateral sides, each hand and foot, and each leg. No study animals displayed obvious symptoms of Bd infection (despite relatively high infection intensities; see Results). After collection, swabs were frozen at −20 C until the time of analysis. DNA was extracted from samples and a quantitative polymerase chain reaction (qPCR) assay was used to detect and quantify Bd. The Qiagen DNeasy Blood and Tissue Kit was used to extract genomic DNA from swabs using the manufacturer's protocol for animal tissue with the following modifications: swabs were incubated for 30 min, vortexed and spun in a centrifuge, and then incubated for another 30 min. Samples were eluted two times with 100 µL of elution buffer for a final elution volume of 200 µL. Our qPCR assay generally followed the methods of Boyle et al. (2004), but included an internal positive control (Hyatt et al., 2007) and bovine serum albumin (final concentration 400 ng/µL, Garland et al., 2010) in each reaction well. Each qPCR run included positive and negative controls and a seven-fold dilution series of plasmid-based Bd standards (Pisces Molecular, Boulder CO). Each extract was tested once (singlicate) to maximize cost efficiency (Kriger et al., 2006). Samples that exhibited > 1 DNA copy were considered positive. Bd load per 5 µL reaction volume was converted to whole-swab loads, and these values were log-transformed before analysis.

2.3. Field stress studies

Immediately upon capture in the field, a timer was started and the time was recorded. Each individual's mouth was opened with a sterile pipette tip and one quarter of a saliva swab (SalivaBio Infant's Swab; Salimetrics) was inserted into the mouth for one minute. Time between capture and first sampling was recorded—the average time was 86.7 ± 3.8 s (mean ± S.E.). The animal was then placed in a sterile Ziploc bag for holding at ambient temperature and 30 min after capture another swab was collected in the same manner. This sampling point was selected based on previous work demonstrating that salivary corticosterone in the focal species peaked ~30 min after initiation of stress (Hammond et al., 2018). After collection, saliva swabs were stored in microcentrifuge tubes and frozen at −20 C until assay. Saliva was not collected from animals with food in their mouths, as this is known to alter salivary GC concentrations (Gibson et al., 1999).

2.4. Captive stress studies

A subset of captured individuals was transported to the captive animal housing facilities at the Pymatuning Laboratory of Ecology and was given 7–10 days to adjust to captivity before being exposed to an adrenocorticotrophic hormone (ACTH) challenge. Between 1000 and 1400 h, animals were removed from their tanks and a baseline saliva sample was collected from each individual, immediately after which ACTH (Sigma Aldrich A0298, 250 µg dissolved in 2 mL of 0.9% saline solution) was administered at 0.45 µg ACTH/g body mass (Graham et al., 2013; Narayan et al., 2011) using an intraperitoneal injection low on the ventral side. Body masses (mean ± S.E.) for the study individuals were 66.9 ± 6.73 g for *R. catesbeiana*, 31.7 ± 1.5 g for *R. clatimans*, and 13.0 ± 0.8 g for *R. sylvatica*. This resulted in injection volumes that generally ranged between ~0.04–0.25 mL, depending on size. Additional saliva samples were collected at 30 min and 4 h after injection.

2.5. Salivary corticosterone sample preparation & assay

Samples were prepared based on the protocol in Hammond et al. (2018). Briefly, after freezing, saliva was spun out of swabs at 7000 rpm for 10 min and the resulting volume was quantified; this was the saliva volume used for concentration calculations. Swabs were then washed with 120 µl of assay buffer and spun again at the same settings, and wash was quantified and combined with original sample. This mixture was treated with trichloroacetic acid (TCA) at 20% of the saliva volume to precipitate out interfering proteins. Samples were then vortexed, incubated at room temperature for 15 min, spun at 6000 rpm for 8 min, and the supernatant was collected and assayed the same day in duplicate using an enzyme immunoassay kit for corticosterone (KGE009; R&D Systems). Plates were read with a microplate spectrophotometer (Epoch; BioTek). The only difference between the methods used here versus in Hammond et al. (2018) is that the dilution step occurred during rather than after spinning out samples, in the form of a wash step. This was found to increase the yield of corticosterone from saliva swabs. Any samples contaminated with notable blood were not assayed, meaning that some individuals did not have data for certain time points. Intra- and inter-assay coefficients of variation (CVs) were 9.4% and 9.9% (manufacturer reported CVs: 6.1% and 6.2%). This assay was previously validated for *R. clamitans* and *R. catesbeiana* (Hammond et al., 2018) and the same analytical and biological validation experiments were conducted for *R. sylvatica* (see electronic supplementary data).

2.6. Statistics

All analyses were conducted in the R programming environment (R Core Team, 2017). Corticosterone concentrations were log-transformed

Table 2
Results from a model averaged GLMM testing for effects of species and other variables on corticosterone in both field and captive settings; significant terms are bolded.

	Estimate	Adjusted S.E.	Z value	P value	Relative Importance
(Intercept)	**2.77**	**0.06**	**48.0**	**< 0.00001**	
Species (*R. clamitans*)	**0.15**	**0.06**	**2.65**	**0.008**	1.00
Species (*R. sylvatica*)	**0.3**	**0.08**	**5.00**	**< 0.00001**	
Time point (30 min)	**0.63**	**0.06**	**10.29**	**< 0.00001**	1.00
Time point (240 min)	**0.22**	**0.08**	**2.87**	**0.004**	
Species (*R. cl*) * Time point (30)	**0.29**	**0.06**	**4.58**	**< 0.00001**	1.00
Species (*R. sy*) * Time point (30)	−0.10	0.07	1.38	0.17	
Species (*R. cl*) * Time point (240)	0.06	0.09	0.66	0.51	
Species (*R. sy*) * Time point (240)	0.08	0.11	0.73	0.47	
Setting (field)	**0.11**	**0.04**	**2.67**	**0.008**	1.00
Setting (field) * Time point (30)	**−0.26**	**0.06**	**4.63**	**< 0.00001**	1.00
Bd load	0.05	0.03	1.89	0.06	0.62
Bd load * Species (*R. cl*)	−0.03	0.04	0.72	0.47	0.62
Bd load * Species (*R. sy*)	**−0.21**	**0.05**	**4.59**	**< 0.00001**	
SVL	−0.05	0.03	1.90	0.06	0.08
		S.D.	X^2	P value	
Site		0.07	2.52	0.11	
Individual		**0.16**	**22.02**	**< 0.00001**	

prior to analyses in order to meet assumptions for models. For all models, continuous variables were mean-rescaled prior to analysis to allow for comparisons of effect size. For each model a model averaging approach was used to account for uncertainty and model selection bias (Gruner et al., 2017; McDermott Long et al., 2017; Thiele and Markussen, 2012). A full model containing all the below listed variables was first constructed using the lmer function in the lme4 package (Bates et al., 2007). From this model, a set of all possible sub-models (containing all combinations of variables) was constructed using the dredge function of the MuMIn package in R (Barton, 2009). All models with an AICc value (the change in the Akaike information criterion, adjusted for sample size) that differed by less than four from the best ranked model were included in model averaging, which was conducted using the model.avg function. Significance of random effects was assessed using a likelihood ratio test. All models were validated using graphical and statistical tests in the package 'DHARMa' (Hartig, 2019). Our presentation of the model results focuses on variables in the final, model-averaged model.

The models were as follows. First, a GLMM was constructed to test for interspecific differences in corticosterone and for general patterns across all species. This model contained log-transformed corticosterone values as the response variable, and setting (field or captive), time point (0 min, 30 min, or 240 min, treated as a factor because the relationship between time and corticosterone was expected to be non-linear), an interaction between setting and time point, Bd load, an interaction term between Bd load and setting, an interaction term between Bd load and time point, sex, snout-to-vent length (SVL), and day of year as fixed effects. It also contained interactions between species and the following variables: Bd load, setting, and time point. Individual identity and collection site were included as random effects. Second, separate GLMMs were constructed for each species using all of the same variables except for the interaction terms involving species. Model averaging was then applied to each of these four models (all species, *R. catesbeiana* only, *R. clamitans* only, and *R. sylvatica* only) as described above.

Mass and a body condition score (mass divided by SVL) were also tested in models instead of SVL, but SVL was found to explain the most variance. GLMMs were also constructed that contained as response variables the difference between stress-induced and baseline corticosterone concentrations (the magnitude of the stress response), and for captive studies the difference between samples collected at baseline and those collected four hours later (the magnitude of recovery), however, the predictors of interest had less explanatory power in these models therefore these models were not focused on for the purposes of this study. Hour of capture and delay time to the first saliva sample were not included in GLMMs due to co-variance issues with fixed effects that were of primary interest (hour of collection co-varied with setting because in the field samples were collected at night and in captivity samples were collected during the day; the delay between capture and first sampling co-varied with sampling time point). However, for each species correlation tests were conducted to test for relationships between baseline corticosterone and hour of sampling, and between baseline corticosterone and the delay time between capture and sampling. No significant relationships were found for any species (Kendall's rank correlation tau, all $p > 0.12$; Table S3). Because of aforementioned co-variance issues, which are problematic for GLMMs, and because no significant correlations were found, these variables (hour of sample collection and delay between capture and sampling) were not included as fixed effects in GLMM averaging.

3. Results

3.1. Relationships between corticosterone and phenotypic and environmental traits

Across settings there were significant species-related differences in corticosterone concentrations. In general, *R. catesbeiana* ($n = 59$ F/16 M) had significantly lower baseline and stress-induced corticosterone than *R. clamitans* ($n = 71$ F/31 M) and *R. sylvatica* ($n = 6$ F/46 M; Tables 2 and S4; Fig. 1). Sex, SVL, and collection date did not explain significant variation in corticosterone in the field or in captivity when looking at all species together (Table 2), but when modelling exclusively data from *R. catesbeiana* there was a significant effect of SVL and date: larger *R. catesbeiana* exhibited lower corticosterone concentrations, and later in the season *R. catesbeiana* exhibited lower corticosterone concentrations (Table 3). Corticosterone of *R. clamitans* and *R. sylvatica* did not appear to be predicted by SVL, or date (Tables 4 and 5). SVLs (mean ± S.D.) for each species were 93.7 ± 20.0 mm for *R. catesbeiana* (range: 55.9–140.2 mm), 70.4 ± 9.4 mm for *R. clamitans* (range: 34.7–92.5 mm), and 48.8 ± 5.0 mm for *R. sylvatica* (range: 27.0–56.9 mm). There was no significant effect of sex in any species, but the *R. sylvatica* studied were disproportionately male, so it is difficult to draw any conclusions about sex in this species.

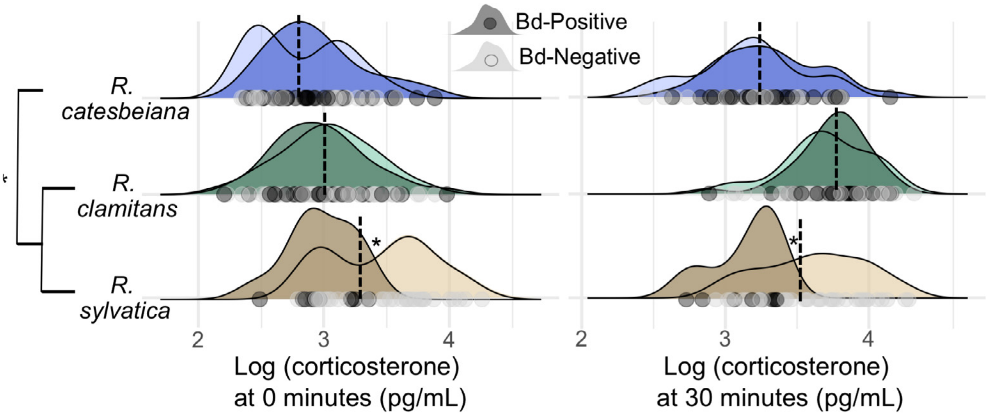

Fig. 1. Density plots showing corticosterone concentrations in the field for Bd positive and Bd negative animals at baseline (left) and after handling stress (right). Values are shown for *R. catesbeiana* (blue, top), *R. clamitans* (green, middle), and *R. sylvatica* (brown, bottom). For each species Bd positive animals are shown in the darker shade and Bd-negative animals are shown in the lighter shade. Dotted lines indicate average corticosterone concentrations for each species at each time point. Asterices indicate significant differences in corticosterone concentrations.

Table 3
Results from a model averaged GLMM testing for effects of Bd and other environmental and phenotypic variables on FGM levels in both field and captive settings in *R. catesbeiana*; significant terms are bolded.

	Estimate	Adjusted S.E.	Z value	P value	Relative Importance
(Intercept)	**2.95**	**0.08**	**37.2**	**< 0.00001**	
Time point (30 min)	**0.46**	**0.05**	**8.54**	**< 0.00001**	1.00
Time point (240 min)	0.12	0.08	1.54	0.12	
SVL	**−0.09**	**0.03**	**3.18**	**0.002**	0.70
Date	**−0.13**	**0.05**	**2.86**	**0.004**	0.67
		S.D.	X^2	P value	
Site		0.10	2.34	0.13	
Individual		0.00	0	1	

Table 4
Results from a model averaged GLMM testing for effects of Bd and other environmental and phenotypic variables on FGM levels in both field and captive settings in *R. clamitans*; significant terms are bolded.

	Estimate	Adjusted S.E.	Z value	P value	Relative Importance
(Intercept)	**2.95**	**0.05**	**58.0**	**< 0.00001**	
Time point (30 min)	**0.76**	**0.04**	**19.8**	**< 0.00001**	1.00
Time point (240 min)	**0.20**	**0.06**	**3.68**	**0.0002**	
Date	0.09	0.06	1.54	0.12	0.15
		S.D.	X^2	P value	
Site		0.11	3.24	0.07	
Individual		**0.12**	**4.88**	**0.03**	

3.2. Corticosterone in the field and in captivity

There were significant differences in corticosterone in captive versus field settings (Table 2; Fig. 2). Specifically, across all species (Table 2) but most evidently in *R. sylvatica* (Table 5), there was a pattern of lower baseline corticosterone in the field than in captivity, and higher stress-induced corticosterone concentrations in response to ACTH in captivity than in response to handling in the field (Figs. 2 and 3).

3.3. Relationships between Bd and GCs in the field and in captivity

For Bd-positive animals in the field, the number of Bd DNA copies per swab (mean ± S.E.) was 126910 ± 1625 for *R. catesbeiana*, 2343 ± 18 for *R. clamitans*, and 22952 ± 324 for *R. sylvatica*. Baseline and stress-induced corticosterone concentrations did not significantly vary with Bd load in field or captive settings for *R. catesbeiana* (*n* = 35 Bd-positive and 28 Bd-negative in the field; *n* = 13 Bd-positive and 15 Bd-negative in captivity) or for *R. clamitans* (*n* = 32 Bd-positive and 59 Bd-negative in the field; *n* = 14 Bd-positive and 37 Bd-negative in captivity; Tables 3 and 4; Figs. 1–3). In captivity, no species showed any significant differences in 4-hour-post-stress corticosterone between Bd-positive and Bd-negative animals (Tables 3-5).

In the field and in captivity, *R. sylvatica* individuals with higher Bd loads exhibited significantly lower corticosterone concentrations than uninfected/less infected animals (*n* = 10 Bd-positive and 34 Bd-negative in the field; *n* = 11 Bd-positive and 19 Bd-negative in captivity; Table 5; Figs. 1–3). Both baseline and stress-induced corticosterone concentrations were lower in more Bd infected individuals. The effect of Bd was most evident at baseline in the field, and was less evident during stress

4. Discussion

Our results document species- and context-dependent differences in the relationships between Bd infection and corticosterone concentrations in three congeneric, co-occurring amphibian species. While most previous work has documented positive relationships between Bd infection and GCs, we found no relationships between Bd infection and corticosterone in two species (*R. catesbeiana* and *R. clamitans*), and largely negative relationships in *R. sylvatica*, such that Bd-infected animals of this species tended to have lower corticosterone concentrations. These relationships were to some extent dependent on setting and on whether baseline or stress-induced corticosterone were measured.

4.1. Environmental and phenotypic traits related to corticosterone

There were significant differences in corticosterone between species, with *R. catesbeiana* exhibiting lower salivary corticosterone concentrations than *R. clamitans* and *R. sylvatica*. Studies of these species' plasma GCs have identified similar interspecific patterns, though little work has focused on *R. clamitans* in this context (Crespi et al., 2015; Falso et al., 2015; Hall et al., 2017 Mendonça et al., 1985; Stephens and Mccurdy, 2008). More in-depth examinations of stress physiology in these species (e.g. receptors, binding proteins) is required to understand interspecific differences. Sex was not predictive of corticosterone in any species, though in *R. sylvatica* the sample size of females was extremely limited. All species were studied during their reproductively active periods so this was somewhat surprising given differences between male and female reproductive behaviors in these species (males spend large amounts of energy vocalizing to attract mates, and are generally thought to experience high glucocorticoid levels during this period, Emerson, 2001).

Table 5
Results from a model averaged GLMM testing for effects of Bd and other environmental and phenotypic variables on FGM levels in both field and captive settings in *R. sylvatica*; significant terms are bolded.

	Estimate	Adjusted S.E.	Z value	P value	Relative Importance
(Intercept)	**3.06**	**0.11**	**28.4**	**< 0.00001**	
Bd load	**−0.15**	**0.05**	**3.02**	**0.003**	1.00
Time point (30 min)	**0.73**	**0.08**	**9.03**	**< 0.00001**	1.00
Time point (240 min)	**0.41**	**0.09**	**4.80**	**< 0.00001**	
Setting (field)	**0.23**	**0.08**	**2.99**	**0.003**	1.00
Time point (30 min) * Setting (field)	**−0.56**	**0.10**	**5.57**	**< 0.00001**	1.00
Bd load * Setting (field)	**−0.11**	**0.05**	**2.13**	**0.03**	0.25
SVL	−0.10	0.18	0.53	0.59	0.10
		S.D.	X^2	P value	
Site		0.06	0	1	
Individual		**0.23**	**22.15**	**< 0.00001**	

In the field, corticosterone concentrations tended to be higher at baseline and lower during stress in comparison to captivity. There were differences between field and captive contexts that help explain this: sample collection took place at night in the field, and during the day in captivity, and a handling stressor was used in the field while an ACTH stressor was used in captivity. Additionally, while we did not find any significant correlations between baseline salivary corticosterone and delay to first sampling (the time between initiation of handling and insertion of the swab into the animal's mouth), there were positive patterns for all species (Table S3). Increased baseline corticosterone in the field in comparison to captivity could be related to the increased sampling delay time in the field, when logistics can make it more challenging to rapidly insert the swab into the animal's mouth. Future studies incorporating salivary corticosterone should control for sampling delay times between settings; even a 30–60 s difference could contribute to altered salivary corticosterone concentrations, which appear to change along timelines that are more similar to plasma corticosterone than to fecal or urinary corticosterone. Differences between field and captive settings were evident across all species, but appeared to be significantly stronger in *R. sylvatica*. The magnified difference between field and captive settings in *R. sylvatica* could be related to the larger temperature differences in field vs. captive settings for this species: because *R. sylvatica* breeds at the very start of spring and we were working in an indoor, shared-use, captive facility, it was not possible to maintain the facility at temperatures as low as outdoor low temperatures during this period (though temperatures were within the outdoor ambient temperature range). Also, samples were collected during reproduction for all study species, but in contrast to the other focal species, *R. sylvatica* are explosive breeders. Explosive breeding behavior likely results in higher energy requirements, which could cause elevated baseline corticosterone in the field setting in comparison to the captive setting

4.2. Context-dependent relationships between Bd and corticosterone

Our studies documented no significant relationships between Bd infection and corticosterone in two of the three focal species. For *R. catesbeiana,* this was not surprising. This species is generally considered to be a competent Bd reservoir that shows little evidence for negative impacts from Bd, despite high infection intensities like those documented in this study (Gahl et al., 2012; Schloegel et al., 2010). In *R. clamitans*, a species that is thought to be less Bd-tolerant than *R. catesbeiana*, we similarly found no links between corticosterone and Bd-infection. It could be that *R. clamitans* at our field sites in Northwest Pennsylvania are more tolerant to their local Bd strain, as suggested by Gahl et al. (2012). *R. clamitans* is a widespread, generalist species, and we know of no accounts of Bd-linked declines of this species in the wild. In addition, *R. clamitans* individuals (like the study individuals of all species) did not exhibit symptoms of chytridiomycosis, and also exhibited the lowest infection intensities of the three focal species. In this study we were interested in characterizing potential relationships between sublethal Bd infections and changes in physiology during the breeding season, when such changes might have especially strong fitness consequences. No such changes were evident in *R. catesbeiana* and *R. clamitans*. Potentially if *R. clamitans* had exhibited higher infection intensities our results may have been different; future work could focus

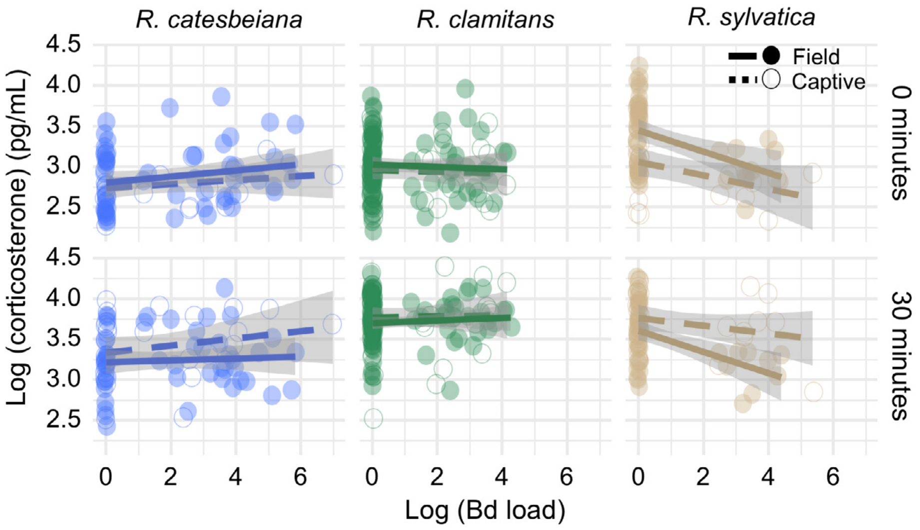

Fig. 2. Relationships between Bd load and baseline and stress-induced corticosterone in field and captive settings. Solid lines and filled points depict field samples (handling stressor) and dashed lines and open points depict captive samples (ACTH stressor). Values are shown for *R. catesbeiana* (blue, left), *R. clamitans* (green, middle), and *R. sylvatica* (brown, right) at 0 min (top) and 30 min (bottom) after stress. Lines of best fit and 95% confidence intervals (grey shading) are shown. Bd load is measured in DNA copies per swab.

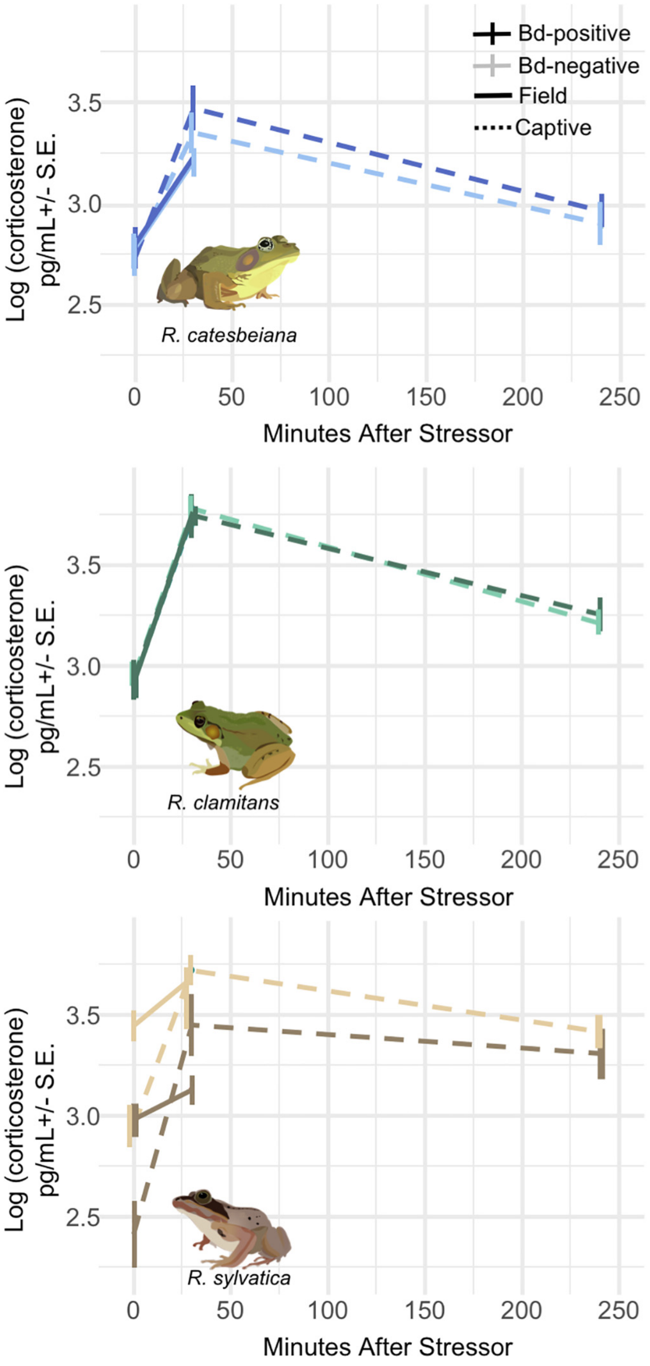

Fig. 3. Stress responses for Bd-positive and Bd-negative animals in the field and in captivity. Values are shown for *R. catesbeiana* (blue, top), *R. clamitans* (green, middle), and *R. sylvatica* (brown, bottom). Solid lines depict field samples (handling treatment) and dashed lines depict captive samples (ACTH treatment). Bd positive animals are shown in the darker shade and Bd-negative animals are shown in the lighter shade for each species. Samples at 240 min after stress were only able to be collected in captivity.

on sampling this species in early spring, when infection intensities tend to be highest (unpublished data).

In *R. sylvatica* we found that Bd-infected animals exhibited lower baseline and stress induced corticosterone. Differences in corticosterone between infected and uninfected *R. sylvatica* were significantly stronger in the field than in captivity. This underscores the importance of studying relationships between infection and GCs in multiple contexts, because GCs respond to a variety of energetic demands. Our findings ran counter to our prediction that Bd-positive *R. sylvatica* would exhibit increased baseline corticosterone. While tolerance/resistance to Bd has not been assessed in our focal populations, *R. sylvatica* has been used as a “model” Bd-susceptible species in multiple studies (Bradley et al., 2015; Eskew et al., 2018; Greenspan et al., 2012a,b) and the study individuals exhibited relatively high infection intensities in comparison to values detected in surveys of the study sites in 2017–2019 (unpublished data). It is difficult to compare Bd loads across studies due to differences in Bd strains and units of measurement (Longo et al., 2013). Our Bd loads were measured in DNA copies and thus cannot be directly compared to previous experimental work on *R. sylvatica* that uses zoospore equivalents, however, if units are comparable within an order of magnitude (Cádiz et al., 2019; Longo et al., 2013), then our Bd load values match or exceed those that have been associated with high histology infection scores or mortality in other studies of this species (Eskew et al., 2018; Greenspan et al., 2012a). One possible explanation for our results is that Bd-positive *R. sylvatica* were experiencing infection-induced chronic stress to such an extent that their hypothalamic-pituitary-interrenal axis was inhibited via negative feedback, resulting in lower baseline corticosterone. Significant work in other species has documented lower baseline and stress-induced GCs in animals experiencing chronic stress (Barton et al., 1987; Cyr and Romero, 2007; Müller et al., 2009; Rich and Romero, 2005; Torres-Medina et al., 2018). However, the magnitude of the stress response did not differ with Bd infection in *R. sylvatica*, which does not support this hypothesis. Moreover, no individuals exhibited clinical symptoms of infection. It is unclear whether Bd infections for the focal individuals were recently incurred or long-term infections, which makes it difficult to assess the likelihood of chronic stress. Notably, recent work exploring gene expression differences in Bd-infected *R. sylvatica* revealed upregulation of genes related to the GC response even over a relatively short period of infection (10 days; Eskew et al., 2018). In combination with literature suggesting that Bd may cause increases in GCs (Table 1), this supports the idea that infections of various lengths could induce a state of heightened physiological stress in wood frogs. Our results underscore the value of considering timeframe in experimental studies: circulating GCs may be expected to be high in the early stages of exposure to a chronic stressor, but low as exposure time increases. More generally, these results highlight the importance of taking care when interpreting GC results. Low GCs are not necessarily indicative of health, just as high GCs are not necessarily maladaptive.

There are other possible explanations for the patterns we document, which our correlational data cannot distinguish amongst. It could be that in *R. sylvatica* Bd inhibits the hypothalamic-pituitary-interrenal axis via other mechanisms, that animals with lower corticosterone are behaviorally or physiologically different (e.g. Chatfield et al., 2013) in ways that make them more Bd-susceptible, or that high corticosterone directly or indirectly confers a protective effect against Bd. In the future, it would be valuable for more studies to experimentally test the impacts of high baseline GCs on Bd susceptibility, using both exogenous glucocorticoids and more naturalistic stress regimens, and to assess relationships between Bd and baseline and stress-induced GCs separately. It is also notable that *R. sylvatica*’s breeding season takes place in early spring, when environmental temperatures fall within the optimal thermal niche for Bd (Piotrowski et al., 2004; Stevenson et al., 2013). This could have contributed to the patterns we documented for this species but not the other focal species, which breed during the summer, when ambient temperatures can fall above Bd’s upper thermal limit (Piotrowski et al., 2004; Stevenson et al., 2013). It is likely that Bd-related changes in GC physiology (or GC-related changes in Bd dynamics) in *R. sylvatica* are mediated by temperature. Nevertheless, in comparison to the other focal species, *R. sylvatica* does seem to exhibit stronger relationships between Bd and GC during its breeding season, when such changes could have particularly costly impacts on fitness.

4.3. Conclusions

Altogether, our results suggest that corticosterone concentrations may be related to infection with Bd in one susceptible species, *R. sylvatica*, though not in the most obvious or expected manner. Anthropogenic change (e.g. noise pollution, salt pollution) has also been shown to cause changes in GCs in *R. sylvatica* (Hall et al., 2017; Tennessen et al., 2014). Extensive work has established causal links between environmental change and increased GCs, and between increased GCs and immunosuppression across taxonomic groups (Busch and Hayward, 2009; McEwen, 1997; Padgett and Glaser, 2003). Because such changes can make hosts more physiologically susceptible to disease while simultaneously altering their movement patterns, population densities, and levels of contact with humans, together they can result in increased likelihood of intra- and interspecific disease transmission (Daszak et al., 2001). An improved understanding of ties between GCs and disease could allow for better management and prediction of outbreaks, which may be critical in the management and conservation of amphibian species. Recent work has documented various sublethal impacts of Bd on amphibian physiology and behavior (e.g. Chatfield et al., 2013; Table 1); such sublethal changes could contribute to significant long-term impacts on populations (Walls and Gabor, 2019). Our results suggest that baseline and stress-induced GCs could be considered as potential fitness-relevant endpoints in studies of chytridiomycosis, but that much remains to be discovered about the cause-and-effect relationships between GCs and infection with Bd.

5. Data accessibility

Data associated with this manuscript can be accessed online via figshare at https://doi.org/10.6084/m9.figshare.9775835.v1.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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