Effects of corticosterone on infection and disease in salamanders exposed to the amphibian fungal pathogen *Batrachochytrium dendrobatidis*

Chris W. Fonner[1], Shreya A. Patel[1], Shelby M. Boord[1], Matthew D. Venesky[2], Sarah K. Woodley[1,*]

[1]**Department of Biological Sciences, Duquesne University, Pittsburgh, PA 15282, USA**
[2]**Department of Biology, Allegheny College, Meadville, PA 16335, USA**

ABSTRACT: Although it is well established that glucocorticoid hormones (GCs) alter immune function and disease resistance in humans and laboratory animal models, fewer studies have linked elevated GCs to altered immune function and disease resistance in wild animals. The chytrid fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) infects amphibians and can cause the disease chytridiomycosis, which is responsible for worldwide amphibian declines. It is hypothesized that long-term exposure to environmental stressors reduces host resistance to *Bd* by suppressing host immunity via stress-induced release of GCs such as corticosterone (CORT). We tested whether elevation of CORT would reduce resistance to *Bd* and chytridiomycosis development in the red-legged salamander *Plethodon shermani*. Plasma CORT was elevated daily in animals for 9 d, after which animals were inoculated with *Bd* and subsequently tested for infection loads and clinical signs of disease. On average, *Bd*-inoculated animals treated with CORT had higher infection abundance compared to *Bd*-inoculated animals not treated with CORT. However, salamanders that received CORT prior to *Bd* did not experience any increase in clinical signs of chytridiomycosis compared to salamanders not treated with CORT. The lack of congruence between CORT effects on infection abundance versus disease may be due to threshold effects. Nonetheless, our results show that elevation of plasma CORT prior to *Bd* inoculation decreases resistance to infection by *Bd*. More studies are needed to better understand the effects of CORT on animals exposed to *Bd* and whether CORT variation contributes to differential responses to *Bd* observed across amphibian species and populations.

KEY WORDS: Behavior · Chytridiomycosis · Disease · *Plethodon shermani* · Glucocorticoid · Stress hormone · Immunity

INTRODUCTION

Increased glucocorticoid hormones (GCs) are a hallmark of the vertebrate stress response. After exposure to a real or perceived threat, circulating GCs increase and coordinate a variety of behavioral and physiological responses that help an animal avoid, counter, or cope with a stressor (Sapolsky et al. 2000).

Stress-induced elevations of GCs modulate metabolism, brain function, reproduction, and immune function. Studies in humans and rodent models have found that GCs have widespread effects on the immune system, including changes in natural killer cell activity, lymphocyte production, T-cell ratios, antibody production, and cytokine production (Webster Marketon & Glaser 2008). Generally, acute in-

creases in plasma GCs activate immune function, while chronic elevations in plasma GCs suppress immune function (Dhabhar 2014). Exact effects are modulated by context, age, sex, energetic stores, and early life experiences (Sapolsky et al. 2000, Meaney et al. 2007). Importantly, suppression of immune function by chronic stress has been associated with an increased ability of pathogens to infect hosts and produce disease (reduced disease resistance of the host) (Cohen et al. 1991, Kiecolt-Glaser et al. 1996, Godbout & Glaser 2006, Tait et al. 2008).

Compared to humans and domesticated rodent models, much less is known about stress-immune interactions in wild vertebrates (Martin 2009). Whereas many studies have measured GCs in wild animals across a number of contexts (e.g. predation, disease, habitat alteration), very few studies have demonstrated a causal relationship between GCs and disease resistance in wild animals. Although many results from domesticated rodent species should apply to wild animals, differences may exist due to the decoupling of stress responses from fitness in domesticated rodent models (Martin 2009). Understanding stress-immune interactions in wild animals is important because emerging infectious diseases are increasing in wildlife populations, impacting biodiversity as well as human health via zoonotic diseases (Daszak et al. 2000, Jones et al. 2008, Blaustein et al. 2012, Tompkins et al. 2015). It is proposed that increased exposure to environmental stressors might contribute to the rise in infectious diseases in some cases (Carey 2000).

Amphibians represent an important group to study stress-immune interactions because they have been adversely affected by several infectious agents (Daszak et al. 1999, Blaustein et al. 2012). One pathogen is the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), which has been found all over the world. *Bd* zoospores infiltrate keratinized amphibian skin and develop into zoosporangia, which then produce and discharge motile zoospores which can re-infect amphibian skin (Berger et al. 1998, Longcore et al. 1999, Skerratt et al. 2007). Once infected, the disease called chytridiomycosis often develops, with clinical signs including hyperkeratosis (lesions), skin sloughing, reduced locomotory activity, body mass drop, and death due to disruptions in respiration and osmoregulation (Voyles et al. 2009). Recently, a second pathogenic chytrid fungus, *B. salamandrivorans* (*Bsal*), has been discovered in Europe that is particularly lethal for salamanders, driving the extirpation of fire salamander populations (Martel et al. 2013, 2014). There is great concern that *Bsal* will reach the Americas with devastating consequences on salamander populations.

Resistance to both *Bd* infection (presence of *Bd* in skin cells) and chytridiomycosis (development of disease) varies among and within amphibian species (reviewed by Venesky et al. 2014). A long-standing hypothesis for the variation in resistance to *Bd* is that species and populations differ in exposure to environmental stressors (Carey et al. 1999). In amphibians, a large number of environmental stressors, including food restriction, capture, handling, contaminant exposure, thermal variation, and exposure to predator cues, resulted in elevated plasma corticosterone (CORT), the dominant amphibian glucocorticoid hormone (Carr 2010, Ricciardella et al. 2010, Woodley & Lacy 2010, Narayan et al. 2012, 2013). In turn, there is evidence that elevated plasma CORT is immunosuppressive in amphibians via inhibition of antimicrobial peptide (AMP) production, antibody proliferation, and altered white blood cell levels (Bennett et al. 1972, Rollins-Smith & Blair 1993, Ramírez et al. 1996, Simmaco et al. 1997, Belden & Kiesecker 2005, Davis & Maertz 2008, Groner et al. 2014, Falso et al. 2015). Thus, it is hypothesized that CORT-induced immunosuppression reduces resistance to pathogens including *Bd* (Carey et al. 1999, Rollins-Smith 2001). Due to the drastic declines experienced by amphibian populations, investigations into how the stress response affects resistance to *Bd* are critical, and may explain why certain amphibians are more resistant than others.

Several studies have examined links between stressors, plasma CORT, and amphibian resistance to infection and disease; however, there is no consensus on the relationship between CORT, infection, and disease. A study of larval treefrogs found that 3 wk of exposure to CORT prior to infection with a trematode parasite resulted in increased parasite loads (Belden & Kiesecker 2005). Also, amphibians infected with chytrid had elevated CORT levels compared to noninfected amphibians (Kindermann et al. 2012, Gabor et al. 2013, Peterson et al. 2013), although it is not clear whether infection increased levels of circulating CORT or vice versa. In contrast, studies exposing larval anurans to natural stressors such as predator cues and high conspecific densities did not alter resistance to ranavirus, another serious pathogen affecting amphibian populations (Gray et al. 2009, Reeve et al. 2013). However, these natural stressors did not alter plasma CORT, which may explain the lack of effect on resistance to ranavirus. Finally, treatment of anuran larvae with CORT did not affect resistance to *Bd* (Searle et al. 2014). Given the mixed

findings on the role of GCs, infection, and disease in amphibians, more experimental studies examining the effects of elevated CORT are needed.

In this study, we examined the effects of experimental CORT elevation on resistance to *Bd* in the red-legged salamander *Plethodon shermani*. We chose red-legged salamanders for 2 main reasons. First, the stress physiology of this salamander species and related plethodontid salamanders is relatively well studied, with information about the effects of CORT on behavior, metabolic rate, and wound healing (Wack et al. 2012, 2013, Thomas & Woodley 2015). In particular, Wack et al. (2010) developed and validated a minimally invasive method for elevating plasma CORT to physiologically relevant levels using dermal patches, which is very useful for studies of CORT effects. Second, free-living red-legged salamanders do become infected with *Bd* in both field and laboratory studies. In the field, 6% (4 out of 65 individuals) of red-legged salamanders from a site in southwestern North Carolina (USA) were positive for *Bd* infection, although there were no obvious clinical signs of disease and there is no evidence of declining numbers of red-legged salamanders in this area (Kiemnec-Tyburczy et al. 2012). Several salamander species of the genus *Plethodon* have been infected by *Bd* in laboratory studies and developed chytridiomycosis (Chinnadurai et al. 2009, Vazquez et al. 2009, Venesky et al. 2015). Using red-legged salamanders as our model, we expected that repeated CORT elevations prior to inoculation with *Bd* would cause increased infection abundance compared to subjects not exposed to CORT. We also expected that CORT elevation would increase clinical signs of chytridiomycosis in *Bd*-inoculated subjects, including reduced body mass, increased skin sloughing, reduced locomotory activity, and increased mortality.

MATERIALS AND METHODS

Salamander collection and husbandry

Adult male red-legged salamanders were collected in August of 2012 from Macon County, North Carolina, with appropriate permits from the North Carolina Department of Wildlife and the Nantahala National Forest (12-SC00294, NAN404802). In the laboratory, animals were housed individually in plastic boxes (16 × 16 × 5 cm) lined with moistened unbleached paper towels with a 14:10 light:dark cycle (lights off at 17:00 h) at 20°C, which is appropriate for *Bd* growth in culture and within the preferred temperature range of red-legged salamanders (Spotila 1972). Animals were fed 1 wax worm every 2 wk. This feeding regimen is sufficient for these salamanders to maintain body mass because plethodontid salamanders have very low metabolic rates (Feder 1983). For example, female dusky salamanders only need the equivalent of 12 worms yr^{-1} to maintain body weight and reproduce (Fitzpatrick 1973, Feder 1983). After salamanders had been housed for about 8 mo in the laboratory, we began our experiment.

Overview of experimental design

We randomly assigned 40 individuals of *Plethodon shermani* to 4 treatment groups: (1) animals treated with CORT for 9 d and then exposed to *Bd* in synthetic spring water (CORT + *Bd*), (2) animals treated with oil vehicle for 9 d and then exposed to *Bd* in synthetic spring water (Oil + *Bd*), (3) animals treated with CORT for 9 d and then exposed to synthetic spring water without *Bd* (CORT + Sham), and (4) animals treated with oil vehicle for 9 d and then exposed to synthetic spring water without *Bd* (Oil + Sham). After exposure to *Bd* or the sham inoculum (synthetic spring water), animals were monitored for clinical signs of chytridiomycosis. See Fig. 2 and below for details on the timing of procedures. The initial sample size for each treatment was 10, although sample sizes decreased over the course of the study due to *Bd*-induced mortality (see below). We used sample sizes of 10 for each group based on Vazquez et al. (2009) who found that sample sizes of 12 were sufficient to detect effects of temperature on *Bd*-induced mortality in plethodontid salamanders (*Plethodon* sp.). Body masses measured the day before hormone treatment began did not differ among treatment groups (range of 1.98 to 3.63 g; ANOVA p = 0.89). All procedures were approved by Duquesne University's Institutional Animal Care and Use Committee.

Transdermal elevation of CORT

Dermal patches containing CORT were used to transdermally elevate plasma CORT in salamanders, with control subjects receiving patches containing sesame oil vehicle (Fig. 1). Oil is a standard vehicle for dissolving steroid hormones, with no evidence of toxicity. Patches (1.5 × 3 mm) were cut from filter paper (Whatman, cat no. 1820–070) and applied to the dorsal surface between the forelimbs of each salamander using clean forceps. Patches adhere well

Fig. 1. Photograph showing the daily dermal patch application to a red-legged salamander *Plethodon shermani*. Patches delivered corticosterone (in sesame oil) to treatment groups; control animals were given patches with only sesame oil

to the moist salamander skin. With a pipette, 1.25 µg of CORT was applied to a patch in a volume of 2.5 µl (cat. no. Q1550-000, Steraloids; 0.5 mg ml^{-1}). Controls received patches that contained 2.5 µl of sesame oil vehicle. After 45 min, the patch was gently removed with forceps.

Patches were applied once daily in the morning for 9 consecutive days prior to inoculation with either *Bd* or sham inocula. We chose to apply patches for 9 consecutive days based on a previous study in a related species of salamander which showed that wound healing (which requires inflammation to proceed) was delayed by 9 d of consecutive CORT treatment compared to controls treated with oil vehicle (Thomas & Woodley 2015). We chose to stop daily treatment at inoculation because we wanted to minimize disturbance of animals once they were inoculated with *Bd* to avoid potential contamination from *Bd*-inoculated animals to sham-inoculated controls.

The use of dermal patches to elevate plasma corticosterone has been validated for use in red-legged salamanders (Wack et al. 2010). In animals treated with oil patches for 30 to 45 min, CORT levels were similar to levels in animals that received no patches. In animals treated with CORT patches for 30 to 45 min, CORT remained elevated for approximately 4 h after CORT patch application and were back to baseline levels by 8 h. It is also important to note that differences in adult body masses did not contribute to

differences in the plasma CORT achieved by the CORT patches.

To mimic stress-induced levels of CORT, we used a dose of 1.25 µg CORT. Although the use of patches in red-legged salamanders was previously validated (Wack et al. 2010), we did not specifically include patches with a CORT dose of 1.25 µg. Thus, we did an additional patch study to determine the plasma levels of CORT in animals that received dermal patches containing 1.25 µg of CORT. Such patches produced plasma CORT levels of 70 ± 14.56 ng ml^{-1} (SEM, n = 4). This level is higher than baseline levels found in free-living males (plasma CORT in free-living males in the early breeding season ranged from 20 to 62 ng ml^{-1}, with a mean of 38 ± 3.8 ng ml^{-1}, n = 12, unpublished data). Also, plasma CORT in animals given 1.25 µg CORT patches were higher than that in oil-patched controls (26 ± 10 ng ml^{-1}, n = 5). Thus, our pilot experiment confirms that our patches were delivering elevated levels of CORT compared to baseline, but were still at ecologically relevant levels.

Inoculation with *Bd*

Our methods for inoculation with *Bd* were based on previous studies that found that salamanders of the genus *Plethodon* became infected and exhibited clinical signs of chytridiomycosis after exposure to 1 × 10^{7} zoospores of *Bd* isolate JEL 197 for 8 to 24 h (Chinnadurai et al. 2009, Vazquez et al. 2009). Although these concentrations of *Bd* are unlikely to be observed in the field, the ability to induce chytridiomycosis in the laboratory allows us to test the effects of CORT on infection and disease. JEL 197 was isolated by Dr. Joyce Longcore in 1997 from a captive blue poison dart frog (Longcore et al. 1999). The isolate was passaged 3–4 times yr^{-1} and then cryopreserved in 2005. We received a sample in 2011 that we passaged every 3 to 4 wk in tryptone broth before use in this experiment (June 2013).

Bd was cultured and harvested using standard methods. Briefly, zoospores of the *Bd* isolate JEL197 were grown in 1% tryptone broth at 23°C until clumps of thalli were visible, at which point culture was transferred to 1% tryptone agar and subcultured for 1 wk at 23°C. Zoospores were harvested by flooding plates with 3.0 ml of sterile water. After 30 min, water containing zoospores was collected and filtered using a 20 µm nylon mesh to isolate zoospores. Zoospores were diluted with sterile synthetic spring water to achieve a concentration of 6.9 × 10^{7}

zoospores in 15 ml. Sham inocula were prepared similarly except that plates were not inoculated with *Bd*.

After 9 d of patch treatment (described above), salamanders were inoculated with either *Bd* zoospores or a sham inoculum. For inoculation, each animal was placed into a sterile petri dish containing 15 ml of either *Bd* inoculum or sham inoculum at 20°C. After inoculation for 20 h, animals were transferred back to their containers.

Measuring *Bd* infection

Subjects were swabbed 1 d before (baseline) and on Days 10 and 17 after *Bd* or sham inoculation, in order to quantify *Bd* infection (Fig. 2). Each subject was swabbed (Rayon swabs, cat. no. MW113, Medical Wire) 30 times along the ventral surface from tail to neck. After drying for 15 min, swabs were sealed in a 1.5 ml sterile microcentrifuge tube and stored at −20°C. Three animals (1 in the CORT + Bd group and 2 in the Oil + Bd group) died between Days 13 and 17; for these animals, we collected swabs from the carcasses within 24 h of death. These swabs were grouped with those animals swabbed on Day 17. Hereafter, we refer to this set of swabs as Days 13−17. Swabs collected from recently deceased animals did not have particularly high *Bd* infection loads (8, 30, and 48 zoospore equivalents swab^{-1}) compared to swabs from surviving *Bd*-inoculated animals, so inclusion of swabs from recently deceased animals should not bias the results.

DNA of *Bd* zoospores from swabs was extracted using MO BIO Powerlyzer UltraClean Microbial DNA Isolation kit (cat. no. 12255-50, MO BIO Laboratories). Extraction procedures followed the kit protocol, with minor modifications. Firstly, 300 µl of Glass Microbead Solution were pipetted into each microcentrifuge tube containing an individual swab, after which the tube was vortexed for 5 s and the swab removed using sterile forceps. Secondly, Microbead tubes were homogenized using a Disruptor Genie for 45 s at 2000 rpm. Samples were eluted into a final volume of 50 µl.

DNA extractions were analyzed via real-time qPCR Taqman assays (ABI StepOnePlus 7500), according to previously validated methods (Boyle et al. 2004, Hyatt et al. 2007). Primers specific to highly conserved regions of rDNA found in nearly all *Bd* strains were used to detect infection levels, with the primer sequences as follows: ITS1-3 Chytr (5′-CCT TGA TAT AAT ACA GTG TGC CAT ATG TC-3′) and 5.8S Chytr (5′-AGC CAA GAG ATC CGT TGT CAA A-3′).
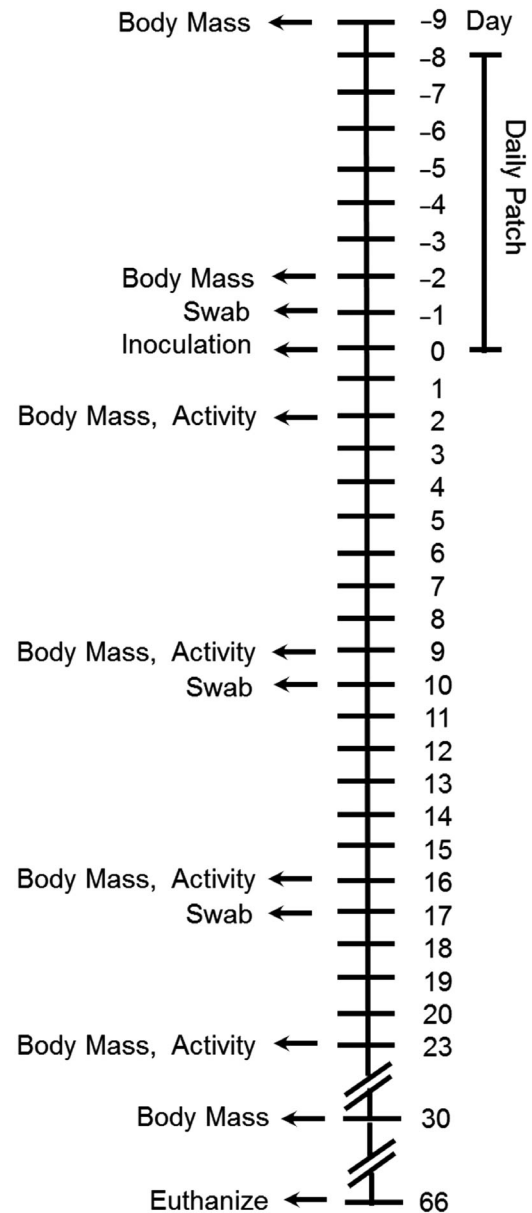

Fig. 2. Timeline showing the experimental procedures. Prior to the start of the experiment, patches delivering corticosterone (or oil, for control groups) were applied to red-legged salamanders *Plethodon shermani* daily for 9 d. On Day 0, groups were inoculated with *Batrachochytrium dendrobatidis* (*Bd*; see 'Materials and methods' for details of all treatment groups). Body mass and activity were then measured on the indicated days. Swabs were collected on days following body mass measurements

Two batches of qPCR were performed. First, we performed qPCR on all baseline samples and samples from Day 10. After finding that baseline samples and samples from sham-inoculated animals were negative, we next ran samples from *Bd*-inoculated animals that were collected on Day 10 and Days 13−17.

In each batch, samples were distributed across plates so that treatments were equally represented within a given plate.

Each sample was run in triplicate, along with known *Bd* standards prepared using the same strain as that used in the experiments (Batch 1: 100, 10, 1, 0.1 zoospore equivalents; Batch 2: 50, 10, 2, and 0.4 zoospore equivalents). Samples were considered positive for *Bd* infection if 2 out of 3 replicates amplified before the cycle corresponding to the lowest point on the standard curve. Values from the qPCR were multiplied by 10 to account for the dilution factor such that values represent zoospore equivalents swab^{-1}. Validation parameters for qPCR analyses indicated optimum PCR amplification (Batch 1: 3 plates, R^2 = 0.98, qPCR efficiency = 87%; Batch 2, 3 plates, R^2 = 0.98, qPCR efficiency = 95%). Negative controls (*Bd*-free MOBIO-extracted swabs) were also included within each qPCR plate in order to assess the possibility of contamination/false positives during each run. Negative controls were all *Bd*-negative.

Sample sizes were 10 group^{-1} on each swab date with the following exceptions. The sample size for Oil+*Bd* was 8 because we had technical difficulties (did not get a swab sample or DNA extraction was unsuccessful) for 2 animals. The sample size for CORT+*Bd* was 9 because we did not get a swab from 1 animal.

Assessing clinical signs of chytridiomycosis

Animals were checked daily, noting details such as mortality, skin condition, behavior, and any other unusual signs (like dropping a tail). Animals that did not survive were either found dead (n = 10) or were euthanized because they had dropped a tail (n = 1), were writhing with a reddened tail and body mass drop of 19% (n = 1), had lost more than 20% of body mass (n = 2), or had no righting response (n = 1). By Day 66, there had been no change in survival for 24 d so the remaining animals were euthanized.

To measure the effects of inoculation on body mass, we first measured body masses of each animal 2 d before *Bd* or sham inoculation. Body masses did not differ among groups at this time (range of 1.9 to 3.9 g, mean + SEM: 2.7 + 0.07, n = 40). We then measured body mass on 2, 9, 16, 23, and 30 d after inoculation and calculated the percent of initial body mass change (measured 2 d before inoculation).

Locomotory activity was measured at 2, 9, 16, and 23 d post exposure (Fig. 2). Because salamanders are crepuscular, locomotory activity was measured in the early evening, at least 2 h after lights were normally turned off. Testing was performed under dim, incandescent lighting. For measurement of locomotory activity, each subject was transferred from its home box to a testing chamber (24 × 24 × 2 cm, cleaned with Alconox® detergent, then rinsed with ethanol, and then further disinfected with bleach before and after use) lined with a single layer of unbleached paper towel moistened with 15 ml of synthetic spring water. A time-lapse digital camcorder (Sony DCR-VX2000) recorded animal movements for 2 s every 30 s for 75 min. Later, recordings were observed (excluding the first 15 min which represents an acclimation period), and activity levels were measured by an investigator blind to treatments. To quantify activity, each testing chamber was divided into 4 quadrants, and each time a subject's head was in a different quadrant from one scan to the next was counted as 1 movement.

Statistical analyses

To evaluate resistance to *Bd*, we determined *Bd* infection abundance (zoospore genome equivalents of the *Bd*-infected salamanders as well as the salamanders that were exposed to, but not infected with, *Bd*). We focused our statistical analysis on *Bd* infection abundance because it encompasses aspects of both prevalence (the proportion of animals that had detectable levels of *Bd*) and intensity (the number of zoospore equivalents in *Bd*-positive animals) and can be analyzed in a single statistical model, the zero-inflated negative binomial model. Zero-inflated models assume that the response variable is a function of a binomial process (infected versus non-infected) and a count process (negative-binomial distributed infection abundance). We confirmed that the zero-inflated negative binomial model best fit the data by comparing various models (with or without zero-inflation, with or without different error distributions [negative binomial, Poisson, logistic, and Gaussian]) using the Akaike information criterion. Analysis was done using the 'glmmADMB' function in the 'glmmADMB' package in R statistical software. Animals swabbed before inoculation (baseline) and sham-inoculated subjects (on Day 10) had undetectable zoospore equivalents; therefore, only *Bd*-inoculated subjects, post inoculation, were statistically analyzed.

Locomotory activity and body mass data were homoscedastic with a normal distribution and thus were analyzed using ANOVA. For locomotory activity, the number of times the location of each animal

changed relative to the previous scan was summed and analyzed with a 2-way repeated measures ANOVA, with inoculation (*Bd* or sham) and patch application (CORT or Oil) as between-subjects factors. For body mass, the percent of initial body mass was calculated and analyzed with a 2-way repeated measures ANOVA, with inoculation (*Bd* or sham) and patch application (CORT or Oil) as between-subjects factors. We did not include body mass data from Day 30 in the statistical analysis because sample sizes were very low by Day 30 as survival declined.

Whether animals shed their skin at least 1 time over the course of the experiment was analyzed with a Fisher's exact test comparing *Bd* inoculation vs. sham inoculation, and Oil + *Bd* vs. CORT + *Bd*.

Survival among groups was analyzed using log rank (Mantel-Cox) tests comparing *Bd* inoculation vs. sham inoculation and Oil + *Bd* vs. CORT + *Bd*.

Finally, to assess whether *Bd* infection abundance was correlated with clinical signs of disease, we compared *Bd* infection abundance with survival, body mass lost, and locomotory activity within individuals using non-parametric correlations.

RESULTS

Bd infection

Before inoculation, all subjects had undetectable levels of *Bd* zoospores. Also, sham-inoculated subjects had undetectable *Bd* infection on Day 10. After inoculation with *Bd*, several animals had detectable levels of zoospores, with infection intensities ranging from 1 to 706 zoospore equivalents. Although there was no effect of patch treatment on infection abundance by Day 10 ($\chi^2_{1,13} = 0.0001$, p = 0.98), there was an effect of patch treatment on infection abundance on Days 13–17 ($\chi^2_{1,13} = 4.7$, p = 0.029), with *Bd*-inoculated subjects treated with CORT patches having higher infection abundance compared to *Bd*-inoculated subjects treated with Oil patches (Fig. 3).

Chytridiomycosis

By Day 43, only 40% of *Bd* exposed subjects were still alive compared to 85% of sham-inoculated sub-
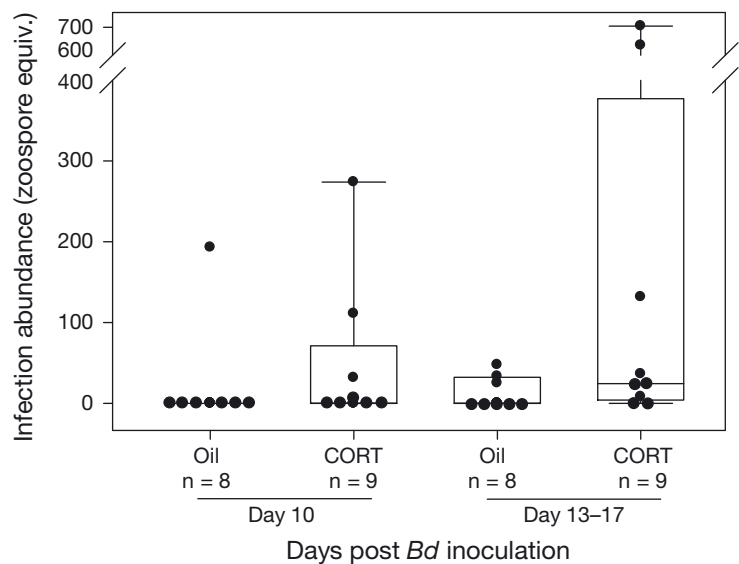

Fig. 3. *Batrachochytrium dendrobatidis* (*Bd*) infection abundance (genomic *Bd* zoospore equivalents per swab) from *Bd*-inoculated red-legged salamanders *Plethodon shermani* on Days 10 and 13–17 post-inoculation. Box: 25th–75th percentile; line: median; whiskers: 10th – 90th percentile; points laid over the boxplots show the individual data points. Prior treatment with corticosterone (CORT) increased infection abundance in *Bd*-inoculated animals by Days 13–17

jects (log-rank Mantel Cox: $x^2 = 10.42$, p < 0.001; Fig. 4). However, there was no difference in survival between CORT + *Bd* subjects and Oil + *Bd* subjects (log-rank Mantel Cox: $x^2 = 0.93$, p = 0.34; Fig. 4). All animals still alive on Day 43 survived with no signs of chytridiomycosis for an additional 23 d, at which point they were euthanized.

Inoculation with *Bd* resulted in a decrease in body mass over time (Fig. 5), as evidenced by an interaction between day and inoculation ($F_{3,93} = 9.4$, p < 0.001) as well as an overall effect of inoculation on body mass ($F_{1,31} = 24.9$, p < 0.001). For example, by Day 23, animals inoculated with *Bd* had 87% of the body mass measured 2 d prior to inoculation. There was no effect of patch treatment on the change in body mass (day × patch interaction: $F_{3,93} = 0.82$, p = 0.49; effect of patch: $F_{1,31} = 0.33$, p = 0.57; Fig. 5). There was an interaction between inoculation and patch (day × inoculation × patch: $F_{3,93} = 3.97$, p < 0.01; inoculation × patch: $F_{1,31} = 4.15$, p = 0.05; Fig. 5). By Day 30, sample sizes were low in some treatment groups because of mortality, but body masses of the surviving animals had recovered to initial body masses.

Locomotory activity declined over time (effect of day: $F_{3,90} = 3.77$, p = 0.032; Fig. 6), but there was no effect of inoculation treatment ($F_{1,30} = 0.87$, p = 0.36) or patch treatment ($F_{1,30} = 0.73$, p = 0.40). Also, all
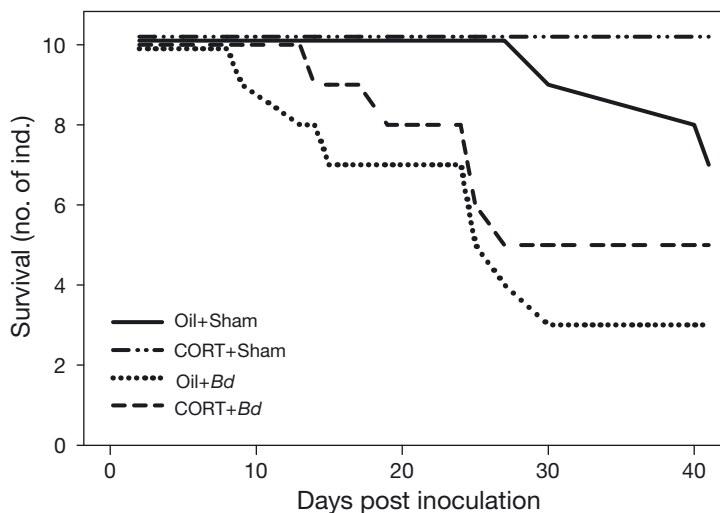
Fig. 4. Number of surviving red-legged salamanders *Plethodon sher-mani* after inoculation with *Batrachochytrium dendrobatidis* (*Bd*) or sham inocula (see 'Materials and methods' for details of treatment groups). Inoculation with *Bd* reduced survival. There was no effect of prior treatment with corticosterone (CORT; see 'Results' for details)
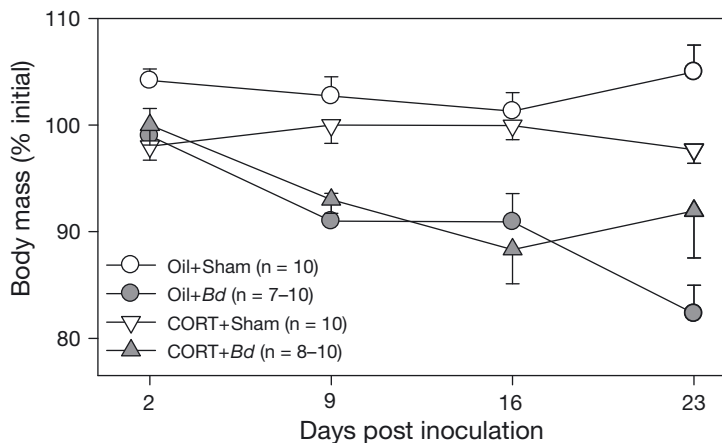

Fig. 5. Mean percent (and SEM) of initial body mass of red-legged salamanders *Plethodon shermani* after inoculation with *Batracho-chytrium dendrobatidis* (*Bd*) or sham inocula (see 'Materials and methods' for details of treatment groups). By Day 23, subjects ino-culated with *Bd* lost more body mass than sham-inoculated subjects. There was no effect of prior corticosterone (CORT) treatment (see 'Results' for details)

interactions among patch treatment, inoculation, and day were nonsignificant (all p > 0.34).

Nine out of 10 of CORT + *Bd* subjects sloughed their skin and 9 out of 10 Oil + *Bd* subjects sloughed their skin at least once throughout the experiment. In comparison, none of the 10 CORT + Sham or 10 Oil + Sham subjects sloughed their skin (Fisher's exact test: p < 0.001). Among *Bd*-inoculated animals, treat-ment with the CORT patch did not affect the number

of animals that sloughed their skin (Fisher's exact test, CORT + *Bd* vs. Oil + *Bd*: p = 0.76). Limb lifting was observed in 2 Oil + *Bd* sub-ject and 4 CORT + *Bd* subjects. None of the subjects exhibiting limb lifting survived. Also, none of the 10 CORT + Sham or 10 Oil + Sham subjects exhibited limb lifting.

Three animals treated with Oil patches and inoculated with synthetic spring water (Sham) died (1 each on Days 30, 41, and 42). None of these animals exhibited skin slough-ing, limb lifting, or declining body mass (body masses of the 3 that died were 100% on Day 23, 106% on Day 30, and 97% on Day 30, respectively, of their baseline values). The only clinical sign noted was dry skin, which was observed for several days prior to death in the animal that died on Day 30.

Correlations of disease with *Bd* infection abundance

Within subjects, infection abundance on Day 17 was negatively correlated with loco-motory activity on Day 16 (ρ = −0.60, p = 0.023, n = 14). However, infection abundance on Days 13–17 was not correlated with body mass lost by Day 16 (ρ = −0.37, p = 0.18, n = 15) or survival (ρ = −0.33, p = 0.19, n = 17). The mismatch between infection abundance on Days 13–17 and survival is due in large part to the fact that 3 of the animals in the CORT + *Bd* group that were *Bd*-positive on Days 13–17 survived to the end of the study. Also, while all 3 of the animals in Oil + *Bd* group that tested positive for *Bd* died by the end of the study, 2 of the Oil + *Bd* animals that tested negative for *Bd* on Days 13–17 died later after developing clinical signs of chytridiomycosis.

DISCUSSION

Using red-legged salamanders, we tested whether prior treatment with CORT resulted in increased infection abundance and disease development when inoculated with a fungal pathogen. Animals tested for *Bd* infection prior to *Bd* inoculation as well as ani-mals inoculated with a sham inoculation had no detectable *Bd* zoospore equivalents. In contrast, many animals were positive for *Bd* after being inocu-
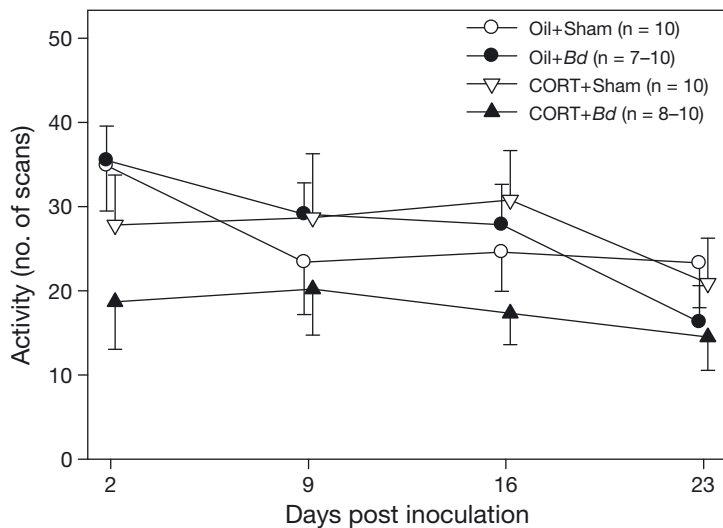
Fig. 6. Mean locomotory activity (and SEM) of red-legged salaman-ders *Plethodon shermani* after inoculation with *Batrachochytrium dendrobatidis* (*Bd*) or sham inocula (see 'Materials and methods' for details of treatment groups and a description of how activity was assessed). Points are slightly offset to increase clarity. Treatment groups did not vary in activity (see 'Results' for details)

lated with *Bd*. Importantly, *Bd*-inoculated animals treated with CORT had a greater infection abundance by Days 13–17 compared to *Bd*-inoculated animals treated with oil vehicle. Inoculation with *Bd* induced clinical signs of chytridiomycosis, including loss of body mass, skin sloughing, and mortality, whereas sham-inoculated controls did not exhibit these clinical signs. However, despite the effect of CORT on *Bd* infection abundance, prior treatment with CORT did not increase expression of clinical signs of chytridiomycosis. These results are discussed further below.

CORT increased *Bd* infection

We experimentally elevated plasma CORT to physiologically high levels using a dermal patch method that was validated in prior studies and again with a pilot study (described in the Methods). We treated animals with patches for 9 d, a time period sufficient to delay wound healing in a related salamander spe-cies (Thomas & Woodley 2015), indicating that 9 d were sufficient to suppress some aspects of immune function. With our patch regimen, we found that treatment with CORT prior to inoculation with *Bd* resulted in a greater *Bd* infection abundance by Days 13–17 after exposure to *Bd*. That is, more animals exposed to CORT + *Bd* were infected and at slightly higher zoospore equivalents compared to Oil + *Bd*

animals. However, it should be noted that most of the infected animals had relatively low zoospore equivalents, and only a few had very high zoospore equivalents.

Our results are consistent with results in larval treefrogs, for which 3 wk of exposure to CORT prior to infection with a trematode parasite resulted in increased parasite loads (Belden & Kiesecker 2005). Although we did not measure specific aspects of immune func-tion in our study, our results are consistent with previous studies showing that CORT treatment is immunosuppressive in verte-brates, including amphibians (Bennett et al. 1972, Rollins-Smith & Blair 1993, Ramírez et al. 1996, Simmaco et al. 1997, Davis & Maertz 2008, Dhabhar 2014, Groner et al. 2014, Falso et al. 2015). Of particular importance for amphibians are AMPs, which are a vital defense against *Bd* infection (Rollins-Smith & Conlon 2005). Treatment with CORT sup-pressed expression of AMPs in frogs (Sim-maco et al. 1997), although another study found the opposite result (Tatiersky et al. 2015). Alternatively, an increase in plasma CORT could inhibit lymphocyte action (Wiegers et al. 1993, Miller et al. 1994, Engler et al. 2004, Sterzer et al. 2004). In frogs, soluble factors produced by *Bd* impair lympho-cytes, contributing to the ability of *Bd* to evade host immune responses (Fites et al. 2013, 2014). Thus, prior chronic elevation of CORT may further dampen immunity, allowing *Bd* to better evade amphibian immune responses and resulting in increased infec-tion abundance.

Another possibility for our results is that CORT in-fluenced the skin directly. In larval anurans (*Bombina orientalis* and *Bufo boreas*), treatment with CORT for 15 d inhibited skin sloughing, which resulted in build-up of the stratum corneum layer of the epider-mis (Hayes 1995). If this effect occurred in our study, it is possible that CORT-treated animals had a thicker stratum corneum when inoculated with *Bd*, which may have somehow influenced the ability of *Bd* to in-vade and infect the underlying epidermal skin layers.

Our results differ from a previous study, which found no effect of exogenous CORT elevation on *Bd* infection in larval (*Anaxyrus boreas*, *Lithobates catesbeianus*, and *Rana cascadae*) and post-meta-morphic anurans (*R. cascadae*) (Searle et al. 2014). Our study also differs from a study on the amphibian ranavirus pathogen, which found that exposure to natural stressors like food deprivation and predator cues had no effect on resistance to ranavirus in wood

frogs *L. sylvatica* (Reeve et al. 2013). Our study differs from the above studies in many ways, including species, regimen of CORT exposure, strain of *Bd*, dose and duration of *Bd* inoculation, and developmental stage, making it difficult to determine whether methodological differences explain the contrasting results. Clearly, more studies are necessary to determine the role of CORT in resistance to *Bd* and other pathogens.

CORT had no effect on chytridiomycosis

Inoculation of salamanders with *Bd* resulted in many signs of chytridiomycosis. All of the *Bd*-inoculated animals that died exhibited clinical signs of chytridiomycosis, including skin sloughing, body mass loss, and limb lifting. In our experience, it is very rare to observe skin sloughing, so the high amount of skin sloughing was an important clinical sign, and could be the result of reduced consumption of skin sloughs and/or increased skin sloughing. Although there was no overall effect of inoculation on locomotory activity, *Bd* abundance was negatively correlated with locomotory behavior, suggesting that *Bd* reduced locomotory activity, which could be interpreted as a lethargy or malaise. Sham-inoculated animals displayed no signs of chytridiomycosis at any point during the study. Although 3 sham-inoculated animals died between Days 30 and 43 of the study, the absence of infection as measured by qPCR and clinical signs of chytridiomycosis indicate that they did not die from *Bd* exposure.

Despite differences in infection abundance between the Oil + *Bd* and the CORT + *Bd* groups, groups did not differ in the expression of clinical signs of chytridiomycosis. Although CORT might have increased the expression of signs of chytridiomycosis that we did not measure, such as appetite, anti-predator defenses, and reproductive efforts, there was no effect on body mass loss, skin sloughing, or mortality. In fact, more animals in the Oil + *Bd* group died than in the CORT + *Bd* group, although this was not statistically significant. The mismatch between infection abundance and mortality is due in part to some of the infected animals in the CORT + *Bd* group surviving to the end of the study, while all of the infected Oil + *Bd* animals died. In addition, some of the Oil + *Bd* animals that tested negative for zoospore equivalents on Day 17 went on to die later. These results might suggest that the CORT + *Bd* animals were more tolerant of infection, or better able to clear infection, but this needs more testing.

There are several explanations for why prior CORT did not increase the expression of chytridiomycosis despite the increased *Bd* abundance. If CORT treatment increased skin thickness as described above (Hayes 1995), the increased skin thickening may prevent zoospores from infiltrating deeper into the epidermis such that additional clinical signs of chytridiomycosis are reduced. Alternatively, there may be a threshold of *Bd* infection required for onset of chytridiomycosis. A threshold theory to describe development of chytridiomycosis has been proposed (Stockwell et al. 2010, Vredenburg et al. 2010), whereby past a certain zoospore infection level, animals will develop chytridiomycosis regardless of infection magnitude. This threshold could signify the amount of zoospores infecting the skin that, via disruption of osmoregulation, will decrease electrolyte concentrations and impair cardiac function (Voyles et al. 2009). The threshold may be affected by multiple factors, including host species, life history stage, and *Bd* virulence. In this way, even though prior CORT elevation increased *Bd* infection loads, it is possible that both CORT- and Oil-treated groups exceeded the minimum zoospore load needed for disease development, and therefore exhibited similar degrees of chytridiomycosis. Supporting this argument, infection loads were not significantly correlated with body mass loss or mortality. However, there was a negative correlation with locomotory activity, suggesting that more careful examination of onset to reduced activity (and other signs) might be informative.

Finally, it is possible that the subjects in our study may have acquired an immune response to *Bd* which may have reduced the impacts of CORT on disease development. Recent evidence suggests that amphibians can develop immunity in response to *Bd* exposure (McMahon et al. 2014). Our subjects were collected from the wild, and *Bd* has been documented at low levels at our collection site (zoospores loads were typically 3–4, with a range of 1 to 57, Kiemnec-Tyburczy et al. 2012). Thus, although *Bd* was undetectable in our subjects prior to inoculation, the salamanders in our study may have been previously exposed to *Bd* and cleared the *Bd* infection in the field. Interestingly, a subset of the *Bd*-inoculated subjects recovered from chytridiomycosis, as evidenced by recovery of body mass, cessation of skin sloughing, and long-term survival. Although speculative, perhaps a pre-exposure to *Bd* helped individual salamanders to clear chytridiomycosis compared to *Bd*-naïve animals.

Finally, it should be noted that our prior CORT treatment might have altered the endogenous CORT

response to infection via negative feedback on the hypothalamic-pituitary-adrenal axis. Increased plasma GCs are part of the acute phase response to infection and functions to mobilize energy etc. to mount an immune defense (Vermes & Beishuizen 2001). The 9 d of CORT treatment may have inhibited an endogenous CORT response induced by infection. If so, then a reduced CORT response coincident with infection may have contributed to the increased *Bd* infection abundance. Additional studies that elevate or suppress CORT after exposure to *Bd* would be informative to better understand the role of CORT prior to and during infection, given the complex interactions existing between plasma CORT outcomes after exposure to *Bd*.

CONCLUSION

Although it is well established that plasma GCs affect immune function in humans and domesticated laboratory models, few studies have linked elevated GCs to altered resistance to infection and disease in wild animals. To our knowledge, our study is the first to provide evidence that repeated CORT elevation prior to exposure to a pathogen increases infection in an amphibian. However, it should be noted that the effects were nuanced, with most of the infected animals having relatively low zoospore equivalents, and only a few having very high zoospore equivalents. Furthermore, the effects of CORT on *Bd* infection did not translate into increased expression of clinical signs of chytridiomycosis, perhaps due to threshold effects or potential immunity due to possible prior exposure to *Bd* in the field. Our findings could explain some of the within- and between-species variation in resistance to *Bd*, but more studies are necessary to untangle the complex interactions existing between plasma CORT, infection, and disease development. Future studies should examine the effects of CORT on immune responses (including AMPs) and the development of infection and disease after *Bd*-exposure in more species and should also examine responses to *Bd* in animals that are stressed in different ways, such as via food scarcity, habitat modification, or captivity. Given the concern over *Bsal*, a chytrid fungus that is particularly virulent for salamander species (Martel et al. 2013), studies should also examine whether prior or concomitant exposure to stressors similarly affects resistance to *Bsal*. Further research into mechanisms underlying resistance to disease is vital to guide conservation strategies.

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