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The Effects of Stressors and Stress Hormones on Behavior, Physiology, and Disease Susceptibility in Terrestrial Salamanders

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THE EFFECTS OF STRESSORS AND STRESS HORMONES ON BEHAVIOR, PHYSIOLOGY, AND DISEASE SUSCEPTIBILITY IN TERRESTRIAL SALAMANDERS

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By

Christopher Wesley Fonner

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ABSTRACT

THE EFFECTS OF STRESSORS AND STRESS HORMONES ON BEHAVIOR, PHYSIOLOGY, AND DISEASE SUSCEPTIBILITY IN TERRESTRIAL SALAMANDERS

By

Christopher Fonner

August 2015

Dissertation supervised by Dr. Sarah K. Woodley

Amphibians are exposed to numerous environmental stressors, which may contribute to population declines being experienced by amphibians worldwide. Upon exposure to stressors, animals will release the glucocorticoid hormone, corticosterone (CORT), which typically mediates physiological and behavioral responses for resisting or coping with the stressor. Additionally, amphibians are at risk from a chytrid fungal pathogen, *Batrachochytrium dendrobatidis* (*Bd*). After *Bd* infection, amphibians may develop the potentially fatal disease chytridiomycosis. To better understand how CORT mediates responses to different stressors, and possible immunosuppressive effects of CORT on *Bd* susceptibility, I performed several experiments. First, I tested the predation stress hypothesis in terrestrial salamanders by exposing animals to acute and prolonged predator kairomones, and measuring anti-predator behaviors and plasma CORT. The predation stress hypothesis predicts that, upon predator cue exposure, both anti-predator behaviors and plasma CORT will increase. Despite expression of anti-predator behaviors from an acute exposure, there

was no CORT response after acute or prolonged exposure to predator kairomones. Secondly, I tested the effects of prolonged thermal elevation on body mass, locomotory activity, plasma CORT, and immunity, in order to examine behavioral and physiological consequences of prolonged temperature shifts for amphibians. After 3 weeks at 24°C, male and female salamanders lost more body mass compared to animals housed at 17°C, a preferred temperature. There were also differences in immunity, with relatively more monocytes and fewer neutrophils and lymphocytes in animals held at 24°C. However, prolonged thermal elevation did not affect activity or plasma CORT when measured after 3 weeks at 24°C. Finally, I examined whether repeated plasma CORT elevations affected *Bd* susceptibility and chytridiomycosis development. Chronic CORT treatment simulates chronic exposure to stressors, which may reduce *Bd* resistance. I found that prior, repeated CORT elevations increased *Bd* infection abundance after *Bd* exposure, with no discernable effect on expression of chytridiomycosis symptoms. Additionally, I observed that prior CORT elevations increased incidence of skin sloughing after *Bd* exposure. Collectively, these studies show that CORT responses to putative stressors may be context-dependent. Furthermore, chronic CORT elevations may contribute to pathogen susceptibility in amphibians, and may partially explain the variable *Bd* resistance among different species.

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Chapter 1

Introduction

SPECIFIC AIMS

Amphibian populations are experiencing worldwide declines, with hundreds of species classified as "rapidly declining" and approximately 43% of all amphibians listed as in danger of extinction (Stuart et al., 2004; Hoffmann et al., 2010; Blaustein et al., 2011). A multitude of environmental factors /stressors may be facilitating these declines, including climate change, habitat loss, pollutants, and pathogens (Stuart et al., 2004; Hayes et al., 2010; Rohr and Raffel, 2010; Blaustein et al., 2011). In response to stressful stimuli in the environment, vertebrates, including amphibians, undergo a physiological stress response involving release of glucocorticoids like corticosterone (CORT), which can help animals to avoid or cope with stressors (Sapolsky, 2002; Carr, 2011). Although a short-term increase in CORT can be beneficial, a long-term, chronic CORT elevation may have detrimental effects, including immune suppression (Dhabhar, 2000; Dhabhar, 2002).

Amphibian species are exposed to a number of stressors in their environments. Factors, including pesticide exposure, capture and handling, temperature variation, predation, and pathogen exposure, have caused an elevation of plasma CORT in several amphibian species (Hopkins et al., 1997; Glennemeier and Denver, 2002; Hayes et al., 2006; Denver, 2009; Woodley and Lacy, 2010; Carr, 2011; Narayan et al., 2012; Narayan et al., 2013; Narayen & Hero, 2014). In verterbrates, prolonged or repeated elevation of CORT may result in negative physiological consequences, including suppression of immunity, reproduction, and growth (Dhabhar, 2002; Sapolsky, 2002; Wingfield and Sapolsky, 2003; Belden, 2005). Collectively, results indicate that the triggers and effects of CORT are context-dependent. Additionally, although CORT elevations have been shown to modulate

some behavioral processes, such as anti-predator and mating behaviors (Moore and Miller, 1984; Thaker et al., 2009; Clinchy et al., 2010), this effect does not always occur, given that an acute CORT elevation did not affect locomotory activity (Ricciardella et al., 2010), and chronic CORT elevations did not alter mating activity in salamanders (Bliley and Woodley, 2012). Furthermore, the ability to cope with and resist certain environmental stressors may be influenced by both the evolutionary and developmental history of a particular organism (Hopkins et al., 2014).

A potential result of concurrent exposure to environmental stressors and chronic elevations of CORT in amphibians is increased susceptibility to infection and disease (Martin, 2009; Rollins-Smith et al., 2011). In particular, a chytrid fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) infects amphibian hosts and can cause the disease chytridiomycosis in susceptible species (Berger et al., 1998; Voyles et al., 2009). With numerous population declines linked to *Bd* infection (Berger et al., 1998; Bosch et al., 2001; Lips et al., 2006), research concerning the actions of CORT on *Bd* susceptibility are vital (Figure 1.1).

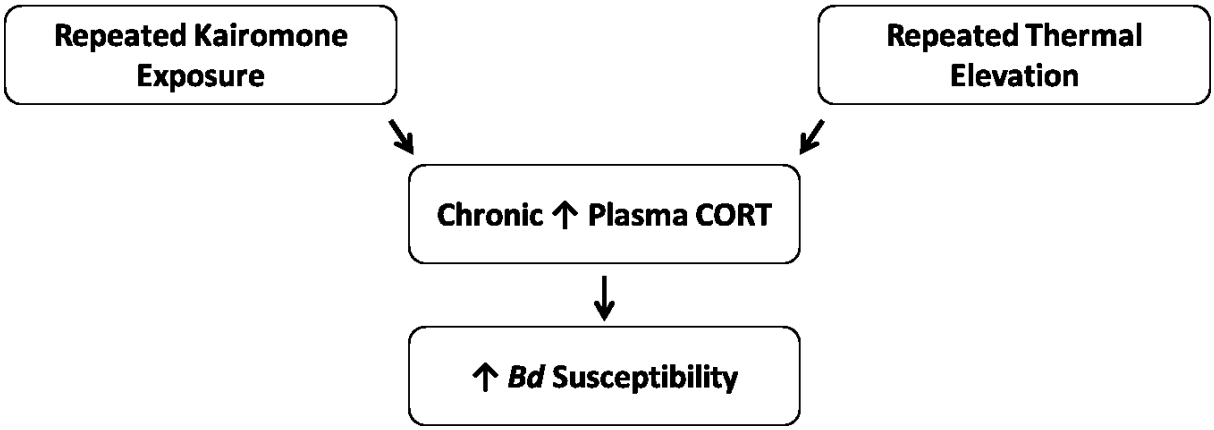

Figure 1.1: Overview of the proposed effects of predator kairomones and temperature on CORT, and how chronic CORT elevation may affect susceptibility to *Bd* infection.

Although much research has been done on effects of stressors in other vertebrates, relatively few studies have examined how environmental stressors affect behavioral and

hormonal responses in amphibian species. In particular, the role of CORT has been investigated in relatively few amphibian species, and under few circumstances. Given the alarming species declines and extinctions currently occuring, research into the exact mechanisms by which different natural stressors influence amphibian behavior and physiology are needed.

In order to better understand the actions of CORT in response to these environmental factors, I used plethodontid salamanders as an animal model for several reasons. Firstly, the behavioral processes of these salamanders are easily characterized, allowing for the observation of stress-induced behavioral changes. Secondly, they are susceptible to *Bd* and plasma CORT can be easily manipulated via dermal patches, such that they are an ideal model for investigating how a long-term CORT elevation may increase susceptibility to *Bd* infection and disease development. Finally, the extremely low-energy lifestyle of plethodontid salamanders (Feder, 1983; Pough, 1983) provides an interesting contrast to results from species with higher-energy lifestyles.

I performed a series of experiments in order to discover how CORT is involved in behavioral and physiological responses to predator-derived kairomones, thermal elevation, and susceptibility to the chytrid fungus. The following sections describe the specific aims of my research, including the specific background and main results concerning each aim.

AIM 1: Describe behavioral and endocrine responses to predator kairomones in a plethodontid salamander.

The predation-stress hypothesis states that exposure to predators and/or predator cues will cause an increased plasma elevation of glucocorticoid hormones, such as CORT, which act in modulating behavioral and physiological responses to cope with stressful stimuli

(Boonstra et al., 1998; Lima, 1998). Although a short-term CORT response to environmental stimuli is initially beneficial, a more prolonged exposure can cause long-term elevation of plasma CORT, thus leading to pathological effects such as suppression of reproduction and immunity (Sapolsky, 2002; Carr, 2011). Therefore, I tested the effects of both acute and prolonged exposure to predator kairomones in Allegheny Mountain dusky salamanders. After an acute exposure to predator kairomones, plasma CORT levels were unaltered compared to non-predator kairomone controls, although locomotory activity and mating behavior was reduced. A prolonged exposure to predator kairomones had no effect on locomotory activity or plasma CORT. These data suggest that exposure to predator kairomones does not cause an increase in plasma CORT in Allegheny Mountain dusky salamanders. It is possible that a CORT response to such stimuli is uncoupled to reduce potentially negative energetic or immunological effects of elevated CORT.

AIM 2: Describe behavioral and endocrine responses to thermal elevation in a plethodontid salamander.

Fluctuations in global temperature are proposed to affect various fitness components in ectotherms, including energy availability, CORT surges, and body mass/size (Feder, 1978; Gardner et al., 2011; Baudron et al., 2014). Specifically, ectothermic species like amphibians are predicted to be severely affected by thermal elevation (Wells, 2010), with consequences including increased susceptibility to pathogens and increased metabolic expenditures (Fitzpatrick et al., 1971; Fitzpatrick and Brown, 1975; Pounds et al., 2006; Narayan et al., 2012). With CORT responsible for mediating various metabolic processes (Mommsen et al., 1999; Norris and Carr, 2013), studies focusing on how long-term temperature elevation affects baseline CORT levels, as well as amphibian innate immunity, are vital. Therefore, I

tested the effects of prolonged temperature elevation (24°C) in Allegheny Mountain dusky salamanders. A prolonged temperature elevation caused a loss of body mass and changes in white blood cell differentials, but had no effect on baseline locomotory activity or plasma CORT levels when measured 3 weeks after housing at the elevated temperature. These results indicate that, similar to predator kairomone exposures, a long-term thermal elevation did not affect CORT responses in dusky salamanders, although the drop in body mass is consistent with increased metabolic rates. It is possible that CORT responses to some putative stressors may be inhibited in Allegheny Mountain dusky salamanders, in order to avoid costly energetic expenditures.

AIM 3: Examine the effects of stress hormones on susceptibility of salamanders to *Bd* infection and occurrence of the disease chytridiomycosis.

The emergence of *Bd* and chytridiomycosis outbreaks have been associated with multiple amphibian population declines (Rohr and Raffel, 2010; Blaustein et al., 2011). It is hypothesized that, due to a prolonged elevation of CORT induced by environmental stressors and subsequent immunosuppression (Ramirez et al., 1996; Simmaco et al., 1997; Davis and Maerz, 2008; Groner et al., 2014), environmental stressors may increase susceptibility to *Bd* infection and disease. Therefore, I tested the effects of prior CORT treatment on *Bd* infection loads and development of chytridiomycosis in red-legged salamanders. Via application of dermal patches, plasma CORT was elevated in subjects for 9 consecutive days before exposure to *Bd* inocula. *Bd*-exposed animals with prolonged CORT elevation had higher infection loads compared to animals exposed solely to *Bd*. However, while *Bd*-exposed animals exhibited symptoms of lethal chytridiomycosis, a prior repeated CORT elevation did not significantly increase signs of chytridiomycosis. An additional experiment was conducted

using a lower *Bd* dosage and longer CORT treatment under similar conditions, in order to assess whether the previously-used high dosage masked any potential CORT-driven effects on disease development. In this second experiment, animals did not display any discernable symptoms of chytridiomycosis. However, although symptoms of chytridiomycosis were not evident, *Bd*-exposed subjects treated with CORT patch for 14 days showed a higher incidence of skin sloughing on a given day than *Bd*-exposed subjects treated with oil patch. These data indicate that prior and concurrent CORT elevations may contribute to increased skin sloughing, which may be a beneficial mechanism in resisting and/or clearing *Bd* infections. Overall, these results show that a chronic elevation of CORT can influence susceptibility to *Bd* infection, which may explain, in part, the varying susceptibility to *Bd* infection of different amphibian species.

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Chapter 2

Testing the predation stress hypothesis: behavioral and hormonal responses to predator cues in Allegheny Mountain dusky salamanders

Christopher W. Fonner and Sarah K. Woodley

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ABSTRACT

The predation stress hypothesis posits that exposure to predators and/or predator cues causes release of glucocorticoid hormones which coordinate behavioral responses that facilitate predator avoidance. We measured responses to short-term and repeated exposure to predator-derived kairomones in Allegheny Mountain dusky salamanders (*Desmognathus ochrophaeus*). Salamanders expressed predator avoidance behaviors (reduced locomotion, reduced mating behavior) in the presence of predator kairomones. However, after short-term exposure to predator kairomones, plasma glucocorticoids were similar to levels after exposure to controls. After repeated exposure to predator-derived kairomones, locomotory activity and plasma glucocorticoids were similar compared to controls. There was no evidence of habituation to predator kairomones. Overall, results did not support the predation stress hypothesis in Allegheny Mountain dusky salamanders in either an acute or chronic context. Use of glucocorticoids to mediate antipredator responses may occur when predation pressure is unpredictable, and when energetic and opportunity costs of linking glucocorticoids to anti-predator responses are low.

INTRODUCTION

Predation has both direct and indirect effects on the fitness of prey species. Failure to avoid a predator can lead to death or serious injury, and animals possess a range of defenses against predators, including developmental, behavioral, and morphological responses (Mccollum and Leimberger, 1997; Lima, 1998). However, predator defense strategies can themselves affect fitness by being energetically costly or leading to missed opportunities to forage or mate (Lima, 1998; Clinchy et al., 2004; Creel et al., 2007; Clinchy et al., 2010). Given the clear fitness consequences of predator defenses, it is important to understand the physiological mechanisms that mediate predator defense strategies. The predation stress hypothesis posits that exposure to a predator and/or predator cues triggers a physiological stress response that includes release of glucocorticoid (GC) hormones via activation of the hypothalamic-pituitary-adrenal/interrenal (HPA/I) axis (Boonstra et al., 1998; Lima, 1998). Glucocorticoids act within minutes to hours to have direct effects on fitness via physiological and behavioral stress responses that help the animal avoid, counter, or cope with the risk of predation (Sapolsky, 2002). Although activation of the stress system and release of GCs in response to short-term or occasional exposure to predators is most likely beneficial, activation of the stress system and prolonged elevation of plasma GCs in response to prolonged predator pressure may induce pathological changes. For example, continual elevation of plasma GCs leads to suppression of reproduction, immune function, and muscle wasting (Sapolsky, 2002).

The predation stress hypothesis generates several predictions. First, acute, short-term exposure to predator cues leads to rapid increases in plasma GCs roughly concomitant with expression of predator avoidance behaviors. Second, prolonged exposure to predator cues leads to chronically elevated plasma GCs (i.e., high baseline), as well as constitutive

expression of predator avoidance behavior. Third, elevated plasma GCs triggers increased expression of predator avoidance behavior.

In support of the predation stress hypothesis, a single brief exposure to predators or predator cues resulted in increased concentrations of glucocorticoid hormones in a number of species (Boonstra et al., 1998; Kagawa and Mugiya, 2000; Canoine et al., 2002; Woodley and Peterson, 2003; Remage-Healey et al., 2006; Thaker et al., 2009; Amaral et al., 2010; Narayan et al., 2013; Davis and Gabor, 2015). Furthermore, elevation of plasma GCs increased expression of antipredator behaviors (Kalynchuk et al., 2004; Thaker et al., 2009; Trompeter and Langkilde, 2011). Repeated exposure to predator cues in laboratory rats, wild birds, and wild mammals was associated with chronically elevated plasma GCs and either blunted or sensitized GC responses to new stressors (Scheuerlein et al., 2001; Clinchy et al., 2004; Clinchy et al., 2010; Newman et al., 2012). In these studies, altered patterns of GCs were associated with reduced body condition and reduced reproductive output (Clinchy et al., 2004; Sheriff et al., 2009; Clinchy et al., 2010).

In other studies, however, support for the predation stress hypothesis is lacking. In elk (*Cervus elaphus*), long-term predation pressure from wolves was not associated with elevated baseline GCs (Creel et al., 2009). In some populations of tadpoles (*Rana temporaria*, *Lithobates sylvaticus*) prolonged exposure to predator cues was not associated with elevated plasma GCs (Dahl et al., 2012; Reeve et al., 2013). In Belding's ground squirrels (*Spermophilus beldingi*) and guppies (*Poecilia reticulata*), populations with increased predation risk had reduced baseline GCs (Mateo, 2007; Fischer et al., 2014). Finally, in San Marcos salamanders (*Eurycea nana*), GCs increased in response to higher risk fish predators but not to low risk predators or water controls, despite expression of antipredator behavior towards both high and low risk predators (Davis and Gabor, 2015). In these examples, there may be selection to uncouple antipredator responses from HPA/I

activation if HPA/I activation incurs unsupportable costs. In the case of overwintering elk, elevated GCs may lead to prohibitively high metabolic costs (Creel et al., 2009). In Belding's ground squirrels, elevated GCs may interfere with the ability to prepare for hibernation (Mateo, 2007). In several species of tadpoles, elevated GCs can slow growth (Hayes et al., 1993; Glennemeier and Denver, 2002; Belden, 2005; Dahl et al., 2012), and suppression of a GC response may be necessary to allow sufficient growth of tadpoles before the pond dries out.

We tested predictions of the predation stress hypothesis in adult Allegheny Mountain dusky salamanders (*Desmognathus ochrophaeus*). Dusky salamanders are members of salamander species assemblages found in stream habitats. Predation by larger species of salamanders on smaller species of salamanders has shaped the community structure (Hairston, 1986). Predators emit kairomones (chemical cues transmitted among species) that are detected by prey. A ubiquitous response of amphibians to predator-derived kairomones is decreased locomotory activity, which allows prey to escape the notice of predators (Epp and Gabor, 2008; Relyea and Edwards, 2010; Johnson and Sullivan, 2014).

To test predictions of the predation stress hypothesis, we examined whether kairomones derived from a predatory salamander elicit hormonal and behavioral stress responses compared to kairomones from a non-predatory salamander. In Experiment 1, we examined hormonal and behavioral responses to acute, short-term exposures to predator kairomones versus non-predator kairomones. We predicted that acute exposure to predator kairomones would elevate plasma corticosterone (CORT, the primary GC in amphibians) and decrease locomotory activity and mating behaviors. In Experiment 2, we confirmed and expanded on hormone results found in Experiment 1 by measuring acute CORT responses to kairomones at 2 additional time points and without any previous exposure to kairomones. In Experiment 3, we examined hormonal and behavioral responses to prolonged, repeated,

exposures to predator kairomones, with the prediction that chronic exposure to predator kairomones over several days would be associated chronically elevated plasma GCs (i.e., high baseline), as well as constitutive expression of predator avoidance behavior.

METHODS

Animals

Adult Allegheny Mountain dusky salamanders were collected May and June of 2010 from two adjacent runs in Westmoreland County, PA: 40.147126 N, 79.208593 W (Experiments 1 and 3) or in September of 2013 from Brooks Run in Cameron County, PA: 41.409132 N, 78.055232 W (Experiment 2). After collection, salamanders were housed individually in the laboratory in plastic home boxes (16 x 16 x 5 cm) lined with moist unbleached paper towels with a 14:10 light:dark cycle (lights off at 1700 h) at 16°C. Animals were fed wax worms every two weeks. All protocols were approved by Duquesne University's Institutional Animal Care and Use Committee. Permits for animal collection were obtained from the Pennsylvania Fish and Boat Commission.

Preparation of kairomone rinses

Predator-derived kairomones were obtained from the spring salamander (*Gyrinophilus porphyriticus*). Spring salamanders are large salamanders that prey on smaller salamander species, including on adult *D. ochrophaeus* (Formanowicz and Brodie, 1993; Petranka, 2010). Spring salamanders were collected from Westmoreland County, PA, 40.147126 N, 79.208593 W (Experiments 1 and 3) or Cameron County, PA 41.409132 N, 78.055232 W (Experiment 2).

Our control was kairomones from the red-backed salamander (*Plethodon cinereus*), a salamander species that does not prey on Allegheny Mountain dusky salamanders (Petranka,

2010). Red-backed salamanders were obtained from Westmoreland County, Pennsylvania, 40.095833 N, 79.295833 W). We used rinses from a non-predator salamander rather than just water as our control because we wanted to differentiate between predator-specific responses versus general responses to other salamanders. Studies indicate that behavioral responses to rinses from non-predatory (heterospecific or conspecific) salamanders are similar to responses to water controls (Ricciardella, 2008; Johnson and Sullivan, 2014).

Both spring salamanders and red-backed salamanders were fed wax worms every two weeks. Following a method used previously (Bliley and Woodley, 2012), we collected kairomones by placing each predator or non-predator in *dd*H_20 (110 mL for *G. porphyriticus*; 35 mL for *P. cinereus*) for approximately 24 h at 16°C. Kairomones were used in experiments immediately after preparation. In order to have sufficient volumes of kairomones, we pooled kairomones from two individual predators or from six individual non-predators before use.

Due to the larger body size of spring salamanders compared to red-backed salamanders, the concentration of predator-derived kairomones was greater than the concentration of kairomones from non-predators, as measured by Pierce BCA™ proteins assay kits. To verify that responses of prey to the predator kairomones were not simply due to an increased amount of material, we compared the locomotory activity of salamanders tested in the presence of non-diluted predator kairomones with activity in the presence of predator kairomones diluted to the same concentration as non-predator kairomones. Locomotory activity was estimated by scanning testing chambers once every minute for 60 minutes and summing the number of scans in which the location changed relative to the previous scan. Both diluted and non-diluted predator kairomones decreased locomotory activity compared to non-predator kairomones (diluted predator kairomones: 25 ± 2.4^{a}; predator kairomones: $22 \pm 2.2^{a;}$ non-predator kairomones: 31 ± 2.1^{b} (mean ± SEM); groups

that do not share a letter are significantly different; repeated measures ANOVA: $F_{2,56} = 3.75$, $p = 0.03$, $n = 30$).

Experiment 1: Behavioral and hormonal responses to short-term exposure to kairomones

Experiment 1 (performed in June 2010) tested the prediction that short-term exposure to predator kairomones would be associated with increased plasma CORT and increased expression of predator avoidance behaviors compared to exposure to non-predator kairomones. The same set of animals (20 males and 20 females) was used for each test, and the order of testing was locomotory activity, mating behavior, and hormone sampling. Because salamanders are crepuscular, all testing was started in the early evening, approximately 2 h after lights were turned off. Dim incandescent lighting provided light when observing behaviors.

To measure locomotory activity, each subject was transferred from its home box to a testing chamber (24 x 24 x 2 cm) lined with a single layer of unbleached paper towel moistened with 15 mL of either predator or non-predator kairomones. A time-lapse digital video camera (Sony DCR-VX2000) recorded for two seconds every minute. After 75 minutes, subjects were returned to their home boxes. At a later time, the footage was observed and activity (excluding the first 15 minutes because it represents an acclimation period) was scored by an investigator blind to the treatments. To quantify activity, each chamber was divided into four quadrants. Each time the salamander's head was in a different quadrant from one 2 second scan to the next was counted as one movement. Each animal was tested one time on predator kairomones and another time on non-predator kairomones. To avoid an order effect, half of the animals were exposed to predator kairomones on the first test, and half were exposed to non-predator kairomones on the second test. At least 3 days separated the activity tests.

One week after the last activity test, mating behavior was assessed. To measure mating behavior, male-female pairs were placed in testing chambers (24 x 24 x 2 cm) lined with a single layer of unbleached paper towel moistened with 15 mL of either predator or non-predator kairomones. Behaviors were observed by the investigators using scan sampling methods (2 second scans every 10 minutes) for 4.5 hours after pairing. Mating behaviors measured during this time included: contact (time to first contact between the two salamanders), first observation of tail-straddling (an advanced stage of courtship), spermatophore deposition by a male salamander, and female insemination. After the observation period, subjects were left in chambers overnight (approximately 10 more hours), after which chambers were checked for further spermatophore depositions and female inseminations, which remain detectable for at least 12 hours after insemination (Verrell and Houck, 1988). After the mating tests, subjects were returned to home boxes. Mating was measured only a single time, with 10 pairs tested in the presence of predator kairomones and 10 pairs tested in the presence of non-predator kairomones.

One week after mating tests were completed, differences in plasma CORT upon exposure to predator or non-predator kairomones were assessed. To do so, subjects were gently placed in testing chambers lined with unbleached paper towels moistened with 15 mL of either predator or non-predator kairomones. After 45 minutes of exposure to the treatments, subjects were decapitated and the blood was collected in a heparinized capillary tube. We chose to measure hormones at 45 minutes because previous studies found that changes in locomotory behavior were evident by 45 minutes after exposure to predator kairomones (Ricciardella, 2008). The tube was centrifuged and the plasma fraction (typically 2 to 8 uL) was frozen in a heparinized microfuge tube at -20°C until assaying for CORT concentrations.

All tests were completed within one month. Subjects were exposed to kairomones no more than three times over the course of the experiment, with approximately one week between each exposure.

Experiment 2: Hormonal responses to short-term exposure to kairomones

Experiment 2 (conducted December 2013) further tested the prediction that exposure to predator kairomones leads to increased plasma CORT in the time frame that predator avoidance behaviors were expressed. Also, we wanted to confirm that the three exposures to kairomones over a period of several weeks prior to the hormone sampling in Experiment 1 did not influence responses to predator-derived kairomones (for example, perhaps habituation was occurring in Experiment 1). Methods were similar to those described in Experiment 1 except that we only measured hormonal responses to kairomones, not behavioral responses. Also in contrast to Experiment 1, subjects in Experiment 2 were exposed to kairomones (either from a predator or non-predator) only a single time before collecting blood to measure hormones. Finally, we measured plasma CORT levels at 30 minutes or 3 hours after initial exposure to kairomones. We chose these time points to confirm that we had not missed an increase before 45 minutes in Experiment 1, and also because we found that some predator avoidance behaviors (i.e., decreased courtship and mating behaviors) to predator kairomones were evident from 45 minutes to 4 hours after initial exposure to kairomones.

Experiment 3: Behavioral and hormonal responses to prolonged exposure to kairomones

Experiment 3 (conducted in September 2010) was performed to test the prediction that prolonged exposure to predator cues leads to chronically elevated plasma GCs (i.e., high baseline), as well as constitutive expression of predator avoidance behavior. To do so, we needed to separate responses to the prior treatment from responses to the current

environment. Thus, we first exposed animals to treatments for at least 10 days and after which we exposed them to non-predator kairomones (presumably a relatively neutral, control environment) and tested them for behavior or CORT. It was also important to know whether animals habituated to the prolonged exposure to predator cues. Thus, we also tested animals in the presence of predator kairomones. If animals had habituated to the predator cues, they would no longer suppress locomotion compared to the groups that were not repeatedly exposed to predator cues.

Subjects were housed individually in 14 cm diameter petri dishes. The bottom of each petri dish was lined with unbleached paper towel moistened with 5 mL of either predator kairomones (n = 12) or non-predator kairomones (n = 11). The kairomone treatments were renewed one time per d for 28 consecutive days (a static renewal design) by replacing the petri dish bottom every day with a fresh bottom lined with fresh kairomones. We also included an additional "no treatment" control group in which subjects (n = 11) were left undisturbed in 14 cm diameter petri dishes lined with towel moistened with 5 mL of water until testing. We included a "no treatment" control to determine whether the daily replacement of petri dish bottoms related to the kairomone treatments affected responses.

After 10 days of treatments, testing for differences in locomotory activity began. (We did not measure mating behavior in this experiment.) We chose 10 days to start testing because in another species of dusky salamander (*D. ocoee*), physical handling every day for 10 days resulted in decreased baseline locomotory activity (Bliley and Woodley, 2012). Each behavioral test occurred at least 8 hours after the previous renewal of the kairomone or non-kairomone treatments. To measure locomotory activity, subjects were transferred from the petri dishes to testing chambers (24 x 24 x 2 cm) that were lined with paper moistened with 15 mL of a fresh application of predator kairomones or non-predator kairomones. A time-lapse digital camera recorded for 2 seconds every minute as described for Experiment 1.

At the end of the recording, subjects were returned to their petri dishes. At a later time, the footage was observed and activity was scored as described for Experiment 1. Each subject was tested one time in the presence of predator kairomones and another time in the presence of non-predator kairomones, with treatments counter-balanced and separated by 4 days.

After 28 days of treatments (two weeks after the last behavioral test), subjects were gently placed in testing chambers lined with paper towels moistened with 15 mL of either predator or non-predator kairomones. After 45 minutes, trunk blood was collected for hormone assays as described above. We were unable to collect blood samples from three of the smaller animals. Thus, sample sizes for plasma CORT were reduced relative to those for activity tests.

Hormone assays

Plasma CORT was assayed at the Endocrine Technology Laboratory at the Oregon National Primate Research Center. A double ether extraction was performed on up to 3μL of plasma, after which concentrations of CORT were obtained via standard radioimmunoassay protocols (Resko et al., 1980; Gruenewald et al., 1992). Recoveries averaged 90% and sensitivity of the radioimmunoassay was 5 pg (1.5 ng/mL for 3 uL of sample). The assay was validated for Allegheny Mountain dusky salamanders by demonstrating parallelism from 0.5 to 4 uL at 0.5 uL increments. All samples from a single experiment were assayed together. The intra-assay coefficients of variation (CV) were 11.2% (Experiment 1), 11.2% (Experiment 2), and 12.1% (Experiment 3). The inter-assay CV was 16.0%. All plasma samples in Experiments 2 and 3 were detectable by the hormones assays. Twenty-five percent of the plasma samples in Experiment 1 were non-detectable and those samples were assigned the lowest detectable value of the assay.

Statistical analyses

Statistical analyses were performed using PASW (Predictive Analytics Software) 18. Data analyzed with parametric statistics met assumptions of normality and homoscedasticity. For analysis of locomotory activity, the number of times the location of each subject changed relative to the previous scan was summed. In Experiments 1 and 3 (behavior was not measured in Experiment 2), the locomotory activity of each subject was measured once with predator kairomones and again with non-predator kairomones in a counterbalanced order. Therefore, in Experiment 1, activity was analyzed using a 2-way repeated measures ANOVA with treatment (predator kairomone or non-predator kairomone) as the repeated, within subjects factor and sex as the between subjects factor. In Experiment 3, activity was measured using a 3-way repeated measures ANOVA with prior treatment (no treatment, non-predator kairomone, or predator kairomone for at least 10 days) and sex as between subjects factors, and with treatment at the time activity was being measured (i.e., tested in the presence of non-predator kairomones or predator kairomones on the substrate) as the repeated, within subjects factor.

For analysis of courtship and mating behaviors in Experiment 1, the percent of times that an individual male salamander reached a particular behavior (averaged over two mating opportunities) was calculated. Data were analyzed using a Fisher's exact test.

Experiment 1, CORT values were analyzed with Mann-Whitney U-tests because values were not normally distributed. In Experiments 2 and 3, CORT data were homoscedastic and normally distributed, and therefore were analyzed with ANOVA. In Experiment 2, values were detectable and were assayed with a 2-way ANOVA with treatment (predator kairomone or non-predator kairomone) and time (30 minutes or 3 hours) as between subjects factors. To determine whether mass contributed to plasma CORT levels, we included mass as a covariate in the model. In Experiment 3, all CORT values were detectable

and results were analyzed using a 3-way ANOVA, with prior repeated treatment (no treatment, non-predator kairomone, or predator kairomone), kairomone treatment on the testing substrate (non-predator kairomone or predator kairomone), and sex as factors. One CORT value was excluded from analysis because it was 10 times higher than the other values.

RESULTS

Experiment 1: Behavioral and hormonal responses to short-term exposure to kairomones

In the presence of predator kairomones, subjects exhibited decreased levels of activity compared to control subjects exposed to non-predator kairomones (repeated measures ANOVA: Figure 2.1; $F_{1,38} = 10.9$, $p = 0.002$). There was also a significant difference in activity levels between sexes, as females were less active compared to male subjects (Figure 2.1; $F_{1,38} = 9.8$, $p = 0.003$). The interaction between sex and treatment was not significant ($F_{1,38} = 0.002$, $p = 0.96$).

In the presence of predator kairomones, subjects exhibited decreased levels of courtship and mating behaviors compared to subjects exposed to non-predator kairomones. Specifically, fewer male-female pairs initiated contact (by 45 minutes) or engaged in tail straddling, spermatophore deposition and insemination in the presence of predator kairomones, compared to non-predator kairomone controls (Table 2.1, Fisher's Exact Test: contact, $p = 0.035$; tail straddling, $p = 0.01$; spermatophore deposition, $p = 0.04$; female insemination, $p = 0.04$). Also, by the next day, fewer male-female pairs had deposited spermatophores in the presence of predator kairomones compared to non-predator kairomone controls (Table 2.1, Fisher's Exact Test: spermatophore next day, $p = 0.04$).

Subjects exposed to predator kairomones for 45 minutes prior to blood collection did not have altered plasma CORT levels compared to subjects exposed to non-predator

kairomones (Figure 2.2; $U = 152.5$, $p = 0.30$). There was no significant difference in plasma CORT levels between male and female subjects (Figure 2.2; $U = 129.0$, $p = 0.08$).

Experiment 2: Hormonal responses to short-term exposure to kairomones

Mass did not explain differences in plasma CORT levels ($F_{1,26} = 2.6$, $p = 0.12$) and was excluded from subsequent ANOVAs. Exposure to predator kairomones for 30 minutes or 3 hours did not elevate plasma CORT relative to non-predator kairomones (Figure 2.3), (effect of $F_{1,27} = 0.86$, $p = 0.36$). Plasma CORT levels were similar at 30 minutes versus 3 hours of kairomone exposure ($F_{1,27} = 0.04$, $p = 0.85$). The interaction between kairomone and time was nonsignificant ($F_{1,27} = 2.2$, $p = 0.16$).

Experiment 3: Behavioural and hormonal responses to prolonged exposure to kairomones

Levels of activity (Figure 2.4) were similar after prior, prolonged treatment to non-predator kairomones, predator kairomones, or no treatment, regardless of testing substrate ($F_{2,28} = 1.1$, $p = 0.34$). However, all subjects were less active when tested in the presence of predator kairomones (Figure 2.4; $F_{1,28} = 13.8$, $p = 0.001$). Female subjects were significantly less active than males (Figure 2.4; $F_{1,28} = 4.9$, $p = 0.035$). Interactions were nonsignificant (all $p > 0.30$).

Plasma CORT levels (Figure 2.5) were similar among treatment groups, with no effect of prior treatment ($F_{2,18} = 0.9$, $p = 0.42$), concurrent/acute treatment ($F_{1,18} = 0.8$, $p = 0.40$), or sex ($F_{1,18} = 0.5$, $p = 0.48$). All interactions were nonsignificant.

DISCUSSION

Our results did not provide support for the predation stress hypothesis in Allegheny Mountain dusky salamanders in either acute or chronic contexts. After single, short-term

exposure to predator kairomones, both males and females were significantly less active and courted/mated less than when tested in the presence of predator kairomones. However, plasma CORT levels were not altered in the time frame in which behavioral responses were evident, indicating that CORT was not driving the behavioral changes. When exposed daily to predator kairomones for more than 10 days (simulating chronic exposure), there was no change in baseline locomotory activity or baseline plasma CORT when tested in the presence of non-predator kairomones. The lack of change in plasma GCs and behavior after prolonged exposure to predator kairomones was not due to habituation because subjects still decreased activity when tested on a fresh application of predator kairomones. Taken together, neither a single nor prolonged exposure to predator kairomones altered plasma CORT relative to control treatments, failing to support key predictions of the predation stress hypothesis. These results are discussed further below.

Behavioral and hormonal responses to short-term exposure to kairomones

Exposure to predator kairomones resulted in dramatic behavioral changes. Within 1 hour of predator kairomone exposure, locomotory behavior of both males and females was reduced. Reduced locomotory activity is a common response to predator exposure in amphibians and other vertebrates (Wells, 2007), and functions to reduce notice by predators (Rohr and Madison, 2001). Exposure to predator kairomones also affected mating and courtship, with fewer male-female pairs in physical contact by 45 minutes after pairing in the presence of predator kairomones, and fewer spermatophore depositions and fewer inseminations by 4 hours. The suppressive effects of predator kairomones on courtship and mating probably also function to reduce notice by predators, although delay in the progression of courtship potentially reduces the probability of successful mating.

Despite the strong behavioral responses to a relatively short-term, concurrent exposure to predator kairomones, there was no change in plasma CORT when sampled 30 minutes, 45 minutes, or 3 hours following placement onto a substrate moistened with predator kairomones (Experiments 1 and 2). Although the mean plasma CORT level in Experiment 1 was slightly higher in animals exposed to predator versus non-predator kairomones, a power analysis showed that sample sizes of 108 in each group would be necessary to reach statistical significance (alpha of 0.05, power of 0.8). Also, Experiment 2 found no effect of a single exposure to predator kairomone on plasma CORT at 30 minutes or 3 hours. In Allegheny Mountain dusky salamanders, capture induced an increase in plasma CORT within 30-60 minutes (Woodley et al., 2014), indicating that dusky salamanders have the capacity to elevate plasma CORT by 30 or 45 minutes after the onset of a stimulus. We cannot rule out the possibility that exposure to predator kairomones may have elevated plasma CORT at time points between 45 minutes and 3 hours, or later than 3 hours, but it is clear that exposure to predator kairomones does not elevate plasma CORT from 30 to 45 minutes after onset of exposure, which is the time frame for which reduced locomotory activity and delayed male-female contact in the mating trials are evident. Also, plasma CORT was not elevated at 3 hours, which is in the same time frame as the suppressive effects of predator kairomones on spermatophore deposition and insemination. The difference levels of plasma CORT between Experiments 1 and 2 may due to seasonal differences in when the studies were done (June versus December).

Another prediction of the predation stress hypothesis is that exogenous elevation of plasma CORT would increase expression of predator avoidance behavior. Although we did not test that prediction in the current study, a separate study from our laboratory demonstrated that treatment of male Allegheny Mountain dusky salamanders with CORT patches (which produce elevated plasma CORT (Wack et al., 2010)) failed to alter

locomotory behavior from 1 to 4 hours post-patch treatment (Ricciardella et al., 2010). The CORT patch study was conducted in the context of handling stress, which also produces a decrease in locomotory behavior, similar to predator kairomone exposure. Thus, acute exposure to predator kairomones does not elevate plasma CORT, and acute exogenous elevation of plasma CORT does not alter locomotory behavior, indicating that the predation stress hypothesis, in an acute context, is not supported in Allegheny Mountain dusky salamanders.

Behavioral and hormonal responses to prolonged exposure to kairomones

A prediction of the predation stress hypothesis is that populations exposed to chronic predation pressure will have chronically elevated plasma GCs and altered behaviors such as increased vigilance and other predator avoidance behaviors. In Allegheny Mountain dusky salamanders, daily exposure to predator kairomones for 10 days did not constitutively reduce locomotory activity or produce a high baseline plasma CORT. This is evident in Figures 2.4 and 2.5 when tested in the presence of non-predator kairomones. The same pattern is seen when tested in the presence of predator kairomones. Activity of subjects in the "no treatment" control were similar to those in subjects repeatedly exposed to predator or non-predator kairomones, indicating that the static-renewal design of the study did not itself alter locomotory activity. Daily exposure to predator kairomones over many days might produce habituation, where animals fail to respond after a period of time. Habituation would be expected in situations where the likelihood of predation varies seasonally or according to predator satiety (Raderschall et al., 2011). Habituation might preclude the ability of predator kairomones to elicit an increase in CORT. However, we found no evidence for habituation. When salamanders were exposed to predator kairomones every day for 10 days, and then tested on substrate moistened with predator kairomones, they expressed decreased activity,

similar to decreased activity demonstrated in salamanders that had received no prior treatment, or those that had been repeatedly exposed to non-predator kairomones. Thus, our results indicate that prolonged exposure to predator kairomones fail to alter baseline plasma CORT or baseline levels of locomotory activity.

Discussion of why CORT was not elevated upon exposure to predator kairomones

There are many, non-mutually exclusive, explanations for why we failed to observe a CORT response after acute or chronic exposure to predator kairomones. Due to the costs of predator defenses, many animals adjust their predator defense strategies according to the frequency of encountering predators and the risk involved (Lima and Bednekoff, 1999; Cockrem and Silverin, 2002; Monclus et al., 2009). The risk-sensitive predator avoidance hypothesis and risk allocation hypothesis (versions of the predation stress hypothesis) state that behavioral and GC responses to predators/ predator cues are reduced when the risk of predation is low (Lima and Bednekoff, 1999; Muller et al., 2006; Monclus et al., 2009). Thus, it could be that we failed to measure increased plasma CORT because the risk of predation cued by predator kairomones was low. Perhaps if we had exposed Allegheny Mountain dusky salamanders to chemical cues derived from injured conspecifics, or a combination of chemical and visual cues from a predator, there may have been a CORT response. However, exposure to predator kairomones induced strong changes in both locomotory activity and mating behavior, suggesting that the salamanders perceived the predator kairomones as a risk worth avoiding.

Another related possibility for the absence of a CORT response is that animals habituated to the predator kairomones such that they no longer perceived the predator kairomones as representing a risk. This could have occurred in Experiment 1 because subjects were exposed to predator kairomones three times over the course of one month (in

order to complete behavioral testing) prior to a final exposure after which we collected blood and measured CORT. The three exposures to predator kairomones may have led to habituation, a low perceived risk, and consequently no CORT response. However, in Experiment 2, subjects were exposed to predator kairomones (at least in the laboratory) only once and there was still no CORT response. In addition, in Experiment 3, subjects were less active when tested on predator kairomones even after more than 10 days of daily exposure, indicating that subjects did not habituate to predator kairomones. Together, our results indicate that the lack of CORT response to predator kairomones was not because of habituation to the predator kairomones.

A third explanation may have to do with the predictability of the predation risk. The HPA/I axis is more responsive to unpredictable threats than to predictable threats (Sapolsky, 2002). Allegheny Mountain dusky salamanders have co-occurred with *Gyrinophilus* predators for millions of years, and predation by larger salamander species has shaped salamander community assemblages (Hairston, 1986). Given that predation threat is likely to be a predictable aspect of salamander assemblages, mechanisms underlying responses to kairomones derived from predatory species of salamanders may not involve the HPA/I axis. It is useful to compare our results with findings in Fijian ground frogs (*Platymantis vitianus*), which show a large elevation in urinary CORT metabolites within 1 hour of exposure to the sight and smell of Cane toads (*Rhinella marina*) (Narayan et al., 2013). Cane toads are a very recent predator of Fijian frogs, and cane toad cues may represent an unpredictable feature of their environment, thereby eliciting a CORT response. Similarly, San Marcos salamanders (*E. nana*) mounted a CORT response after exposure to high risk fish predators but not after exposure to low risk predators (Davis and Gabor, 2015). Future studies should examine responses to predictable versus unpredictable predators to further explore the predation stress hypothesis.

A fourth explanation is that there may be costs to linking plasma CORT elevations with anti-predatory behaviors. In Allegheny Mountain dusky salamanders, as with many vertebrates, the sensitivity of HPA/I is reduced during the mating season (Romero, 2002; Ricciardella et al., 2010), and linking predator avoidance responses to a seasonally variable hormone may be maladaptive. Furthermore, elevation of plasma CORT can increase oxygen consumption, suggesting energetic costs to CORT responses (Durant et al., 2008; Preest and Cree, 2008; Wack et al., 2012). The energetic costs associated with a CORT response may outweigh its the potential benefits, especially in species that are energetically constrained (Mateo, 2007; Creel et al., 2009), or which are highly specialized for low energy lifestyles like Allegheny Mountain dusky salamanders (Feder, 1983).

Conclusions

Taken together, our results in Allegheny Mountain dusky salamanders do not support the predation stress hypothesis after either short-term or more prolonged exposure to predator cues. As detailed above, we think it is unlikely that dusky salamanders viewed the predator kairomones as low threat or that they habituated to the predator kairomones. Instead, we suggest that the behavioral responses to predators are unlinked from changes in plasma CORT in Allegheny Mountain dusky salamanders because of potential costs of CORT responses and/or because the predator is a predictable part of the environment. Clearly, the predation stress hypothesis does not explain all responses to all predators in all vertebrate groups. Further investigation of the predation stress hypothesis in species with differing patterns of predation pressure and energy requirements will help elucidate the contexts in which the predation stress hypothesis applies.

Attributions

Sarah Woodley and I conceived of and designed the experiments described in this chapter, as well as performed the statistical analyses. I performed almost all experimental procedures with the following exceptions. Sarah Woodley and Jess Thomas helped with blood collection, the Endocrine Services Laboratory of the Oregon National Primate Research Center performed the hormone assays, and undergraduates Paige Langhals, Katie Ratay, and Rachel Michael aided in counting of white blood cells.

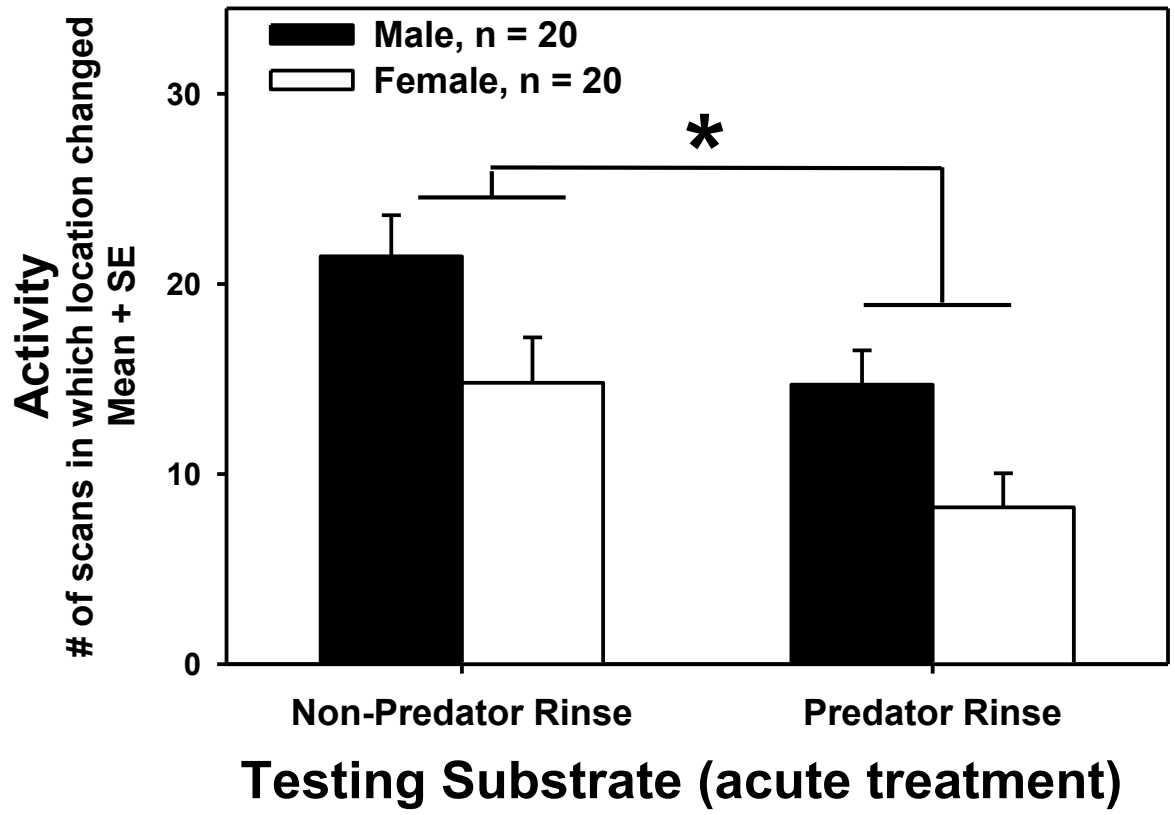

Figure 2.1. Activity of male and female Allegheny Mountain dusky salamanders tested in the presence of non-predator kairomones or predator kairomones for 1 hour. Asterisk indicates that predator kairomone exposure significantly decreased activity levels in both males and females, $p = 0.002$.

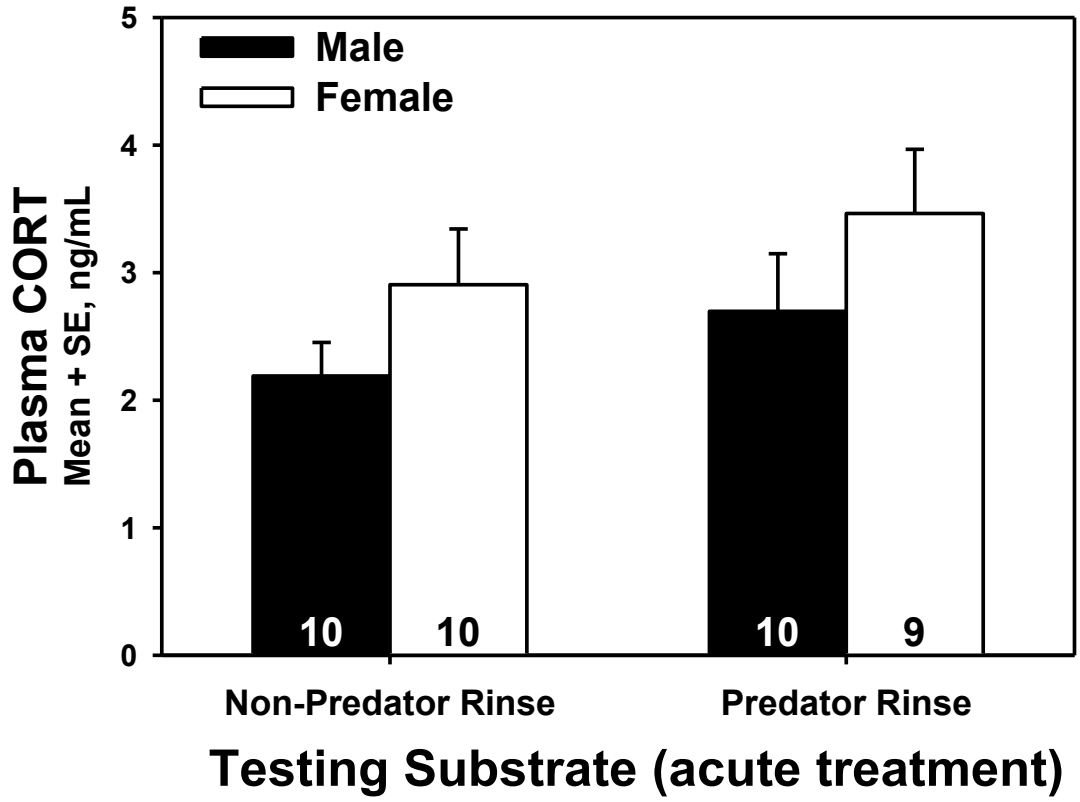

Figure 2.2. Plasma CORT levels of male and female Allegheny Mountain dusky salamanders exposed for 45 minutes to either non-predator kairomones or predator kairomones. No significant difference in CORT levels between the two treatments was present. Sample sizes are indicated within bars.

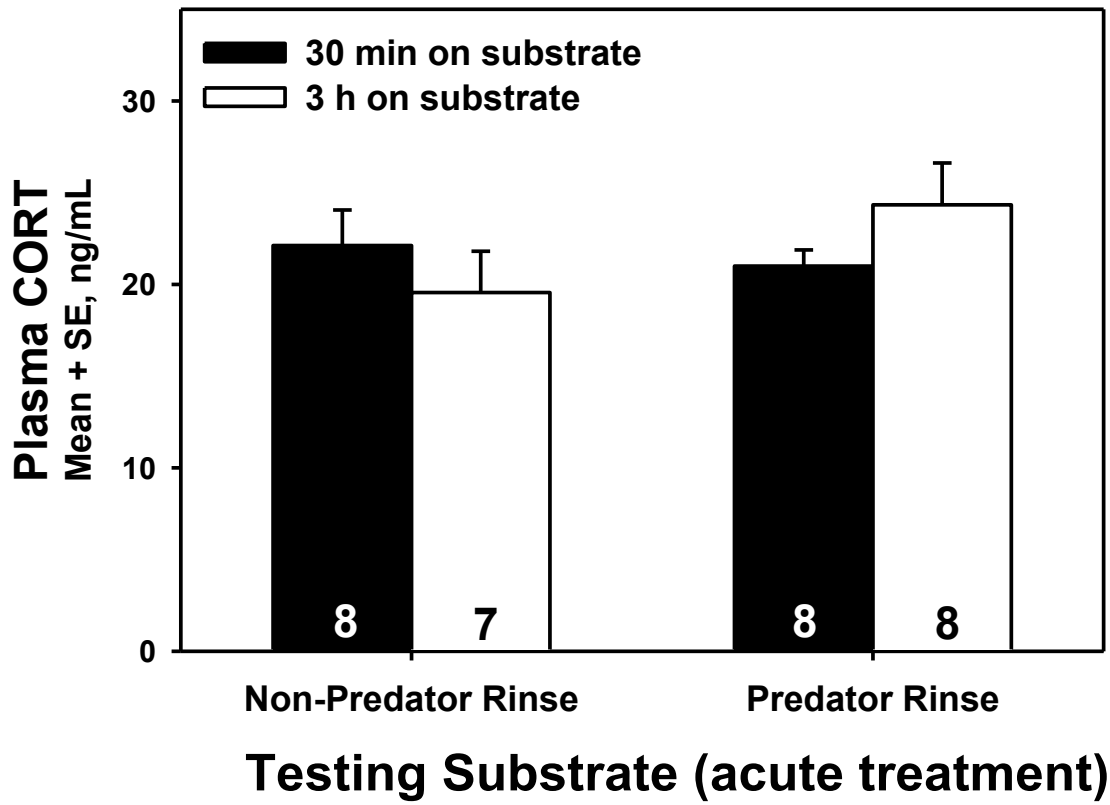

Figure 2.3. Plasma CORT levels of male Allegheny Mountain dusky salamanders exposed to either non-predator kairomones or predator kairomones for either 30 minutes or 3 hours. No significant differences in CORT levels among the treatments were present. Sample sizes are indicated within bars.

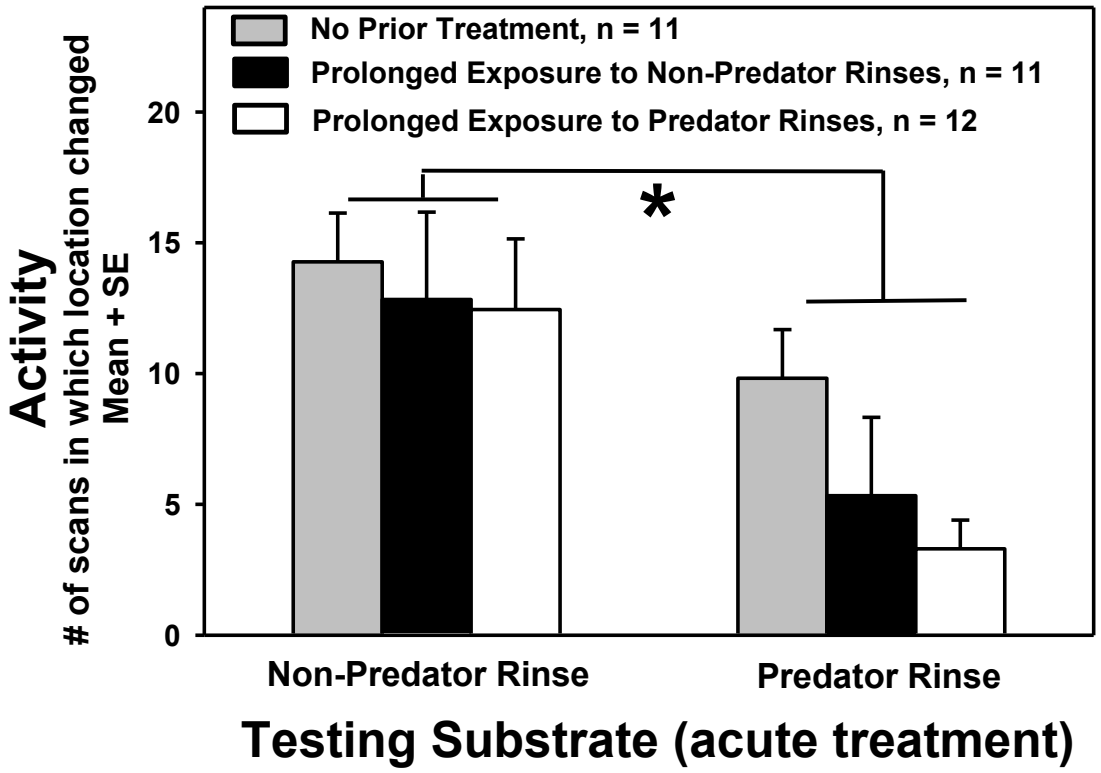

Figure 2.4. Activity of male and female Allegheny Mountain dusky salamanders after prolonged exposure to predator kairomones. Subjects were either left undisturbed (no treatment) or were exposed to non-predator kairomones or predator kairomones every day for at least 10 days. Then, activity was measured in the presence of predator kairomones or non-predator kairomones. Although there was no significant effect of prior, prolonged treatment on activity, activity was reduced when tested in the presence of predator rinses compared to non-predator rinses (asterisk, $p = 0.001$).

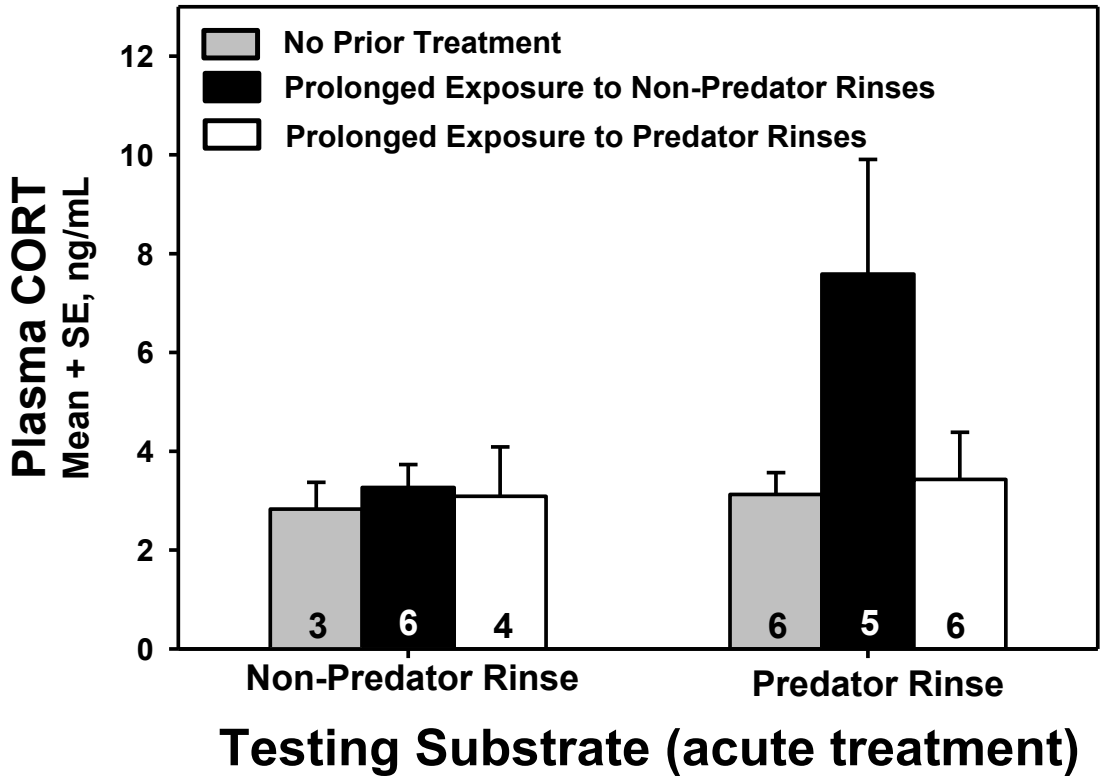

Figure 2.5. Plasma CORT levels of male and female Allegheny Mountain dusky salamanders after prolonged exposure to predator kairomones. Sample sizes are within bars. Subjects were either left undisturbed (no treatment) or were exposed to either non-predator kairomones or predator kairomones every day for at least 10 days. On the final day, subjects were exposed to either non-predator kairomones or predator kairomones and blood was collected 45 minutes later. There was no effect of prior, prolonged treatment on CORT levels, nor was there an effect of the short-term, acute exposure to predator kairomones compared to non-predator kairomones.

Table 2.1. The number of male-female pairs that initiated different courtship and mating behaviors when exposed concurrently to either predator kairomones or non-predator kairomones (Experiment 1). S.D.: spermatophore deposition.

	Concurrent treatment	Contact by 45 minutes	Tail straddling by 4 hours	S.D. by 4 hours	Insemination by 4 hours	S.D. by next day	Insemination by next day
Male-Female Pairs	Non-Predator Kairomone	7/10*	8/10*	7/10*	7/10*	10/10*	9/10
	Predator Kairomone	2/10	2/10	2/10	2/10	6/10	6/10

*Significant difference in courtship and mating activities between kairomone and non-kairomone treatments.

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Chapter 3

Too hot to handle: how elevated temperature affects behavioral, hormonal, and immunological variables in Allegheny Mountain dusky salamanders

ABSTRACT

Global climate change is predicted to severely impact numerous species around the world, including ectothermic species like amphibians. Amphibians in montane habitats are expected to experience dramatic temperature shifts in the future with adverse effects on population viability. I hypothesized that a prolonged thermal elevation would elicit behavioral and physiological changes in Allegheny Mountain dusky salamanders. After being housed for 3 weeks at 24°C, male and female salamanders lost more body mass compared to animals housed at 17°C, a preferred temperature. There were also differences in white blood cell differentials, with relatively more monocytes and fewer neutrophils and lymphocytes in animals held at 24°C. However, animals housed for at least 3 weeks at 24°C and 17°C had similar levels of plasma corticosterone (CORT) and locomotory activity at a given temperature. Collectively, our data indicate that a thermal elevation to ecologically relevant temperatures has effects on body mass and immune cells but no effect on locomotory activity or plasma CORT in Allegheny Mountain dusky salamanders.

INTRODUCTION

Temperature is a critical factor that affects animal behavior and physiology, and global warming is expected to have dramatic impacts on animal populations (Feder, 1978; Angilletta et al., 2004; Hickling et al., 2006; Gardner et al., 2011; Baudron et al., 2014). Long-term responses to global temperature changes include shifted habitat distributions (Galbreath et al., 2009) and reduced mean body size in a population (Gardner et al., 2011; Baudron et al., 2014).

Amphibians may be particularly susceptible to thermal fluctuations because they are ectotherms and they are prone to temperature-linked evaporative water loss (Wells, 2010). Temperature may also influence susceptibility to infection and disease when amphibians are exposed to pathogens like ranavirus and chytrid fungi (Pounds et al., 2006). At the organismal level, metabolic rate is positively associated with body temperature (Fitzpatrick et al., 1971; Fitzpatrick and Brown, 1975; Narayan et al., 2012). Increased metabolic rate can lead to reduced growth if animals are energetically limited. Metabolically-induced energy expenditures can also limit activity, reproduction, maintenance or storage. For example, warmer temperatures cause frogs to find refuge and decrease activity (Pough et al., 1983). Decreased activity levels may negatively affect migration patterns, geographic dispersal, and foraging (Roe and Grayson, 2008).

Intermediary metabolism (particularly lipid and glucose metabolism) is mediated in part by corticosterone (CORT), a glucocorticoid hormone released via the hypothalamic-pituitary-adrenal/interrenal (HPA/I) axis (Mommsen et al., 1999). Additionally, plasma CORT elevations directly increased metabolic rates as measured by oxygen consumption in both lizards and salamanders (Preest and Cree, 2008; Wack et al., 2012). Increases in CORT in amphibians are induced by a number of environmental factors such as capture and handling, food deprivation, and pollution (Carr, 2011). Only a few studies have examined how plasma CORT may be

affected by thermal elevation. One study found that adrenal output of CORT in frogs (*Rana esculenta*) increased at temperatures above 20°C (Jurani et al., 1973). Other studies of cane toads (*Rhinella marina*) found that acute and repeated exposures to very high temperatures (35°C) resulted in increased plasma CORT (Narayan and Hero, 2014a; Narayan and Hero, 2014b). However, these temperatures were extreme, resulting in disorientation and/or death.

Temperature fluctuations have been shown to have variable effects on different aspects of ectothermic immunity. For instance, colder temperatures have been shown to decrease amphibian immune functions, including anti-microbial peptide efficacy, lymphocyte counts/proliferation, and antibody production (Cone and Marchalonis, 1972; Maniero and Carey, 1997; Matutte et al., 2000), while also increasing immune activity of neutrophils and nonspecific cytotoxic T-cells (Le Morvan et al., 1996; Raffel et al., 2006). Compared to lower temperatures, fewer studies have focused on immunological changes due to elevated temperatures, with those studies showing that, at both warmer and cooler temperatures, relative numbers of neutrophils increased and relative numbers of lymphocytes decreased in newts (Bennett and Daigle, 1983). Other studies have also observed that, after being kept at lower temperatures, serum complement levels in amphibians can increase when switched to a warmer temperature (Green and Cohen, 1977; Maniero and Carey, 1997). However, studies examining long-term effects of elevated temperature on amphibian immunity are still needed.

Due to current data projecting seasonal temperature shifts, investigations focusing on behavioral and physiological responses of amphibians to thermal shifts are vital. In this study, we examined the effects of long-term temperature elevation on the lungless salamander *Desmognathus ochrophaeus* (Allegheny Mountain dusky salamanders). These animals are ideal subjects because they are dependent on cutaneous respiration for gas exchange and may be

especially sensitive to temperature elevations, which are predicted to occur in Appalachian habitats where *D. ochrophaeus* reside (Milanovich et al., 2010; Ohlberger, 2013). We hypothesized that a long-term thermal elevation above an optimal temperature would result in changes in plasma CORT, behavior, body weight, and innate immunity. Therefore, we predicted that, in response to a chronic elevated temperature, subjects would exhibit increased levels of plasma CORT, along with changes including increased body/organ weight loss and increased locomotory activity. Changes in white blood cell counts were also expected, specifically in regards to proportions of neutrophils and lymphocytes.

METHODS

Animals

Twenty-four adult male and 24 adult female Allegheny Mountain dusky salamanders were collected in May and June of 2011 from Elk Rock Run in Dunbar, PA: 38° 57′ 04″ N; 79° 34′ 40″ W. After collection, animals were housed individually in the laboratory in plastic home boxes (16 x 16 x 5 cm) lined with moistened unbleached paper towels with a 14:10 light:dark cycle (lights off at 1700 h) at 16°C. Animals were fed wax worms every two weeks. All protocols were approved by Duquesne University's Institutional Animal Care and Use Committee. Permits for collection of animals were obtained from the Pennsylvania Fish and Boat Commission. *Temperature Treatments*

Experiments were done in two rooms in the Animal Care Facility at Duquesne University. One room was set to 17°C and the other to 24°C. These temperatures were chosen because they are ecologically relevant, with 17°C representing a temperature that might be typically experienced during the summer, with 24°C representing a maximal summer

temperature. All animals were placed into the 17°C room for two weeks before transferring half of the animals to 24°C room. Both temperature and humidity were monitored daily in both rooms. Although ambient humidity was significantly different between the two rooms, humidity measurements inside of the home boxes were identical (99%) between the two temperature treatments (data not shown). Therefore, humidity was not considered to be a factor in the results.

Initial sample sizes were n = 24 for both 24°C and 17°C treatments. Over the course of the experiment, one animal escaped from the 24°C treatment and did not survive, and six animals from the 24°C treatment died. Slight discrepancies in samples sizes in the results are due to missing data.

Behavioral and hormonal responses to thermal treatment

Locomotory activity was measured after three weeks of housing at either 17°C or 24°C (November and December of 2011). Each subject was tested one time at 17°C and another time at 24°C. At least 7 days separated the activity tests. On a given night of testing, half of the subjects were tested at 17°C and the other half was tested at 24°C, with sex and treatment distributed evenly across the two temperatures.

Because salamanders are crepuscular, locomotory activity was performed in the early evening, approximately 2 hours after lights were turned off. Dim, incandescent lighting provided light during behavioral observation. To measure locomotory activity, each subject was transferred from its home box to a testing chamber (24 x 24 x 2 cm) lined with a single layer of unbleached paper towel moistened with 15 mL of synthetic spring water. A time-lapse digital video camera (Sony DCR-VX2000) recorded for two seconds every minute for 75 minutes. When the testing on a given night was completed, animals were immediately returned to their

home boxes in the room where they had been previously housed. At a later time, the footage was observed and locomotory activity (excluding the first 15 minutes because it represents an adjustment period) was scored by an investigator blind to the different treatments. To measure activity, each chamber was divided into four quadrants, and each time the animal's head was in a different quadrant from one 2 second scan to the next was scored as one movement.

One month after locomotory activity tests were completed, subjects were euthanized in order to assess how long-term temperature treatment affects plasma CORT levels. For blood collection, animals were decapitated, a drop of trunk blood was used to make a blood smear (using standard procedures), and the remaining blood was collected in a heparinized capillary tube. The tube was centrifuged (typically 2 to 10 μL) and the plasma fraction was frozen in a heparinized microfuge tube at -20°C until assaying for plasma CORT concentrations.

Organ weights

Subjects were weighed at the start of the study, one month after the start of the experiment, and one day before euthanasia. To measure body weights, animals were blotted dry with scientific tissue wipes before weighing to remove excess moisture. On day of euthanasia, livers, gonads, and abdominal fat bodies (both sides) were removed and weighed.

Hormone assays

Plasma CORT was assayed at the Endocrine Services Laboratory at the Oregon National Primate Research Center. A double ether extraction was performed on up to 3μL of plasma, after which concentrations of CORT were obtained via standard radioimmunoassay protocols (Resko et al., 1980; Gruenewald et al., 1992). Functional sensitivity of the radioimmunoassay was <0.50

ng/mL, and the intraassay coefficient of variation (CV) was 10.6%. This assay was validated in Allegheny Mountain dusky salamanders previously by demonstrating parallelism from 0.5 to 4 µL at 0.5 µL increments.

Blood smears and white blood cell differentials

Blood smears were dried and stained using Wright-Giemsa stain according to manufacturer's protocols (Polysciences, Inc.), and coverslips were affixed using Permount solution. Utilizing a compound light microscope set at 400X magnification, slides were analyzed for white blood cells by an investigator blind to treatments. Standard methods for determining white blood cell differentials were used (Davis et al., 2008). To scan slides, the microscope stage was moved horizontally in 1mm increments, in order to avoid counting cells more than once. The number of each type of white blood cell in each field of view was counted until a total of 100 white blood cells were counted. The number of each type of white blood cell was converted into a percentage of total white blood cells. Of the 17 slides prepared from animals in the 24°C, 3 slides were not analyzed due to cells being too distorted to count. A neutrophil:lymphocyte ratio was also calculated for each individual, as an elevated ratio is thought to be an indicator of physiological stress.

Statistical analyses

Statistical analyses were performed using PASW (Predictive Analytics Software) 18. Most data met assumptions of parametric analyses and were analyzed using analysis of variance (ANOVA). Data that did not satisfy assumptions of normality and homoscedasticity were analyzed with nonparametric tests.

For analysis of baseline locomotory activity, the number of times the location of each subject changed relative to the previous scan was summed. Activity was analyzed using a 3-way repeated measures ANOVA, with housing temperature (24°C or 17°C) and sex as between subjects factors, and with testing temperature at the time activity was being measured (i.e. short-term temperature change to 24°C or 17°C) as the repeated, within subjects factor.

For analysis of body weight change, the percent change in body weight relative to day 1 of the experiment was measured over the course of the study. Weight change was analyzed using a 2-way repeated measures ANOVA, with housing temperature (24°C or 17°C) and sex as between-subjects factors, and with weight change throughout the experiment as the repeated, within subjects factor. Organ weights were analyzed using univariate ANOVA, with housing temperature (24°C or 17°C) and sex as fixed factors, and with final body weight at end of experiment as a covariate.

Plasma CORT values and white blood cell differentials were analyzed using a 2-way ANOVA, with housing temperature (24°C or 17°C) and sex as between subjects factors.

RESULTS

Regardless of whether subjects were held long term at 24°C or 17°C, there was no significant difference in locomotory activity at a given temperature (Figure 3.1, $F_{1,38} = 0.2$, $p = 0.67$). However, there was an effect of the testing temperature on locomotory activity, wherein subjects were more active at 24°C compared to 17°C (Figure 3.1, $F_{1,38} = 5.3$, $p = 0.027$). There was no significant difference in locomotory activity between male and female subjects ($F_{1,39} = 0.0001$, $p = 0.88$). All interactions were non-significant.

There was a significant difference in percent body weight change between the long-term temperature treatments. Specifically, subjects housed at 24°C lost more weight by the end of the study compared to subjects housed at 17°C (Figure 3.2, $F_{1,37} = 4.6$, $p = 0.039$). There was no difference in percent body weight change between male and female subjects ($F_{1,39} = 2.8$, $p = 0.10$). Additionally, relative fat body mass was marginally smaller in subjects housed at 24°C than in subjects housed at 17°C (Table 3.1, fat bodies: marginal mean ± SEM for 17°C: 0.014 ± 0.002; for 24°C: 0.009 ± 0.002; $F_{1,29} = 3.8$, $p = 0.061$). There were no significant effects of temperature on relative testis, ovary, or liver masses (Table 3.1).

Regardless of whether subjects were housed long-term at 24°C or 17°C, there was no significant difference in plasma CORT levels measured approximately 8 weeks after placement at the housing temperature (Figure 3.3, $F_{1,37} = 0.1$, $p = 0.73$). There was no significant difference in plasma CORT levels between male and female subjects ($F_{1,37} = 0.8$, $p = 0.39$), and the interaction between sex and temperature was also non-significant ($F_{1,37} = 0.5$, $p = 0.47$).

No basophils or eosinophils were observed during white blood cell counting. There was an effect of treatment on the relative numbers of lymphocytes and monocytes; specifically, subjects kept long-term at 24°C had relatively fewer lymphocytes, and relatively more monocytes, than subjects kept long-term at 17°C (Table 3.2: $F_{1,34} = 4.3$, $p = 0.047$; $F_{1,34} = 18.3$, $p < 0.001$, respectively) . There was a marginally significant effect of treatment on numbers of neutrophils and the N:L ratio, in that subjects kept long-term at 24°C had relatively fewer neutrophils, and a lower N:L ratio, than subjects kept long-term at 17°C (Table 3.2: $F_{1,34} = 4.1$, $p = 0.051$; $F_{1,34} = 3.1$, $p = 0.089$, respectively). Males had significantly more monocytes compared to females (mean ± SEM for males: 21.1 ± 2.1; for females: 16.1 ± 1.3; $F_{1,34} = 4.9$, $p = 0.034$) but there was no effect of sex for the other white blood cells (neutrophils; $F_{1,34} = 0.55$, $p = 0.46$;

lymphocytes: $F_{1,34} = 1.7$, $p = 0.20$). There were no significant interactions between sex and housing temperature for the white blood cell counts (all $p > 0.28$).

DISCUSSION

Overall, we found no effect of long-term housing temperature elevation on locomotory activity or plasma CORT in *D. ochrophaeus* salamanders. However, subjects tested at 24°C were more active than when tested at 17°C, regardless of the temperature they were housed at long-term. Additionally, housing at temperature of 24°C caused a drop in body mass compared to housing at the 17°C temperature. Leukocyte differentials were affected by a long-term temperature elevation, with relative numbers of monocytes increasing and relative numbers of both neutrophils and lymphocytes decreasing at the higher temperature. These results are discussed further below.

Effects of temperature elevation on locomotory activity

Although housing temperature did not alter locomotory activity, subjects were more active when tested at 24°C than when tested at 17°C. This indicates that our methods can detect changes in locomotory activity. Changes in locomotory activity have been shown previously in response to thermal elevation, with increases as ecologically relevant temperatures increase, although decreases in activity and righting responses occur as animals reach critical thermal maxima ranging from 30°C to 40°C (Pough et al., 1983; Narayan and Hero, 2014a).

Previous studies show that Allegheny Mountain dusky salamanders held long-term at different ambient temperatures experience metabolic acclimation within 1 to 2 weeks (Fitzpatrick et al., 1971; Feder, 1985). For example, salamanders housed for 3 weeks at 20°C

had lower oxygen consumption compared to those housed at 15°C when tested at a particular temperature. Despite thermal acclimation of oxygen consumption, locomotory performance, typically measured as maximal speed or stamina, does not seem to acclimate (Feder, 1986; Bennett, 1990). Our work is different from other studies in that we examined spontaneous locomotory activity. Nevertheless, our finding of an absence of thermal acclimation of spontaneous locomotory behavior is consistent with these previous studies of locomotory performance.

Plasma CORT

Plasma CORT levels were similar between subjects housed at 24°C and those housed at 17°C. This result contrasts with previous studies showing that an acute thermal elevation at 30°C, 35°C, and 40°C, along with a repeated thermal shock elevation of 35°C, elevated CORT, suggesting that thermal shock induces chronic stress in animals (Narayen and Hero, 2014a,b). Our study focused on more ecologically relevant temperature ranges and found no evidence of changes in CORT. It is possible that, given that blood was collected for CORT measurement 8 weeks after the start of the experiment, plasma CORT levels increased at an earlier time point in the study, then decreased back to baseline levels after a certain amount of time. However, 2 months of long-term thermal elevation simulates the predicted future climate shift, and showed no acclimation of a CORT response to the new temperature.

Although CORT increases oxygen consumption (Durant et al., 2008; Wack et al., 2012) and affects intermediary metabolism (Mommsen et al., 1999; Norris and Carr, 2013), temperature, which is tightly linked to metabolic rate in ectotherms, did not cause an increase in plasma CORT. Previous studies have shown that CORT elevations can potentially incur

energetic costs (Durant et al., 2008; Preest and Cree, 2008), and may be particularly harmful for species with a low-energy lifestyle, including *D. ochrophaeus* salamanders (Feder, 1983). Therefore, a lack of CORT response to changing temperatures may be adaptive.

Body mass and organ weights

Subjects held at 24°C lost more body mass during the 8 weeks of the experiment compared to subjects held at 17°C. Relative fat body mass was marginally significantly smaller in animals housed at 24°C. The drop in body mass is probably the result of increased metabolic rate due to the higher ambient temperature (Gatten et al., 1992). The reduction in fat bodies also suggests that higher temperature increased metabolic rates, with potential energy stores in fat bodies being diverted elsewhere throughout the body. Interestingly, reductions in body mass and size have been proposed to occur as a direct consequence of global warming (Daufresne et al., 2009). For example, warmer temperatures over a few generations have been associated with decreased body size in plethodontid salamanders, although it is not clear if the change is due to genetic changes or phenotypic plasticity in response to environmental variation (Caruso et al., 2014).

White blood cell differentials

Environmental temperature acts as a mediating factor for numerous processes, including cardiovascular function, respiration, and immune function (Rome et al., 1992). In response to a long-term temperature elevation of 24°C, animals exhibited changes in white blood cell differentials. Specifically, thermal elevation increased the relative numbers of monocytes and decreased relative numbers of lymphocytes, with a trend for relative neutrophil numbers to be

decreased as well. Previous studies have observed changes in both white blood cell function and number in ectothermic species at elevated temperature. For example, a thermal elevation of 30°C decreased phagocytic function (but not numbers) of blood granulocytes in fish (Collazos et al., 1995) and monocytes were shown to increase in wall lizards at a temperature elevation of 25°C (Mondal and Rai, 2001).

The changes in white blood cell differentials observed in my study might reflect a negative response to a long-term temperature elevation, which closely mimics a seasonal temperature change to spring/summer temperatures. Lymphocytes and neutrophils are critical aspects of innate immunity; given our data indicating a decrease in lymphocytes at elevated temperatures, any reduction at warmer temperatures may make amphibian species more susceptible to infection and disease, such as the chytrid fungus. In fact, growth of *Bd* increases from 17-24°C, with higher temperatures indicative of higher growth (Piotrowski et al., 2004). Furthermore, the seasonal-acclimation hypothesis, which posits that amphibian immune cell counts will decrease at a prolonged decrease in temperature until animals can acclimate to the colder temperatures (Raffel et al., 2006), might be applicable in explaining why lymphocytes may have decreased renewal and production at the elevated temperature. In this manner, any deviation away from an optimal temperature may result in a decline in immunological function, an effect observed by Raffel et al. in Red-Spotted Newts (2006), after which immune cell numbers should recover and acclimate to the new temperature.

From an energetic perspective, activation of the immune response is costly, as has been demonstrated previously (Bonneaud et al., 2003). Due to the relative decreases observed in neutrophils and lymphocytes observed in my study, it is possible that, similar to the lack of elevation of plasma CORT, certain white blood cells will cease renewal and proliferation in an

attempt to suppress excessive expenditures of energy. Given that Allegheny Mountain dusky salamanders are adapted to a low-energy lifestyle (Feder, 1983), as in the proposed mechanism for inhibition of surges in plasma CORT, certain immune functions that are costly to maintain may be inhibited until long-term acclimation can occur. Future studies should examine how an acute thermal elevation affects white blood cells; additionally, research into the effects of a prolonged drop in temperature on innate immunity in *D. ochrophaeus* salamanders may be useful in determining if the seasonal-acclimation hypothesis can explain these immunological data.

Conclusions

Collectively, our data indicate that a thermal elevation has effects on body mass and immune cells but no effect on locomotory activity or plasma CORT in Allegheny Mountain dusky salamanders. The losses in body mass and fat bodies suggest that metabolic rates were increasing, although these changes weren'tassociated with changes in CORT. In addition to the general decreases in lymphocytes and neutrophils, the lack of a CORT response suggests that, similar to a prolonged exposure to predator kairomones, salamanders may be uncoupling hormonal responses that are detrimental and too energetically expensive. These results lend further credence to the hypothesis that, depending on context and duration of exposure, amphibian behavioral and hormonal responses to putative stressors will vary. Further examination into how acute and prolonged temperature shifts affect other important processes, such as mating activities and disease susceptibility, can provide insight into the specific consequences of long-term temperature changes.

Attributions

Sarah Woodley and I conceived of and designed the experiments described in this chapter, as well as performed the statistical analyses. I performed almost all experimental procedures with the following exceptions. Sarah Woodley helped with blood collection, and the Endocrine Services Laboratory of the Oregon National Primate Research Center performed the hormone assays.

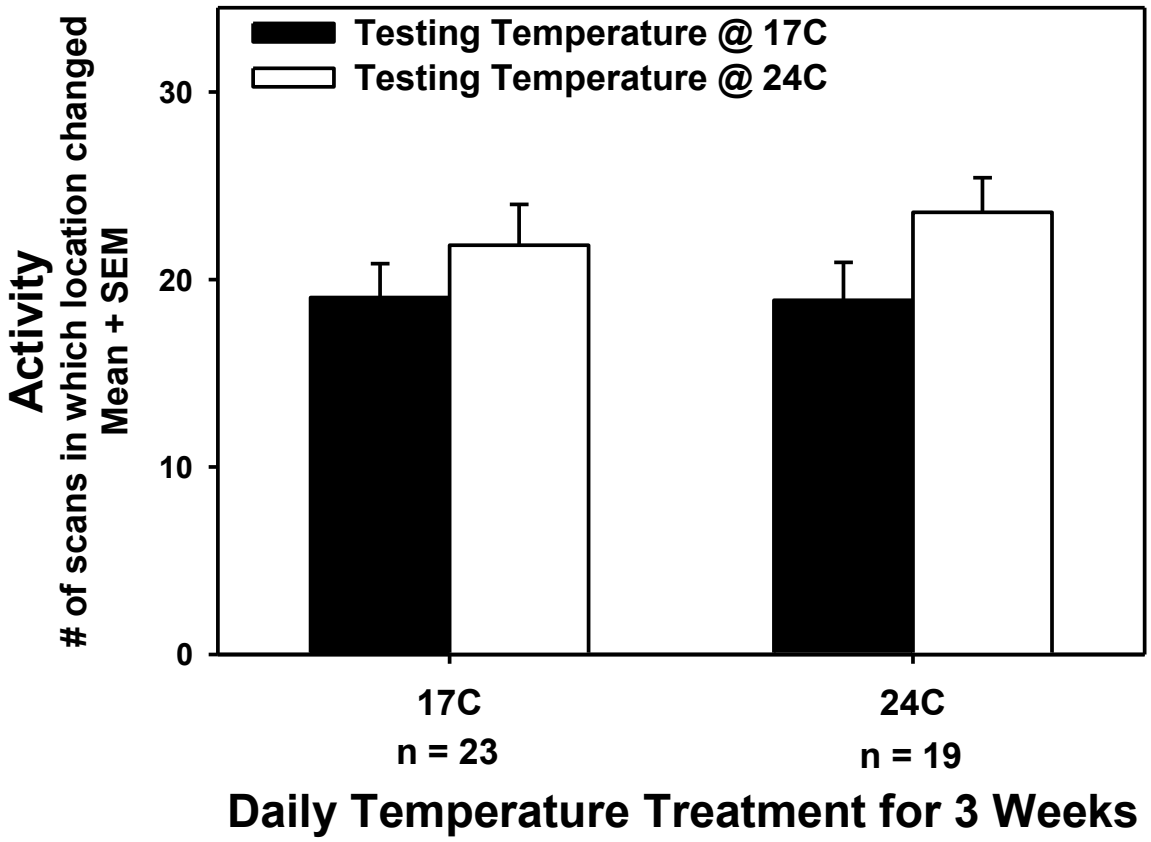

Figure 3.1. Locomotory activity of Allegheny Mountain dusky salamanders, tested in response to three weeks of long-term temperature exposure, as well as short-term exposure to a warmer temperature for 1 hour. Animals exposed to long-term temperature treatments displayed no significant changes in baseline locomotory activity, whereas animals tested short-term at 24°C were more active than animals at 17°C.

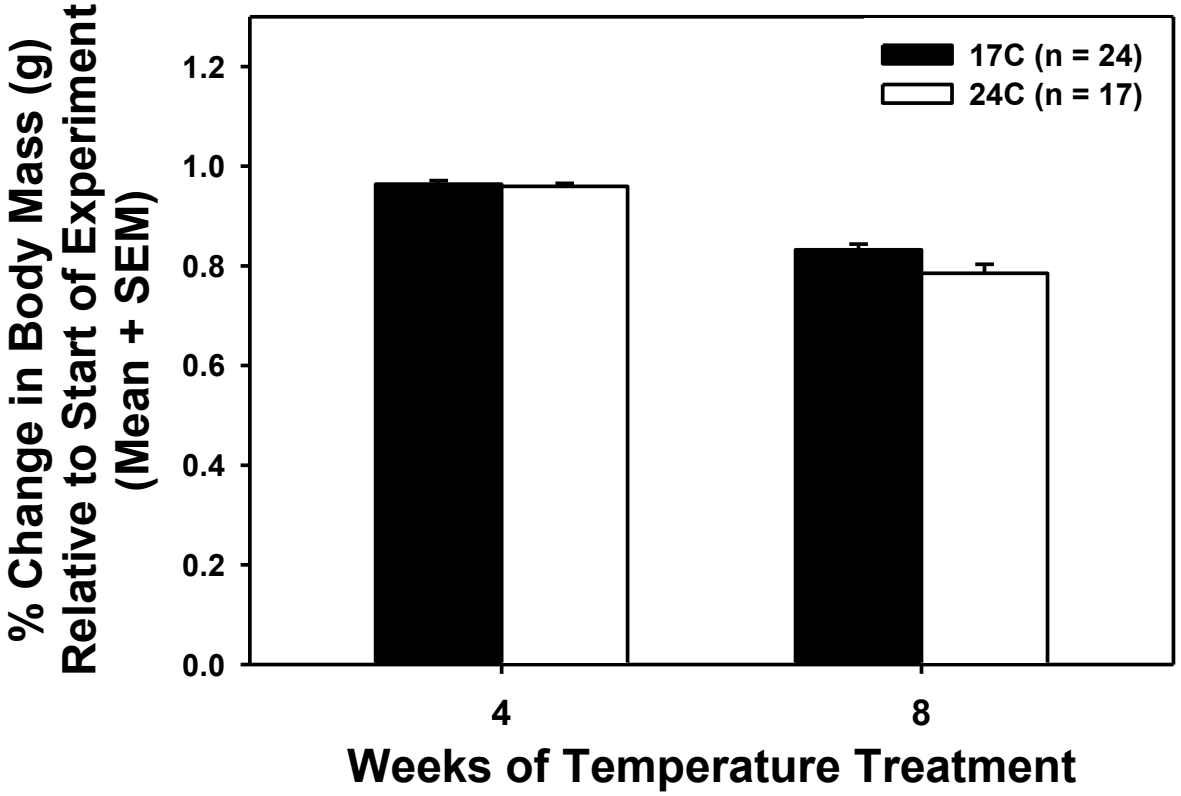

Figure 3.2. Percent change in body weights of Allegheny Mountain dusky salamanders, measured in response to long-term temperature exposure for eight weeks. Animals exposed long-term to the warmer temperature lost significantly more body weight than animals at the lower temperature.

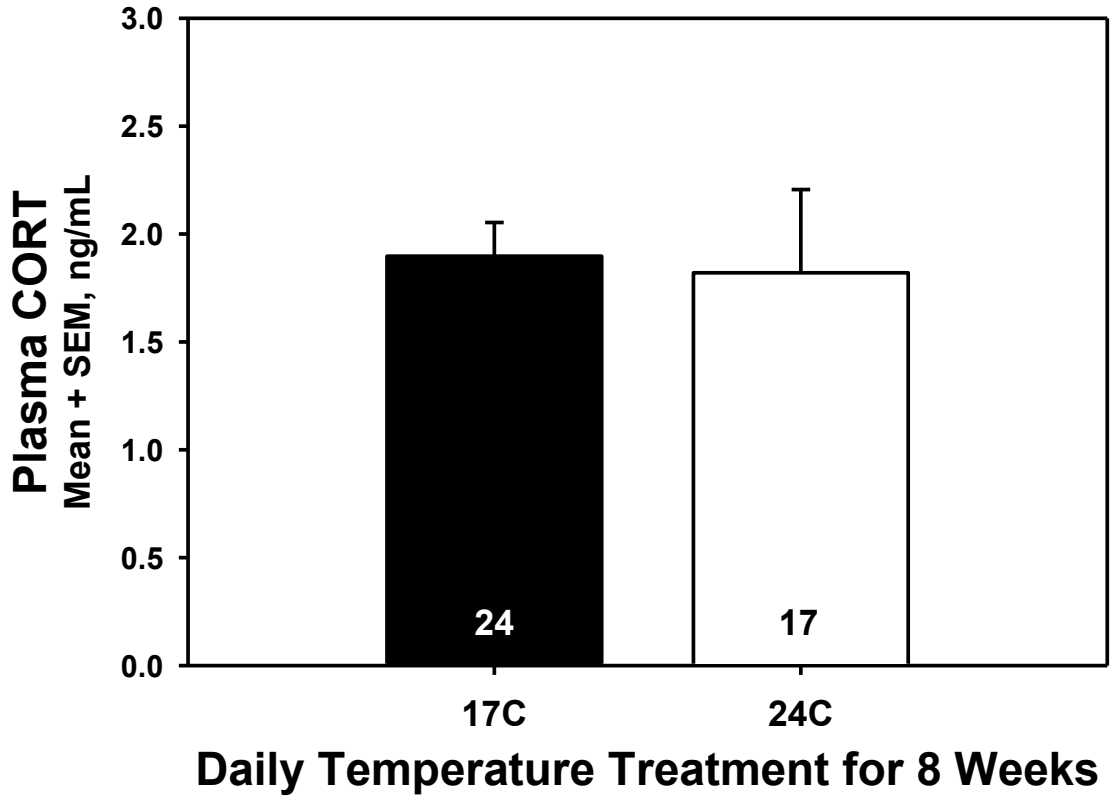

Figure 3.3. Plasma CORT levels of Allegheny Mountain dusky salamanders, measured in response to long-term temperature exposure for eight weeks. No significant difference in CORT levels between the two treatments was present.

Table 3.1. Marginal mean ± SEM of organ mass after correction for differences in body mass. *P*-values correspond to the effect of long-term temperature treatment (eight weeks). Fat bodies were marginally significantly smaller in animals exposed long-term at the warmer temperature. Sample sizes for each organ are indicated in bold and parentheses.

	17°C Treatment	**24°C Treatment**	***P*-value**
Liver (g)	0.023±0.002 **(23)**	0.019±0.002 **(17)**	0.107
Fat Bodies (g)	0.014±0.002 **(20)**	0.009±0.002 **(14)**	**0.061**
Testes (g)	0.002±0.001 **(8)**	0.002±0.001 **(6)**	0.684
Ovaries (g)	0.038±0.005 **(11)**	0.038±0.007 **(6)**	0.957

Table 3.2. Mean ±SEM of percent of each type of white blood cell. *P*-values correspond to the effect of long-term temperature treatment (eight weeks) in ANOVAs. Lymphocyte counts decreased and monocyte counts increased at the warmer temperature.

	17°C Treatment n = 24	**24°C Treatment n = 14**	***P*-value**
Neutrophils (%)	9.0±1.3	4.9±1.3	0.051
Lymphocytes (%)	75.8±1.4	70.3±2.2	0.047
Monocytes (%)	15.2±1.0	24.8±2.4	<0.001
N:L Ratio	0.13±0.02	0.07±0.02	0.089

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Chapter 4

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

ABSTRACT

Amphibian species and populations across the world are declining dramatically due to many factors including disease. The chytrid fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) infects amphibians and can cause the disease chytridiomycosis. *Bd*-associated declines have been reported worldwide, and research into the mechanisms underlying *Bd* susceptibility is vital to guide conservation strategies. It is hypothesized that long-term exposure to environmental stressors exacerbates the virulence and lethality of *Bd* by suppressing host immunity via stress-induced release of glucocorticoid stress hormones like corticosterone (CORT). We tested whether elevation of plasma CORT prior to *Bd* inoculation would increase *Bd* susceptibility and chytridiomycosis development in red-legged (*Plethodon shermani*) salamanders. Plasma CORT was experimentally elevated daily in animals for 9 consecutive days using dermal patches, after which animals were inoculated with *Bd* and subsequently tested for infection loads and disease symptoms. *Bd*-inoculated animals treated with CORT had higher infection abundance compared to *Bd*-inoculated animals not treated with CORT. However, while *Bd*-inoculated animals showed signs of chytridiomycosis, including loss of body mass, skin sloughing, and mortality, prior CORT elevation did not increase disease symptoms. The lack of congruence between the effects of CORT on infection abundance versus disease may be due to threshold or other effects. Overall, our results show that elevation of plasma CORT prior to *Bd* inoculation increases

susceptibility to *Bd*, and may help explain the differential susceptibility observed across species and populations.

INTRODUCTION

Amphibian populations are undergoing worldwide declines, with approximately 30% of species currently classified as endangered, and numerous extinction events in the last 35 years (Stuart et al., 2004; Hoffmann et al., 2010). Several causes contribute to these declines, including habitat modification, climate change, (Collins and Crump, 2009) environmental contamination, invasive species, and pathogens (Beebee and Griffiths, 2005; Rohr and Raffel, 2010; Blaustein et al., 2011)). A pathogen closely linked to amphibian declines is the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*). *Bd* zoospores infiltrate keratinized amphibian skin and develop into zoosporangia, which then produce and discharge motile zoospores, which can then re-infect amphibian skin (Berger et al., 1998; Longcore et al., 1999). Once infected, the disease called chytridiomycosis often develops, with symptoms including hyperkeratosis, skin sloughing, lethargy, body mass drop, and death due to disruptions in respiration and osmoregulation (Voyles et al., 2009). *Bd* has been implicated in precipitous declines in amphibian populations as well as several extinction events (refs). Recently, a second pathogenic chytrid fungus, *Batrachochytrium salamandrivorans* (*Bsal*), has been discovered in Europe that is particularly lethal for salamanders, driving the extinction of fire salamander populations (Martel et al., 2013; Martel et al., 2014). There is great concern that *Bsal* will reach the Americas with devastating consequences on salamander populations.

Amphibians possess a number of immune defenses against *Bd* infection and the development of chytridiomycosis. For example, many amphibians produce antimicrobial

peptides (AMPs) or have cutaneous microflora that are inhibitory against *Bd* (Rollins-Smith and Conlon, 2005; Harris et al., 2009; Rollins-Smith, 2009). Species that are more *Bd*-resistant expressed a mixture of inhibitory AMPs and more efficient anti-*Bd* skin bacteria, whereas declining and at-risk species possessed relatively poor anti-*Bd* inhibitory peptides and less effective anti-*Bd* skin bacteria (Woodhams et al., 2006; Woodhams et al., 2007; Lam et al., 2010). Also, antibodies found in the skin mucous of frogs can inhibit *Bd* as well (Ramsey et al., 2010). Despite these immune defenses, *Bd* can evade host immune defenses via inhibition of lymphocyte-mediated immune responses, which may explain why infection with *Bd* can be so deadly (Fites et al., 2013; Fites et al., 2014).

Susceptibility to both *Bd* infection and chytridiomycosis development varies among and within amphibian species (Savage et al., 2011; Woodhams et al., 2011; Venesky et al., 2014). Numerous species of anurans become infected with *Bd* and develop fatal chytridiomycosis (Berger et al., 1999), resulting in rapid population declines (Berger et al., 1998; Daszak et al., 1999; Bosch et al., 2001; Lips et al., 2006). In contrast, other anuran species tolerate *Bd* infections and show little to no signs of chytridiomycosis (Hanselmann et al., 2004; Beard and O'Neill, 2005; Rothermel et al., 2008). Similar to anurans, salamander species are differentially susceptible to *Bd* infection and development of chytridiomycosis. Declines of several species of plethodontid salamanders were linked to *Bd* emergence in Mexico (Cheng et al., 2011), although salamanders in the Appalachian Mountains appear to be resistant to *Bd* in nature, with an infection prevalence below 2% for both museum and current-day sampling (Cummer et al., 2005; Kiemnec-Tyburczy et al., 2012; Muletz et al., 2014).

One hypothesis for the variation in susceptibility to *Bd* is that species and populations differ in exposure to environmental stressors (Carey et al., 1999). Exposure of vertebrates to

stressors typically results in elevated circulating glucocorticoid hormones which coordinate behavioral, metabolic, and immune responses (Sapolsky, 2002; Carr, 2010). While a short-term surge in plasma glucocorticoid hormones is beneficial by promoting stress responses, a long-term increase in plasma glucocorticoid hormones can lead to a variety of maladaptive effects, including immune suppression (Sapolsky et al., 2000; Dhabhar, 2014).

In amphibians, a large number of environmental stressors, including capture, handling, contaminant exposure, predator cues, thermal variation, and pathogen infection (including *Bd* and ranavirus), resulted in elevated plasma corticosterone (CORT), the dominant amphibian glucocorticoid hormone (Hopkins et al., 1997; Glennemeier and Denver, 2002; Hayes et al., 2006; Denver, 2009; Carr, 2010; Woodley and Lacy, 2010; Narayan et al., 2012; Narayan et al., 2013).

In turn, there is evidence that elevated plasma CORT is immunosuppressive in amphibians via inhibition of AMP production, lymphocyte production and antibody proliferation (Ramirez et al., 1996; Simmaco et al., 1997; Davis and Maerz, 2008; Groner et al., 2014). Thus, it is hypothesized that CORT-induced immunosuppression increases susceptibility to pathogens including *Bd* (Carey et al., 1999; Martin, 2009; Ribas et al., 2009; Rollins-Smith et al., 2011). Due to the drastic declines experienced by amphibian populations, investigations into how the stress response affects susceptibility to *Bd* are critical, and may explain why certain species are more susceptible than others. Over the course of 2 experiments, we examined the effects of experimental CORT elevation on susceptibility to *Bd* in the red-legged salamander. Both experiments utilized similar methodology, but used different doses of *Bd* and a different CORT treatment regimen in order to better ascertain how CORT elevation influences susceptibility at varying doses. Specifically, we expected that repeated CORT elevations prior to inoculation with

Bd would cause increased infection (zoospore abundance) compared to subjects not exposed to CORT. We also expected that CORT elevation would increase chytridiomycosis symptoms in *Bd*-inoculated subjects, manifested by reduced locomotory activity, reduced body mass, increased skin sloughing, and increased mortality.

METHODS

Animals

Adult male red-legged salamanders were collected in August of 2012 from Macon County, North Carolina: 83° 33′ 37″ N longitude; 35° 11′ 13″ W latitude (Experiment 1) or in August of 2013 from the same location (Experiment 2), with appropriate permits from the North Carolina Department of Wildlife and the Nantahala National Forest. In the laboratory, animals were housed individually in plastic home boxes (16 x 16 x 5 cm) lined with moistened unbleached paper towels with a 14:10 light:dark cycle (lights off at 1700 h) at 20°C, which is an optimal temperature for *Bd* growth, and is also within the preferred temperature range of red-legged salamanders (Spotila, 1972). Animals were fed wax worms every two weeks. Animals were assigned randomly to treatments, with sample sizes of 10 animals/treatment (Experiment 1) or 12 animals/treatment (Experiment 2). For Experiment 1, sample sizes decreased over the course of the study due to *Bd*-induced mortality (see later). All procedures were approved by Duquesne University's Institutional Animal Care and Use Committee.

Treatments

Transdermal elevation of CORT

Using previously validated methods (Wack et al., 2010), dermal patches containing CORT were used to transdermally elevate plasma CORT in salamanders, with control subjects receiving patches containing sesame oil vehicle. Patches (1.5 x 3 mm) were cut from filter paper (Cat No. 1820–070, Whatman) and applied to the dorsal surface between the forelimbs of each salamander using clean forceps. 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 Incorp.; 0.5 mg/mL). Controls received patches that contained 2.5 µL of sesame oil vehicle. After 45 min, the patch was gently removed with forceps. This method of transdermal delivery elevates plasma CORT to high physiological concentrations in *P. shermani* for approximately 4 hours after CORT patch application. In oil patch controls, CORT levels are similar to levels in animals that received no patches (Wack et al., 2010). For Experiment 1, patches were applied one time per day in the morning for 9 consecutive days prior to inoculation with either *Bd* or sham inocula. For Experiment 2, patches were applied once per day in the morning for 7 consecutive days prior to inoculation, and for 7 consecutive days post-inoculation, in order to further suppress amphibian immunity.

Inoculation with Bd

Bd was cultured and harvested using standard methods (Rollins-Smith et al., 2002). Briefly, zoospores of the *Bd* isolate JEL197 (Longcore et al., 1999) 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 sub-cultured for 1 week at 23°C. Zoospores were harvested by flooding plates with 3.0 mL of sterile water. After 30 minutes, water containing zoospores was collected and filtered using a 20 µm nylon mesh to isolate zoospores. Zoospores were diluted with sterile synthetic spring water (SSW) to achieve a concentration of 6.9×10^7 zoospores (Experiment 1)

or 1.0 x 10^6 zoospores (Experiment 2) in 15 mL. Sham inocula were prepared similarly except that plates were not inoculated with *Bd*.

After 7 or 9 days of patch treatment, 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 hours, animals were transferred back to home boxes. Our doses and duration of *Bd* inoculation were based on pilot studies in our lab with reference to studies in related species (Chinnadurai et al., 2009; Vazquez et al., 2009).

Measuring Bd infection and disease development

Bd detection

In Experiment 1, subjects were swabbed the day before (baseline) and on days 10 and 17 after *Bd* or sham inoculation, in order to quantify *Bd* infection. In Experiment 2, subjects were swabbed the day before (baseline) and on days 7, 14, 21, and 28 post-inoculation. Each subject was swabbed (Rayon swabs, MW113 - Medical Wire) 30 times along the ventral surface from tail to neck. After drying for 15 minutes, swabs were sealed in a 1.5mL sterile microcentrifuge tube and stored at 20°C.

For Experiment 1, there were 3 animals that died between day 13 and day 17; for these animals, we collected swabs from the carcasses within 24 hrs of death. These swabs were grouped with those animals swabbed on day 17. Hereafter, we refer to this set of swabs as day 13-17. For Experiment 2, one animal died on day of inoculation.

DNA of *Bd* zoospores from swabs was extracted using MO BIO Powerlyzer UltraClean Microbial DNA Isolation kit (Cat. #: 12255-50, MO BIO Laboratories, Inc.). Extraction procedures followed the kit protocol, with minor modifications. Firstly, 300 µL of Glass

Microbead Solution was 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 45s at 2,000rpm. 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'-CCTTGATATAATACAGTGTGCCATATGTC - 3') and 5.8S Chytr (5'-AGCCAAGAGATCCGTTGTCAAA - 3'). 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 (samples were non-detectable in all runs). Samples were considered positive for *Bd* infection if 2 replicates out of the 3 amplified above the lowest zoospore standard (either 0.1 or 0.4, depending on batch). To account for qPCR dilution, each sample was multiplied by a factor of 10.

Extracted DNA was qPCR amplified. For Experiment 1, our samples sizes were 10 per group per swab date with the following exceptions. The sample size for *Bd*+Oil was 8 because we had technical difficulties (did not get a swab or DNA extraction was unsuccessful) for 2 animals. The sample sizes for *Bd*+CORT was 9 because we did not get a swab from one animal. Two batches of qPCR were performed. First, we ran all baseline samples and day 10 samples in triplicate, along with known *Bd* standards in a 1:10 serial dilution of 100, 10, 1, and 0.2 zoospore equivalents. After finding that baseline samples and samples from sham inoculated animals were negative, we next analyzed samples from *Bd*-exposed animals that were collected on day 10 and

17 (including 3 animals that died between days 10 and 17). Upon further optimization of the qPCR protocol, we ran samples from batch 2 in triplicate against known *Bd* standards in a 1:5 dilution of 50, 10, 2, and 0.4 zoospore equivalents. Samples that amplified below the 0.4 standard was considered non-detectable and assigned a value of 0. In each batch, samples were distributed across plates so that treatments were equally represented within a given plate. 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%). See Figures 4.11 and 4.12 for representative standard curves.

For Experiment 2, our sample sizes were 12 per group per swab date with the following exceptions. The sample size for *Bd*+Oil was 11 because one animals died on day of inoculation. The sample sizes for both Sham+CORT and *Bd*+CORT were 11 because of technical difficulties (DNA extraction was unsuccessful) for 1 animal in each group. All samples were run in triplicate against known *Bd* standards in a 1:5 dilution of 50, 10, 2, and 0.4 zoospore equivalents. Samples that amplified below the 0.4 standard was considered non-detectable and assigned a value of 0. Samples were distributed across plates so that treatments were equally represented within a given plate. Validation parameters for qPCR analyses indicated optimum PCR amplification (10 plates, $R^2 = 0.99$, qPCR efficiency = 101%). See Figure 4.13 for a representative standard curve.

Chytridiomycosis symptoms

For Experiment 1, body masses of each animal were measured the day before patching treatments began (initial body masses were not different among treatment groups) and once a week after inoculation. Animals were checked daily for general health and incidence of skin sloughing. Animals that did not survive were either found dead (n = 10) or were euthanized

because they had dropped a tail or had tail ulcers (n = 2), 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 days so the remaining animals were euthanized. For Experiment 2, body masses were measured before patching treatments began (initial body masses were not different among treatments) and once a week after inoculation, with animals being checked daily for general health and daily incidence of skin sloughing.

Locomotory activity was measured at days 2, 9, and 16 (Experiment 1) and days -7, 7, and 21 (Experiment 2) after *Bd* inoculation. Because salamanders are crepuscular, locomotory activity was measured in the early evening, at least 2 hours 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 x 24 x 2 cm) 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 two seconds every thirty seconds for 75 minutes. Afterwards, recordings were observed (excluding the first 15 minutes 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 four quadrants, and each time a subject's head was in a different quadrant from one scan to the next was counted as one movement.

Statistical analyses

For Experiment 1, data analyzed using parametric statistics met assumptions of homoscedasticity and normality. Animals swabbed before inoculation (baseline) and sham-inoculated subjects (at day 10) had undetectable zoospore equivalents; therefore, only *Bd*-

inoculated subjects, post inoculation, were statistically analyzed. To compare *Bd* infection abundance (zoospore equivalents) between treatments, we used a zero-inflated negative binomial general linear model ANOVA to test differences in zoospore equivalents (R statistical software). Specifically, we used the "glmmADMB" function in the "glmmADMB" package in R. Zero-inflated models assume that the response variable is a function of a binomial process (CORT patch vs. no CORT patch) and a count process (negative-binomial distributed infection abundance) (Zeileis et al., 2007). In addition to analyses of infection abundance, the % of individuals infected in *Bd*-inoculated groups was analyzed using a Fisher's exact test. Infection intensity (measure of infection only in animals that were *Bd* positive) was analyzed using Mann-Whitney U-tests. For Experiment 2, effects of inoculation and patch treatment on *Bd* infection abundance were analyzed using Mann-Whitney U-tests.

For locomotory activity (lethargy), the number of times the location of each animal changed relative to the previous scan was summed and analyzed with a 2-way ANOVA, with inoculation (*Bd* or sham) and patch application (CORT or Oil) as between-subjects factors in both experiments..

In both experiments, the percent change in body mass was analyzed with a 2-way repeated measures ANOVA, with inoculation (*Bd* or sham) and patch application (CORT or Oil) as between-subjects factors. For Experiment 1, we only analyzed data up to day 23 because sample sizes rapidly declined after day 23 as survival declined.

For both experiments, incidence ofskin sloughing was analyzed with a Fisher's exact test comparing *Bd* inoculation vs. sham inoculation, and *Bd*-Oil vs. *Bd*-CORT.

Survival among groups was analyzed in Experiment 1 using Log Rank (Mantel-Cox) tests comparing *Bd* inoculation vs. sham inoculation and *Bd*-Oil vs. *Bd*-CORT. Survival was not analyzed in Experiment 2 due to only one animals dying on day of inoculation.

Finally, to determine whether *Bd* infection abundance was correlated with disease symptoms, we compared *Bd* infection abundance with survival, body mass lost, and locomotory activity using non-parametric correlations for Experiment 1.

RESULTS

Experiment 1

Bd infection abundance

Before inoculation, all subjects had non-detectable levels of *Bd* zoospores. Sham-inoculated subjects had non-detectable *Bd* infection at day 10 (Table 4.1). By day 10 and day 13-17, there was no effect of patch treatment on the percent of animals infected (Figure 4.2 and Table 4.2, Fisher's exact $test_{day\ 10}$: $p = 0.18$; Fisher's exact $test_{day\ 17}$: $p = 0.12$). 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 day 13-17 ($\chi^2_{1,13} = 4.7$, $p = 0.029$), with *Bd*-inoculated subjects treated with CORT patches having higher infection loads compared to *Bd*-inoculated subjects treated with Oil patches (Figure 4.1). Infection abundance on day 17 was not correlated with body mass lost by day 16 ($\rho = -0.37$, $p = 0.18$, $n = 15$) or survival ($\rho = -0.33$, $p = 0.19$, $n = 17$). However, infection abundance on day 17 was negatively correlated with locomotory activity on day 16 ($\rho = -0.60$, $p = 0.023$, $n = 14$). There was no effect of patch treatment on infection intensity by either day 10 (Figure 4.3, Mann-Whitney $U\text{-}test_{day10}$: $U = 1.0$, $p = 0.48$) or day 17 (Figure 4.3, Mann-Whitney $U\text{-}test_{day17}$: $U = 10.0$, $p = 0.91$).

Chytridiomycosis symptoms

By day 43, only 40% of *Bd* exposed subjects were still alive compared to 85% of sham inoculated subjects (Log-Rank Mantel Cox: Figure 4.4; $x^2 = 10.42$, $p < 0.001$). However, there was no difference in survival between *Bd*+CORT subjects and *Bd*+Oil subjects (Log-Rank Mantel Cox: Figure 4.4; $x^2 = 0.93$, $p = 0.34$). All animals still alive at day 43 survived with no signs chytridiomycosis for an additional 23 days at which point they were euthanized.

After inoculation through day 23, subjects inoculated with *Bd* had lost significantly more body mass than sham-inoculated subjects (Figure 4.5, $F_{1,31} = 24.9$, $p < 0.001$). However, there was no effect of patch treatment on the change in body mass (Figure 4.5, $F_{1,31} = 0.33$, $p = 0.57$).

Locomotory activity was similar among treatments (Figure 4.6), with no effect of inoculation treatment ($F_{1,31} = 1.3$, $p = 0.26$) or patch treatment ($F_{1,31} = 0.5$, $p = 0.49$). The interaction between inoculation and patch treatment was also non-significant (Figure 4.6; $F_{1,31} = 0.7$, $p = 0.42$).

Ninety % of *Bd*-inoculated subjects sloughed their skin, compared to 0% of the sham-inoculated subjects (Fisher's exact test: Table 4.3; $p < .001$). In *Bd*-inoculated animals, treatment with CORT patch did not affect the number of animals that sloughed their skin (Fisher's exact test, *Bd*+CORT vs. *Bd*+Oil: $p = 0.76$).

Experiment 2

Bd infection abundance

All subjects at baseline, and all sham-inoculated subjects were negative for *Bd* infection. A few subjects exposed to *Bd* had very low levels of *Bd* on days 7 and 14 but all were negative for *Bd* on days 21 and 28 (Table 4.4). There was no effect of inoculation on infection abundance

by either day 7 (Figure 4.7, Mann-Whitney U-test$_{day7}$: $U = 207.0$, $p = 0.064$) or day 14 (Figure 4.7, Mann-Whitney U-test$_{day14}$: $U = 252.0$, $p = 0.30$). There was no effect of patch treatment on infection abundance by either day 7 (Figure 4.7, Mann-Whitney U-test$_{day7}$: $U = 49.0$, $p = 0.50$) or day 14 (Figure 4.7, Mann-Whitney U-test$_{day14}$: $U = 55.0$, $p = 0.32$).

Chytridiomycosis symptoms

Before inoculation through day 49 (Figure 4.8), there was no effect of inoculation treatment ($F_{1,42} = 0.3$, $p = 0.62$) or patch treatment ($F_{1,42} = 0.1$, $p = 0.78$), on change in body mass. The interaction was also non-significant.

Before inoculation through day 21 (Figure 4.9), there was no effect of inoculation treatment ($F_{1,43} = 0.1$, $p = 0.81$) or patch treatment ($F_{1,43} = 0.9$, $p = 0.34$), on locomotory activity. The interaction between inoculation and patch treatment was also non-significant ($F_{1,43} = 0.2$, $p = 0.69$).

87% of *Bd*-inoculated subjects sloughed their skin, compared to 12.5% of the sham-inoculated subjects (Figure 4.10, Fisher's exact test: $p < 0.001$). Treatment with CORT patch increased incidence of skin sloughing on a given date, with *Bd*-inoculated subjects treated with CORT patch sloughing their skin more on a given date compared to *Bd*-inoculated subjects treated with Oil patches (Fisher's exact test: $p < 0.001$).

DISCUSSION

Using red-legged salamanders as an amphibian model, we tested whether prior treatment with CORT resulted in increased infection abundance and disease development when inoculated with *Bd*. In Experiment 1, animals tested for *Bd* infection prior to *Bd* inoculation as well as

animals inoculated with a sham inoculation had no detectable *Bd* zoospore equivalents. However, several animals inoculated with *Bd* were positive for *Bd* by days 10. Furthermore, *Bd*-inoculated animals treated with CORT had a greater infection abundance by day 13-17 compared to *Bd*-inoculated animals treated with oil vehicle. Inoculation with *Bd* induced symptoms of chytridiomycosis, including loss of body mass, skin sloughing, and mortality, whereas sham-inoculated controls were symptom-free. Despite the effect of CORT on *Bd* infection abundance, prior treatment with CORT did not increase expression of symptoms of chytridiomycosis. In Experiment 2, using a lower dose of *Bd* compared to Experiment 1, neither inoculation with *Bd* or CORT patch treatment had an effect on infection abundance, body mass, mortality, and locomotory activity. However, exposure to *Bd* induced skin sloughing, and CORT elevation in *Bd*-inoculated subjects increased skin sloughing on a given day. These results are discussed further below.

Bd infection – Experiment 1

In both experiments, we did not measure plasma CORT in our subjects because of the difficulty of collecting plasma samples non-invasively. However, transdermal delivery of CORT has been validated for red-legged salamanders (Wack et al., 2010; Wack et al., 2012), resulting in high physiological levels of plasma CORT in CORT-patched animals and baseline levels of CORT in oil-patched animals. With our CORT patch regimen, we mimicked patterns of plasma CORT evident in free-living animals in high-stress environments and found that treatment with CORT prior to inoculation with *Bd* resulted in a greater *Bd* infection abundance by day 13-17. We did not find an effect of prior CORT elevation on *Bd* infection abundance by day 10 of our study, perhaps because *Bd* needed more than 10 days to reach levels detectable by our methods.

Although we did not measure specific aspects of immune function, it is possible that the prior CORT treatment was immunosuppressive, as has been found in previous studies (Dhabhar and Mcewen, 1997; Sapolsky et al., 2000; Dhabhar, 2002; Davis and Maerz, 2008). Of particular importance for amphibians are AMPs, which are a vital amphibian defense against *Bd* infection (Rollins-Smith and Conlon, 2005). Although there is some evidence that treatment with CORT suppresses expression of antimicrobial peptides in frogs (Simmaco et al., 1997), more studies are needed to determine whether CORT treatment inhibits activity or production of anti-*Bd* AMPs. Furthermore, it is possible that an increase in plasma CORT could inhibit lymphocyte action, as has been indicated previously in other vertebrate species (Wiegers et al., 1993; Miller et al., 1994; Engler et al., 2004; Sterzer et al., 2004). A recent study in frogs demonstrated that soluble factors produced by *Bd* impair lymphocytes, contributing to the ability of *Bd* to evade host immune responses (Fites et al., 2013; Fites et al., 2014). Thus, prior chronic elevation of CORT may further dampen immunity, allowing *Bd* to better evade amphibian immune responses and resulting in increased infection abundance.

Another possibility is that CORT impacts *Bd* infection via the effects of CORT on amphibian skin. In a study by Hayes (1995), treatment with CORT for 15 days resulted in build-up of the stratum corneum layer of the epidermis. If this effect occurred in our study, it is possible that CORT may contribute to increased infection by increasing the thickness of the stratum corneum, thereby providing *Bd* zoospores with more nutrient sources for growth and proliferation. Additionally, as a result of CORT treatment, the increased skin thickening may prevent zoospores from infiltrating deeper into the epidermis such that additional symptoms of chytridiomycosis are reduced. This suggested mechanism might explain why we found increased infection without increased symptoms.

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-metamorphic anurans (*R. cascadae*) (Searle et al., 2014). Our study differs from Searle et al. (2014) in many ways, including species, regimen of CORT exposure, strain of *Bd*, developmental stage, etc., making it difficult to determine whether methodological differences explain the contrasting results. However, one important difference is the infection severity. The infections were relatively mild in Searle et al. (2014), with no effect of *Bd* exposure on survival. The low level of infection and symptoms could have prevented putative effects of CORT being manifested. In our study of red-legged salamanders, infections resulted in relatively high zoospore equivalents and 60% mortality in *Bd*-inoculated animals. Clearly, more studies are necessary to determine the role of CORT in susceptibility to *Bd*.

Bd infection – Experiment 2

There was no significant effect of either *Bd* inoculation or patch treatment on infection abundance in Experiment 2. Compared to Experiment 1, infection abundance in Experiment 2 was substantially lower, which may also have contributed to the lack of effect of *Bd* inoculation. A key difference between the experiments was the lower inoculum dosage used in Experiment 2 (1×10^6 zoospores/animal). A lower dosage was used in this experiment because we wanted to test whether animals would exhibit CORT-driven increases in chytridiomycosis development in response to a lower dosage (see infection-disease threshold discussion below). At the higher dosage used in Experiment 1, a significant effect of inoculation was observed in *Bd* infection abundance. Therefore, it appears there is a dose-effect of *P. shermani* susceptibility to *Bd* infection, and that a threshold for significant infection exists between 1×10^6 zoospores/animal

and 6.9 x 10^7 zoospores/animal. It is unclear how ecologically relevant this difference in dosages might be for free-living animals, but perhaps future studies should aim to determine the specific dosage required for significant *Bd* infection in *P. shermani*.

Chytridiomycosis – Experiment 1

Inoculation of salamanders with *Bd* resulted in many signs of chytridiomycosis, including skin sloughing, body mass loss, and mortality. Sham-inoculated animals displayed no symptoms of chytridiomycosis at any point during the study. Despite the symptoms of chytridiomycosis observed in *Bd*-inoculated salamanders, symptoms were not exacerbated by prior exposure to CORT. Although CORT might have increased the expression of symptoms we did not measure, such as appetite, anti-predator defenses (Parris et al., 2004; Berger et al., 2005), and reproductive efforts, there was no effect on mortality, a key fitness component.

There are several explanations for why prior CORT did not increase the expression of chytridiomycosis despite the increased *Bd* abundance. First, there may be threshold of *Bd* infection required for onset of chytridiomycosis. A threshold theory to describe development of chytridiomycosis has been posited previously (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 (Mcconnell, 2007; 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 manifestation of disease symptoms, including body mass loss, locomotory activity, or mortality.

Another explanation for the lack of CORT treatment on disease development in *Bd*-inoculated animals relates to the relatively benign conditions of the controlled laboratory environment which could have mitigated the effects of increased *Bd* infection. In more natural conditions with limited food availability, thermal variability, predator-prey interactions, pollutants, and competition(Blaustein and Kiesecker, 2002; Relyea and Edwards, 2010; Blaustein et al., 2011), increased *Bd* abundance may translate into increased signs and symptoms of chytridiomycosis. Finally, it is possible that the subjects in our study may have developed immunity 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 in the areas surrounding our collection sites (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* exposed 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.

CORT, *Bd* infection, and chytridiomycosis likely interact in complex ways. Previous studies found that infection with *Bd* and development of chytridiomycosis was associated with elevated plasma CORT (Kindermann et al., 2012; Gabor et al., 2013; Peterson et al., 2013).

However, these studies were correlational and could not determine whether differences in CORT were a result of infection and disease, or were a cause of infection and disease. Our study indicates that CORT can increase susceptibility to *Bd*, but it is likely that infection with *Bd* also causes an increase in endogenous plasma CORT. Indeed, acute elevation of plasma glucocorticoids is a component of the acute phase response that is triggered by infection (ref) and presumably facilitates immune defenses. It should also be noted that our prior CORT treatment might have altered the endogenous CORT response to infection via negative feedback on the HPI axis. Clearly, more studies are necessary to untangle the complex interactions existing between plasma CORT, susceptibility to *Bd,* and outcomes after exposure to *Bd*.

Chytridiomycosis – Experiment 2

Contrary to original predictions, neither *Bd* inoculation nor CORT patch treatment had a significant effect on body mass, locomotory activity, or mortality in Experiment 2. As stated previously, a probable explanation for these results is that our inoculation dose of 1×10^6 zoospores/animal did not infect *P. shermani*. At the higher dosage in Experiment 1, substantial development of chytridiomycosis was observed, with *Bd*-inoculated animals exhibiting loss of body mass and mortality compared to sham-inoculated animals.

Although the dose of *Bd* was insufficient to infect animals, there was a significant effect of repeated CORT elevations on skin sloughing. As mentioned beforehand, prolonged CORT treatment for 15 days increased the thickness of outer skin layers of anuran amphibians (Hayes, 1995). With our CORT patching regimen of 14 days, it is possible that the stratum corneum was thickened. Upon exposure to *Bd* inocula, animals demonstrated increased skin sloughing, with pretreatment of CORT resulting in even more sloughing compared to oil patch treatment.

Animals exposed to *Bd* continued to slough skin for up to 55 days post-inoculation. It is possible that the sloughing response to *Bd* exposure I results in clearing the relatively low dosage of *Bd* zoospores from the skin, an effect supported by the low infection abundance measured by qPCR.

Conclusions

To our knowledge, our study is the first to provide evidence that repeated CORT elevation prior to exposure to *Bd* increases *Bd* infection in an amphibian. The effects of CORT on infection did not translate into increased expression of symptoms of chytridiomycosis, perhaps due to threshold effects, the relatively benign conditions of captivity, 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 susceptibility to *Bd*. In order to better understand the scope of the effects of stress and stress hormones on susceptibility to chytrid pathogen, future studies should examine the effects of CORT on *Bd* susceptibility in more species and should also examines responses to *Bd* in animals that are stressed in other ways, such as via predator exposure, food scarcity, competition, or habitat modification. Given the concern over *Bsal*, a chytrid fungus that is particularly virulent for salamander species, it is important to also examine whether prior or concomitant exposure to stressors also affects susceptibility to *Bsal*.

Attributions

Sarah Woodley and I conceived of and designed the experiments described in this chapter, as well as performed the majority of the statistical analyses. I performed most of the experimental procedures with the following exceptions. Shreya Patel aided in quantifying the disease symptoms for Experiment #1. Shelby Boord performed the DNA extractions and qPCR

reactions for Experiment #1. Chris Garbark aided in quantifying the disease symptoms for Experiment #2. Finally, Matthew Venesky performed the statistical analyses for *Bd* infection abundance described in Experiment #1.

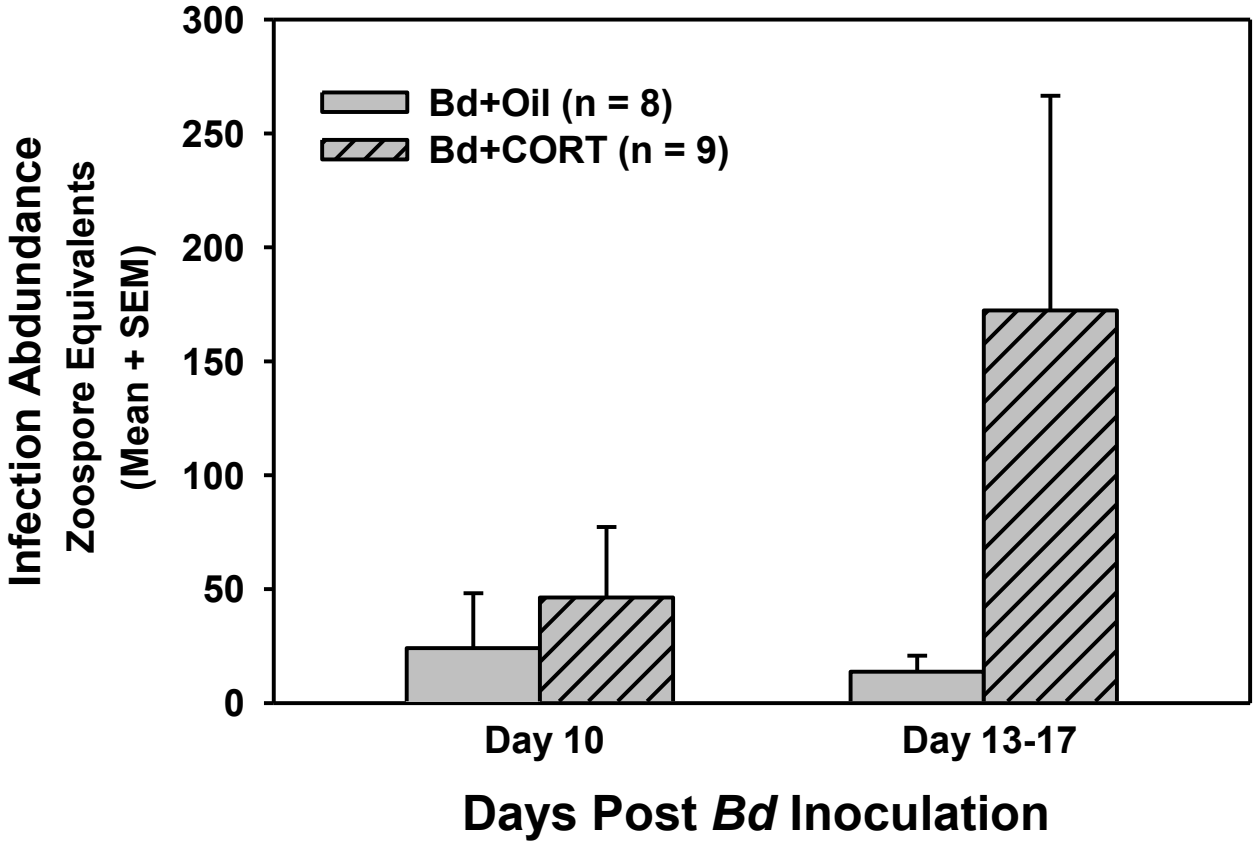

Figure 4.1. Mean genomic *Bd* zoospore equivalents from *Bd*-inoculated salamanders at days 10 and 13-17 (Experiment 1). Prior treatment with CORT increased infection abundance in *Bd*-inoculated animals by day 13-17 (see text for details)

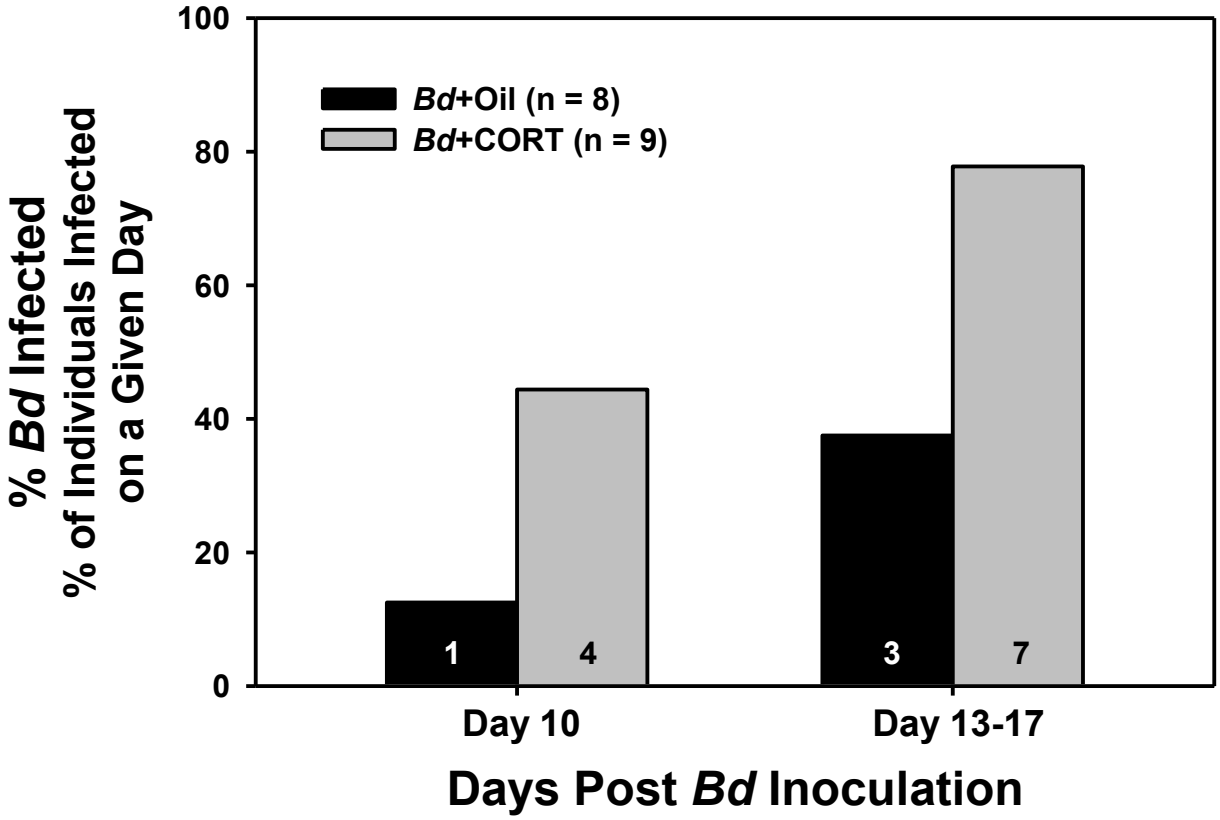

Figure 4.2. Percentage of individuals positive for *Bd* infection out of number of individuals inoculated with *Bd* within each treatment at days 10 and 13-17. There was no effect of prior treatment with CORT on % infection at either time point (see text for details)

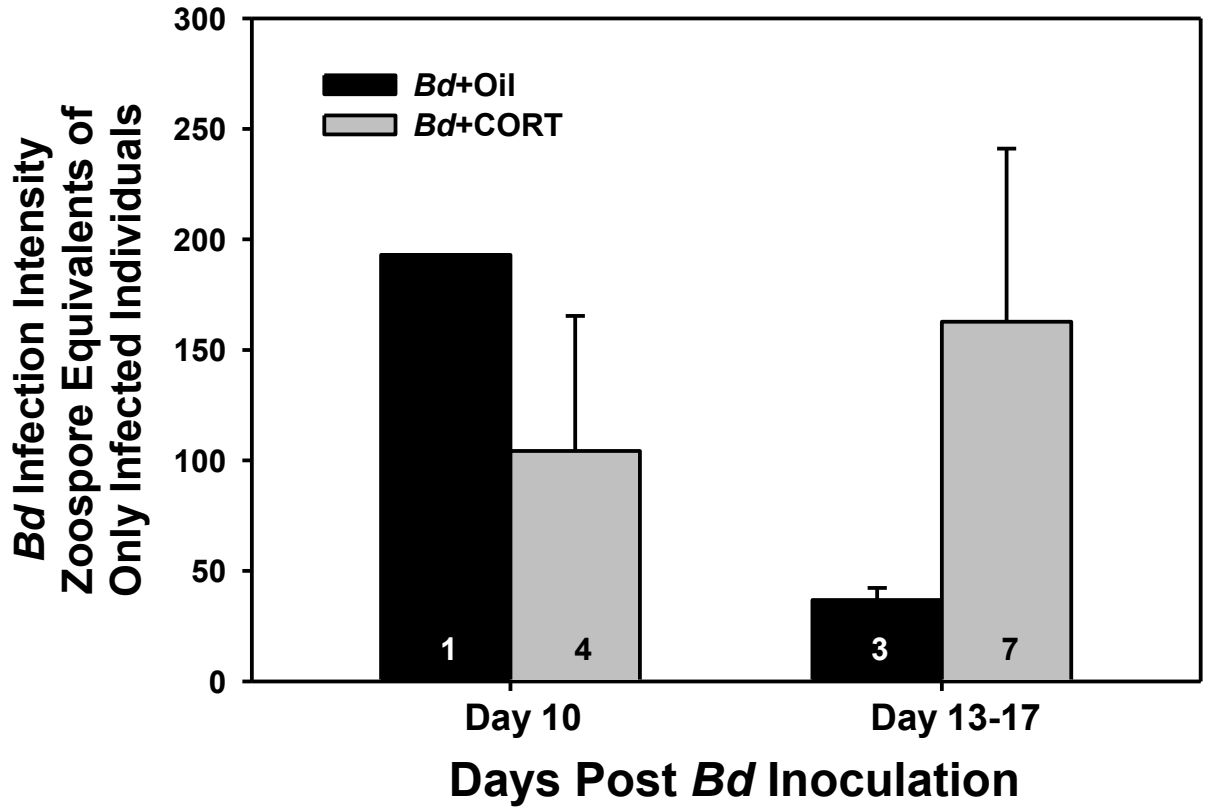

Figure 4.3. Mean genomic *Bd* zoospore equivalents of *Bd*-positive salamanders at days 10 and 13-17. Prior treatment with CORT did not affect infection intensity at either time point (see text for details)

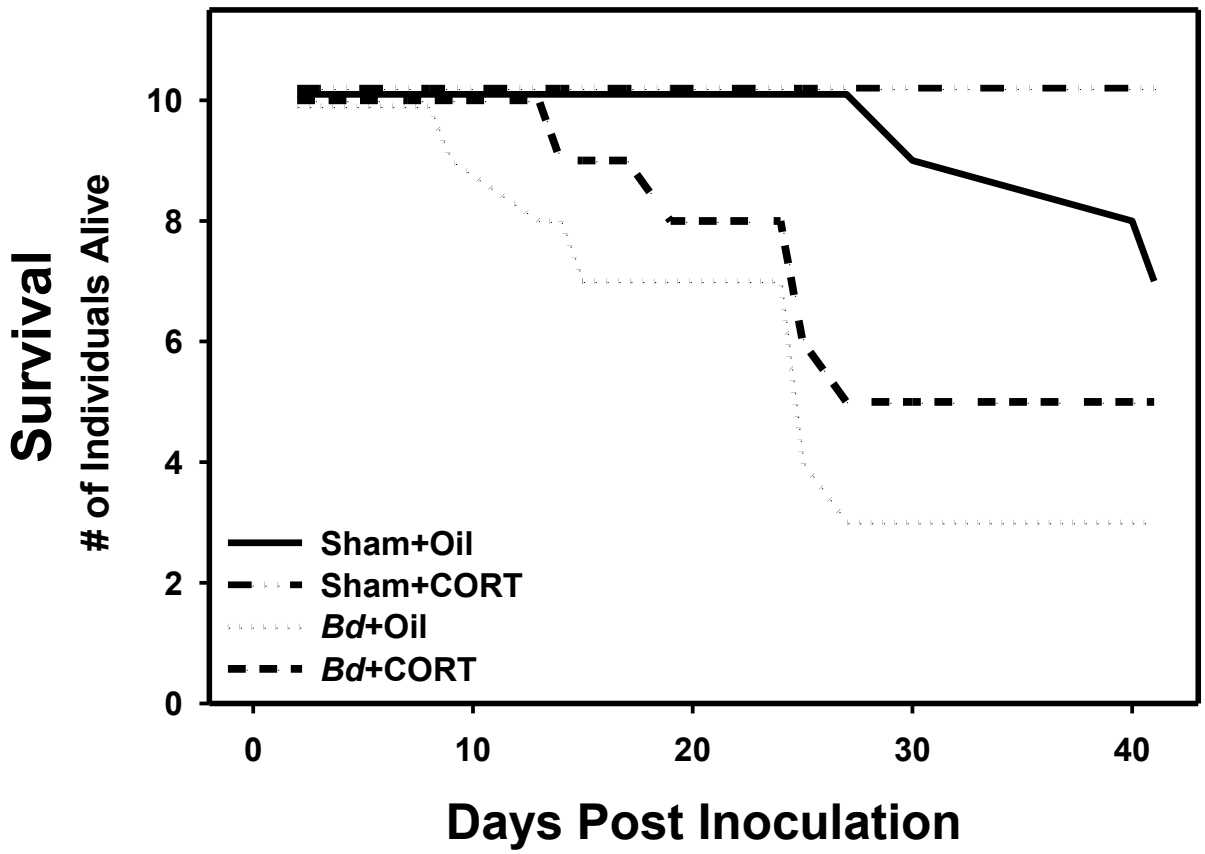

Figure 4.4. Number of surviving salamanders after inoculation with *Bd* or sham inocula. Inoculation with *Bd* reduced survival. There was no effect of prior treatment with CORT (see text for details)

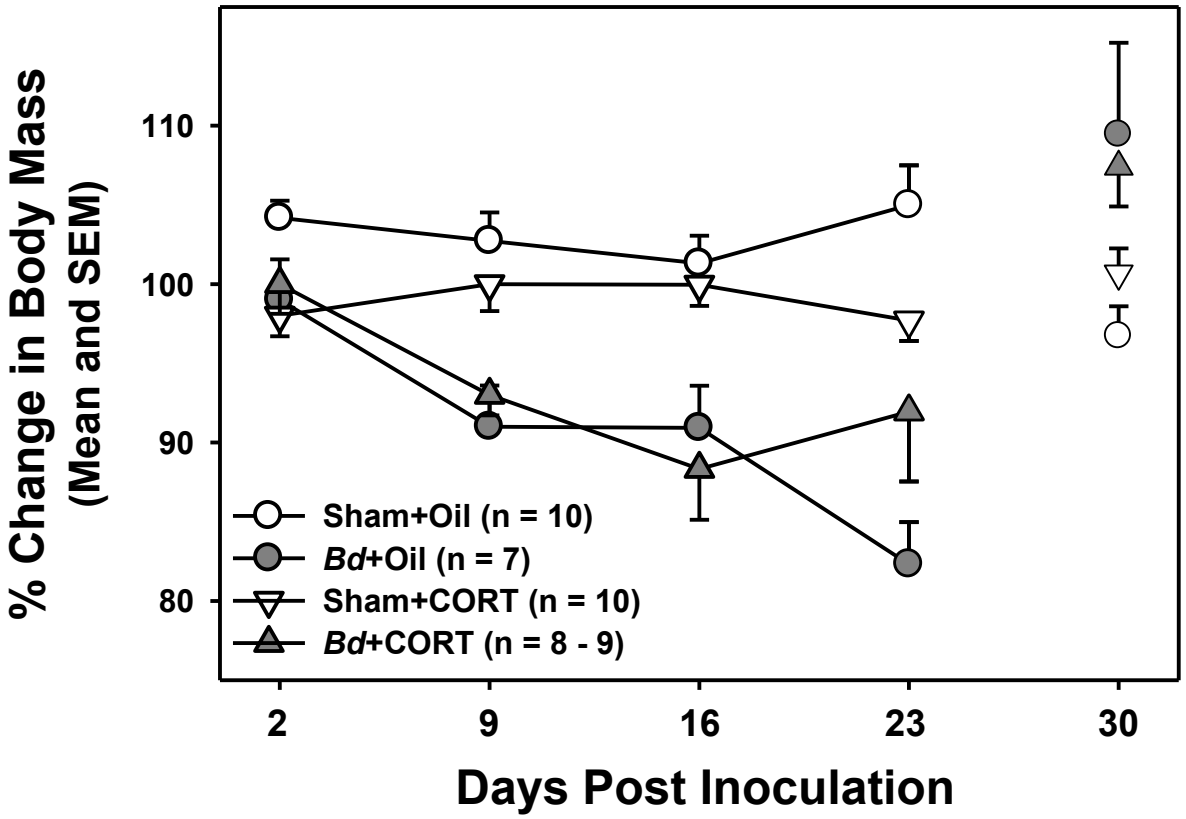

Figure 4.5. Percent body mass change of salamanders after inoculation with *Bd* or sham inocula. By day 23, subjects inoculated with *Bd* lost more body mass than sham-inoculated subjects. There was no effect of prior CORT treatment. Sample sizes for days 2-23 are shown. Sample sizes for day 30 are 3, 5, 10, and 9 (highest to lowest value) (see text for details)

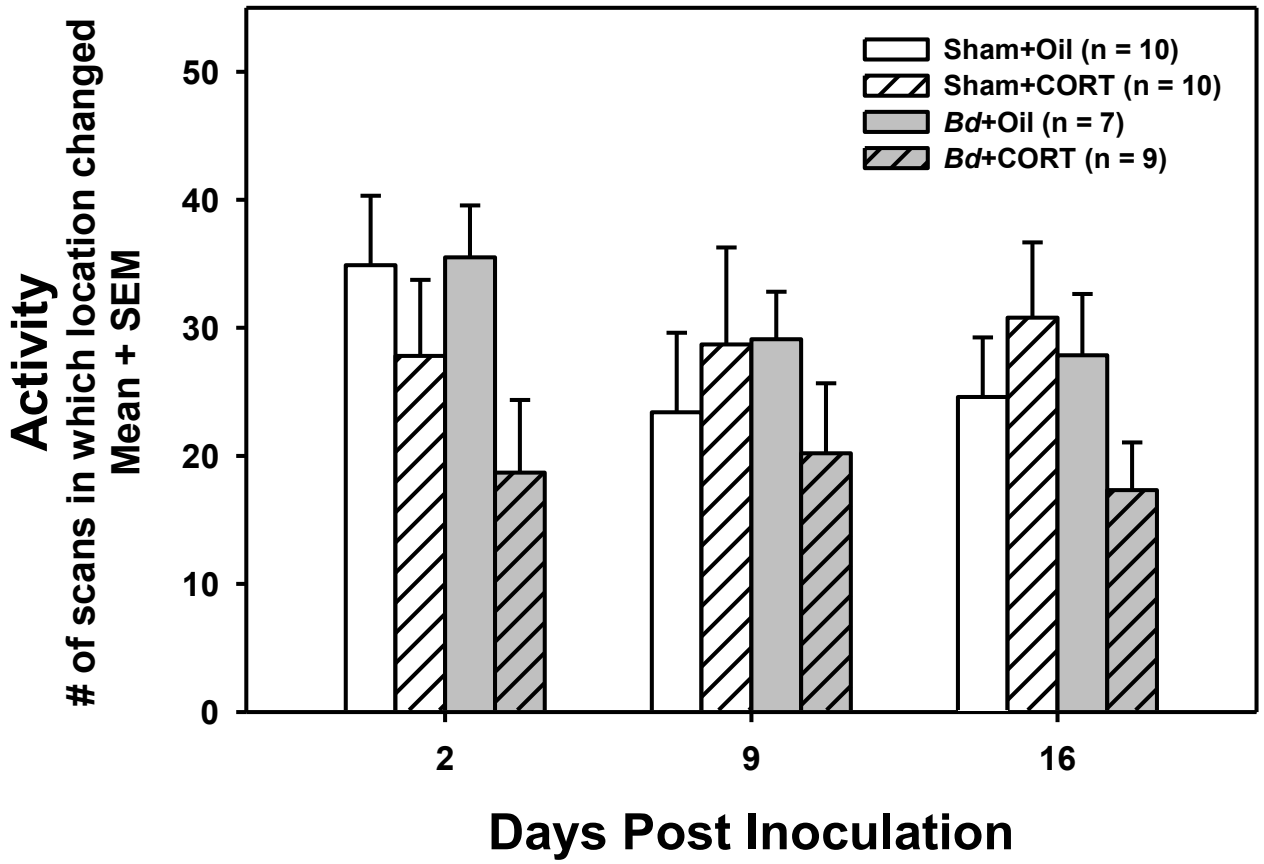

Figure 4.6. Locomotory activity of salamanders after inoculation with *Bd* or sham inocula (Experiment 1). Treatment groups did not vary in activity (see text for details)

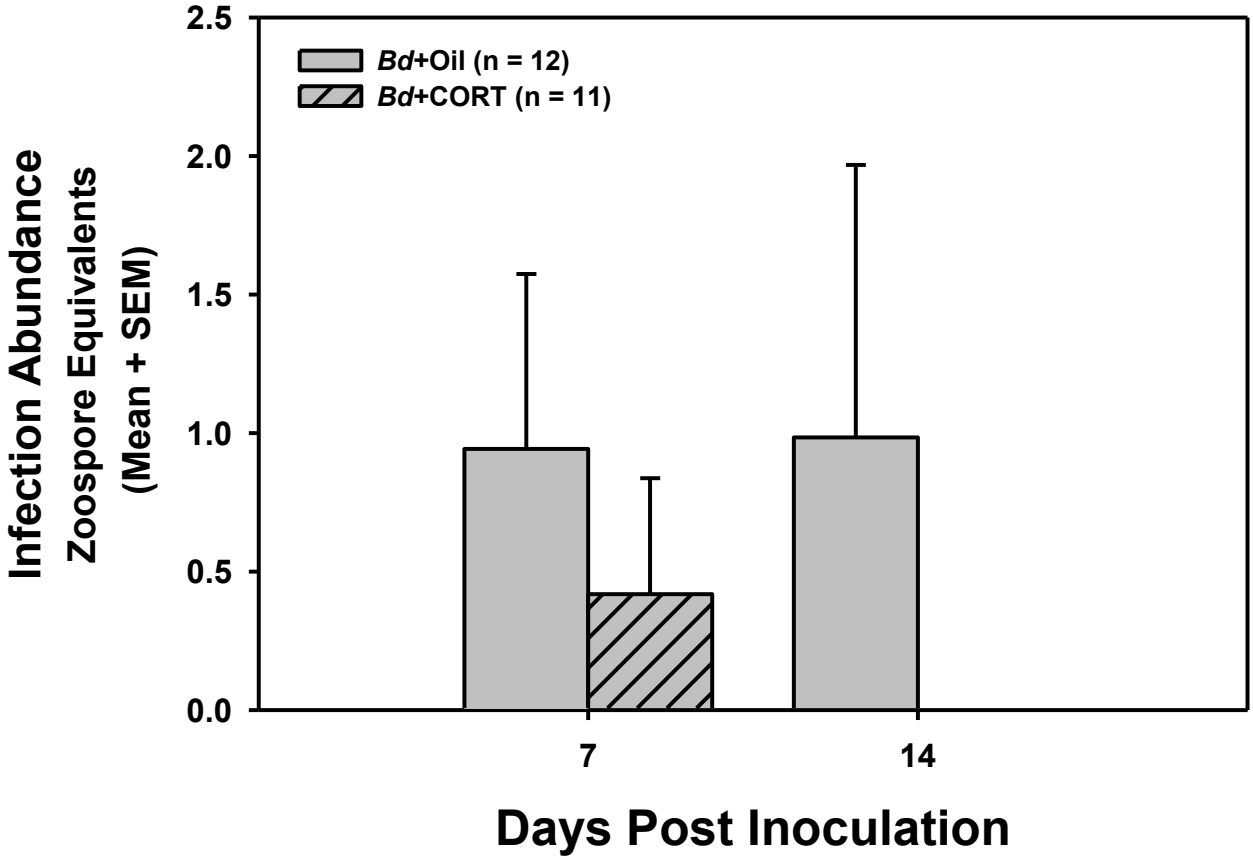

Figure 4.7. Mean genomic *Bd* zoospore equivalents from *Bd*-inoculated salamanders at days 7 and 14 (Experiment 2). Subjects at baseline, days 21, and 28 were negative for *Bd* infection, as were sham-inoculated subjects. There was no effect of inoculation or patch treatment on *Bd* infection abundance (see text for details).

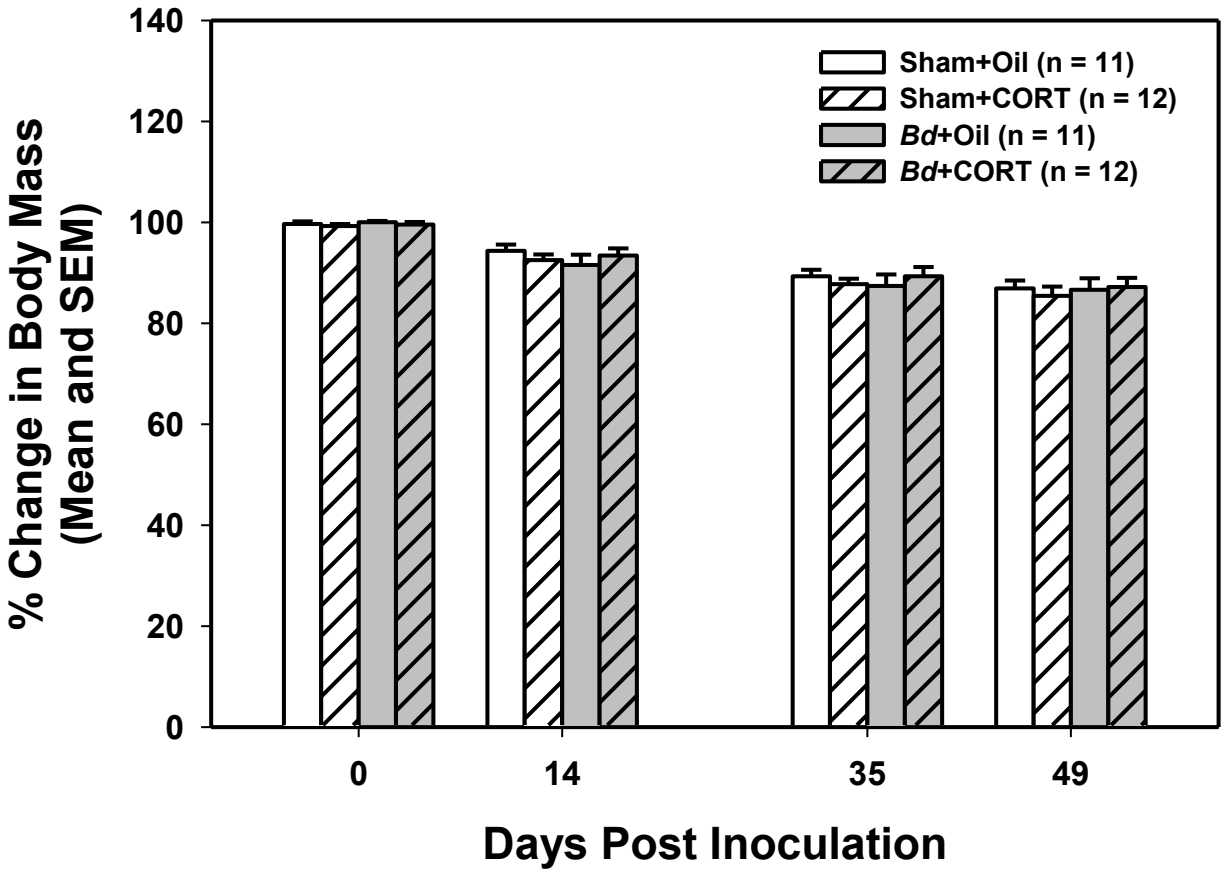

Figure 4.8. Percent body mass change of salamanders after inoculation with *Bd* or sham inocula (Experiment #2). There was no effect of either *Bd* inoculation or CORT treatment on body mass loss throughout the study (see text for details).

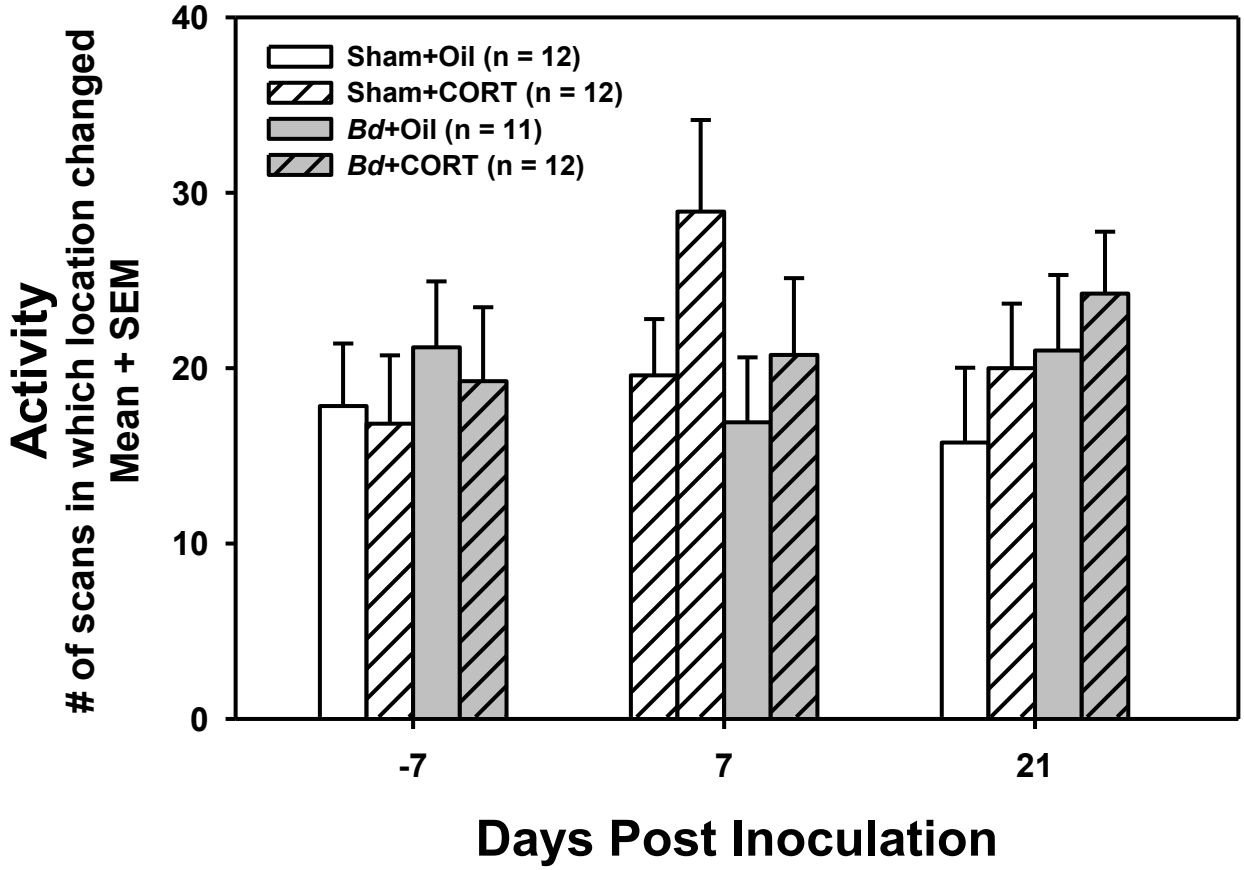

Figure 4.9. Locomotory activity of salamanders (Experiment #2) after inoculation with *Bd* or sham inocula. Treatment groups did not vary in activity (see text for details).

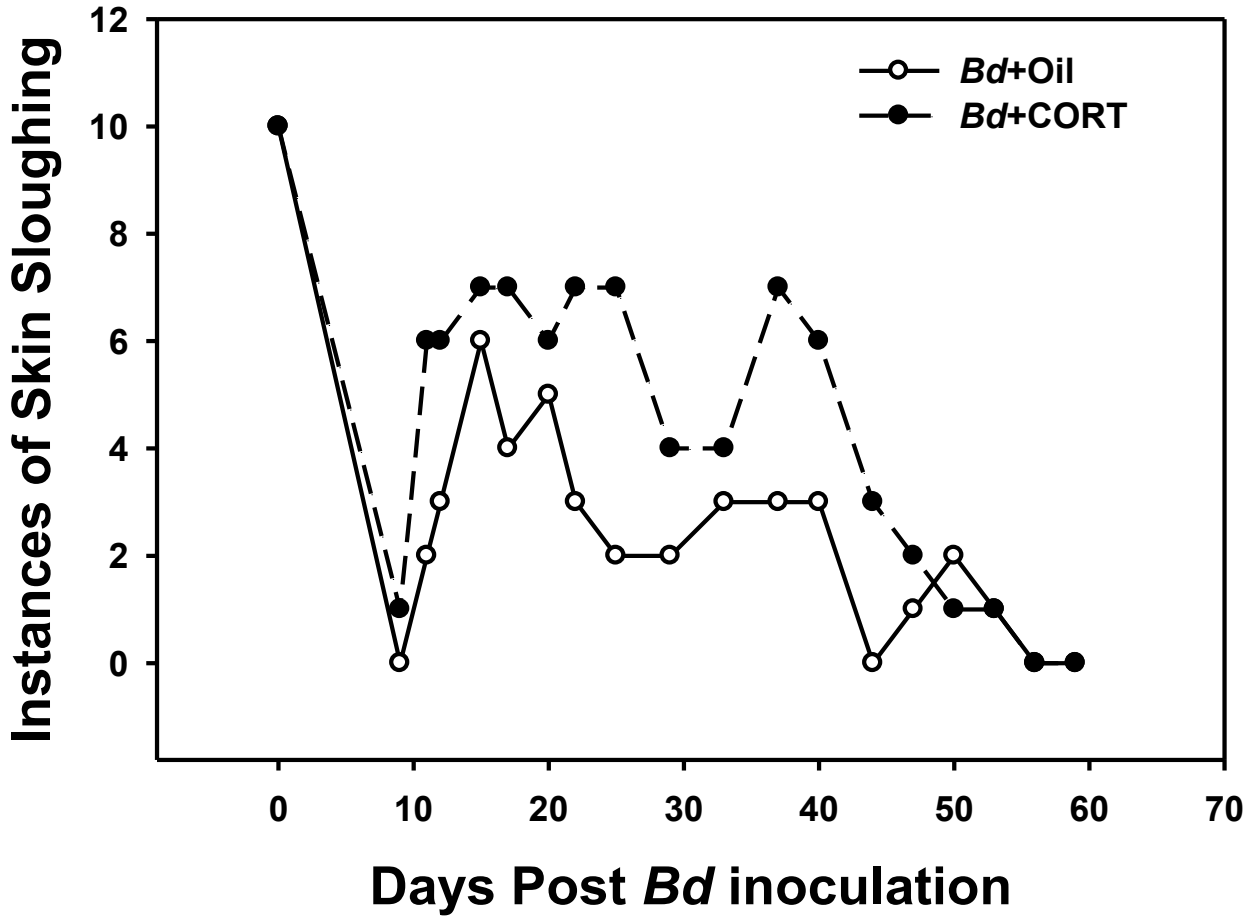

Figure 4.10. Number of animals that sloughed their skin on a given day in in *Bd*-inoculated salamanders (Experiment #2). For clarity, sloughing data from every 3 days is shown. Treatment with CORT increased instances of skin sloughing in *Bd*-inoculated animals on a given day.

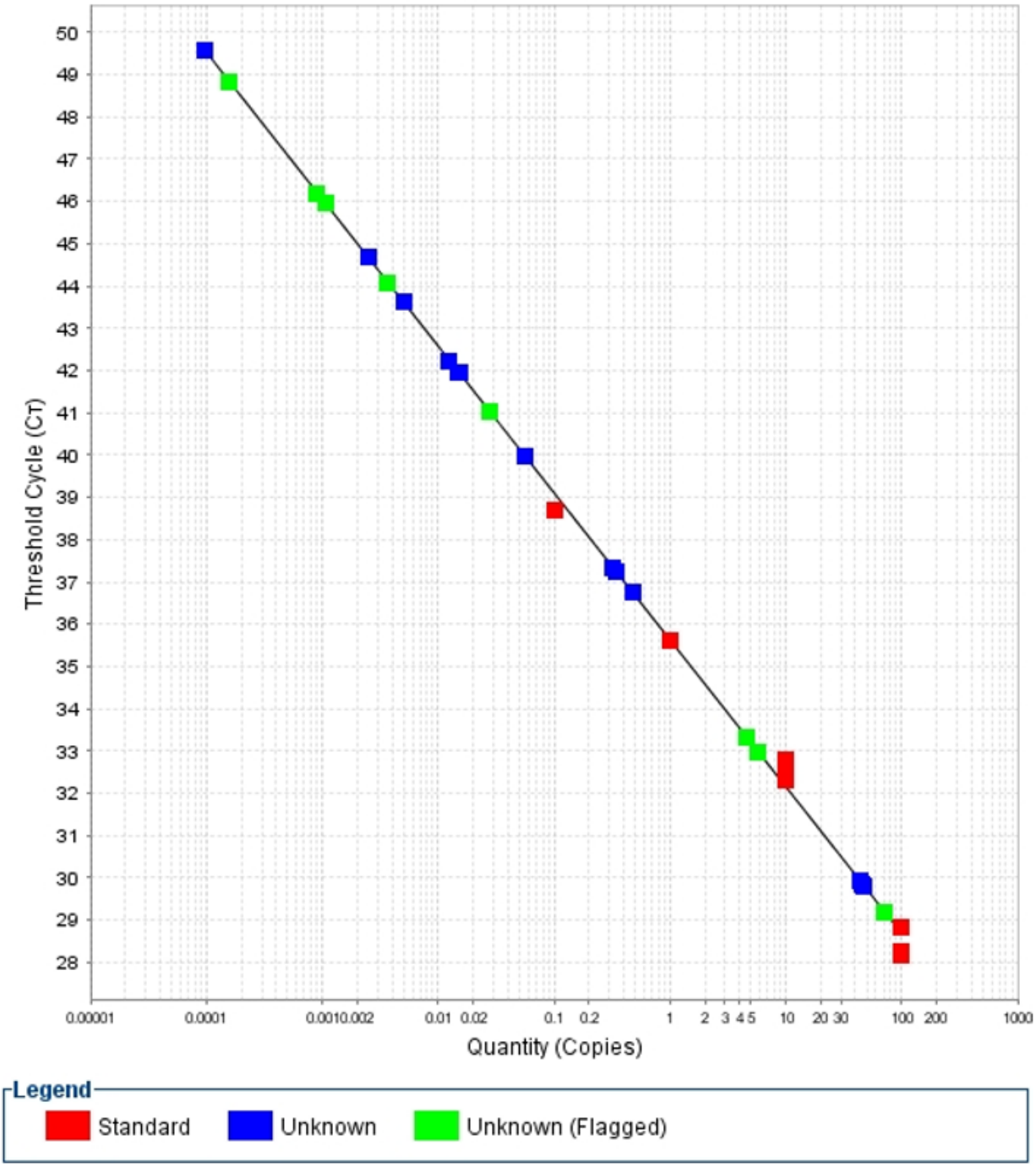

Figure 4.11. Representative standard curve from Batch #1 of qPCR analyses (Experiment 1). Standards range from 100, 10, 1, and 0.1 zoospore equivalents. $R^2 = 0.98$, % efficiency = 87%.

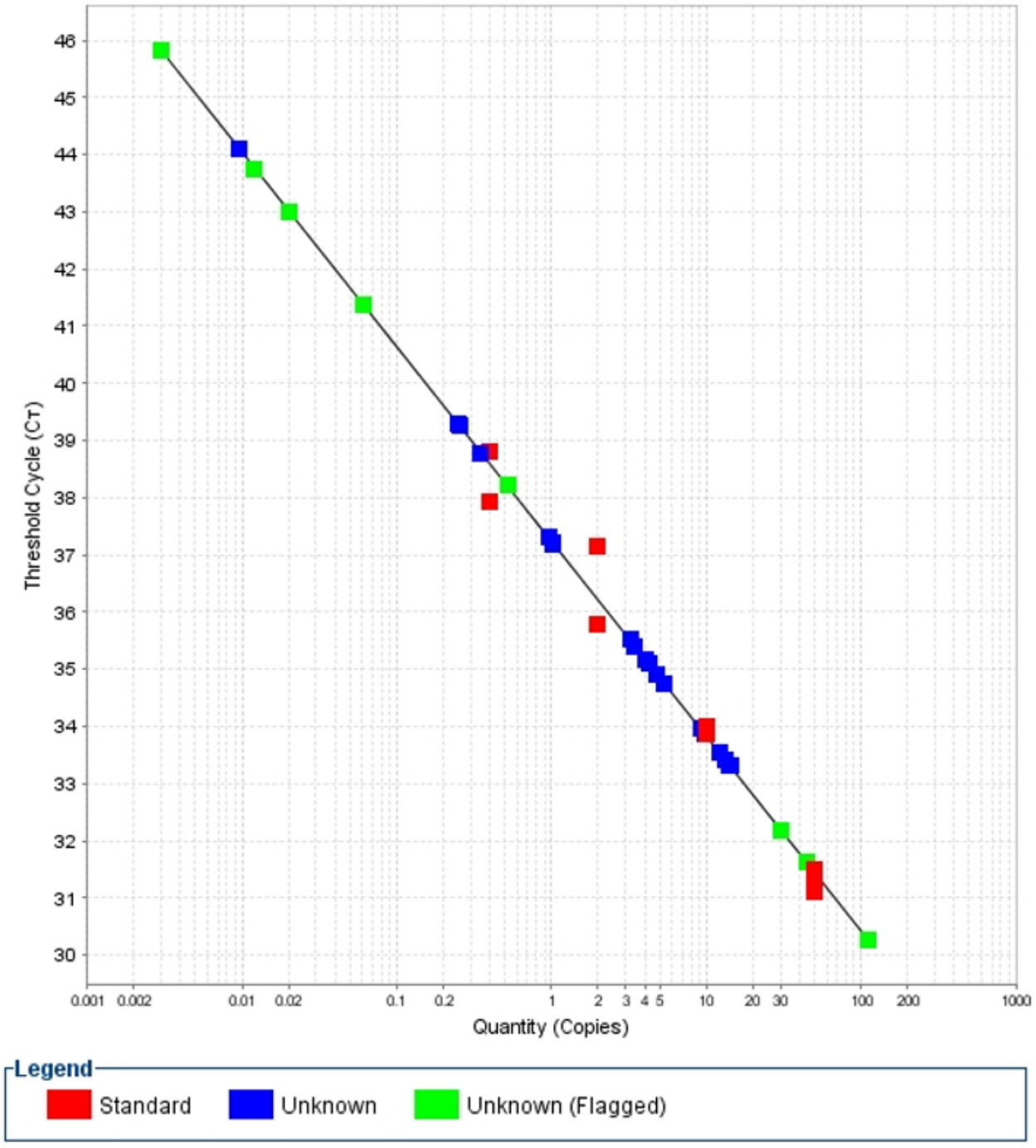

Figure 4.12. Representative standard curve from Batch #2 of qPCR analyses (Experiment 1). Standards range from 50, 10, 2, and 0.4 zoospore equivalents. $R^2 = 0.98$, % efficiency = 95%.

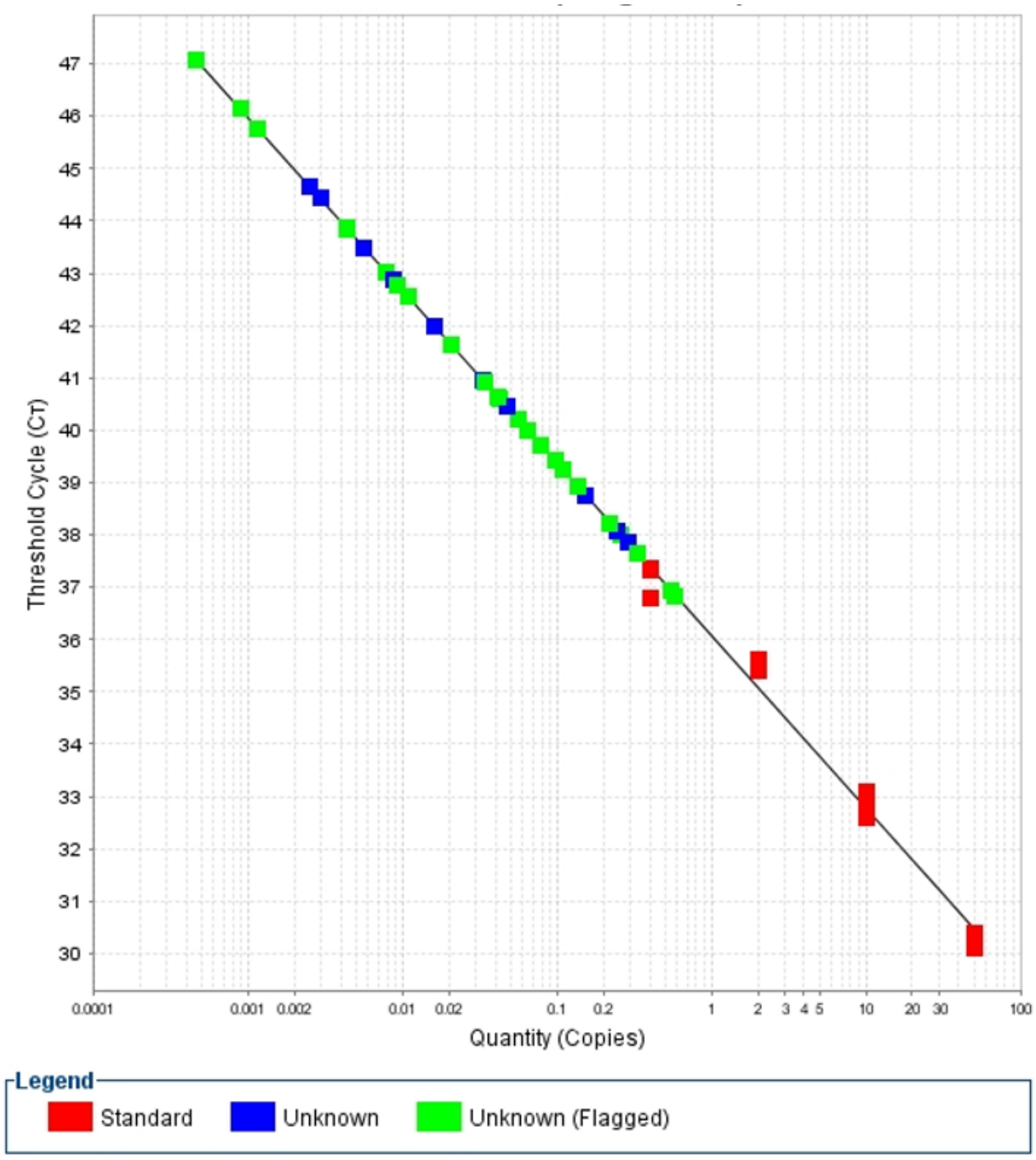

Figure 4.13 Representative standard curve of qPCR analyses (Experiment 2). Standards range from 50, 10, 2, and 0.4 zoospore equivalents. $R^2 = 0.99$, % efficiency = 101%.

Table 4.1**.** The number of salamanders that tested positive for *Bd* infection in batch 1 of Experiment 1 of the qPCR analysis. The number in parenthesis shows the total number of animals tested.

Treatment	Baseline	Day 10
***Bd*+CORT**	0 (10)	8 (9)
***Bd*+Oil**	0 (10)	3 (8)
Sham+CORT	0 (10)	0 (10)
Sham+Oil	0 (10)	0 (10)

Table 4.2. The number of salamanders that tested positive for *Bd* infection in batch 2 of Experiment 1 of the qPCR analysis. The number in parenthesis shows the total number of animals tested.

Treatment	Day 10	Day 17
***Bd*+CORT**	4 (9)	7 (9)
***Bd*+Oil**	1 (8)	3 (8)

Table 4.3. The number of salamanders in each treatment group that sloughed their skin at least one time throughout Experiment 1. The number in parentheses shows the sample size.

Treatment	Number that Sloughed
***Bd*+CORT**	9 (10)
***Bd*+Oil**	9 (10)
Sham+CORT	0 (10)
Sham+Oil	0 (10)

Table 4.4. The number of salamanders that tested positive for *Bd* infection in Experiment 2 of the qPCR analysis. The number in parenthesis shows the total number of animals tested. No baseline or sham inoculated animals tested positive for *Bd*. No *Bd*-exposed animals tested positive on days 21 and 28.

Treatment	**Day 7**	**Day 14**
***Bd*+CORT**	1 (11)	0 (11)
***Bd*+Oil**	2 (12)	1 (12)

Table 4.5. qPCR data from Experiment 1 (Batch 1) of individual salamanders, with corresponding treatments and Ct means (maximum of 3 wells). Samples that amplified below the 0.1 *Bd* standard were considered Non-Detectable (N.D.) and assigned a value of 0, and samples that amplified above the 100 *Bd* standard were assigned a value of 100. All baseline and sham-inoculated samples were 0. Equivalents shown in the table were multiplied by 10 to account for qPCR dilution.

ID	Treatment	Ct Mean Day 10	Zoospore Equivalents Day 10
107	*Bd*+CORT	N.D.	0
102	*Bd*+CORT	31.95	167.98
106	*Bd*+CORT	36.95	25.79
135	*Bd*+CORT	31.07	200.04
133	*Bd*+CORT	36.94	8.23
143	*Bd*+CORT	33.15	41.63
129	*Bd*+CORT	34.57	62.04
120	*Bd*+CORT	37.11	5.97
100	*Bd*+CORT	35.38	17.54
122	*Bd*+Oil	36.86	12.58
148	*Bd*+Oil	N.D.	0
157	*Bd*+Oil	N.D.	0
103	*Bd*+Oil	35.13	20.24
161	*Bd*+Oil	29.86	441.34
114	*Bd*+Oil	N.D.	0
159	*Bd*+Oil	N.D.	0
160	*Bd*+Oil	N.D.	0

Table 4.6. qPCR data from Experiment 1 (Batch 2) of individual salamanders, with corresponding treatments and Ct means (maximum of 3 wells). Samples that amplified below the 0.4 *Bd* standard were considered Non-Detectable (N.D.) and assigned a value of 0, and samples that amplified above the 50 *Bd* standard were assigned a value of 50. Equivalents shown in the table were multiplied by 10 to account for qPCR dilution.

ID	Treatment	Ct Mean Day 10	Zoospore Equivalents Day 10	Ct Mean Day 17	Zoospore Equivalents Days 13-17
107	*Bd*+CORT	30.41	273.89	28.89	500
102	*Bd*+CORT	33.71	110.83	33.43	131.5
106	*Bd*+CORT	N.D.	0	31.36	416.48
135	*Bd*+CORT	33.84	31.44	37.43	7.94
133	*Bd*+CORT	N.D.	0	34.35	22.67
143	*Bd*+CORT	N.D.	0	N.D.	0
129	*Bd*+CORT	37.24	1.02	35.34	36.26
120	*Bd*+CORT	N.D.	0	N.D.	0
100	*Bd*+CORT	N.D.	0	35.36	24.16
122	*Bd*+Oil	N.D.	0	34.38	29.68
148	*Bd*+Oil	N.D.	0	34.94	47.55
157	*Bd*+Oil	N.D.	0	N.D.	0
103	*Bd*+Oil	N.D.	0	N.D.	0
161	*Bd*+Oil	30.84	192.97	33.58	33.15
114	*Bd*+Oil	N.D.	0	N.D.	0
159	*Bd*+Oil	N.D.	0	N.D.	0
160	*Bd*+Oil	N.D.	0	N.D.	0

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Chapter 5

Conclusions

Environmental stressors have widespread effects on animal behavior and physiology. Although the typical stress response to acute and chronic stressors involves behavioral and physiological modifications including elevation of plasma CORT, numerous studies have observed conflicting effects of responses to stressors. With amphibian species undergoing worldwide population declines and extinctions, research into how amphibians respond to different natural stressors is essential. My initial studies examined how salamanders respond to predator kairomones and thermal elevation under both an acute and prolonged exposure. I showed that animals responded to acute predator kairomone exposure with changes in locomotory and mating activity. However, plasma CORT did not increase as originally predicted after acute predator kairomone exposure, and prolonged exposure to both predator kairomones and thermal elevation failed to elicit a plasma CORT response. Through the results of these two studies, I provided evidence that stressors do not induce identical behavioral and hormonal responses; specifically, plasma CORT responses may be decoupled depending on the context and severity of a given stressor.

Increases in CORT can also influence susceptibility to infection and disease, especially in regards to *Bd* infection. Previous studies have investigated the role of CORT in susceptibility to *Bd*. However, due to low infection levels and correlational evidence, a direct link between CORT elevation and significant infection abundance has not yet been determined. Therefore, the bulk of my thesis has focused on examining the mechanistic link between prolonged CORT elevations and changes in *Bd* infection abundance and chytridiomycosis development in red-legged salamanders. I found that treatment with CORT caused a significant increase in *Bd* infection,

with no CORT-driven changes in disease development observed. This effect on infection is the first direct evidence of CORT on changes in *Bd* susceptibility, and shows that a chronic stress response plays a key role in determining resistance to pathogen infection. Furthermore, in more natural conditions, the effect of CORT on progression of chytridiomycosis might be exacerbated due to combination of multiple environmental stressors.

In the following sections, I elaborate on the findings and significance of my research, and I provide details on future experiments that can build upon my current results. My data concerning effects of predator kairomones and thermal elevation will first be discussed, after which I discuss the impact of my work examining how repeated CORT elevations increases susceptibility to *Bd* infection.

Effects of predator cues and temperature elevation on behavior and physiology

In my experiments, an acute exposure to predator cues caused a decrease in both locomotory activity and mating levels in Allegheny Mountain dusky salamanders. After exposure to predatory stimuli, numerous vertebrates will reduce activity and mating levels in order to avoid a predator (Wells, 2010). However, potentially detrimental costs can arise from prolonged suppression of activity, including missed opportunities to forage and reproduce. In accordance with the predation stress hypothesis, it is thought that induction of these anti-predator behaviors is mediated by plasma CORT. I found that, after an acute exposure to predator cues, there was no change in plasma CORT, indicating that CORT is not causing suppression of locomotory and mating activity. Although previous studies have shown that GCs like CORT are increased after an acute predator stimulus (Remage-Healey et al., 2006; Thaker et al., 2009; Amaral et al., 2010; Narayan et al., 2013; Davis and Gabor, 2015), this effect was not evident in

our study. Overall, these findings suggest that the predation stress hypothesis does not predict how Allegheny Mountain dusky salamanders respond to an acute predator cue exposure.

Contrary to our initial predictions, after a prolonged exposure to predator kairomones, salamanders exhibited no changes in locomotory activity and plasma CORT. A lack of change in plasma GCs after long-term predator exposure has been observed previously in other vertebrates, including amphibians (Creel et al., 2009; Dahl et al., 2012; Reeve et al., 2013). There are several reasons for my results. First, the lack of a CORT response may be due to predictability of the predator threat, wherein animals may reduce or uncouple a CORT response if predation is frequently encountered, as it seems to be in Allegheny Mountain dusky salamanders. Furthermore, it may be beneficial for animals to uncouple CORT responses in certain unncessary situations, in that CORT is a metabolic hormone and may incur substantial energetic costs (Durant et al., 2008; Preest and Cree, 2008; Wack et al., 2012). Taken together, my results, in addition to the previously mentioned studies, indicate that anti-predator behaviors of *D. ochrophaeus* are not associated with plasma CORT elevation, likely due to predictability of predator threat and maladaptive costs of CORT elevations.

The lack of a plasma CORT elevation was also observed in my temperature elevation study, in which a prolonged thermal elevation of 24°C had no effect on plasma CORT levels when measured at 8 weeks, despite changes in body mass and white blood cell differentials.. Because CORT is associated with increased metabolic rates (Wack et al., 2012) in salamanders, , an additional increase in metabolism induced by elevated CORT in response to elevated temperature may prove to be detrimental and result in severe energetic costs. Therefore, by inhibiting any long-term elevation of baseline CORT, animals can avoid potentially maladaptive energetic costs.

Future Directions

To further test the predation stress hypothesis, studies should investigate how CORT responds to predator kairomones from an unpredictable, high-risk predator of Allegheny Mountain dusky salamander populations. Furthermore, other vertebrate species should be examined in regards to behavioral and hormonal responses to varying predation predictability, in order to better understand the exact situations in which CORT responds to predation in different species.

To further understand the role of CORT in responses to temperature shifts, more studies should examine CORT responses to changing temperatures in more ecologically relevant contexts, such as diurnal and seasonal variation. It is possible that plasma CORT acclimates to temperature, thereby mitigating possible adverse effects of CORT.

Effects of plasma CORT on susceptiblity to Bd infection disease development

Susceptibility to *Bd* infection varies widely among amphibian species and can be attributed to a number of different factors, including differences in immune defenses like AMPs and skin microflora (Woodhams et al., 2006; Woodhams et al., 2007; Lam et al., 2010). Furthermore, it is traditionally thought that differences in exposure to environmental stressors may explain differential susceptibility to *Bd* (Carey et al., 1999). A major aspect of my research has examined how the red-legged salamander responds to *Bd* infection after a prolonged treatment/elevation of plasma CORT. I found that a prior, repeated elevation of plasma CORT increased *Bd* infection abundance in *Bd*-inoculated animals, with no CORT-induced effects observed on chytridiomycosis development. Furthermore, in a separate study, I found that

prolonged CORT elevation increased rate of skin sloughing in *Bd*-inoculated salamanders. These novel effects of CORT treatment on susceptibility to *Bd* are discussed further below.

The increase in *Bd* infection abundance after CORT treatment may be a result of immunosuppression, as multiple studies have demonstrated that CORT treatment can suppress/impair both innate and adaptive aspects of amphibian immunity, including AMPs and lymphocytes (Bennett et al., 1972; Simmaco et al., 1997; Fites et al., 2013; Fites et al., 2014; Groner et al., 2014). In addition, prior CORT treatment may have acted directly on amphibian skin itself by causing buildup of the stratum corneum, which has been documented in a previous study (Hayes, 1995). Through this mechanism, skin thickening may function in providing more nutrients in which to sustain growth and proliferation of *Bd* zoospores. However, exposure to *Bd* triggers increased sloughing, which may help to rid animals of zoospores.

The lack of any CORT-driven effects on disease development was surprising, given the significant increases in infection abundance. However, one probable explanation is that a threshold exists for progression from infection to a disease state, in which a certain number of zoospores must be exceeded on the skin in order to induce a disease state. This threshold effect has been previously theorized (Stockwell et al., 2010; Vredenburg et al., 2010) and may have occurred in my study, in that *Bd*-inoculated subjects had zoospore loads past the threshold and displayed similar symptoms of chytridiomycosis. Another explanation for a lack of effect of CORT treatment on chytridiomycosis can be attributed to the benign laboratory conditions in which the studies took place. Finally, because we obtained animals from sites where *Bd* has been found (Kiemnec-Tyburczy et al., 2012), it is possible that animals may have previously encountered *Bd* in the field and developed resistance to *Bd*. A pre-exposure to *Bd* in the field

may have lessened the effects of CORT treatment on disease development, compared to *Bd*-naïve salamanders.

Future Directions

Although I found that prolonged treatment with CORT caused increases in *Bd* infection abundance, there are numerous avenues of research for exploration of this novel result. For example, although chronic elevations of CORT have been associated with immunosuppressive effects, acute, low-dose elevations of CORT have been shown to enhance innate immunity (Dhabhar and Mcewen, 1999). Therefore, it would be interesting to test whether an acute elevation of plasma CORT can enhance immunity and decrease *Bd* infection abundance at varying dosages in red-legged salamanders. Additionally, corticosterone is not the only hormone that can mediate changes in immunity. For example, other hormones, including testosterone, may function to suppress immunity and likely plays a role in mediating overall susceptibility to *Bd*. Finally, future studies should examine how CORT elevation affects susceptibility to *Bsal*, which has been shown to be more virulent and deadly for salamander species. Perhaps CORT plays some role in affecting how dangerous *Bsal* is to different salamander species.

Another important question that has yet to be investigated fully is how *Bd* susceptibility varies between male and female amphibians. Due to a number of factors, including differences in reproductive organs, hormones, and body mass, it seems logical that a sex difference may exist in regards to *Bd* infection and development of chytridiomycosis. Furthermore, it may be interesting to examine how *Bd* infection affects important amphibian behaviors like mating/courtship. Perhaps animals can sense that a potential mate that is carrying significant *Bd* infection levels and will not initiate mating activities, a phenomenon that may incur substantial

costs to fitness. Alternatively, if male-female pairs initiate courtship activities and one animal is infected, it seems likely that this infection can be transmitted to the mating partner, which may signify an alarming potential spread of *Bd*. If answered, these important questions may further shed light on the specific mechanisms and behaviors that are influencing the increased spread of *Bd*.

Conclusion

The data presented in my thesis have provided significant contributions to a variety of research fields, including stress physiology, animal behavior, and disease physiology. From our findings concerning how plasma CORT responds to predator kairomones and thermal elevations, it is evident that not all stressors stimulate the stress response in the same way. In fact, depending on the duration, type, and context in which the stressor occurs, both CORT and behavioral processes will respond in different ways. Additionally, my research showing how CORT influences susceptibility to *Bd* infection is the first evidence of such an effect. These data on *Bd* infection are innovative contributions to the chytrid fungal field, and can be useful in determining if differences in plasma CORT may explain the variability in *Bd* susceptibility among amphibian species. Given the high degree of amphibian population declines occurring throughout the world, investigations into the specific mechanisms explaining these reductions are vital. Collectively, my results provide key explanations on the effects of stressors on amphibian species, and provide a strong foundation for future studies on how different natural stressors affect amphibian stress responses and susceptibility to infection and disease.

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