How Previous Stressors Affect Disease Resistance in Red-Backed Salamanders (Plethodon cinereus)

By
Sean Michael Collins
April, 2018



Allegheny College
Department of Biology

How Previous Stressors Affect Disease Resistance in Red-Backed Salamanders (Plethodon cinereus)

By
Sean Michael Collns
April, 2018

A Senior Comprehensive Project in Partial Fulfillment of the Requirements for a Bachelor of Science Degree from Allegheny College

I hereby recognize and pledge to fulfill my responsibilities as defined in the Honor Code and to maintain the integrity of both myself and the College community as a whole.

Pledge

Approval by the Senior Thesis Committee:

Dr. Matthew Venesky

Dr. Scott Wissinger

Acknowledgements

I'd like to give a heartfelt thank you to Dr. Matthew Venesky for allowing me to work under him these past few years. Along with being a great mentor, I'd like to think that Dr. Venesky and I have become very good friends in these past few years, which is something I have appreciated greatly. Dr. Venesky has put up with countless questions about amphibians, grad school, laboratory techniques, and who knows what else from me and still seems more than happy to answer any question I have to this day. I don't think I can describe how thankful I am to have gained a mentor and a friend like Dr. Matthew Venesky.

I'd also like to thank Dr. Scott Wissinger for taking the time to be my second reader and helping me further develop my scientific writing and speaking skills.

To my friends and the Venesky COMP group thanks for all your support and help with collecting animals, lab work, and the entire writing process

I would also like to thank the biology department for giving the resources and tools that have led me to become the student I am today.

"*Aut viam inveniam aut faciam*"

Table of Contents

Abstract	page 5
Introduction	page 6
Methods	page 11
Results	page 16
Discussion	page 22
Works Cited	page 29

1.Abstract

External climate factors influence the rate of physiological processes in ectotherms, and thus greatly influences their day to day actions. Not only does temperature affect the efficiency of their physiological processes, but it can also elevate an organism's stress levels. With an increasingly changing climate, ectotherms are going to be put out of their optimal range for their physiology more often, causing more and more stress. Corticosterone (hereafter CORT) is a main hormone of the stress response in amphibians that may help them survive a particular situation and has been shown to have immuno-suppressing effect, when chronically elevated. My experiment examined how stress affects disease resistance, using two separate lab experiments, a control temperature group and a high temperature group, and a No CORT group and CORT group. I used the amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (hereafter *Bd*), a fungal pathogen that may be responsible for many worldwide amphibian population declines. After a 10-day period the stressors were stopped, every animal was housed at the same temperature, and I exposed every animal to an infectious dose and *Bd*. There was no difference in mass loss during the stress period between the CORT and No CORT group, but the CORT group did lose significantly more mass than the No CORT group following the *Bd* exposure. The high temp group lost significantly more mass following the 10 day temperature exposure period compared to the low temp group, and the low temp group lost significantly more mass following the exposure to *Bd*. I found no statistically significant differences in infection abundance post treatment between the CORT/ No CORT groups and the Low Temp/High Temp groups. My results suggest that the established protocol the CORT dosing cycle I used may not cause the immediate physiological changes in metabolic rate or the immune response. My results also suggest that if an ectotherm experiences a temperature increase, that doesn't put them out of their

preferred temperature range, their physiological processes could be enhanced in the short term allowing them to be better prepared for an impending infection. Future studies that build of this one could add a sham *Bd* component with same treatment groups and same timeline, with an ELISA both after the stress period and post *Bd* exposure. In order better understand the long-term effects of stress on body condition, circulating CORT and disease resistance.

2.Introduction

An ectotherm's internal temperature is dependent on the ambient temperature of their environment, and therefore the efficiency and effectiveness of their physiological processes, such their immune response, will also be affected and correlate with the ambient temperature of their environment (Berger et al. 2004, Carey. 2000, Forrest and Schlaepfer. 2011, Homyack et al. 2010, Richards-Zawacki. 2010; Woodhams et al. 2003). Warmer temperatures have been shown to increase metabolic rate in ectotherms (Homyack et al. 2010, Wang et al. 2002). If organisms experience extreme temperatures or are exposed to temperatures outside of their preferred range for too long, they can start to experience negative effects which include changes in metabolic rate, stress levels, immunity, fitness loss, and ultimately changes in population abundance (Carey. 2000, Gómez et al. 2008, Homyack. et al. 2010, Kingsolver et al. 2013). Drastic short-term increase or long-term exposure to an increased temperature can lead to elevated stress hormones (e.g., glucocorticoids), which can have both positive and negative effects on an organism (Peterson et al. 2013, Homyack et al. 2010, Kingsolver et al. 2013, Murone and Venesky. unpublished, Murone et al. 2016, Ricciardella et al. 2010). Based on individual temperature preferences, each individual species will handle temperature fluctuations differently,

and therefore handle elevated stress levels differently based on the soundness of their own stress response.

An organism's response to a stressor is mediated by the HPA (Hypothalamo-Pituitary Adrenal) axis (Ricciardella et al. 2010, Warne et al. 2011). The HPA axis, when activated interprets external factors into developmental processes that will in turn help the organism survive a typical situation, e.g. releasing glucocorticoids and catecholamines, specifically corticosterone (hereafter "CORT") the main glucocorticoid (hereafter "GC") in ectotherms (Ricciardella, et al. 2010, Wack et al. 2012, Warne et al., 2011). Basal levels of GCs are thought to support basic physiological processes, like vascular tone and blood sugar levels (Norris and Carr. 2013, Wack et al. 2012). GC levels can change seasonally in response to life history events or in preparation for a stressful event that requires specific behavioral and energetic changes and can also suppress other processes that aren't essential for the particular situation (Licht et al. 1983, Romero. 2002, Wack et al. 2012). In response to a stressor, GC levels can peak minutes to hours after exposure. Within that time GCs can increase glucose availability, metabolic rate, antipredator behavior, affect B and T cell production, lymphocyte, antibody and cytokine production, and suppress disease resistance and thyroid and gonadal axis function (Fonner et al. 2016, Hardy et al. 2005, Murone et al. 2016, Pickering, et al. 1987, Ricciardella et al. 2010, Wack et al. 2012, Warne et al. 2011).

The duration and intensity of a stressor, specifically acute vs chronic stress, can in turn affect the duration and concentration of circulating GCs, which can have varied effects on many physiological processes (Fonner et al. 2016, Hardy et al. 2005, Murone et al. 2016, Peterson et al. 2013, Pickering et al. 1987, Warne et al. 2011). The response to acute stress is believed to prepare the cardiovascular, musculoskeletal, and neuroendocrine systems for the fight or flight

response, as well as enhance the immune response to prepare for wounds or infection (Dhabhar and Mcewen. 1997, Dhabhar. 2014, Fonner et al. 2016). The acute stress response is generally believed to be adaptive and have mostly positive effects (Dhabhar and Mcewen, 1997; Dhabhar, 2014; Fonner et al., 2016). On the contrary, chronic stress is generally known to be maladaptive, as it suppresses the innate and adaptive immune response, suppresses disease resistance and reproductive function, has adverse effects on metabolic rate, body condition, and developmental success (Dhabhar and Mcewen. 1997, Dhabhar. 2014, Fonner et al. 2016, Glaser and Kiecolt-Glaser. 2005, Hardy et al. 2005, Peterson et al. 2013, Pickering et al. 1987, Warne et al. 2011). Although there have been a few studies that contradict much of what the previous literature shows, that chronic stress can be beneficial, specifically in speeding up developmental rates, which in some ecological contexts could be beneficial, as well as possibly enhancing adaptive immunity (Murone et al., 2016; Peterson et al., 2013; Warne et al., 2011) The varied effects of GCs may suggest that, the stress response attempts to suppress any non-essential physiological process to divert as much energy as possible to dealing with the current situation. However, the stress response seems to be both species and context dependent (Murone et al. 2016, Peterson et al. 2013, Ricciardella et al.2010, Warne et al. 2011).

Amphibians, a taxonomic group that has been under increasing stress due to the changing climate and increased disease prevalence, are found across the globe (Carey. 2000, Richards-Zawacki. 2010, Woodhams et al. 2003). Warmer temperatures have been shown to increase the robustness of an ectotherm's immune response, assuming the increase in temperature does not put the animal outside their preferred range, and in addition organisms may also seek out warmer microclimates to increase their internal temperature to further speed up their physiology, (Carey. 2000, Forrest and Schlaepfer. 2011, Karavian and Venesky. 2016, Richards-Zawacki. 2010,

Woodhams et al. 2003). However, it is also possible that by increasing its internal temp, an organism could be exposing itself to temperature and moisture stress and the increase in temperature could in turn damage the organism, and thus render the possible increase in the immune response a moot point (Carey. 2000, Gómez et al. 2008, Homyack et al. 2010, Kingsolver et al. 2013). The benefits of increasing one's body temp also depends on the duration of the increase in temperature, whether the organism perceives the situation as a chronic or acute stressor. These factors could have either positive or negative effects on the robustness of their immune response (Dhabhar and Mcewen. 1997, Fonner et al. 2016, Murone et al. 2016, Peterson et al. 2013, Warne et al. 2011).

Batrachochytrium dendrobatidis, (hereafter "*Bd*"), also known as the amphibian chytrid fungus is a fungal pathogen that infects amphibians causing chytridiomycosis (Murone et al. 2016). Once infected, amphibians develop hyperkeratosis, a hardening of the stratum corneum, which interferes with cutaneous functions and eventually can lead to cardiac arrest (Murone et al. 2016, Voyles et al. 2011). *Bd* is believed to be involved in mass amphibian population declines throughout the world (Carey, 2000; Rohr et al. 2008). Chytrid Fungus prefers cooler temperatures as it grows best from around 17℃ to 25℃, with an optimal temperature of a 23℃, it starts to die at 30℃ and above (Johnson et al. 2003, Piotrowski et al. 2004, Richards-Zawacki. 2010).

In amphibians, temperature and elevated CORT levels are known to affect disease resistance (Fonner et al. 2016, Murone et al. 2016 Peterson et al. 2013). Lower temperatures are generally known to be immunosuppressive by reducing T-lymphocyte proliferation, circulating eosinophils, and serum complement activity (Carey et al. 1999, Maniero and Carey. 1997, Raffel et al. 2006). Lower temperatures are also known to increase the pathogenicity of certain

pathogens, such as ranavirus and chytrid fungus (Berger et al. 2004, Carey et al. 1999, Maniero and Carey. 1997, Raffel et al. 2006). On the contrary, higher temperatures are known to increase the effectiveness of the amphibian immune response by causing a behavioral fever, an increased metabolic rate, and increased concentration of immune cells (Carey et al. 1999, Karavian and Venesky. 2016 Maniero and Carey. 1997, Raffel et al. 2006). As one can expect higher temperatures are known to decrease the pathogenicity of certain pathogens, such as ranavirus and chytrid fungus (Berger et al. 2004, Carey et al. 1999, Karavian and Venesky. 2016 Maniero and Carey. 1997, Raffel et al. 2006). Although less is known on how temperature affects plasma CORT levels, compared to what is known about how exogenous CORT application affects plasma CORT levels. There is evidence in previous literature, that elevated plasma CORT is immunosuppressive in amphibians via inhibition of antimicrobial peptide production, antibody proliferation, and altered white blood cell levels (Bennett et al. 1972, Rollins-Smith and Blair 1993, Ramirez et al. 1996, Simmaco et al. 1997, Belden and Kiesecker 2005, Davis and Maertz 2008, Groner et al. 2014, Falso et al. 2015).

3. Methods

3.1 Experimental Objective and Hypotheses

The overall objective of this experiment was to examine how previous chronic stressors, in the form of temperature and exogenous CORT application, affected circulating levels of CORT and *Bd* resistance using two separate lab experiments. More specifically, previous literature has shown that exogenous CORT application does indeed raise circulating CORT as well as affect disease resistance. I wanted to compare the effect of temperature on circulating CORT levels and disease resistance to an established protocol and data on exogenous CORT application. If the high temperature treatment acts as a stressor, then circulating CORT levels will increase more

than the cold treatment, based in warm environments amphibians' physiological processes run faster, and that they prefer cooler temperatures (Carey, 2000; Homyack et al., 2010). In part two if chronic stress is immunosuppressive, then the high temp and CORT treatment groups will have a less robust resistance to *Bd* (Fonner et al., 2016; Murone et al., 2016; Peterson et al., 2013; Warne et al., 2011).

3.2.1 Study Species

Plethodontid salamanders are abundant and found throughout the United States their stress response and immune response has been the subject of much study, making it a great model organism for this study, because of the large amount of reference material (Fonner et al. 2016, Homyack et al. 2010, Ricciardella et al. 2010, Wack et al., 2012). *Plethodon cinereus*, the Red-Backed Salamander, is a terrestrial animal, but still is susceptible to a pathogen that tends to prefer an aquatic environment like Chytrid Fungus, examining how this interaction is influenced by stress is a topic that still needs more study.

3.2.2 Animal Husbandry

Red-backed salamanders were caught in Crawford County, PA in Fall 2017. Salamanders were housed in individual plastic containers with 1 folded unbleached paper towel dampened with 12 ml of aged tap. Salamanders were held in a 18.5°C environmental chamber (± 1C) on a 12:12 light:dark photoperiod for approximately 2.5 weeks before the experiment starts. There were weekly bedding and container changes for every salamander, Salamanders were also fed approximately every 7-10 days with 25 flightless fruit flies.

3.3 Examining the Effects of Dermal Corticosterone Application on Circulating Corticosterone

24 salamanders were randomly assigned to one of two treatment groups (n=12 per group), which were no CORT and CORT. Preparation and application of exogenous CORT followed the protocol of previous literature (Murone and Venesky, unpublished; Murone et al., 2016; Wack et al., 2012). Dermal patches were used for exogenous application of CORT, 1.5 x 3mm cut filter paper saturated in .5µg of CORT dissolved in a total volume of 2.5µg solution of sesame oil (Murone and Venesky, unpublished; Wack et al., 2012). Patches were applied on the dorsal side of the salamander between their two front legs (Wack et al., 2012). New patches were be applied every other in the morning for 10 days, totaling 5 treatments (Murone et al., 2016). The schedule that Murone et al 2016 used for application was used for this experiment, because this study builds in part off of what they did and the circulating CORT values need to be comparable to their study (Murone and Venesky, unpublished; Murone et al., 2016). The no CORT treatment group was given the same patches just saturated with sesame oil to control for any effects that the application process has on the salamanders' stress levels. Both groups were housed in the same environmental chamber at 18.5℃.

3.4 Examining the Effect of Temperature Exposure on Circulating CORT

For the second experiment 24 additional and previously unused salamanders were randomly assigned to one of two treatment groups (n=12 per group), which were low temperature (18.5℃) and high temperature (23.5℃). The temperature treatment groups were put in their respective temperatures for the same 10 days as the CORT treatment groups. The low temperature group acted as a control for the effect of temperature, 18.5℃ is well within the preferred temperature of red-backed salamanders, the high temperature group was housed in a separate incubator. The temperatures chosen were based on critical thermal min and max for the salamanders.

3.5 Quantification of CORT levels

The extraction and quantification procedures of CORT levels that I used are justified in previous literature (Gabor et al., 2013; Murone et al., 2016). After the 10-day treatment period, each salamander from both experiments was transferred to individual petri dishes filled with 50 mL of water for 60 minutes, (Gabor et al., 2013; Murone et al., 2016). Then each sample was analyzed for the total (free and conjugated) CORT that was released into the water sample (Gabor et al., 2013; Murone et al., 2016). First, CORT was extracted by passing water through a sterilized 60 mL syringe into C18 solid phase extraction columns then these filters were then stored at -20℃ until CORT extraction (Gabor et al., 2013; Murone et al., 2016). Before CORT analysis, the columns were primed with 4 mL of HPLC-grade methanol into borosilicate vials, and the methanol was evaporated under a stream of nitrogen gas at 37℃ using an Evap-O-Rac (Gabor et al., 2013; Murone et al., 2016). Samples were then re-suspended in 20 µL of 100% ethanol, vortexed for one minute, and then 380 µL of EIA buffer will be added for a total volume of 400 µL (Gabor et al., 2013; Murone et al., 2016). From there the samples were pipetted into an CORT EIA kit and each sample was run in duplicate (Gabor et al., 2013; Murone et al., 2016)

3.6 Examining Stress on Disease Clearance

After the 10-day treatment period and water collection for the CORT assay, I, gave every salamander an infectious dose of *Bd* and then housed them all at 18.5°C, in order to limit added stress on the salamanders as well as limit the inhibitory effect of temperature on *Bd's* growth rate. I exposed the animals to *Bd* by pipetting 2.0 mL of tryptone containing 8×10^4 zoospores directly onto the animal if it was visible or onto its bedding (Murone et al., 2016). The salamanders stayed on the infected bedding for 48 hours and then were placed into a fresh container with fresh bedding, using a fresh nitrile glove for each salamander to avoid accidental contamination (Murone et al., 2016). After *Bd* exposure, any time I handle a salamander I put on

a fresh glove, to avoid any accidental contamination between animals (Murone et al., 2016). Any equipment that touched a *Bd* infected animal was either autoclaved or placed in a 10% bleach solution to kill the *Bd* (Murone et al., 2016).

3.4.2 Swabbing and qPCR

Every animal in each treatment group was randomly swabbed seven days post *Bd* exposure, by passing a sterile swab along the animal's dorsal side from head to tail 10 times (Murone et al., 2016). To prevent cross-contamination between samples, new nitrile gloves were used for every animal (Murone et al., 2016). DNA extractions and qPCR analyses followed the protocol of previously justified studies (Boyle, et al., 2004; Murone et al., 2016). Samples were run only once, to control cost (Murone et al., 2016). Taqman Exogenous Internal Positive Control (Exo IPC) reagents were added to every reaction well to assess inhibition of PCR reaction (Boyle et al., 2004; Murone et al., 2016). The Exo IPC system uses a standard concentration of an artificial DNA sequence that is added to each reaction well with its own set of primers and a separate fluorescent probe (Boyle et al., 2004; Murone et al., 2016). The strength of this reaction is used to assess the overall reaction inhibition (Boyle et al., 2004; Murone et al., 2016). DNA extractions were diluted at 1:100 ratio and processed in an Applied Biosystems Step One Real-time PCR system (Boyle et al., 2004; Murone et al., 2016). Infection intensity was considered as the number of *Bd* zoospore equivalents per sample (Boyle et al., 2004; Murone et al., 2016). Zoospore equivalents were calculated by multiplying genome equivalents generated by the PCR assay by 80 (Boyle et al., 2004; Murone et al., 2016). A sample was considered positive if zoospore equivalents ware greater than 1 (Murone et al., 2016).

3.5 Statistical Analysis

I ran three statistical models for each experiment, each of which had the same predictor and response variables but differed in the data analyzed (e.g., data on the effects of CORT exposure and data on the effects of temperature exposure). First, I tested whether treatment (CORT/no CORT or low/high temp) affected *Bd* abundance (the *Bd* infection of all individuals exposed to *Bd*, including those that were exposed but not infected). I then conducted linear models to test whether treatment affected the percentage of salamander mass loss. The first statistical models tested whether stressors (CORT or high temp) affected mass loss during the treatment period and before exposure to *Bd* (i.e., the percentage of mass loss during the first 10 days of the treatment period). The second statistical models tested whether the previous application of stressors (CORT or high temp) affected the percentage of mass loss after the salamanders were exposed to *Bd* (i.e., the percentage of mass loss between Day 10 and Day 22). I used a liner model for each of the 6 statistical tests and all analyses were conducted in R statistical software.

4. Results

Unfortunately, I made one or more mistakes in the process of running the water-borne CORT ELISA. I chose not to include that results of the ELISA, because the results did not seem to be viable and I did not want to present false data that would lead to false conclusions.

Effects of Previous Stressors on *Bd* Abundance

The CORT treatment did not have a significant effect on infection level compared to the No Cort treatment, based on the zoospore abundance of only the infected individuals (ANOVA $F_{1,21}$=0.4811, P= 0.6248) (Figure 1). Animals were swabbed 7 days after they were exposed to *Bd*. There was no significant difference between groups, although the number of individuals that became infected was rather small compared to the size of their respective groups (Figure 1). Of the individuals that were infected the CORT treatment had on average 315.13 zoospores per

swab (±127.53 zoospores) and the No CORT treatment had on average 294.91 zoospores per swab (±40.79 zoospores) (Figure 1). Both groups were housed at the same temperature during this period.

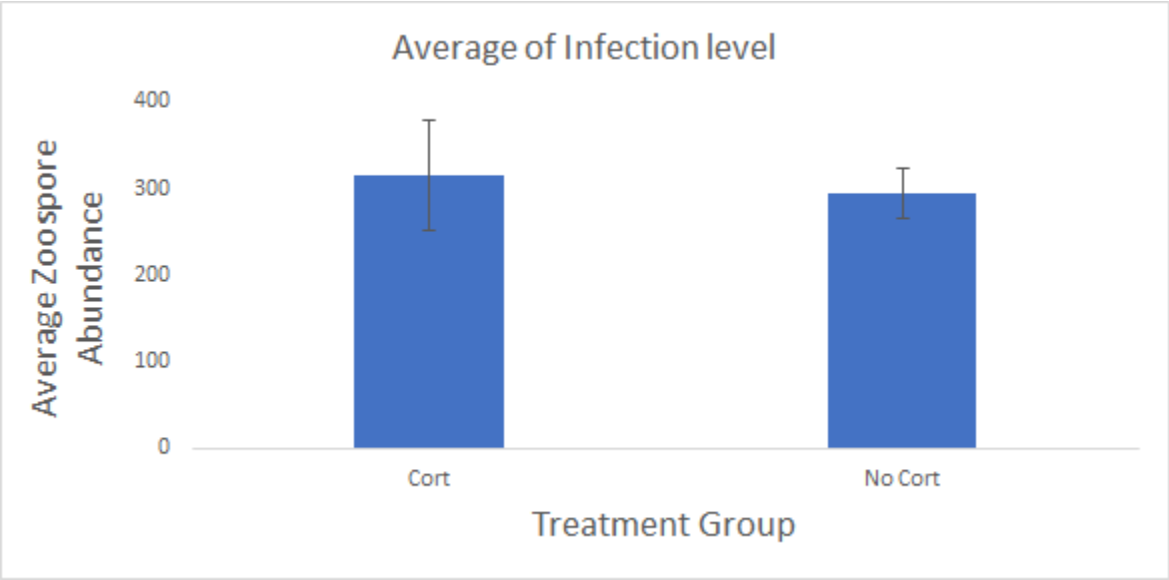


Figure 1. Average infection level of *Bd* positive individuals 7 days post *Bd* exposure (Cort n=4, No Cort n=2). Values indicate genomic *Bd* zoospore equivalents per swab. Statistical significance was based on a P Value of less than .05.

Prior temperature exposure did not seem to have a significant effect on infection level, based on the zoospore abundance of only the infected individuals (ANOVA $F_{1,21}$=0.0743, P= .7879) (Figure 2). The High Temp group had an average of 123.62 zoospores per swab (±94.27 zoospores) and the Low Temp group had an average of 240.26 zoospores per swab (± 194.28 zoospores) (Figure 2). Animals were swabbed 7 days post *Bd* exposure. Animals were held at the same temperature post exposure, in order to minimize the effect of temperature on the growth of the pathogen. The High Temp treatment group was moved back down to the same temperature as the Low Temp group 24 hours prior to *Bd* exposure. The number of infected individuals in each group were in the minority compared to the size of their respective groups.

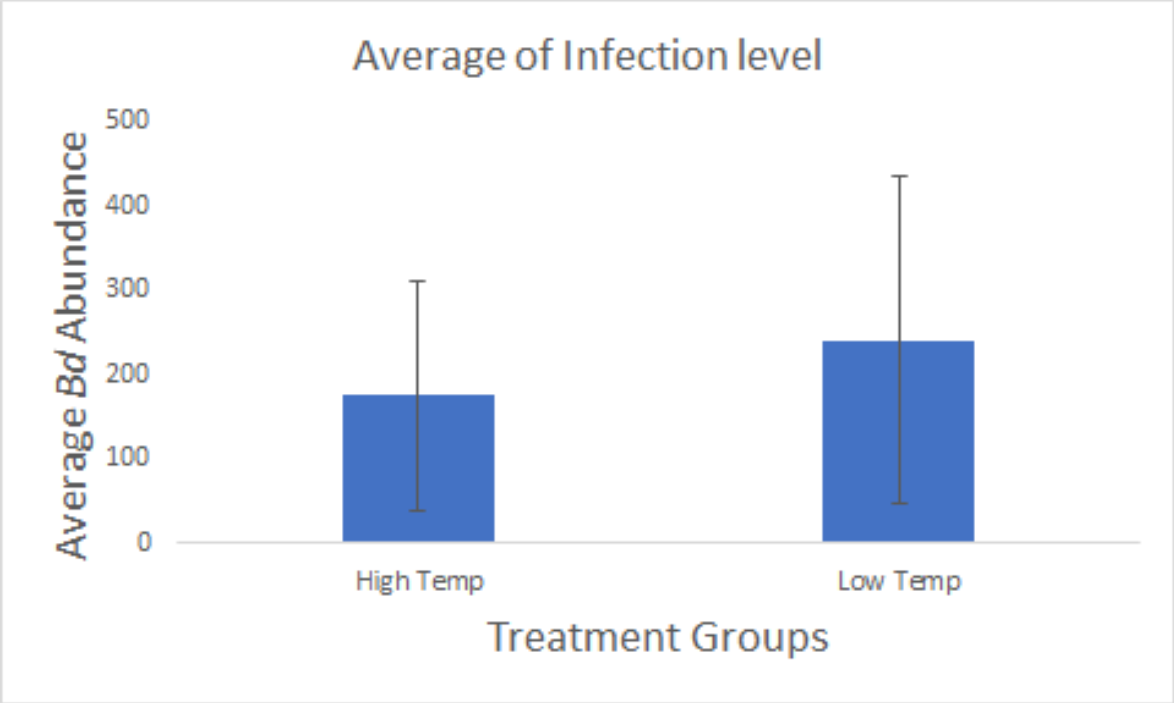


Figure 2. Average infection level of *Bd* positive individuals 7 days post *Bd* exposure (High Temp n=2, Low Temp n=3). Values indicate genomic *Bd* zoospore equivalents per swab. Statistical significance was based on standard error.

Mass Change Post CORT Application Period

During the 10-day CORT application period both groups lost mass, but there was not a significant difference in average percent mass loss between either group at the end of the 10-day treatment period (ANOVA $F_{1,21} = 0.6194$, P= 0.5478) (Figure 3). The No CORT group lost approximately 6 percent of their body mass (±2.21%) and the CORT group lost approximately 5 percent of their body mass (±2.04%) (Figure 3). Both groups were given their respective treatment patches on the same day and during the same time period. Both groups were also held

at the same temperature during this period.

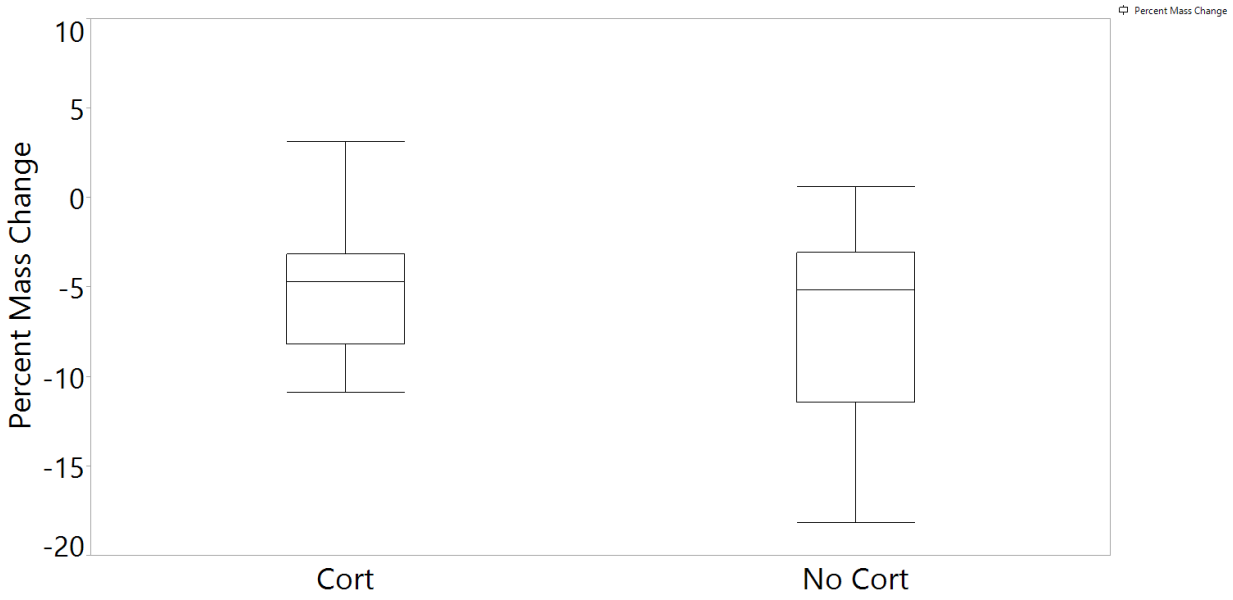


Figure 3. Box and whisker plot for percent mass loss for day 1-10 for each treatment group (No Cort n=12, Cort n=12). Box ends represent 75% and 25% quartiles, the line going across inside the box represents the average percent mass change, and the whiskers represent the lowest and highest observed data point. Values represent percentages. Statistical significance was based on an ANOVA model I.

Mass Change Post Temperature Period

Following the 10-day exposure period there was a statistical difference in average percent mass loss between the High Temperature group and the Low Temperature group (ANOVA $F_{1,21}$= 65.308, P = <0.0001) (Figure 4). The Low Temp group lost about 3.5 percent of their body mass (±1.55%) and the High Temp group lost about 16 percent of their body mass (± 6.33%) (Figure 4). The animals in the temperature were completely different animals from the Cort/No Cort treatment groups.The High Temperature group was housed in a separate incubator from the Low

Temp group for the 10 day exposure period.

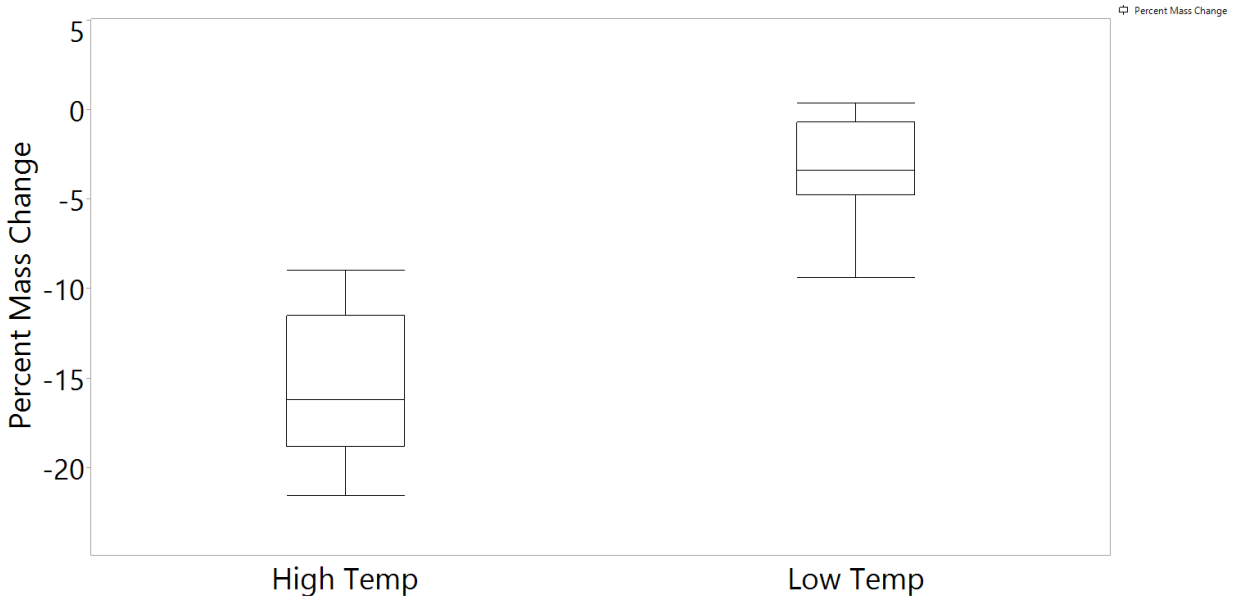


Figure 4. Box and Whisker Plot for percent mass loss for day 1-10 for each treatment group (Low Temp n=11, High Temp n=12). Box ends represent 75% and 25% quartiles, the line going across inside the box represents the average percent mass change, and the whiskers represent the lowest and highest observed data point. Values represent percentages. Statistical significance was based on ANOVA model I.

Mass Change Post *Bd* Exposure

11 days post *Bd* exposure the CORT group lost a significantly more amount of mass than the No CORT group (ANOVA $F_{1,21}$=8.094, P=0.002478) (Figure 5). The No CORT group lost approximately 8 percent of their body mass (±2.63%) and the CORT group lost approximately 12.9 percent of their body mass (±5.04%) (Figure 5). During this time period treatment patches were not given to either group and both groups were housed at the same temperature. Even though there was not a significant difference the trend does seem to show that the CORT group

lost had a higher average mass loss.

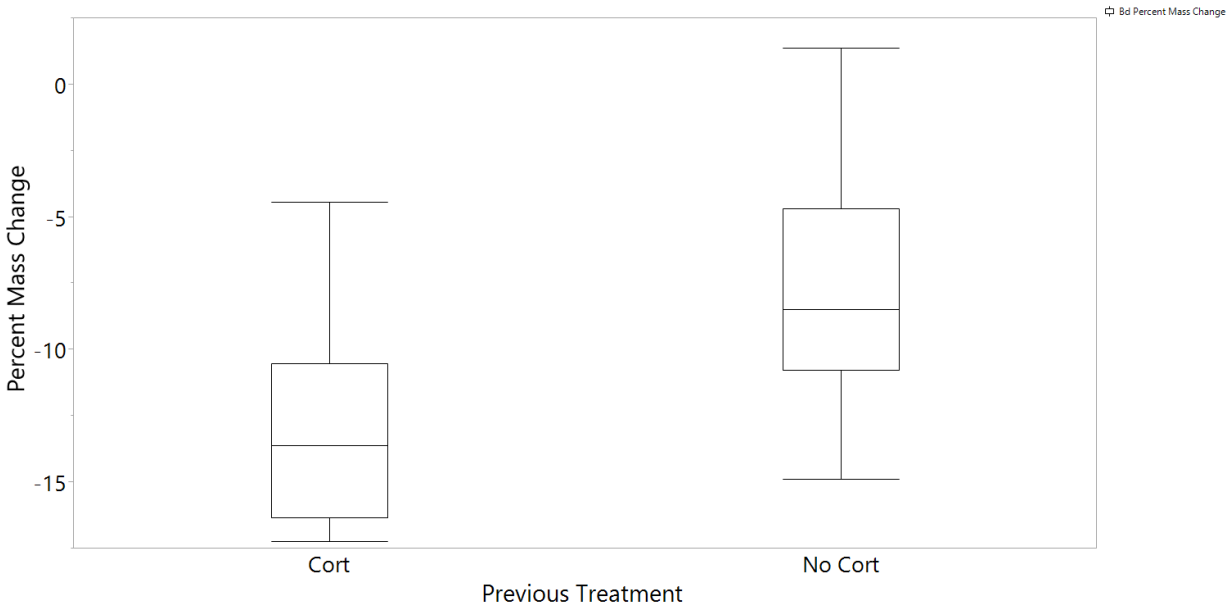


Figure 5. Box and whisker plot for the percent mass loss from day 10-22 for each treatment group (No Cort n=12, Cort n=12). Box ends represent 75% and 25% quartiles, the line going across inside the box represents the average percent mass change, and the whiskers represent the lowest and highest observed data point. Values represent percentages. Statistical significance was based on an ANOVA model I.

There appears to be a significant difference between average percent mass loss of the Low Temp and High Temp groups 11 days post *Bd* exposure (ANOVA $F_{1,21}$= 18.948, P= 0.0002794) (Figure 6). The Low Temp group lost approximately 10.9 percent of their body mass (±4.73%) and the High Temp group actually gained about 1 percent of their body mass back (±0.35%) (Figure 6). During this period both groups were housed at the same temperature in the same environmental chamber. The High Temp group was brought down to the same temperature of the

Low Temp group approximately 24 hours before *Bd* exposure.

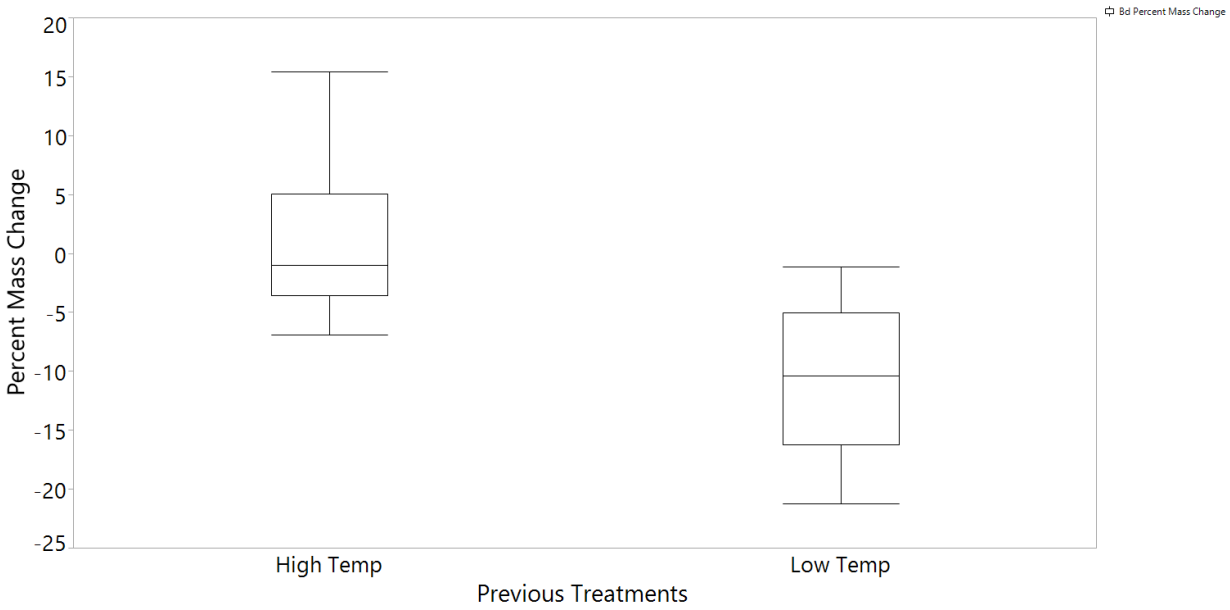


Figure 6. Box and whisker plot for percent mass loss from day 10-22 for each treatment group (Low Temp n=11, High Temp n=12). Box ends represent 75% and 25% quartiles, the line going across inside the box represents the average percent mass change, and the whiskers represent the lowest and highest observed data point. Values represent percentages. Statistical significance was based on an ANOVA model I.

5. Discussion

The relationship between stress and the immune response has been the subject of much recent study, but the relationship is still not completely clear. My study provided an incremental step towards better understanding the relationship between stress and immunity. Even though there were no statistical differences between the infection levels of the four groups, my results still allow for some interesting inferences (Figure 1, Figure 2). Every animal was exposed to *Bd* even though there were a small number of infected individuals, there are still costs associated with exposure and clearance of a pathogen, especially following a chronic stressor.

If the magnitude and duration of the high temperature treatment was not detrimental to the animals, i.e. it did not act as a chronic stressor, it could have served as a primer for their

immune response as well as other physiological processes, which would explain why the high temp group did not lose any mass post *Bd* exposure (Figure 6) (Berger et al. 2004; Karavian and Venesky 2016). Being that red-backed salamanders (*Plethodon cinereus*) are ectotherms their physiology is going to correlate with the ambient temperature of their environment, in a warmer environment their physiological processes are going to run faster and be more effective, assuming that the temperature they are in is not a stressor itself (Homyack et al. 2010; Karavian and Venesky 2016). Whereas, in a colder environment the physiological process of an ectotherm are going to be less effective (Carey et al. 1999, Maniero and Carey. 1997, Raffel et al. 2006). After being exposed to a warm, but not too warm, climate for 10 days, the animals in the high temp group could have had a higher metabolic rate, more potent immune response, higher plasma volume, which would lead to more room for nutrients as well as immune cells (Carey et al. 1999, Homyack et al. 2016; Karavian and Venesky 2016, Maniero and Carey. 1997, Raffel et al. 2006). After being exposed to a cooler environment for 10 days, the Low temp group could have had a relatively lower metabolic rate, less potent immune response, and a lower plasma volume (Carey et al. 1999, Homyack et al. 2016; Karavian and Venesky 2016, Maniero and Carey. 1997, Raffel et al. 2006).

After the 10-day exposure period, when the high temp group was put at the same temperature as the low temp group, the high temp group may have had a supercharged immune system, which in turn would allow them to better fight off a pending infection. So, once the high temp group was exposed to the pathogen, they wouldn't need to drastically increase their metabolic rate in order to meet the increased metabolic demand to fund their immune response. The exposure to the warmer temperature could have already accomplished all of the necessary steps for the animal to be able to mount an immune response against the pathogen, which would

explain why the high temp group didn't lose any mass after being exposed to *Bd* (Figure 6). Whereas the low temp group may have had to play catch up, once they were exposed to *Bd*, and greatly increase their metabolic rate in order to meet the energetic demand required to clear the infection (Figure 6). Because all the treatment groups were not being fed any extra flies after they were exposed to *Bd* and being that the low temp group did not have a primed physiology like the high temp group, they would most likely have had to catabolize their own fat, protein and sugar stores in order to meet their increased energetic demand, causing a significant decrease in their mass (Figure 6). *Plethodon cinereus* also have a microbiome of bacteria on their skin, which have been found to have antifungal properties (Lauer et al. 2007; Louden et al 2013). Being that bacteria are generally warm temperature specialists the 10-day high temperature exposure period could have given the high temp group another advantage over the low temp group in preparing their immune response.

The fact that there is no difference in infection level between the CORT and No CORT groups (Figure 1) and in the high temp and low temp groups (Figure 2) could mean that treatment periods and intensity are not perceived as a chronic stressor by the animals. It has been found that the dose concentration (.5µg) and cycle of CORT has increased circulating CORT levels compared to a control within this species, these levels could not be high enough or maintained long enough to elicit any negative effects on their immune response (Murone and Venesky. unpublished). Being that the CORT and No CORT groups had no statistical difference between percent mass loss during the pre-exposure treatment period (Figure 3), could also mean that the CORT treatment does not cause immediate physiological effects on metabolic rate that we believe, and there is no reason why there couldn't be the same lack of immediate physiological change on disease resistance (Fonner et al 2012; Wack et al. 2012). However, the

CORT group lost a significantly more amount of mass than the No CORT group post *Bd* exposure, which could mean that the physiological effects of the dosing cycle are delayed, or more likely the CORT treatment could serve as primer for the animals' metabolic rate, allowing it to respond to new stressors more rapidly and more powerfully. The CORT dose and dosing cycle I used has been shown to increase circulating levels of CORT in *Plethodon cinereus*. The effect of an added stressor post treatment period, such as exposure to a pathogen, could further increase circulating CORT levels to the point of causing physiological change. So, the CORT group losing significantly more mass post *Bd* exposure than the No CORT group, could be explained by further increase in circulating CORT levels from the *Bd* exposure (Figure 5). Although without valid ELISA results making inferences on circulating CORT based on mass change is really only an educated guess that lacks actual data.

If the ELISA plate I ran had viable results, it would have helped greatly with teasing out the effect that temperature has on circulating CORT levels, and further extrapolating the effect of temperature onto other physiological processes. That being said, the High Temperature group lost a significantly more amount of mass during the pre-exposure treatment period than the Low Temperature group (Figure 4). Post exposure to *Bd,* the low temp group lost a significantly more amount of mass than the high temp group, in fact the high temp group's average mass actually increased post *Bd* exposure (Figure 6). The 10-day high temperature exposure period seemed to not have any lasting effects on metabolic rate based on Figure 6, which could mean that it was not a intense enough stressor to induce maladaptive physiological effects that are associated with chronic stress. Being that these animals are ectotherms, and that there was no significant difference in percent mass loss between the No CORT and CORT groups pre *Bd* exposure (Figure 3), one can infer that the high temperature group's average mass loss was due to their

metabolic rate mirroring the increased temperature, and not due to a change in circulating CORT levels (Homyack et al. 2010; Wack et al. 2012). Although without valid ELISA results, inferring that temperature has a greater effect on metabolic rate than the effect of CORT on metabolic rate, and the stress response as a whole, lacks a fair amount of evidence.

The possibility of a beneficial physiological effect from an increase in temperature, which my results could suggest is dependent on many ecological factors and species-specific physiology (Karavian and Venesky 2016). Some species of amphibians prefer cooler environments, such as salamanders, but other amphibians prefer warmer environments, such as toads. So, the time and intensity of temperature exposure could be very species dependent, because different species may perceive different temperature increases as more or less intense than others based on their life history and preferences. The beneficial effects of an exposure to a warm but not too warm temperature is also dependent of resource availability as well. If a population of ectotherms do not have enough resources to meet the increased energetic requirements that come along with an elevated metabolic rate from an increase in temperature, then they would have to start to catabolize their own fat and protein stores, thus deteriorating their body condition and nullifying any positive effects from the temperature increase (Homyack et al. 2010, Wack et al. 2012,).

Being that this was a study conducted in a lab setting and my animals were fed regularly with more than enough fruit flies to sustain them, the possible beneficial effects from the temperature increase may not be entirely transferable to a real world ecological setting. Stressors in the wild are going to be compounded within an animal so along with a temperature increase an animal could experience predation, intra and inter-specific competition for territory and resources, and a high pathogen abundance. The zone of beneficial temperature increase may be

much smaller in the wild than it would be in a laboratory setting, due to the compounding assortment of stressors an animal can face in the wild. Studies have shown that ectotherms will seek out warmer microclimates when infected, so it seems an animal can perceive the beneficial effects from a moderate temperature increase (Karavian and Venesky 2016)). So, there doesn't seem to be any reason that a ectotherm wouldn't stay in a warmer microclimate if they perceive a high pathogen prevalence in order prime their immune system for a pending infection.

My study may also support the idea that every species responds to the same stressor differently, in a previous study the same relative CORT dose was enough to increase the metabolic rate and decrease in disease resistance of a plethodontid salamander in an acute application (Fonner et al. 2016; Wack et al. 2012). But the same relative CORT dose did not cause a significant difference in mass loss over a 10-day treatment period compared to a control treatment in my study (Figure 3), which could mean that CORT dose did not change their metabolic rate significantly in that time period compared to the control treatment. But the same dosing cycle of CORT that I used in my study, has also been shown in one particular study to increase disease resistance in American toads (*Anaxyrus bufo*) in a previous study (Murone et al. 2016). My data showed no significant difference between disease resistance in my No CORT and CORT groups (Figure 1), which could be due to a low number of infected values, or the CORT dosing cycle did not raise their circulating CORT levels enough to elicit an immediate effect on their immune response. So the species that I used in this study, *Plethodon cinereus*, could have a more robust stress response compared to the species used in the Wack et al. 2012 study, *Plethodon shermani*, and in the Murone et al. 2016 study, which would explain why the same relative stressor did not cause the same physiological changes for the *P.cinereus* used in my study. Or *Plethodon cinereus* could need higher stress levels in order to elicit the same

physiological changes as other amphibians but finding that threshold without exceeding physiologically relevant stress levels is going to be a challenge for future studies.

Imitating physiologically relevant CORT levels in a lab study is paramount in understanding the effects of stress on any physiological process, because if one were to give an animal an astronomical amount of CORT or expose them to a very severe stressor just to solicit a significant effect, their results might not provide much help on how ecological stressors might affect certain physiological processes. Raising an animal's circulating CORT levels past physiologically relevant levels into pharmacological levels could lead to effects that may be contrary to effects from physiologically relevant levels, and thus wouldn't aid in trying to understand the relationship of ecological stressors in a lab setting. For future studies physiologically relevant and pharmacological levels need to be taken under consideration, while I did not have a fully crossed design, the treatment designations I chose, seemed to be most likely in eliciting physiologically relevant CORT levels. Future studies that build of this particular one could have the same experimental design and add sham inoculated (*Bd*-) treatment groups to the experimental design to better compare the effects of stress on mass and disease resistance. Also, future studies could run an ELISA on samples taken after the stress period and after the exposure period on both the *Bd*+ and *Bd*- treatment groups to better understand the relationship between CORT and disease resistance.

Works Cited

Belden LK, Kiesecker JM (2005) Glucocorticosteroid hormone treatment of larval treefrogs increases infection by Alaria sp. trematode cercariae. J Parasitol 91: 686−688

Bennett M, Gaudio C, Johnson A, Spisso J (1972) Changes in the blood of newts, Notophthalmus viridescens, following administration of hydrocortisone. J Comp Physiol 80: 233−237

Berger, L., R. Speare, H. Hines, G. Marantelli, A. Hyatt, K. McDonald, L. Skerratt, V. Olsen, J. Clarke, G. Gillespie, M. Mahony, N. Sheppard, C. Williams, and M. Tyler. 2004. Effect of season and temperature on mortality in amphibians due to chytridiomycosis. Australian Veterinary Journal 82:434-439.

Boyle, D. G., D. B. Boyle, V. Olsen, J. A. T. Morgan, and A. D. Hyatt. 2004. Rapid quantitative detection of chytridiomycosis (Batrachochytrium dendrobatidis) in amphibian samples using real-time Taqman PCR assay. Diseases of aquatic organisms 60:141-148.

Carey, C. 2000. Infectious disease and worldwide declines of amphibian populations, with comments on emerging diseases in coral reef organisms and in humans. Environmental Health Perspectives 108:143-150.

Davis AK, Maertz JC (2008) Comparison of hematological stress indicators in recently captured and captive paedo169 Dis Aquat Org 123: 159–171, 2017 morphic mole salamanders, Ambystoma talpoideum. Copeia 2008: 613−617

Dhabhar, F. S. 2014. Effects of stress on immune function: the good, the bad, and the beautiful. Immunologic Research 58:193-210.

Dhabhar, F. S., and B. S. Mcewen. 1997. Acute Stress Enhances while Chronic Stress Suppresses Cell-Mediated Immunityin Vivo:A Potential Role for Leukocyte Trafficking. Brain, Behavior, and Immunity 11:286-306.

Easterling, D. R., G. A. Meehl, C. Parmesan, S. A. Changnon, T. R. Karl, and L. O. Mearns. 2000. Climate Extremes: Observations, Modeling, and Impacts. Science 289:2068-2074.

Fabian, P., and A. Menzel. 1999. Growing season extended in Europe. Nature 397:659

Falso PG, Noble CA, Diaz JM, Hayes TB (2015) The effect of long-term corticosterone treatment on blood cell differentials and function in laboratory and wild-caught amphibian models. Gen Comp Endocrinol 212: 73−83

Fonner, C., S. Patel, S. Boord, M. Venesky, and S. Woodley. 2016. Effects of corticosterone on infection and disease in salamanders exposed to the amphibian fungal pathogen, Batrachochytrium dendrobatidis. Diseases of Aquatic Organisms :.

Forrest, M. J., and M. A. Schlaepfer. 2011. Nothing a Hot Bath Won't Cure: Infection Rates of Amphibian Chytrid Fungus Correlate Negatively with Water Temperature under Natural Field Settings. PLOS ONE 6:e28444.

Gabor, Caitlin R. , Kristina C. Zabierek, Diana S. Kim, Laura Alberici da Barbiano, Megan J. Mondelli, Nathan F. Bendik, and Drew R. Davis. 2013. A Non-Invasive Water-Borne Assay of Stress Hormones in Aquatic Salamanders. Copeia 104:172-181

Glaser, R., and J. K. Kiecolt-Glaser. 2005. Stress-Induced Immune Function: Implications for Health. Perspectives :.

Gómez, J. J., A. Goy, and M. L. Canales. 2008. Seawater temperature and carbon isotope variations in belemnites linked to mass extinction during the Toarcian (Early Jurassic) in Central and Northern Spain. Comparison with other European sections. Palaeogeography, Palaeoclimatology, Palaeoecology 258:28-58.

Groner ML, Rollins-Smith LA, Reinert LK, Hempel J, Bier ME, Relyea RA (2014) Interactive effects of competition and predator cues on immune responses of leopard frogs at metamorphosis. J Exp Biol 217:

351−358

Hardy, M. P., H. Gao, Q. Dong, R. Ge, Q. Wang, W. R. Chai, X. Feng, and C. Sottas. 2005. Stress hormone and male reproductive function. Cell and Tissue Research 322:147-153.

Hayes, T. B., V. Khoury, A. Narayan, M. Nazir, A. Park, T. Brown, L. Adame, E. Chan, D. Buchholz, T. Stueve, and S. Gallipeau. 2010. Atrazine induces complete feminization and chemical castration in male African clawed frogs (Xenopus laevis). Proceedings of the National Academy of Sciences 107:4612-4617.

Hoffmann, A. A., and P. A. Parsons. 1997. Extreme Environmental Change and Evolution. Cambridge University Press,

Homyack, J. A., C. A. Haas, and W. A. Hopkins. 2010. Influence of temperature and body mass on standard metabolic rate of eastern red-backed salamanders (Plethodon cinereus). Journal of Thermal Biology 35:143-146.

Huntingford, Chris. Philip D Jones, Valerie N Livina, Timothy M Lenton, and Peter M Cox. 2013. No increase in global temperature variability despite changing regional patterns. Nature 500:327

Johnson, M. L., L. Berger, L. Phillips, and R. Speare. 2003. Fungicidal effects of chemical disinfectants, UV light, desiccation and heat on the amphibian chytrid, Batrachochytrium dendrobatidis. Diseases of Aquatic Organisms 57:255-260.

Karavlan, S. A., and M. D. Venesky. 2016. Thermoregulatory Behavior of Anaxyrus americanus in Response to Infection with Batrachochytrium dendrobatidis. Copeia 104:746-751.

Kingsolver, J. G., S. E. Diamond, and L. B. Buckley. 2013. Heat stress and the fitness consequences of climate change for terrestrial ectotherms. Functional Ecology 27:1415-1423.

Lauer, A., M. A. Simon, J. L. Banning, E. André, K. Duncan, and R. N. Harris. 2007. Common Cutaneous Bacteria from the Eastern Red-Backed Salamander Can Inhibit Pathogenic Fungi. Copeia 2007:630-640.

Loudon, A. H., D. C. Woodhams, L. W. Parfrey, H. Archer, R. Knight, V. McKenzie, and R. N. Harris. 2014. Microbial community dynamics and effect of environmental microbial reservoirs on red-backed salamanders (Plethodon cinereus). The ISME journal 8:830.

Licht, P., B. R. McCreery, R. Barnes, and R. Pang. 1983. Seasonal and stress related changes in plasma gonadotropins, sex steroids, and corticosterone in the bullfrog, Rana catesbeiana. General and Comparative Endocrinology 50:124-145.

McKechnie, Andrew E.: Wolf, O.Blair. 2009. Climate change increases the likelihood of catastrophic avian mortality events during extreme heat waves. Biology Letters :.

Murone, J., J. A. DeMarchi, and M. D. Venesky. 2016. Exposure to Corticosterone Affects Host Resistance, but Not Tolerance, to an Emerging Fungal Pathogen. PLOS ONE 11:e0163736.

Murone and Venesky, unpublished. The Effects of Exogenous Corticosterone on Stress Hormone Release in Water in Plethodon cinereus

Norris, D. O., and J. A. Carr. 2013. Vertebrate Endocrinology. Academic Press, .

Ouellet, M., J. Bonin, J. Rodrigue, J. DesGranges, and S. Lair. 1997. Hindlimb deformities (ectromelia, ectrodactyly) in free-living anurans from agricultural habitats. Journal of Wildlife Diseases 33:95-104.

Peterson, J. D., J. E. Steffen, L. K. Reinert, P. A. Cobine, A. Appel, L. Rollins-Smith, and M. T. Mendonça. 2013. Host Stress Response Is Important for the Pathogenesis of the Deadly Amphibian Disease, Chytridiomycosis, in Litoria caerulea. PLOS ONE 8:e62146.

Pickering, A. D., T. G. Pottinger, J. Carragher, and J. P. Sumpter. 1987. The effects of acute and chronic stress on the levels of reproductive hormones in the plasma of mature male brown trout, Salmo trutta L. General and

Comparative Endocrinology 68:249-259.

Piotrowski, J. S., S. L. Annis, and J. E. Longcore. 2004. Physiology of Batrachochytrium dendrobatidis, a Chytrid Pathogen of Amphibians. Mycologia 96:9-15.

Ramírez F, Fowell DJ, Puklavec M, Simmonds S, Mason D (1996) Glucocorticoids promote a TH2 cytokine response by CD4+ T cells in vitro. J Immunol 156: 2406−2412

Ricciardella, L. F., J. M. Bliley, C. C. Feth, and S. K. Woodley. 2010. Acute stressors increase plasma corticosterone and decrease locomotor activity in a terrestrial salamander (Desmognathus ochrophaeus). Physiology & Behavior 101:81-86.

Richards-Zawacki, C. 2010. Thermoregulatory behaviour affects prevalence of chytrid fungal infection in a wild population of Panamanian golden frogs. Proceedings of the Royal Society B: Biological Sciences 277:519-528.

Jason R. Rohr, Thomas R. Raffel, and David B. Wake. 2010. Linking global climate and temperature variability to widespread amphibian declines putatively caused by disease. Proceedings of the National Academy of Sciences of the United States of America 107:8269-8274.

Rohr, J. R., T. Sager, T. M. Sesterhenn, and B. D. Palmer. 2006. Exposure, Postexposure, and Density-Mediated Effects of Atrazine on Amphibians: Breaking down Net Effects into Their Parts. Environmental Health Perspectives 114:46-50.

Rohr, J. R., T. R. Raffel, J. M. Romansic, H. McCallum, and P. J. Hudson. 2008. Evaluating the links between climate, disease spread, and amphibian declines. Proceedings of the National Academy of Sciences 105:17436-17441.

Rohr, J. R., A. A. Elskus, B. S. Shepherd, P. H. Crowley, T. M. McCarthy, J. H. Niedzwiecki, T. Sager, A. Sih, and B. D. Palmer. 2004. Multiple Stressors and Salamanders: Effects of an Herbicide, Food Limitation, and Hydroperiod. Ecological Applications 14:1028-1040

Rollins-Smith LA, Blair PJ (1993) The effects of corticosteroid hormones and thyroid hormones on lymphocyte viability and proliferation during development and 170 Fonner et al.: Corticosterone effect on infection in salamanders 171 metamorpho

Romero, L. Michael. 2002. Seasonal changes in plasma glucocorticoid concentrations in free-living vertebrates. General and Comparative Endocrinology 128:1-24.

Simmaco M, Boman A, Mangoni ML, Mignogna G, Miele R, Barra D, Boman HG (1997) Effect of glucocorticoids on the synthesis of antimicrobial peptides in amphibian skin. FEBS Lett 416: 273−275

Screen, J. A. 2014. Arctic amplification decreases temperature variance in northern mid- to high-latitudes. Nature Climate Change 4:577.

Sokolova, Inna M., and Gisela Lannig. 2008. Interactive effects of metal pollution and temperature on metabolism in aquatic ectotherms. Climate Research 37:181-201.

Voyles, J., E. B. Rosenblum, and L. Berger. 2011. Interactions between Batrachochytrium dendrobatidis and its amphibian hosts: a review of pathogenesis and immunity. Microbes and Infection 13:25-32.

Wack, C. L., S. E. DuRant, W. A. Hopkins, M. B. Lovern, R. C. Feldhoff, and S. K. Woodley. 2012. Elevated plasma corticosterone increases metabolic rate in a terrestrial salamander. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 161:153-158.

Walther, G., E. Post, P. Convey, A. Menzel, C. Parmesan, T. J. Beebee, J. Fromentin, O. Hoegh-Guldberg, and F. Bairlein. 2002. Ecological responses to recent climate change.

Wang, T., M. Zaar, S. Arvedsen, C. Vedel-Smith, and J. Overgaard. 2002. Effects of temperature on the metabolic

response to feeding in Python molurus. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 133:519-527..

Warne, R. W., E. J. Crespi, and J. L. Brunner. 2011. Escape from the pond: stress and developmental responses to ranavirus infection in wood frog tadpoles. Functional Ecology 25:139-146.

Woodhams, D. C., R. A. Alford, and G. Marantelli. 2003. Emerging disease of amphibians cured by elevated body temperature. 55:.

Woodward, F. I. 1987. Climate and Plant Distribution. Cambridge University Press,

.