Different doses of *Batrachochytrium dendrobatidis* Affect Secretion of Corticosterone in *Plethodon cinereus*

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April, 2018



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Department of Biology

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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.

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Pledge

Approval by the Senior Thesis Committee:

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Dr. Matthew D. Venesky

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Dr. Ronald Mumme

Acknowledgements

First and foremost, I need to thank my comp advisor, Dr. Matthew D. Venesky. I've been so grateful for your constant guidance throughout this entire process. I sincerely appreciate you sharing your time, lab, and love of salamanders with me. You have taught me so much throughout my years at Allegheny and I would not have made it this far without your support.

Thank you to my comp group for taking the time to read and comment on my drafts. Your insight and comments truly enhanced my project.

To my Kappa girls, thank you for the laughs and support when times were difficult. It's been a journey that I wouldn't have wanted to do with anyone else. In particular, Katie Mays and Chloe Spadafora, you two have been such an inspiration to me and I truly adore you both.

To my best friend and forever roommate, Maddie Clark. I would never have gotten through college without you. Your friendship over the past 8 years has meant the world to me. No matter where life takes us, I'll always be here for you.

A special thank you to my parents, Mark and Carla, and sisters, Gianna and Isabella, for their constant patience, encouragement and love throughout my time at Allegheny College. Without your guidance, I would not be at the place I am today. You mean the world to me and I will forever be grateful to have such an incredible support system.

Finally, thank you to the Allegheny College Biology Department for providing me with the funds to carry out this experiment. This research would not have been possible without it.

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Abstract

A stressor is any challenge to an organism's homeostasis. To regulate how the body responds to stress, vertebrates have a hypothalamo-pituitary adrenal (HPA) axis which release glucocorticoid (GC) hormones. Vertebrates respond to various stressors by releasing corticosterone (CORT) which is the main GC hormone. Factors such as physiological changes, environmental changes, and infectious diseases affect the body's amount of stress. *Batrachochytrium dendrobatidis* (*Bd*) is a parasitic fungus that causes the disease chytridiomycosis, infecting amphibians worldwide. Previous research has not found a connection between dosages of *Bd* and CORT secretion. Within my experiment, I predicted the high treatment group will have the highest infection load, followed by the low treatment group and control will not have any infection present. My hypothesis was, as *Bd* dosage increases, the more secretion of CORT will be present from each salamander. I exposed Red-backed salamanders (*Plethodon cinereus*) with different doses of *Bd* and observed the amount of CORT secreted. Salamanders were divided into 3 treatment groups: a low dose of *Bd* (1×10^7 zoospores), a high dose of *Bd* (2×10^7 zoospores), and a control group that was not given *Bd*. After applying the above treatments, I analyzed CORT secretion using a water-borne assay enzyme linked immunosorbent assay (ELISA) kit. I then analyzed *Bd* prevalence and *Bd* infection intensity within exposed animals using quantitative polymerase chain reaction (qPCR). As predicted, the results showed the high treatment group had the highest infection load. Additionally, there was a general trend of the low treatment group released the most amount of CORT and the high treatment group released the least amount of CORT. While this experiment did not find the relationship between stress hormones and disease it did, however, add to the knowledge on the

impact this infection has on amphibians. Future studies could determine if the cause of CORT secretion is species specific, disease oriented, or an outside factor of stress.

Introduction

Any challenge to the homeostasis of an organism is considered to be a stressor. These could include, but are not limited to, environmental, social and physical factors. Examples of these variables include factors such as temperature, elevation, predators, handling, reproduction and disease. Competition for food, space, and mating partners are costly conditions and reduces overall fitness of the animal (Liesenjohann *et al*. 2013). In a natural environment, animals encounter these different types of stress at the same time. High stress-induced situations such as severe weather may cause parents to leave breeding sites or abandon reproduction altogether (Helm *et al*. 2017). The costs of stress factors within the environment are very high due to limited resources such as energy, nutrients and time. Environmental stress enhances adaptations and the evolution of changing habitats (Bijlsma *et al*. 2005). This ultimately has a long-term impact on ecological, physiological and genetic processes.

Vertebrates respond to stressors by secreting hormones that are part of the hypothalamo-pituitary adrenal (HPA) axis. The HPA axis is viewed as a complex physiological system that can mediate a large variation in the physiological and behavioral responses in animals (Moore *et al*. 2003). Sensory information activates the HPA axis and releases peptide hormones (Harris *et al*. 2016). The peptide hormones travel down the pituitary portal system to stimulate the adrenocorticotropic hormones (Harris *et al*. 2016). These hormones then circulate and bind to receptors to release glucocorticoid (GC) hormones. The GC hormones are released within the body to handle different types of stress. A stress response which results in an increase of GC

hormone levels also increases glucose levels and changes behavioral activities (Narayan *et al*. 2013). Corticosterone (CORT), a vertebrate stress hormone, promotes physiological and behavioral responses known to affect the body's systems (Narayan *et al*. 2013). For an event to be considered stressful the organism must perceive the event as stressful and thus trigger a response (Harris *et al*. 2016). CORT is the primary GC hormone in vertebrates and is involved in many processes including energy regulation, immunity, and stress levels (Narayan *et al*. 2013). CORT levels increase when vertebrates are exposed to stressful stimuli resulting in short-term effects such as temporary suppression of reproduction, increased foraging, immune system regulation and increased gluconeogenesis (Newcomb *et al*. 2003). Additionally, unpredictable threats or challenges naturally causes an elevation in CORT levels (Wack *et al*. 2010).

If the stressor is long lasting, GC hormones exhibit negative consequences for the animal, such as shutdown of reproduction, chronic suppression of both immune function and growth, and neuronal cell death (Newcomb *et al*. 2003). There is an important distinction between the actions of CORT levels circulating at low to moderate levels and their actions at high levels (Busch *et al*. 2009). At low to moderate levels, CORT influences feeding behavior and acts on the liver, adipose tissue, and muscle to maintain adequate circulating levels of glucose and free fatty acids (Busch *et al*. 2009). Studies have contributed to the profound interest in understanding which stress hormones affect the development of the neuroendocrine system and the balance between energy and food intake (Crespi *et al*. 2005). While studies have attempted to increase CORT levels by using various stressors, they have failed to elevate hormone levels (Searle *et al*. 2014).

Infectious disease is one factor resulting in an increase in stress and therefore an increase of CORT. Due to the lack of known information, recent studies have focused on the relationship between stress and disease. A recent experiment studied the linkage between social stress and

disease. Their conclusions suggest if an animal is deprived of social factors such as contact, space allowance, and order this will trigger high stress levels resulting in a higher risk of disease (Proudfoot *et al*. 2015). Another study focused on disease in amphibians at different elevations and their release rate of CORT (Gabor *et al*. 2015). At higher elevation levels the more severe the disease was found to be. Additionally, with increased elevation, there was an increase in CORT levels. This indicated with higher CORT release rates the severity of disease increased (Gabor *et al*. 2015). Researchers have found two general trends, firstly increased CORT within organisms in physiological or environmental conditions can downregulate the organism's immune system and increase disease risk. Secondly disease activates the HPA axis and therefore increases stress and CORT levels within the body (Kindermann *et al*. 2012).

Amphibians are particularly affected by stress due to habitat destruction, overuse by humans, environmental chemicals, other species, climate change, and infectious diseases (Rollins *et al*. 2017). Amphibians respond to environmental stressors by secreting CORT. CORT has many roles in amphibians by monitoring normal development, mobilization and osmoregulation (Rollins *et al*. 2017). CORT levels generally start to rise in amphibians 3-4 minutes after exposed to a stressor (Harris *et al*. 2016). Acute stressors are considered short-term and last less than 24 hours (Harris *et al*. 2016). An acute stress response may cause behavioral changes to the amphibian in attempts to overcome the stressors (Narayan *et al*. 2013). Long-term stressors (i.e. chronic) can last between a time period from days to weeks resulting in a physiological response from the amphibian (Harris *et al*. 2016). Chronic stress is known to affect an amphibian's life history and suppress reproduction and fitness (Bliley *et al*. 2012).

An emerging pathogen studied in amphibians is *Batrachochytrium dendrobatidis* (*Bd*), a parasitic fungus that causes the disease chytridiomycosis (Murone *et al*. 2016). *Bd* is known to

have a great impact on amphibian global decline and extinction of amphibians in various populations throughout the world (Gabor *et al*. 2015). Amphibian declines have been observed since the 1970s and the organisms could possibly disappear on a global scale. Potentially, population declines can outnumber population increases in status (Green 2003). *Bd* infects tissues within the skin of the amphibian and is spread through colonization of zoospores (Parris *et al*. 2004). Since the infection is confined to the skin, it results in an imbalance of essential ions within the body (Rollins *et al*. 2017). Signs of the infection include: lethargy, abnormal posture, gross lesions, and hemorrhages of the skin (Kiesecker *et al*. 2011). *Bd* also affects processes such as appetite, fitness and reproduction (Gabor *et al*. 2015). Furthermore, there is much uncertainty of the relationship between CORT secretion and response to the infection *Bd*. It has been studied that increased CORT applied to toads pre-infection lowers *Bd* abundance (Murone *et al*. 2016). On the contrary, it has also been shown that exposure to CORT has no effect on *Bd* infection in frogs and toads (Searle *et al*. 2014). CORT is influenced by increasing amphibian resistance to *Bd* infection. In salamanders the infection seems to be of lower virulence (Rollins *et al*. 2017).

There are minimal studies focusing on the repercussions and effects of *Bd* infection on CORT secretion. A related study focused on *Bd* infected populations of Stony Creek frogs in Queensland, Australia and the frog's natural secretion of CORT (Kindermann *et al*. 2012). However, they were unable to determine the relationship between the stress hormone and *Bd* abundance. Amphibians are good model organisms to study because they have been harmfully affected by infectious agents. In particular, Red-backed salamanders (*Plethodon cinereus*) are very resistant to *Bd* and clear the infection quickly (Stockwell *et al*. 2010). These organisms are well studied regarding stress physiology, plasma CORT levels, and infection abundance.

A study conducted by Murone *et al*. (2016) distributed one of three doses of CORT (none, low and high) to toads in addition to one of three doses of *Bd* (none, low and high). The results concluded that toads exposed to CORT had a lower prevalence of *Bd* infection exhibiting an acute stressor (Murone *et al*. 2016). This research focused on the causes of *Bd* through changes in survival, resistance and tolerance. My research instead focused on the consequences of *Bd* on CORT rather than the effects of CORT on *Bd* in Red-backed salamanders. I hypothesized as *Bd* dosage increases among treatment groups, the more natural secretion of CORT will be present from each salamander. The goal of this experiment was to focus on determining the connection between *Bd* and CORT. In other words, how do different doses of *Bd* affect the secretion of CORT in Red-backed salamanders?

Materials and Methods

Animal Collection and Husbandry

Thirty six Red-backed salamanders (*Plethodon cinereus*) were hand collected from under rocks and logs from a deciduous forest in Crawford County, Pennsylvania in the Fall of 2017. Upon collection they were transported to the laboratory at Allegheny College (Pennsylvania) and housed individually in vented plastic containers (110 mm diameter x 75 mm depth). Each container had a non-bleached paper towel substrate that was soaked in 12mL of aged tap water. A bedding change was performed weekly and each salamander was fed approximately 25 fruit flies (*Drosophila melanogaster*) immediately after each bedding change. Prior to, and throughout the duration of this experiment, the salamanders were housed in a walk-in environmental chamber at 18.5°C and on a 12:12 light:dark photoperiod.

Experimental Design

Salamanders (n=36) were randomly placed in 3 groups (n=12/group) equally distributing mass. The *Bd* isolate used in this experiment consisted of a stock solution with 1% tryptone broth kept at 5°C in the laboratory at Allegheny College. Two of the 3 treatment groups were given *Bd* as a lower and higher dose and the third group was the designated control group. Dosages of *Bd* (i.e. low and high) were adapted from Murone *et al*. (2016); group 1 (n=12) was given a low dose of *Bd* (1×10^7 zoospores), group 2 (n=12) was given a high dose of *Bd* (2×10^7 zoospores), and group 3 (n=12), control, was not given *Bd*.

Mass was taken for each salamander before the experiment started. On day 0, salamanders in groups 1 and 2 were placed in respective containers and pipetted the varying levels of *Bd* treatment. High dose salamanders were given 4mL of *Bd* and 4 mL of water. Low dose salamanders were given 2mL of *Bd* and 6mL of water. Control salamanders were given 8mL of water from a petri dish with no *Bd*. Salamanders were exposed to *Bd* at the same time on the same day to avoid unwanted transmission. The bedding exposed to *Bd* was left untouched for 48 hours. After the 48 hour time period (day 2), the *Bd*-exposed bedding was removed and changed to fresh bedding. On day 7, salamanders in all groups were swabbed for DNA extractions (see below) and mass was taken for each salamander. On day 8, water-borne CORT collection (see below) was performed on salamanders in all groups. Finally on day 14, a final mass was taken for each salamander.

Water-borne CORT collection

In previous studies, CORT levels were measured by taking blood samples. This procedure was done under anesthesia and therefore detrimental to the animal's life. A non-

invasive, alternative method of measuring CORT levels is assaying hormones through a water-borne sampling technique. This process does not disturb the organism or require euthanization. In addition, repeated samples can be taken from the same organism over a period of time. Adapted from Gabor *et al*. (2013) and Murone *et al*. (2016), each salamander was individually placed in a petri dish (140 mm diameter x 25 mm depth) filled with 200mL of water. The salamanders sat within the container for 1 hour to collect water-borne hormones from urine samples (Figure 1). After 1 hour, 50mL of water was taken from each container using a 60mL plastic syringe. A C_{18} sep pak column was prepped ahead of time with 4mL of methanol and 4mL of water. The 50mL sample was then pushed through the column and the waste was discarded. Water samples were stored in a freezer at -20°C until ready for extraction.

After thawing samples, they were eluted into an RIA tube using 4mL of methanol. Each eluted sample was placed into a rack in order to dry the material inside. Samples were dried down under N_2 of 37°C. RIA tubes were stored in a freezer at -20°C until ready for re-suspension.

To re-suspend the sample, 20µL of ethanol and 380µL of EIA buffer were added to each sample. To measure the amount of CORT secreted by the salamanders within each water sample an enzyme linked immunosorbent assay (ELISA) kit (Cayman Chemicals Inc.) was used. I followed the manufacturer's instructions for all aspects of the ELISA kit. All samples were duplicated in the well. CORT data is presented as pg/sample/gram of salamander (pg/mL multiplied by 0.40 mL, which accounts for the amount of EIA buffer used to re-suspend the sample).



Figure 1. Salamander sitting in 200mL of water in Petri dish

Swabbing, DNA Extractions, and Quantitative Polymerase Chain Reaction (qPCR)

Protocol was obtained from Murone *et al*. (2016). In order to measure *Bd* intensity on exposed salamanders, each salamander was swabbed for DNA extractions 7 days post-exposure. The salamanders were swabbed 15 times from head to tail with its dorsal side up. The swab was placed into a vial in preparation for dilutions. 40µL of Prepman Ultra was added to each vial and placed in a dry bath at 100°C for 10 minutes. The vials were centrifuged at 13,000 rpm for 3 minutes. In preparation for dilutions, liquid inside vials was pipetted into snap-cap vials for a pure, undiluted DNA sample. 2µL of the undiluted DNA sample was pipetted into another snap-cap vial filled with 198.0µL of sterile, ultrapure reagent water for the double dilution. This snap-cap vial was centrifuged briefly. All snap-cap vials were stored in a freezer box and placed in the freezer until quantitative polymerase chain reaction (qPCR) protocol.

qPCR protocol screens for *Bd* infection and prevalence of the disease. While thawing samples from the freezer, 20μL of MasterMix was added to strip-wells. MasterMix contained the following – 236.28μL sterile ultrapure reagent water, 52.80μL 10x Exo IPC Mix, 26.40μL 50x Exo IPC DNA, 23.76μL ITS-3 Primer, 23.76μL 5.8 S Primer, 33.00μL diluted MGB probe and 660μL Taq Polymerase. 5μL of thawed double diluted samples were added to wells. Additionally, 2μL of thawed zoospore standards 1-7 were pipetted to each of the according wells along with 3μL of ultrapure reagent water. There was also a well containing strictly 5μL of ultrapure reagent water. Entire strip-well was centrifuged and loaded into an Applied Biosystems Step One Real-time PCR System (Applied Biosystems). Standard error graph was produced.

Statistical Analyses

I conducted a general linear model to test for differences in *Bd* infection prevalence between the two pathogen exposure treatments (low *Bd* and high *Bd*). In this model I used a binomial error distribution to account for the fact that salamanders were either infected or not infected and assessed statistical significance ($P < 0.05$). Because of the low prevalence of salamanders infected in the low *Bd* treatment (see below), I could only qualitatively compared differences in *Bd* infection intensity between the two pathogen exposure treatments.

My next two statistical models tested for differences in salamander mass change throughout the experiment. Each linear model tested for differences in the percentage of mass change on Day 7 and Day 14 (the response variable) between the pathogen treatment groups (control, low *Bd*, and high *Bd*).

Results

***Bd* Treatment**

High dose (2 x 10^7 zoospores) and low dose (1 x 10^7 zoospores) treatments were given to salamanders on Day 0 of the experiment. Salamanders were swabbed for infection on Day 7. The infection intensity figure (see below) represents the salamanders exposed and infected by *Bd*. Out of the 24 salamanders that were exposed to *Bd* only 6 (25%) were infected, these numbers were statistically significant (P=0.05). Five out of the 6 salamanders were in the high treatment group and only 1 salamander was in the low treatment group. Infection intensity was greater in the high treatment group compared to the low treatment group (Figure 2). The high treatment group had an average of 560.66 zoospores while the low treatment group had an average of 156.17 zoospores.

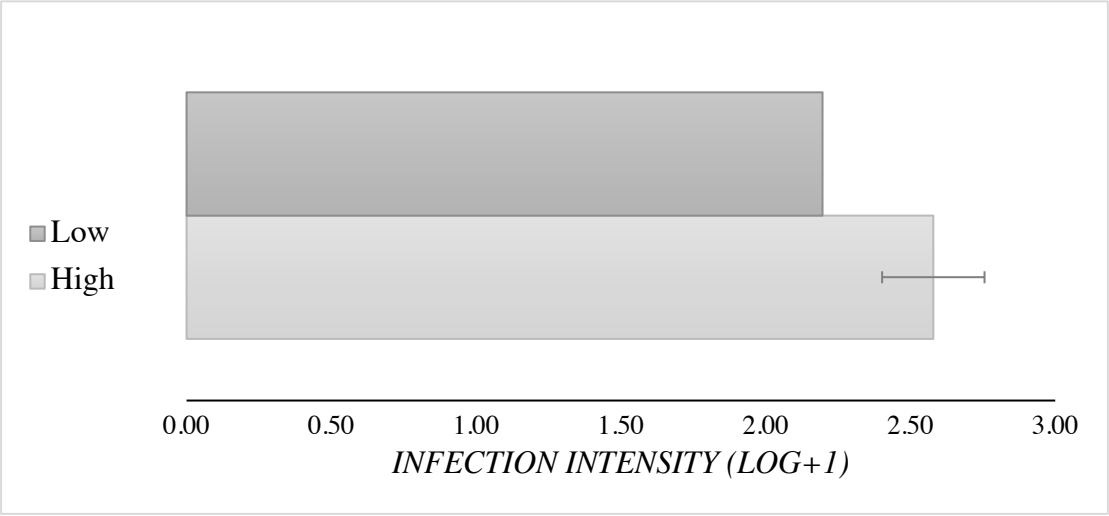


Figure 2. Infection Intensity. Average log-transformed values for *Bd* intensity of the high and low treatment groups. The infection load of Red-backed salamanders (*Plethodon cinereus*) was greater in the high treatment group (2 x 10^7 zoospores) than the low treatment group (1 x 10^7 zoospores). Error bars represent average standard error. Because there was only one salamander in the low treatment group, an error bar for the low treatment group is not applicable.

Change in Mass

Mass was taken for each Red-backed salamander (*Plethodon Cinereus*) before the experiment started (pre-mass), in the middle of the experiment (Day 7), and at the end of the experiment (Day 14). Throughout the duration of the experiment there were three treatment groups – control salamanders were not given infection, low treatment group was given 1 x 10^7 zoospores and high treatment group was given 2 x 10^7 zoospores. Control salamanders gained mass throughout the experiment, while both infected groups lost mass. Day 7 ($F_{3,23}$= 5.136, P= 0.005) and Day 14 ($F_{3,31}$= 11.174, P< 0.0001) values were statistically significant. The low treatment group lost 5.6% on Day 7 and 9.3% on Day 14 while the high treatment group lost the most mass, 6.1% on Day 7 and 12.9% on Day 14 (Figure 3a and b). However, there was no significant difference between the low and high treatment group percent mass change. Animals confirming infection through qPCR were compared by average percent mass loss.

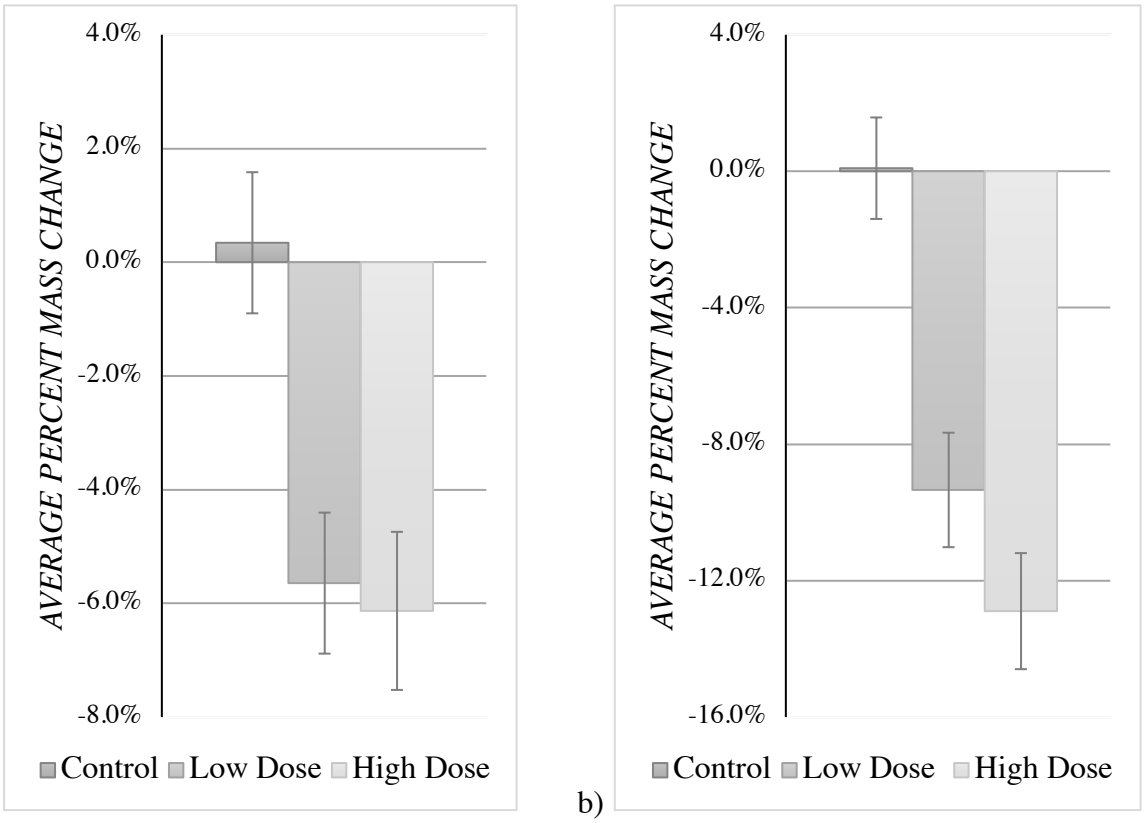


Figure 3. Average Percent Mass Change. Salamander mass was taken before the experiment started (pre-mass), 7 days and 14 days post infection. Control salamanders were not given infection, low treatment group was given 1 x 10^7 zoospores and high treatment group was given 2 x 10^7 zoospores. a) Day 7 and b) Day 14 experimental mass was taken for each Red-backed salamander (*Plethodon cinereus*) and compared to pre-mass. Control group salamanders on both Day 7 and 14 gained mass, while both infected treatment groups lost mass. Error bars represent average standard error.

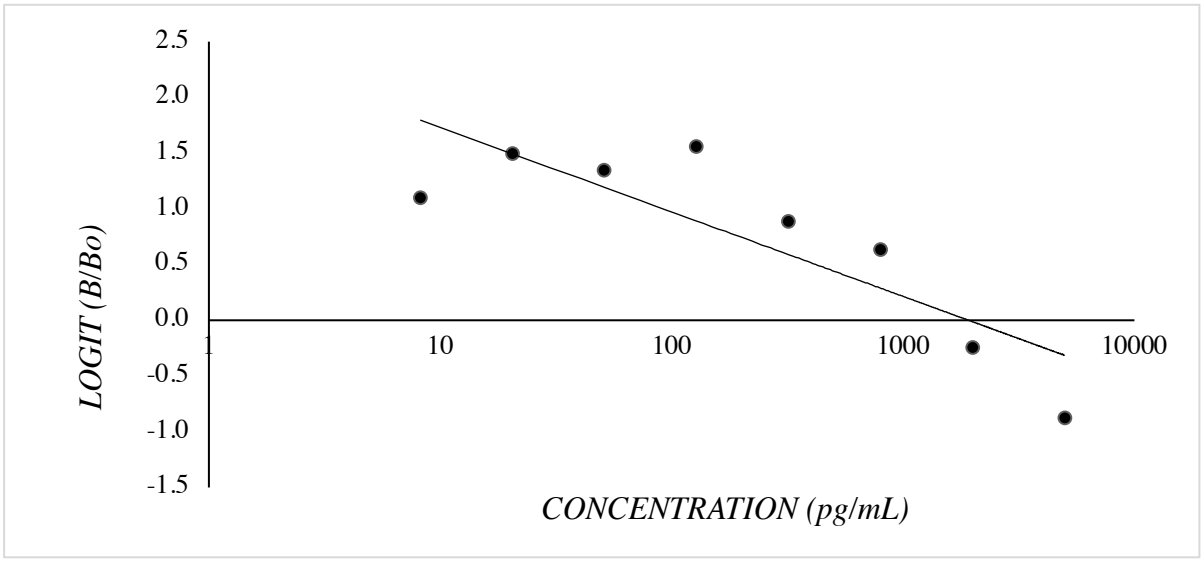
CORT Treatment

The standard curve from CORT analysis was produced with two outliers, standards 5 (128 pg/ml) and 8 (8.91 pg/ml) (Figure 4a). I only used data from salamanders that was within or relatively close to the range of CORT in field collected salamanders– Minimum: 49.1 pg/mL and Maximum 359.7 pg/mL (Gabor, *unpublished data*). Because I had to cut salamanders from the

dataset, I only had 1 salamander from the control treatment group and thus could not run statistics on the dataset (Figure 4b).

The results demonstrate there was a difference between treatment dosage and secretion of CORT in water. Fourteen out of 35 (40%) salamanders exhibited data within or relatively close to this range. The low treatment group salamanders released the most amount of CORT (598.81 pg/g) while the high treatment group salamanders released the least amount of CORT (295.02 pg/g) (Figure 4b).

a)



b)

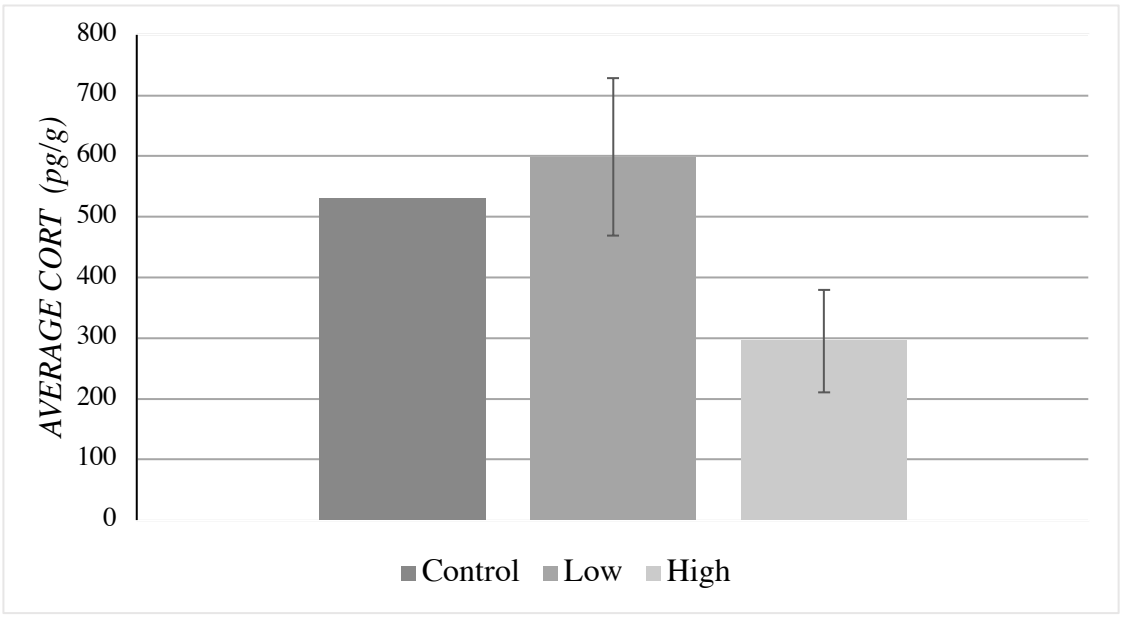


Figure 4. CORT Release for each Treatment Group. a) Standard curve from CORT analysis. The standards line up on the trend line except for two outliers, standards 5 (128 pg/ml) and 8 (8.91 pg/ml). b) Average amount of CORT pg per sample per gram of Red-backed salamanders (*Plethodon Cinereus*) released in water using the ELISA kit after 90 minutes of incubation period. Low treatment group salamanders released the most amount of CORT (598.81 pg/g) while the high treatment group salamanders released the least amount of CORT (295.02 pg/g). Error bars represent average standard error. Because there was only one salamander in the control group, an error bar for the control group is not applicable.

Discussion

Corticosterone (CORT) is the primary glucocorticoid (GC) hormone in vertebrates released from the hypothalamo-pituitary adrenal (HPA) axis during a stress response (Narayan *et al*. 2013). A stress response occurs if the vertebrate is exposed to energy costly factors such as infectious diseases. A field study done on a frog population infected with *Bd* compared infection abundance to baseline CORT concentrations (Kindermann *et al*. 2012). Their results concluded male frogs who tested positive for *Bd* had significantly higher CORT secretion. Another study also looked at *Bd* infected population of tadpoles at different elevations and their release rate of CORT (Gabor *et al*. 2015). At higher elevation levels there were higher infection loads in the tadpoles. Additionally with increased elevation there was an increase in CORT levels. This indicated tadpoles with higher infection loads had higher CORT release rates (Gabor *et al*. 2015). However, there is much uncertainty of the relationship between CORT secretion and response to the infection *Bd*. Using the amphibian Red-backed salamanders (*Plethodon cinereus*), I tested whether different doses of the emerging pathogen, *Bd*, elevated the natural secretion of CORT present from each salamander.

After exposing the high and low treatment groups to their designated doses of *Bd*, I analyzed *Bd* intensity using quantitative polymerase chain reaction (qPCR). Out of the 24

salamanders exposed to *Bd*, only 6 (25%) were infected. Five out of the 6 salamanders were in the high treatment group and only 1 salamander was in the low treatment group. The high treatment group had an average of 560.66 zoospores while the low treatment group had an average of 156.17 zoospores. Similar results were found by Fonner *et al*. (2017) in Red-legged salamanders (*Plethodon shermani*) with their highest *Bd* treatment group reaching 706 zoospores. My study in addition to other disease abundance studies have found high exposure of infection produces a larger infection load compared to low exposure of infection.

When looking at various studies that experimented with *Bd*, I've found this infection to be species-dependent. In other words, depending on the species, there could be multiple and various effects. In my experiment the low dosage treatment group received 1×10^7 zoospores of *Bd* while the high dosage treatment group received 2×10^7 zoospores of *Bd*. Murone *et al*. (2016) also gave amphibians, toads, two different dosages of *Bd*. However, their dosages were much higher. The low dosage group received 2×10^4 zoospores of *Bd* and the high dosage group received 8×10^4 zoospores of *Bd*. Differences between experiments include different model organisms, size of the model organisms and dosages of treatment. Furthermore, their results showed higher dosage of *Bd* had a higher CORT release.

Amphibians are good model organisms to study because they have been harmfully affected by infectious agents. Depending on the species, over a period of time the organisms will completely clear themselves of the infection. In particular, Red-backed salamanders are very resistant to *Bd* and eliminate the infection within their bodies all together over a period of time (Stockwell *et al*. 2010). These organisms are well studied regarding stress physiology, plasma CORT levels, and infection abundance.

One clinical sign of amphibians having the disease chytridiomycosis is body mass loss (Fonner *et al*. 2017). Throughout the duration of the experiment, I took the mass of the salamanders 3 times: before the experiment started (pre-mass), Day 7 and Day 14. The results showed a trend indicating a negative change in mass from salamanders who were exposed to the infection (high and low treatment groups) while salamanders from the control group gained mass (Figure 3a and b). This indicated exposed salamanders lost significantly more mass and then non-exposed salamanders. I have found a positive correlation between loss of body mass and *Bd* infection. However, previous laboratory experiments have found different results. Researchers who have taken the mass of the organisms throughout the duration of the experiment have not always found a loss or change of mass post-infection (Kriger *et. al* 2007). While body mass loss can be an indicator of infection it is not always a symptom.

Another clinical sign of amphibians having the disease chytridiomycosis is skin sloughing (Fonner *et al*. 2017). During my experiment, one of my salamanders in the high treatment died due to infection intensity and had symptoms of skin sloughing. Looking at the skin cells under a microscope it was very evident the large amount zoospores the salamander had on its body. A similar study by Fonner *et al*. (2017) looked at effects of CORT in Red-legged salamanders exposed to *Bd* found an abundance of salamanders exhibiting skin sloughing with high infection loads. Skin sloughing is extremely physiologically and energetically costly in amphibians because of ion and body mass loss (Wu *et al*. 2018). Smaller animals are more susceptible to skin sloughing due to higher infection loads and therefore increased ion loss (Wu *et al*. 2018). Due to these clinical signs of infection, we can infer that salamanders in the high treatment group of my experiment had high infection loads.

Within this experiment, I measured CORT levels by assaying hormones through water-borne sampling in addition to an enzyme linked immunosorbent assay (ELISA) kit (Cayman Chemicals Inc.). This is a very effective technique which many other studies (Gabor *et al*. 2013, 2015, Murone *et al*. 2016) have done similarly. The ELISA kit used in this experiment produced a standard curve. The standard curve from CORT analysis had two outliers, standards 5 (128 pg/ml) and 8 (8.91 pg/ml) (Figure 4a). These two standards did not follow the linear line of the other standards, therefore inhibiting comparisons of CORT data. However, CORT analysis was still possible for the other six standards with valid data (Figure 4b). The results have shown a general trend of a lower dosage of *Bd* increased natural CORT release in the water, while the higher dosage of *Bd* released the least amount of CORT in water (Figure 4b). These results contradict my hypothesis. This could be due to reaching a possible capacity of infection abundance. If *Bd* reached a certain threshold in the salamander's body, metabolic rate, oxygen consumption, and other bodily functions could perform opposite activities (Stockwell *et al*. 2010). A study conducted on toads found high infection loads survived longer than lower infection loads (Stockwell *et al*. 2010). This information indicated the relationship between infection load and survival does not determine time of mortality. Mortality appeared to be affected by the threshold of the infection load. The threshold amount is unknown and could differ between organisms. However once this threshold is reached, researchers have concluded physiological functions that aid in the healing of infection or prevent the infection from spreading are defeated and therefore terminated (Searle et al. 2011).

The results suggested the higher dosage of *Bd* released the least amount of CORT could indicate another complication within the treatment group. The salamanders within the high treatment group could have potentially not been healthy enough to secrete CORT. The infection

could have been too much or the dosage was too high for their bodies to handle it. Once a disease overtakes an organism's regular bodily systems, they are less likely to produce hormones such as CORT. Stressors take a toll on organism's bodies primitively. Thus, when adding a demanding stressor such as infectious disease, the body has to prioritize which system to direct it's energy to.

Due to two outliers in my standard curve and having to eliminate data from salamanders that did not fall between the range of 49.1 pg/mL to 359.7 pg/mL (Gabor, *unpublished data*), it is possible cross-contamination was factor, in addition to inaccuracy of pipetting skills. If I could have successfully had all eight standards fit the line, there could have possibly been more CORT data to analyze. Because I had a small sample size to begin with, it was harder to analyze data smaller than my original amount. Using a small sample size of only 36 salamanders was limitation within my experiment. By using a smaller sample size poses numerous problems with statistical analysis. The statistical analysis is important in determining predictive potential when comparing models (Hernandez *et al*. 2006). With a sufficient sample size, data is more likely to be statistically significant and experiments can easily be replicated (Fitts 2011). With larger sample sizes there is increased model accuracy in the experiment (Hernandez *et al*. 2006). If I had a larger sample size my numbers within each treatment group would have increased and given me more data to analyze.

There is an abundant amount of unknown information regarding stress and disease, more specifically there is much less information on CORT and *Bd*. By adding to this continual effort to find the connection we will eventually find the linkage between the two factors. One study in particular made an important conclusion, rather than associating *Bd* susceptibility to CORT secretion, *Bd* could be driving CORT levels (Gabor *et al*. 2015). However, there is much

unknown knowledge regarding these theories. There needs to be ample experimentation to detect the relationship.

This experiment was important in various ways in adding to the information regarding stress physiology and disease. While this experiment did not find the confident connection between stress hormones and disease it did, however, add to the knowledge on the impact this disastrous infection has on amphibians. As many studies have shown, *Bd* affects different amphibians in numerous ways. While Gabor *et al*. (2013) found infected toads had a higher CORT release rates than uninfected toads, the infection could have completely different effects on salamanders. This is yet to be determined; however, future research could find a connection. Since the threshold amount is unknown, future studies could focus on finding this value. It could be important knowledge to understand when determining doses of infection. A way to test this theory is giving amphibians various doses of infection. These different levels of infection will help determine the threshold rather than simply stating the infection is present or absent. Finally, these future studies could determine if the cause of CORT secretion is species specific, disease oriented, or an outside factor of stress.

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