Effects of Vitamin D Supplementation in Eastern Redback Salamanders on *Batrachochytrium dendrobatidis* Infection Burden



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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.

Pledge

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Abstract

Food intake is an integral part of maintaining bodily functions such as growth, reproduction, and immunological responses in all species. In many cases, changing environmental conditions may pose challenges such as increased competition, lack of nutritious food sources, or the absence of regular food sources altogether. In addition to caloric intake, vitamins and micronutrients are an important part of maintaining several life processes, including supporting immune functions in many species. One of the vitamins that amphibians specifically rely on is vitamin D. Usually procured through their diets, vitamin D helps maintain several metabolic functions, including calcium metabolism and the ability to enhance T-cell responses. The fungus *Batrachochytrium dendrobatidis* (*Bd*) is a prolific pathogen in amphibian populations that often leads to death through the resulting disease, chytridiomycosis. As such, being able to examine ways in which this disease may be mitigated is extremely important. To effectively study *Bd*, Eastern Redback salamanders (*Plethodon cinereus*) were used because of their ability to clear the infection regularly. This experiment specifically allows for a closer examination of the specific effects of vitamin D supplementation on *Bd* infection burden. The main hypothesis of this study is that salamanders who are both exposed to *Bd* and supplemented with vitamin D will be able to clear their infection more readily than control subjects. Secondary hypotheses include that all infected salamanders will lose mass, and that those supplemented with vitamin D will see less mass loss, comparatively. To test this, groups of red back salamanders both infected with *Bd* and not infected with *Bd* were randomly assigned to two treatment groups; those who are given fruit flies that have been dusted with Rep-Cal Calcium with Vitamin D powder and those given fruit flies which have no nutrient dusting. All salamanders, regardless of treatment group, were massed weekly and given approximately 15-20 fruit flies on weekly feeding days. *Bd* infection was quantified using qPCR analysis, and because no infection was found on any of

the salamanders, the final results revealed no significant differences between diet treatments and mass, or diet treatments and *Bd* exposure. Overall, examining the effects of a simple vitamin supplementation on this prolific fungal infection allowed for a better understanding of vitamin mediated immune response and how that may play a role in larger ecosystem functions.

Introduction

Without adequate caloric intake, the initiation and maintenance of immune, reproductive, digestive and skeletal systems becomes a challenge for the organism and exposes it to an increased risk of infection. In cases of extreme disease, caloric intake is essential to both fight infection and maintain bodily processes at the same time. Infected hosts deploy a range of immune responses, which vary from avoidance behaviors (Rohr et al. 2009) to employing lymphocytes and antibodies in the bloodstream to fight off active infections (Hart 1988). While often effective, these responses are energetically costly and can lead to a decrease in body condition of the host, which may ultimately serve to increase risk of death from infection (Hart 1988). Infected organisms living in a natural ecosystem may have to balance their energy allocation between growth and immune responses, especially in situations where their food sources may be limited due to competition (Budischak et al. 2018). During an active infection, hosts often exhibit an increased resting metabolic rate, such as in a study on house sparrows where infected individuals had nearly doubled their resting metabolic rate at the height of their infection in comparison to control individuals (Martin, Scheuerlein & Wikelski 2003). While increased metabolic rate is only one way to measure the energy cost of infection, there is mounting evidence that in most host species, immune responses require a large energy expenditure, and therefore may affect the feeding behavior of an organism.

Disease is thought to possibly affect feeding behaviors in two ways: either the infected host will exhibit increased feeding behaviors to compensate for the energetic costs of infection, or the infected host will exhibit decreased feeding behaviors because they lack the energy to seek and consume food while infected (Hite, Pfenning & Cressler 2020). A change in feeding behavior is often an early marker of illness in many organisms and thus has been studied in close detail in an attempt to better understand the intricacies of the interaction (Hart 1988). The effects of disease on feeding behaviors is a commonly studied system, as being able to understand the way that infection influences energy consumption and usage can help to predict larger implications for diseases as well as possible treatments. In some animal studies, such as Exton (1997), it is suggested that certain hosts may use anorexia as a defense mechanism for pathogen resistance. In organisms such as mice, force feeding caused hosts to die faster than those allowed to eat as little as they pleased (Murray & Murray 1979). The opposite was seen in a study concerning *Drosophila* that showed this feeding behavior actually led to an increase in infection intensity and death rate (Ayres & Schneider 2009). The act of illness-induced anorexia not only affects the host, but the pathogen as well.

Not only is the amount of feeding being done by an infected host important, but the content of their diet is important as well. High protein diets are generally more effective against infections, as demonstrated by a study conducted on larval insects which found that those provided with a higher protein diet had a higher antibacterial activity (Povey et al. 2009). A study on mice who were given low and high protein diets found that those on a low protein diet treatment ate more often than those on a high protein diet (Budischak et al. 2018). The individuals given a higher protein diet also produced higher levels of an infection specific antigen, suggesting that diet content allowed for a more robust immune response (Budischak et

al. 2018). Micronutrients like vitamin D can also play an important role in supporting several bodily functions, including immune responses through both direct and indirect measures. Vitamin D specifically has been marked as having properties that allow for the enhancement of macrophage activity and thus an increase in immune response (Mora et al. 2008). The most common ways that vitamin D enters the body are through sunlight exposure and diet. In humans, vitamin D is fortified into a large majority of foods and therefore supplements us in months where sunlight may be lacking. Having vitamin D in a human diet also helps to prevent diseases such as rickets by allowing calcium to synthesize more readily (Bouillon et al. 2008). Vitamin D is a precursor compound that aids in synthesizing a hormone called calcitriol which is responsible for enhancing the functions of effector cells such as macrophages in human systems (Prietl 2013).

Amphibians specifically exhibit different caloric needs at different times of their life cycles, and they follow an annual feeding pattern that may change around breeding and hibernation seasons (Ferrie et al. 2014). Wild amphibians' diets are widely variable and depend on what is available in their immediate environment. This makes it challenging to determine exactly how much feeding wild amphibians are doing, let alone the nutritional components making up the food. In infected captive amphibians that are showing decreased body conditions, it is understood that they are either reducing their food intake altogether, have an issue of malabsorption, or are having to increase their energy expenditure because of the burden of disease (Hadfield et al. 2006). Amphibians are also able to obtain vitamin D through UV sunlight exposure, but UV has also been hypothesized as a factor in amphibian decline as climate change continues to degrade the ozone layer (Antwis & Browne 2009). While vitamin D is not the only nutrient that plays an important role in bodily functions, it is one of the most important

supplements to add to captive amphibian's diets due primarily to its ability to prevent a bone degeneration disease called nutritional metabolic bone disease (Antwis & Browne 2009), a common ailment in captive amphibians that results in weakened bones and decreased mobility. A study done on red-eyed tree frogs (*Agalychnis callidryas*) showed that having their diets supplemented with carotenoids, which are the precursors of vitamin D, increased the amount of symbiotic bacteria present on their skin when compared to their counterparts that received unsupplemented diets (Antwis et al. 2014).

Understanding the link between nutrition and immune response plays an important role in assessing specific models of infection. *Batrachochytrium dendrobatidis* (*Bd*) is a fungal pathogen which is responsible for causing chytridiomycosis, and has recently been implicated as a leading cause of amphibian deaths and declines in biodiversity (Kilpatrick et al. 2010). *Bd* is transmitted through zoospores which can only infect their amphibian hosts through direct contact with the skin (Piotrowski, Annis & Longcore 2004). The pathogen then relies on close contact of amphibians, usually within bodies of water, to continue its spread (Piotrowski et al. 2004). In Eastern Redback salamanders (*Plethodon cinereus*), *Bd* infections present as loss of mass, shedding skin, and limb-lifting when compared to their uninfected counterparts (Becker & Harris 2010). However, Redback salamanders are generally resistant to mortality from *Bd* infections and can test negative for the pathogen as soon as 28 days after infection (Hess 2015). Due to their reliable ability to clear the pathogen, Redback salamanders are a common study species for manipulation experiments regarding *Bd.* As disease-caused deaths continue to play a large role in loss of biodiversity, especially in amphibians infected with *Bd*, it is especially important to understand if altering the content of their diets will increase resistance to the pathogen and help to reverse the intense diversity loss *Bd* causes. Vitamin D's numerous benefits and links to aiding

immune responses makes it a valuable element of study in understanding not only *Bd* infection burden, but how we can better inform the diet choices made for captive amphibians. As we consider reintroducing amphibians previously in captivity to their natural environments where *Bd* infection may be more pervasive, it is especially important to try to understand how differences in diet supplementation could affect community immune responses (Antwis et al. 2014).

Objectives and Hypotheses

The objective of this research is to better understand how nutrition supplementation affects an amphibian's ability to clear an infection. Focusing on vitamin D supplementation may be important for understanding the role of micronutrients for infection mitigation and also in informing the kinds of choices being made about captive amphibians' feeding guidelines. Through an ecological lens, the findings of this paper could also be applied to conservation efforts as related to reintroducing organisms to their natural environments and maintaining their health within captive enclosures. In this experiment specifically, I may be able to better understand how nutrition may play a role in aiding Redback salamanders in clearing *Bd* infections. The primary hypothesis of this research is that Redback salamanders who are suffering from a *Bd* infection that are given vitamin D dusted fruit flies will have a lower infection burden versus unsupplemented salamanders. Secondary hypotheses of this research predict that all salamanders who are exposed to *Bd* infection will lose more mass than unexposed individuals. Given the prevalence of *Bd*, it is advantageous to attempt to understand how supplementing a basic diet with common micronutrients could shape possible treatments for the disease.

Methods

Redback salamanders were collected from their natural environment and then allowed to assimilate to a lab setting for 7-14 days while receiving proper husbandry measures as detailed below. After their acclimation period, I began the experimental diet treatments on randomly selected salamanders. In order to collect data to follow through with testing the hypothesis, I utilized swabbing of the dorsal surface of each organism and then was able to assess infection burden through later quantitative polymerase chain reaction (qPCR) testing (Hess 2015). This information will allow for quantification of how much, if any, impact the presence or absence of vitamin D has on infection burden across multiple specimens.

Animal Husbandry and Collection

I collected 24 Eastern Redback salamanders from wooded areas at Robertson Athletic Complex on Allegheny College campus in Meadville, Pennsylvania during the month of October. All salamanders were found underneath rocks or logs and placed into a 6 quart storage container containing leaf litter while field collection was occurring. After collection in the field, salamanders were transported to a laboratory setting in Allegheny College and housed in individual, vented plastic containers on unbleached paper towels that have been soaked in dechlorinated and aged tap water (Hess 2015). During their acclimation time of 7-14 days (dependent on the day they were collected from Robertson) in the lab, all salamanders were provided with weekly bedding changes and feedings of approximately 20 wingless fruit flies (*Drosophila melanogaster*) provided *ad libitum* every week (Hess 2015). They were all housed in a temperature controlled lab set at 18 degrees celsius (± 1 C) and given a 12h light:dark cycle. Upon collection, all salamanders were massed and labeled with their experimental ID number. Exposure to *Bd* took place approximately two weeks after the salamanders' collection date. All

animals were massed thrice more on consecutive weeks of the experiment to monitor any physical changes.

Animal Feeding Treatments

Before exposure to *Bd*, all 24 salamanders were given the same diet of *ad libitum* fruit flies that contain no nutrient dusting (control treatment) during their 7-14 day acclimation window in the laboratory. On the day of *Bd* inoculation, the salamanders were haphazardly split into two groups of 12 to determine feeding treatment, and then split again into two groups of 6 to determine their infection status (Figure 1). If assigned to the vitamin D treatment group, salamanders will be given the fruit flies dusted with Rep-Cal Calcium and vitamin D powder at their weekly feedings beginning the week of *Bd* inoculation. Those in the control group will continue to receive undusted fruit flies at their weekly feeding intervals.

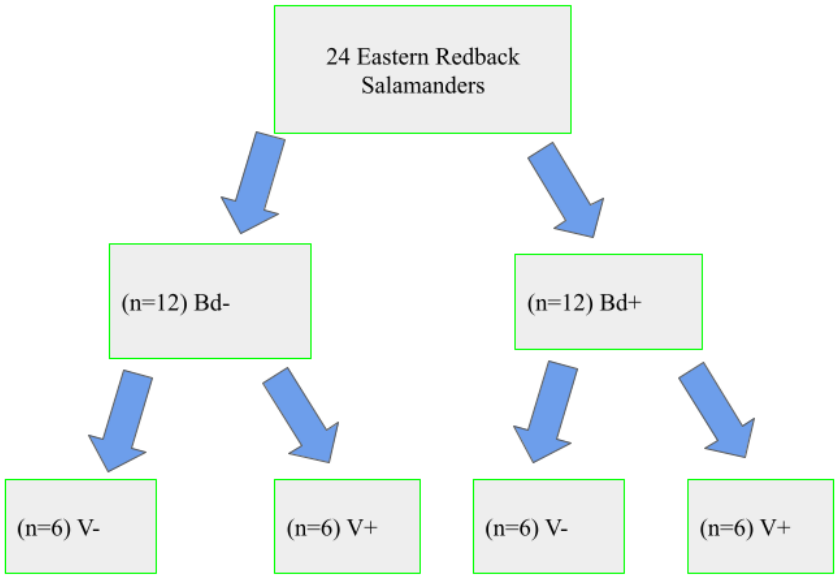

Figure 1. Experimental Design is a 2x2 factorial design. 12 salamanders were given fruit flies dusted with Rep-Cal Calcium with Vitamin D powder (vitamin D treatment); 12 salamanders were given fruit flies with no micronutrient dusting (control). 6 salamanders in each diet group were inoculated with *Bd* (*Bd* +); 6 salamanders in each diet group were inoculated with a broth not containing *Bd* (*Bd* -).

Bd Infection

Bd was obtained from an isolated laboratory growth in a 1% tryptone bath (Hess 2015). For this specific experiment, *Bd* inoculation had to take place over two different days due to low zoospore counts on the first day of inoculation. On the first day of inoculation, every salamander assigned to be infected was given a dose of 2.0 mL, through a pipette, which should contain roughly 87,500 zoospores per mL, on the dorsal surface of the salamander (Hess 2015). Due to the relatively low dose of the first load of *Bd*, the same process was followed four days later with the same group of salamanders; this time given a dose of 4 mL through a pipette which should have contained roughly 75,000 zoospores per mL. In total, all of the infected salamanders received 6 mL of *Bd* with about 475,000 zoospores.

On both infection dates, latex gloves were worn during the infection process and switched out between each animal. The non-infected group of salamanders also received a dose of 5.0 ml through a pipette of just the 1% tryptone bath without any *Bd* zoospores (Hess 2015). 24 hours after the first inoculation, the exposed salamanders were given bedding changes but not fed, as I knew that a second round of inoculation was coming and diet treatments were set to begin after inoculation was completed. 24 hours after the secondary round of inoculation, the salamanders were removed from the containers where they received treatments, all salamanders were moved to a new container with fresh bedding and received their assigned feeding treatment. Once again, gloves were worn and switched out with every transfer of an animal into a clean container, making sure not to touch the outside of the new containers. The *Bd*+ liquid was disposed of into a bleach container inside of the lab space. All *Bd*+ soaked paper towels were disposed of in a red biohazard bin, and the plastic containers that were used in *Bd* exposure were cleaned with bleach water.

Measuring Mass

The mass of each salamander was recorded in grams in a lab notebook on the day of collection prior to any feeding, then on days 7 and 14 post infection, before lastly on day 21 prior to being transferred to the care of Dr.Venesky. This weekly measurement allows for comparison between the *Bd* treatment groups; as some weight loss is expected in the *Bd*+ group due to the infection.

***Bd* Testing**

The swabbing process followed the protocol in Venesky et. al (2015), such that every salamander in the infected treatment group was swabbed with sterile fine tipped swabs 10 times on their dorsal side, including the length of the tail, while rotating the swab. Each swab only has DNA from a single salamander, and gloves were switched out between each animal. This process was conducted 7 days post infection and again 14 days post infection for all 24 of the salamanders. Since redback salamanders are proven effective at clearing *Bd* infection (Lenz 2013), swabbing them 7 days after infection should allow for the salamanders to combat but not completely clear the infection before swabbing. Swabbing again at 14 days post infection will allow for a second data point which may better quantify the specific infection burden of the treatment groups. All swabs were stored at -20 degrees celsius in a vial until further qPCR testing could be conducted to determine exact parasite burden on these days (Hess 2015).

DNA Extraction

DNA was extracted from the swabbed samples taken 7 and 14 days post infection for each salamander. This was done by using 40 μL of Prepman Ultra. Vials including the swabs and Prepman Ultra were placed in a dry bath for 10 minutes before being cooled for 2 minutes and then centrifuged for 3 minutes at 13,000 rpm. Next, 2 μL of extracted DNA was pipetted into an

18 µL reagent water to dilute the sample and centrifuged again until the speed reached 4,000 rpm. This process was then repeated with each DNA sample from each salamander, and then were all stored in the freezer at -20℃ until qPCR could commence.

qPCR

Quantitative polymerase chain reaction (qPCR) was used to quantify the amount of *Bd* present on the salamanders' skin. The standard procedure for this as outlined by Boyles et al. (2004) and Hyatt et al. (2007) requires the use of extracted DNA as detailed in the section previous. To execute qPCR, 20µL of MasterMix containing reagent water, 10x Exo IPC Mix, 50x Exo IPC DNA, ITS-3 Primer, 5.8 S Primer, Taq polymerase, and diluted MGB probe were added to each of the wells of a qPCR plate. In the first row of wells, I added 2 µL of *Bd* zoospore standard and 3 µL of reagent water to the existing MasterMix in the first row of wells. To have a control standard, the last well in this row receives 5 µL of reagent water and no *Bd* zoospore standard. All other wells had 5 µL of the diluted DNA samples added.

Euthanization & Transfer

Euthanization was not required for the termination of this experiment. On day 22 of the experiment, all of the salamanders under my care were transferred to the care of Dr.Venesky, for the sake of using them for future research in the lab. Prior to this transfer, all of the 24 salamanders were massed, given a bedding change and fed.

Statistical Analysis

I conducted a linear model ("lm" in R) to test for main and interactive effects of my experimental treatment groups on the percentage of mass changed throughout the experiment [(initial mass - final mass)/initial mass]. Because none of my salamanders tested positive for Bd (see Results), I used pathogen exposure treatment (exposed, not exposed) as a categorical

predictor. I considered vitamin treatment (added, not added) as a second categorical predictor variable. I assessed significance when the p-value was < 0.05 for all main effects (pathogen exposure, vitamins) and the interaction between these two terms.

Results

Bd Prevalence

The qPCR testing showed that none of the salamanders in the experiment had *Bd* zoospores on their skin at the time of swabbing. All of the 12 salamanders that were exposed to *Bd* came back negative for infection and therefore the infection rate of this experiment as a whole was 0%. As such, I will consider the absence or presence of "*Bd* exposure" as the experimental treatment instead of *Bd* infection.

Pathogen Exposure

As all of the qPCR results came back negative for *Bd* zoospores, pathogen exposure was the next best factor that could be analyzed for results. Pathogen exposure was statistically compared with mass change and diet treatment group. No significant differences were found between mass change and pathogen exposure ($F_{1,23} = 0.418$, $p = 0.525$). Percent mass changes between feeding treatment groups was not significant ($F_{1,23} = 0.3254$ $p = 0.574$). Generally, a majority of the salamanders (n=21) lost mass during the course of the experiment regardless of their diet treatment group, with the exception of the *Bd*- V- group. (Figure 2).

There were also no significant differences recorded between pathogen exposure and the diet treatments ($F_{1,23} = 2.9288$ $p = 0.1025$).

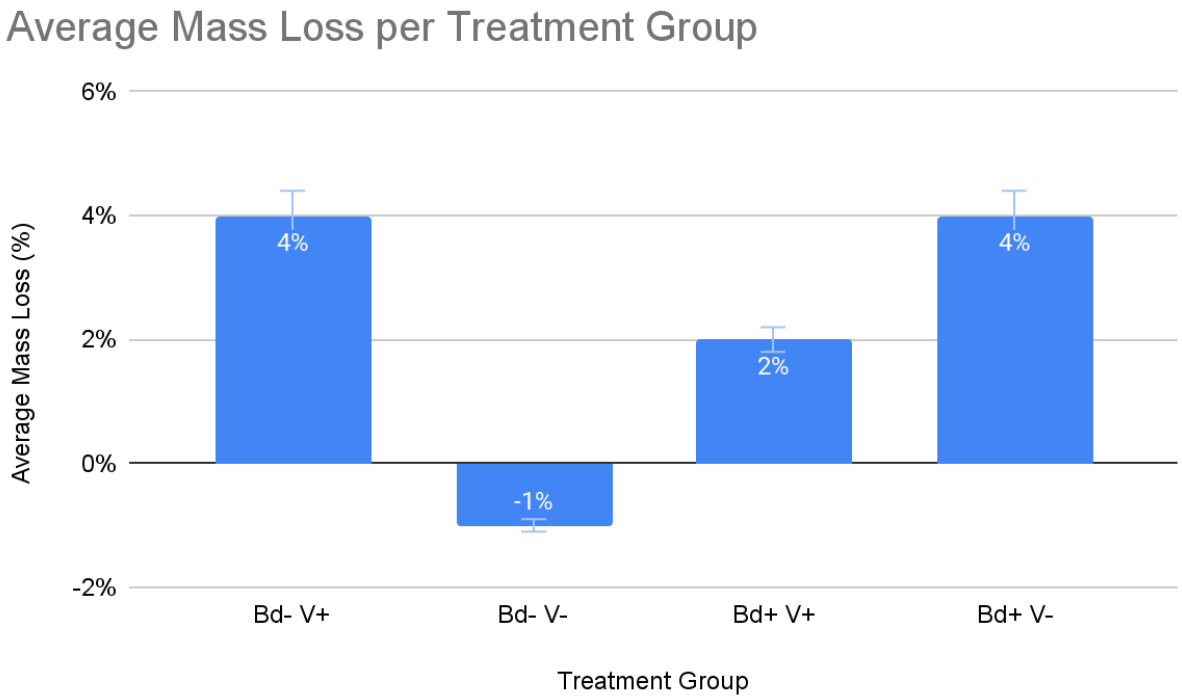

Figure 2. Graphical representation of mass change averages across treatment groups as discussed above. Each salamander was weighed on intake and on their last day of experimentation to calculate their individual mass change over the course of the experiment. Each treatment group (*Bd*- V+, *Bd*- V-, *Bd*+ V+, *Bd*+ V-) then had their mass change percentages combined and averaged to create group totals represented by the bars. Negative percentages indicate an overall mass gain within the group.

Discussion

The large aim of this study was to examine and compare the effects of vitamin D supplementation on Redback Salamanders' ability to clear *Bd* infection. Examining this relationship allows for a better understanding of how vitamin supplementation can play a role in the immune response of these salamanders and amphibians at large. Amphibian populations across the globe are in a decrease as an effect of the *Batrachochytrium dendrobatidis* (*Bd*) fungal infection, which causes death and loss of biodiversity in many ecosystems around the world (Kilpatrick et al. 2010). To facilitate this experiment, Redback salamanders (N=24) were sorted into one of four treatment groups. *Bd*+ individuals were inoculated, and after the 24 hour incubation period all salamanders began their diet treatments. On days 7 and 14 of the

experiment, all of the salamanders were swabbed for infection burden with the use of DNA extraction and qPCR analysis to determine the amount of *Bd* zoospores present on both days of swabbing. Salamanders were massed weekly throughout the experiment. The qPCR analysis on all of the swabs came back negative for *Bd* zoospores.

These negative results could have been a result of a few different things throughout the experimental process. During the initial inoculation period, the zoospores may have never effectively penetrated the skin after the 24 hour time period, so the swabs would have no evidence of infection. It is also possible that the salamanders were able to clear their infection before the first swabbing took place, as Redback salamanders are able to reliably clear *Bd* infection as soon as 28 days after exposure (Hess 2015). Additionally, there could have been human error in the swabbing technique or DNA extraction technique. It is unlikely that there were issues in the process of qPCR analysis, as the standard fit of each plate run was consistent with the machine. Both plates were also run simultaneously with other researchers' samples, and some of their samples came back positive on the same plates that mine did not.

Since the qPCR analysis was unsuccessful, all of the analysis shifted to understanding how mass change related to vitamin D supplementation and exposure to *Bd* and how exposure and supplementation interacted, not necessarily *Bd* infection. Generally, the energetic costs of an infection are large. One study done on butterfly pupae found that, on average, infected individuals had a standard metabolic rate that was 8% higher than uninfected pupae (Freitak et al. 2003). As hypothesized, a majority of the salamanders lost mass over the span of the experiment, with the exception of the three individuals that gained mass. Despite this interesting difference, there was no statistically significant difference found between the diet treatment groups' mass change overall ($p=0.574$). This could be due to the fact that the salamanders in

each treatment group were given a consistent number of fruit flies per feeding regardless of supplementation or not. The salamanders also varied widely in size across all four treatment groups, so in analyzing mass averages the wide range of specimens may have resulted in the insignificance of the comparison results as things evened out (Figure 2). When comparing *Bd* exposure with mass change, the results were also insignificant ($p= 0.525$), which was in opposition to my original hypothesis that the individuals exposed to *Bd* would experience greater mass loss than those who were not exposed to *Bd*. Lastly, my last hypothesized point that salamanders given vitamin D would have a lower pathogen burden was disproven both by the negative qPCR results and the statistical analysis comparing the two being insignificant ($p= 0.1025$).

Due to the inconclusive results of this study, it is still uncertain exactly how vitamin D supplementation could aid in clearing infection. It is possible that vitamins work best when they are in combination, such as in a multivitamin that would contain vitamins A,C and E as well as D. Since amphibians rely on their adaptive immune response when they are actively infected, the process of making the specific antibodies is energetically costly and likely requires specific vitamin components to work effectively. In one case where South African clawed frogs (*Xenopus laevis*) were immunized with dead *Bd* zoospores; specific antibodies to fight the pathogen were found on the skin when tested (Ramsey et al. 2010). These frogs were given ground beef heart as their main source of food, which offers a wider range of vitamin supplementation and protein to facilitate their immune responses as well (Ramsey at al. 2010). While the diet of Redback salamanders is clearly different from the frogs, the feeding of something as simple as fruit flies-whether supplemented with vitamin D or not- may not offer the full extent of nutrition needed to produce an effective immune response. Diet supplementation as a whole is also quite

unquantifiable, as the powder begins to fall off of the food as soon as it's given, and in the case of the salamanders it was impossible to know exactly how long they allowed the fruit flies to move around before being eaten. It is also noted that species specific vitamin D requirements are not known, and it is possible to be at risk of over or under supplementation for the specific species.

The foundational logic behind focusing on only vitamin D supplementation in this project stems from the results of Hobson (2020), where individuals given multivitamin supplementation had the least amount of zoospore presence, but the vitamin A supplemented groups still experienced a lower zoospore count than the control group. Existing literature on vitamins in amphibian diets revealed vitamin D as one of the most important for overall health, as it exists as a precursor to calcium that can be used for growth of bones, skin and intestinal structures in amphibians (Antwis & Browne 2009). Combining these two aspects of research led me to consider vitamin D alone, as *Bd* is a disease which enters through the skin and can cause other skin related issues such as skin-shedding (Becker & Harris 2010). Another study by Antwis et al. (2014) proved that amphibians supplemented with vitamin D precursors presented a more robust beneficial bacterial colony on the skin than those with unsupplemented diets, which would lend itself to a more complete immune response. While the inconclusive results of this study make it difficult to make a case for the importance of vitamin D supplementation related to infection burden or immune response, the vitamin itself is still important. The absence of vitamin D in amphibian diets can cause a disease called Nutritional Metabolic Bone Disease, which leads to weakened bones, limited movement and death of amphibians if untreated (Antwis & Browne 2009).

Understanding the effects of vitamin supplementation on amphibian populations, especially those threatened with *Bd* and other diseases, is important to longevity and conservation. While climate change continues to damage environments around the world and UV exposure becomes more apparent, offering vitamin D diet supplements in amphibian populations can help preserve species that are particularly vulnerable to UV (Antwis & Browne 2009). Diet supplements are also fairly cheap and easily accessible; so those keeping captive amphibians are able to maintain vitamin levels, while those working with wild populations can use supplemental feedings to help promote population growth. Though the results of this specific study were inconclusive on understanding the direct link between vitamin D and *Bd* infection burden, I still believe that vitamin supplementation is a promising avenue to study and understand how diet components are able to aid amphibians in their immune responses. Going forward with this research, I would like to consider a larger sample size and use a similar model, but this time consider two different vitamins against one another. This would possibly better expose the different benefits of the elements in a multivitamin, and perhaps identify which of them is most effective or important in immune response.

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I would like to extend special thanks to Dr.Venesky for all of his help throughout the process of crafting my idea, following through with my experimentation and all of the following writing, processing and understanding. You have exposed me to a field of science I greatly enjoy, and have been such a good wealth of knowledge for me for the last two years. Thanks are also due to my fellow compers, who all worked unbelievably hard for their work to come to fruition while also being an awesome support system and understanding of the specific intricacies of comping within this lab.

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