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The Effect of Chytridiomycosis and Bioaugmentation on the Metabolic Rate of Redbacked Salamanders, Plethodon cinereus

Jonathan Lenz
Carroll College, Helena, MT

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Jonathan Lenz

Department of Natural Sciences, Carroll College, Helena, Montana

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Brandon A. Sheafor 4/02/2013

Dr. Brandon Sheafor (Thesis Director)

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Dr. Sam Alvey (Reader)

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Dr. Kyle Strode (Reader)

Abstract

The infectious disease chytridiomycosis caused by the fungal pathogen *Batrachocytrium dendrobatidis*, *Bd*, contributes to worldwide amphibian decline, but definitive pathogenesis of lethality for the disease is not well understood. Because amphibians respire through their skin (in addition to respiration using lungs), it is speculated that amphibians with chytridiomycosis may show a decrease in metabolic rate, but amphibians bioaugmented with probiotic bacteria may alleviate possible metabolic stress. A species of lungless salamander (*Plethodon cinereus*) was used in this study to analyze the cutaneous effects of *Bd* on metabolic rate *in vivo*. Net weight change, infection load (using a Taqman real time PCR assay), and metabolic data (using an FMS) of salamanders were recorded over the course of a 25 day span. Data demonstrate that salamanders infected with *Bd* may possess a higher overall metabolic rate, but further research must be conducted to confirm statistical significance. Real time Taqman PCR results suggest that salamanders infected with *Bd* were able to clear infections in less than two weeks due to rise and subsequent decline in detection of zoospore genomic equivalence despite showing weight loss and other characteristic symptoms of chytridiomycosis. Further research including more intense infection of salamanders, application of techniques to minimize PCR inhibitors, and recording of metabolic data in a refined experimental environment are needed to better understand these phenomena.

Introduction

There have been five notable mass extinctions on planet earth dating as far back as 435 million years ago to as recently as 65 million years ago (Wake and Vrendenburg, 2008). Amphibians are typically species that have survived mass extinctions, but because of habitat loss, climate change, and emerging infectious diseases, their loss of populations may be on track for the sixth mass extinction (Wake and Vrendenburg, 2008).

Chytridiomycosis is an emerging infectious disease caused by the fungus *Batrachocytrium dendrobatidis* (*Bd*) that infects the skin of an amphibian and is a global threat to amphibian biodiversity (Berger et al., 1999; Crawford et al., 2010; Lips et al., 2006; Rachowicz et al., 2006). Amphibians are indicators of global environmental health, and thus other species of wildlife may be threatened with a decline in the worldwide amphibian population (Collins and Storfer, 2003). Collins (2010) postulates that amphibian decline through chytridiomycosis threatens the extinction of this entire class of organisms; moreover, this infectious disease is unique to most amphibians, and may have risen to prominence as a result of climate change.

Disease is both an ecological and physiological problem for particular species; the interaction between a host and its pathogen represents a simple dynamic at its core physiological role, but raises a greater concern of lethality across an entire class of species when examined macroscopically (Blaustein et al., 2012). Because of the serious and lethal nature that chytridiomycosis imposes on global amphibian populations, a bottom-up approach could be utilized in understanding why it is a threat to an entire class of organisms, and what can be done to prevent amphibians from heading on a course

towards mass extinction (Blaustein et al., 2012; Berger and Speare, 1998). Placing pathogens into an ecological context can be important for understanding disease emergence and should complement more traditional approaches of examining host-pathogen interactions (Belden and Harris, 2007). Despite the primary prominence of chytridiomycosis in South American amphibians, evidence also definitively shows that *Bd* is found in regions of North America, Europe, and Australia (Ouellet et al., 2005; Pullen et al., 2010; Bosch et al., 2007; Kriger and Hero, 2007). Perhaps, the spread of *Bd* across these continents suggests the impact of rising temperatures due to climate change. Pullen et al. (2010) demonstrated that *Bd* prevalence in the state of Virginia varies with seasonal change, but it is not affected by urbanization, and no signs of disease were recorded in areas of Central Virginia; thus, they assumed that local amphibian communities must be resistant to *Bd*. This is important because regional impact of *Bd* provides a pathway for research to investigate why some amphibian populations are resistant and others are not. Bosch et al. (2007) found that in Penelara, Spain, rising temperature has spurred more cases of chytridiomycosis. However, Andre et al. (2008) speculate raised temperature affects a frog's resistance to chytridiomycosis rather than producing a lethal effect on the frog. Thus, *Bd* and the spread chytridiomycosis may be more prevalent in warmer climates, but lethality is less prevalent. Kriger and Hero (2007) found that *Bd* was responsible for many of the unexplained disappearances of stream-breeding amphibian populations in recent decades in Southeast Queensland, Australia. Overall, it is unclear whether both climactic anomalies and disease-related extirpations are working synergistically or separately, but Lips et al. (2008) have found no evidence to

support the hypothesis that climate change has been driving outbreaks of amphibian chytridiomycosis, as it has been posited in the climate-linked epidemic hypothesis.

Chytridiomycosis is a dangerous pathogen because of its high virulence rate among a large number of potential hosts in the amphibian realm (Berger et al., 1999; Pessier et al.,1999). 43% of amphibian species have declined and 32.5% are globally threatened because of the lethality of *Bd* (Lips et al., 2006). Rachowicz et al. (2006) suggest that *Bd* is capable of causing high mortality and population decline without the presence of environmental cofactors (e.g., chemical contaminants, climate change, UV-B radiation) because field mortality rates and disease progression were similar to those in the laboratory in which cofactors were not present. Much information has yet to be determined with regard to *Bd* pathogenesis. *Bd* disrupts cutaneous osmoregulatory function, leading to electrolyte imbalance and death, and the ability of *Bd* to compromise the epidermis explains how a superficial skin fungus can be fatal to many amphibians (Voyles et al., 2009). Peterson (2012) suggests that chytridiomycosis affects cutaneous respiration and metabolism in amphibians, but more data must be collected to support this result.

Researchers understand how *Bd* colonizes an amphibian host. The spherical thalli of *Bd* live within keratinized epidermal cells of amphibians (Pessier et al., 1999). Zoospores are the primary method of dispersal for *Bd*, but these spores are only able to infect a host if they attach directly on the skin of the amphibian (Piotrowski et al., 2004). *Bd* zoospores may spread from amphibian to amphibian by close or direct contact during mating, schooling of larvae or other aggregative behaviors (Piotrowski et al., 2004). Plasma ion suppression, appetite suppression, increased metabolic rate, weight loss, and

increased skin shed frequency occur prior to when individual amphibians display clinical signs of disease such as lethargy, abnormal head lifting, etc. in the species *Lonicera caerulea* (Peterson, 2012). Therefore as a starting point, scientists should look at these characteristic symptoms across all species of amphibians to qualitatively diagnose those with chytridiomycosis.

Janthinobacterium lividum (referred to as *J. liv.*) produces a metabolite called violacein (Becker et al., 2009). Violacein is associated with the survival of amphibians when this bacterial culture colonizes the host prior to *Bd* infection and exposure (Becker et al., 2009). Thus, *J. liv*. acts as a probiotic that promotes the survival of *Bd*-infected amphibians from chytridiomycosis-induced death. Bioaugmentation is simply introducing a microbial strain to a reservoir or host. This suggests that the antimicrobial peptide secretions produced by the amphibians have a select group of bacteria that is resistant to amphibian produced antimicrobial peptides and resist the pathogenic fungi forms (Becker et al., 2009). Conservation strategies are currently being researched with *J. liv*. Futhermore, Muletz et al. (2012) demonstrated that *J. liv.* introduced into natural soil can successfully be transmitted to the skin of *P. cinereus*. The Anti-*Bd* compound, violacein, found in *J. liv.* required low concentrations to be effective in preventing lethality among *Bd*-infected *P. cinereus* as Becker and Harris (2010) demonstrated. Therefore, it is important to examine if cutaneous bioaugmention of *J. liv*. offsets possible metabolically-induced stress of salamanders with *Bd* infections.

Given these symptomatic parameters for amphibians and the desire to explore the pathogenesis of chytridiomycosis infection, the bottom-up approach to understanding the physiological and ecological role of chytridiomycosis in global amphibian populations

can be administered as follows: 1) scientists should identify model amphibian organisms to analyze *Bd* infection in order to develop experiments that effectively isolate variables to determine modes of pathogenesis, 2) specific experiments should recognize and test the current hypotheses of pathogenesis which include plasma ion suppression, appetite suppression, upregulation of immune system response, increased metabolic rate, etc, and 3) comparison of results from these experiments may support or reject these current hypotheses (e.g. climate change, metabolic rate alteration, ion disruption and imbalance, etc) and may build the foundation for analyzing the lethality of chytridiomycosis for the amphibian population in general.

This thesis used specific analysis of a model amphibian with emphasis on cutaneous metabolic respiration based on a bottom-up approach. The model amphibian was *P. cinereus*, commonly referred to as the Red-backed Salamander. In general, *Plethodon* species are at risk of becoming infected with *Bd* and developing chytridiomycosis (Vazquez et al., 2009). Lungless salamanders exclusively rely on cutaneous respiration and buccopharyngeal gas exchange (Feder, 1976), which make them ideal amphibians to study metabolic rate in response to cutaneous infection from *Bd*. This is because *Bd* colonizes on the skin of the salamander, and infection may induce a lower metabolic rate. Metabolic research on lungless salamanders has been conducted by Sheafor et al. (2000), who analyzed the effects of hypoxia on metabolism. This research found that lower levels of oxygen in the environment exposed to the salamander species *Desmognathus fuscus*, increased metabolic rate as well as buccal activity (Sheafor et al., 2000). The *Desmognathus fuscus* salamander is lungless and is in the same family as *Plethodon cinereus*, which is classified in the family *Plethodontidae*. So far, no

research has been conducted that examines the metabolic rates of *Plethodon cinereus* under *Bd*-induced metabolic stress. This thesis will conduct research to advance the knowledge of pathogenesis of chytridiomycosis in amphibians by specifically examining metabolic stress and gas exchange (e.g., O_2 consumption, CO_2 production) during infection.

This thesis examined the metabolic rates of randomly selected Red-backed Salamanders in a controlled experimental setting that ensured proper environment acclimation, optimal *Bd* growth conditions, and nutrient acquisition. I hypothesized that chytridiomycosis will decrease cutaneous gas exchange in Red-backed Salamanders, and that bioaugmentation will alleviate this metabolic decline by inhibiting fungal growth of *Batracochytrium dendrobatidis*. In other words, the Red-backed Salamander will lower its oxygen consumption as a response to *Bd* infection. When the Red-backed Salamander is subjected to bioaugmentation with the probiotic *Janthinobacterium lividum* and then exposed to *Bd*, I hypothesized that metabolic decline will be corrected and may be a reason for survivability of salamanders that confront these unique microbial communities. The objectives of this study were to: 1) analyze the change in mass of Red-backed Salamanders as they are infected with *Bd*, inoculated with the probiotic *J. liv.*, and inoculated with both, and compared to a control group, 2) quantify infection and probiotic load on the skin of each salamander using real-time PCR techniques, and 3) successfully record metabolic measurements over a 25 day span for each salamander and compare metabolic results to possible changes in mass and infection load.

Materials and Methods

Study Species

Plethodon cinereus is a terrestrial salamander with a geographic range spanning across most of the northeastern United States, southern Quebec, and the Maritime Provinces of Canada. Their moist, nutrient-rich skin helps support a diverse community of cutaneous bacteria (Austin, 2000). There is no evidence that *P. cinereus* populations have been adversely affected by chytridiomycosis in nature, but this species can be infected by *Bd* (Harris et al., 2009).

Sampling and housing

Twenty-eight adult Red-backed Salamanders were collected from George Washington National Forest in Harrisonburg, Virginia and relocated to the animal facility at Carroll College in Helena, MT. Salamanders were given four weeks to acclimate to laboratory conditions. *Bd* grows optimally at 17-25 ^{o} C (Piotrowski et al., 2004). Salamanders were housed at a temperature of 18 ^{o} C in containers filled with 50 mL of Provasoli medium. Provasoli medium, artificial pond water, was prepared with following ingredients: 10 L deionized water and 20 mL each of $NaNO_3$, $MgSO_4 * 7H_2O$, $CaCl_2 * 2H_2O$, K_2HPO_4, KCl, and KH_2PO_4. Each salamander was fed 15 infant fruit flies, *Drosophila melanogaster*, every seven days. Containers were cleaned and autoclaved every seven days.

Experimental Design

Twenty-four of the twenty-eight salamanders were randomly chosen to have their metabolic rates measured in the experiment. The other four salamanders were used to calibrate the field metabolic system to ensure adequate recordings of VO_2 and VCO_2 in the flow-through chamber of the Field Metabolic System, FMS (Sable Systems International). Salamanders were randomly placed in either the control group or one of three experimental groups (Table 1). The control group, which contained five salamanders, was not exposed to *Bd* and did not have its microbial community augmented. The first experimental group, which contained seven salamanders, was exposed to *Bd* without bioaugmentation from *J. liv*. The second experimental group, which contained six salamanders, was not exposed to *Bd*, but had its cutaneous bacterial communities augmented with the anti-*Bd* bacterium *J. liv*. The final experimental group (referred to as the combo group), which contained the final six salamanders, was exposed to *Bd* and had its microbial communities augmented with *J. liv*.

Table 1. Experimental Groups Identification for *P. cinereus* Experiment.

Group Name	Number of Salamanders
Control	5
Bd	7
J. liv.	6
Bd/*J. liv*	6

Exposure to *Batrachochytrium dendrobatidis*

A strain of *Batrachochytrium dendrobatidis* (Jel 310) was obtained from James Madison University. *Bd* culture and harvesting were performed using the protocol of Rollins-Smith et al. (2002). Zoospore concentrations were determined via the use of a hemocytometer. The salamanders were exposed to 9.11 x 10^6 *Bd* zoospores in a five mL solution of Provasoli in 50 mL Falcon tubes (Becton Dickinson, Franklin Lakes, New Jersey) for eight hours three days prior to probiotic inoculation as per the protocol described by Becker et al. (2009). Salamanders that were not inoculated with *Bd* were exposed to sterile Provasoli medium under the same conditions. Falcon tubes were turned every 30 minutes to ensure exposure.

Bioaugmentation with *Janthinobacterium lividum*

J. lividum was grown in 1% tryptone (Becton Dickinson, Sparks, Maryland) broth for 48 hours at 26 ^{o}C. Cells were centrifuged at 4500 x g for 10 minutes and resuspended in Provasoli medium. To eliminate the presence of anti-*Bd* metabolites in the bacterium bath, the cells were centrifuged and resuspended in Provasoli medium three additional times. The quantity of cells was determined via spectrophotometry. Salamanders in the positive bioaugmentation groups were bathed in a solution of approximately 1x10^8 cells of *J. liv*. in a five mL solution of Provasoli medium for two hours. Control salamanders that did not have augmented microbial communities were exposed to sterile Provasoli medium under the same conditions. Tubes were rotated every 30 minutes to ensure salamanders were exposed to the bacterium.

Mass, mortality and behavior

All salamanders were weighed to the nearest tenth of a gram on the day of *J. lividum* inoculation and each time that the metabolic rate was measured every five days. Mortality and behavioral changes such as limb lifting and buccal pumping were recorded as qualitative observations (Peterson, 2012). Proportion of mean body mass lost in all salamanders was statistically determined through the use of single factor analysis of variance (ANOVA) using Microsoft Excel.

Bd and J. liv. genomic detection

Salamanders were swabbed on the day of bacterial and/or probiotic inoculation and every time metabolic rate was measured. Swabs were used to detect *Bd* and *J. lividum* concentration using real time polymerase chain reaction (rt-PCR) methods. Each salamander was swabbed ten times up and down its belly and five times on one front and one hind leg to ensure adequate retrieval. Fungal and bacterial DNA isolation were performed using the DNeasy Blood and Tissue Kit using the manufacturers protocol (Qiagen). *Bd* zoospore equivalents were determined with genomic equivalent standards ranging from 10^4 to 1 using the protocol of Boyle et al. (2004) with the Taqman probe ChytrMGB2 5' CGAGTCGAACAAAAT 3'. The primers used for real-time PCR assays amplify a 95 base fragment between the forward primer ITS1-3 (Invitrogen) Chytr 5' CCT TGA TAT AAT ACA GTG TGC CAT ATGTC 3' located in ITS1 and the reverse primer 5.8S Chytr 5' AGC CAA GAG ATC CGT TGT CAAA 3' located in 5.8S (Boyle et al., 2004). The following modifications were applied: each cocktail consisted of 12 µL

of Taq supermix, 2.2 µL of forward primer, 2.2 µL of reverse primer, 0.6 µL of Taq probe, 2.5 µL of H_2O, and 20 µL of extracted DNA totaling 40 µL. 5 µL of the cocktail were used for each rt-PCR reaction.

J. lividum densities were determined with genomic equivalent standards ranging from 10^5 to 1 using the protocol of Muletz et al. (2012) with a SYBR-Green assay. Following Muletz et al. (2012) the forward primer JlivViolaceinF (Eurofins MWG Operon) 5'-TACCACGAATTGCTGTGCCAGTTG-3' at a concentration of 67.5 nmol and the reverse primer JlivViolasceinR 5'-ACACGCTCCAGGTATACGTCTTCA-3' at a concentration 57.7 nmol were used in a cocktail to detect the genomic presence of *J. liv* with the following modifications. Each cocktail for detection consisted of 12.5 µL of SYBR green, 1 µL each of forward and reverse primers, 5.5 µL of H_2O, and 20 µL of extracted DNA totaling 40 µL. 5 µL of the cocktail were used to fill each well before rt-PCR was performed. All rt-PCR measurements were performed in duplicate on a Bio-Rad iQ5 Multicolor Real-Time PCR Detection System.

Metabolic Rate

Oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were measured by flow through respirometry using a Sable System Field Metabolic System (FMS) following the protocol of Sheafor et al. (2000). Salamanders were placed in a 20 mL chamber held at 20^{o}C. Humidified air was pulled through the chamber at a flow rate of 40 mL per minute using a mass flow controller on the FMS. Salamanders were acclimated to the chamber for 30 minutes prior to data acquisition. Barometric pressure, water vapor pressure, percent of CO_2, and percent of O_2 of the gas exiting the chamber

were measured by the FMS for a minimum of thirty minutes, and these data were used to calculate mass specific carbon dioxide production (VCO_2), oxygen consumption (VO_2) and respiratory quotient (RQ) following the protocol of Withers (1977). Metabolic rate data collection and analysis were performed using ExpeData software (Sable Systems). Metabolic rates were determined for each salamander every five days over a twenty-five day span.

Results

The first objective of this study was to analyze the mean mass of each experimental group (*Bd*, *J. liv*., combo, and control) over a 25 day span and determine if weight loss across groups was statistically different using an analysis of a single variable test, ANOVA. The calculated p-value was recorded as 0.757. The calculated F value was recorded as 0.396 and was compared to the critical F value recorded as 3.23. The alpha value was set to a 0.05 confidence level. A visual diagram of the mean net weight loss and ANOVA results are provided, respectively (Figure 2, Table 2).

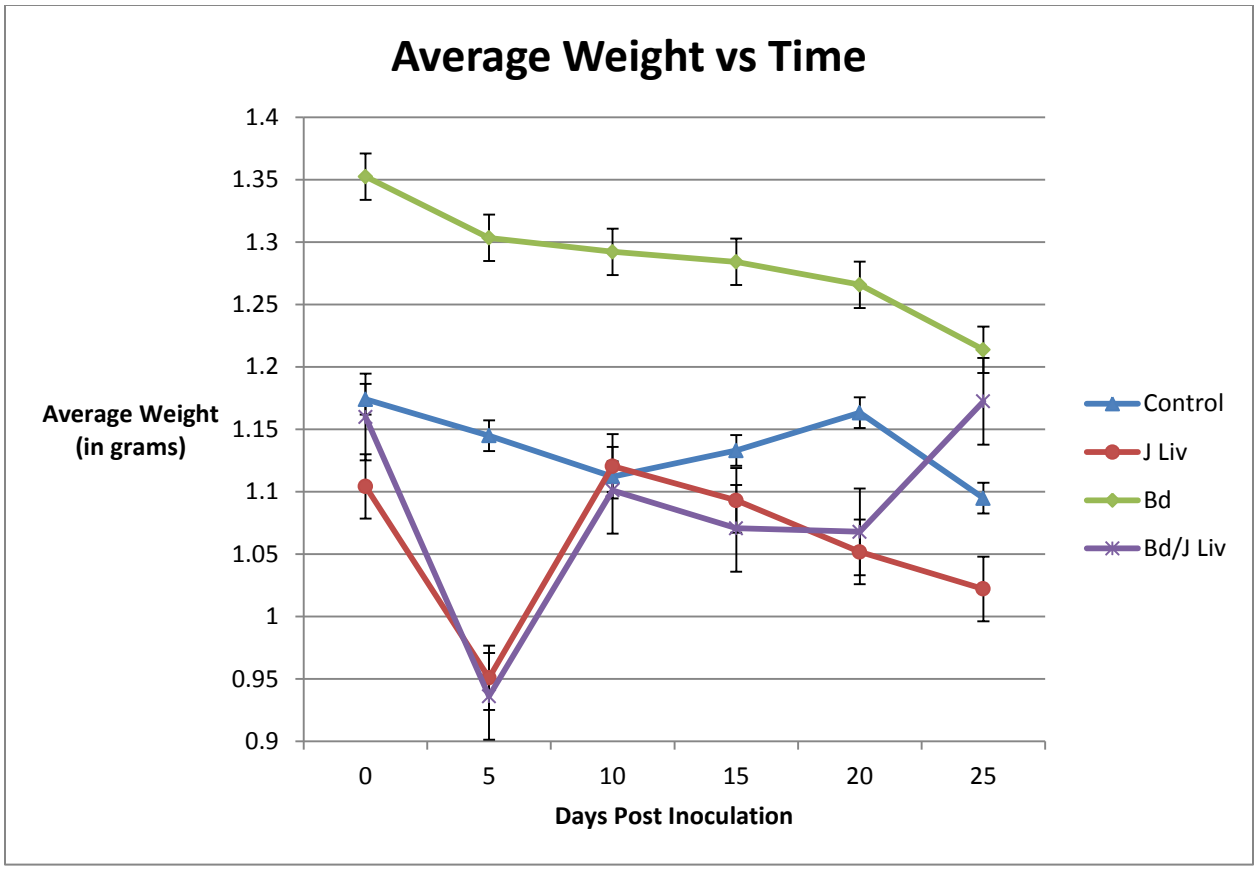

Figure 1. Mean weight loss change for each experimental group over the 25 day span with standard error bars.

Table 2. Analysis of a single variable test used to compare mean net weight loss among each experimental group.

Anova: Single Factor				
SUMMARY				
Groups	*Count*	*Sum*	*Average*	*Variance*
Control	5	-0.3517	-0.07034	0.00201883
Bd	5	-0.39837	-0.079674	0.00245975
J Liv	5	-0.20911	-0.041822	0.00218301
Bd/J Liv	5	-0.3913	-0.07826	0.00901243
ANOVA				
Source of Variation	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.00155234	0.39615756	0.7575381	3.23887152
Within Groups	0.0039185			

The second objective of this study was to quantify the mean amount of *Bd* zoospores (Table 3) and *J. liv.* bacteria (Table 4) using real-time PCR amplification to determine if salamanders were successfully infected and bioaugmented.

Table 3. Genomic equivalence of zoospores for each group to detect presence of *Bd*.

	control	**Bd**	**J. Liv.**	**Bd/J. liv.**
day 0	0	0	0	0
day 5	0	83.8373	0	11656.5
day 10	0	4.53242	0	1.514866
day 15	0	0	0	0
day 20	0	0	0	0

Table 4. Genomic equivalence of *J. Lividum* bacteria using real-time PCR amplification. "Day 20" does not possess data due to computer crash which wiped saved information.

	control	Bd	J. Liv.	Bd/J. Liv.
day 0	0	224.0903	0	0
day 5	0	3422.983	36586.7	206.9
day 10	0	1332.4	34080	41968
day 15	0	71.43	12518.67	2907.52
day 20	n/a	n/a	n/a	n/a

The third objective of this study was to measure metabolic data (VO_2 and VCO_2) using the Field Metabolic System for each salamander over a 25 day span; these data were then compared to mass change and amount of infectivity for each experimental group. I hypothesized that chytridiomycosis would decrease cutaneous gas exchange in Red-backed Salamanders, and that bioaugmentation would alleviate this metabolic decline by inhibiting fungal growth of *Bd*.

The first metabolic parameter was mean oxygen consumption, VO_2 measured in microliters per hour (Table 5). The *Bd* group displayed the highest mean oxygen consumption, but also had the largest standard error in the experiment. The control, *J. liv.*, and *Bd/J. liv.* groups followed in oxygen consumption, respectively. Figure 2 visually demonstrates the changes in VO_2 measurements over the 25 day span.

Table 5. Summary of mean metabolic oxygen consumption for each group over the entire 25 day span of the experiment.

Group	VO_2 (µL/hr)	std error
Control	115.4654	14.27216
J. liv.	110.1563	15.99344
Bd	135.2888	31.96015
Bd/J. liv.	106.3068	11.56416

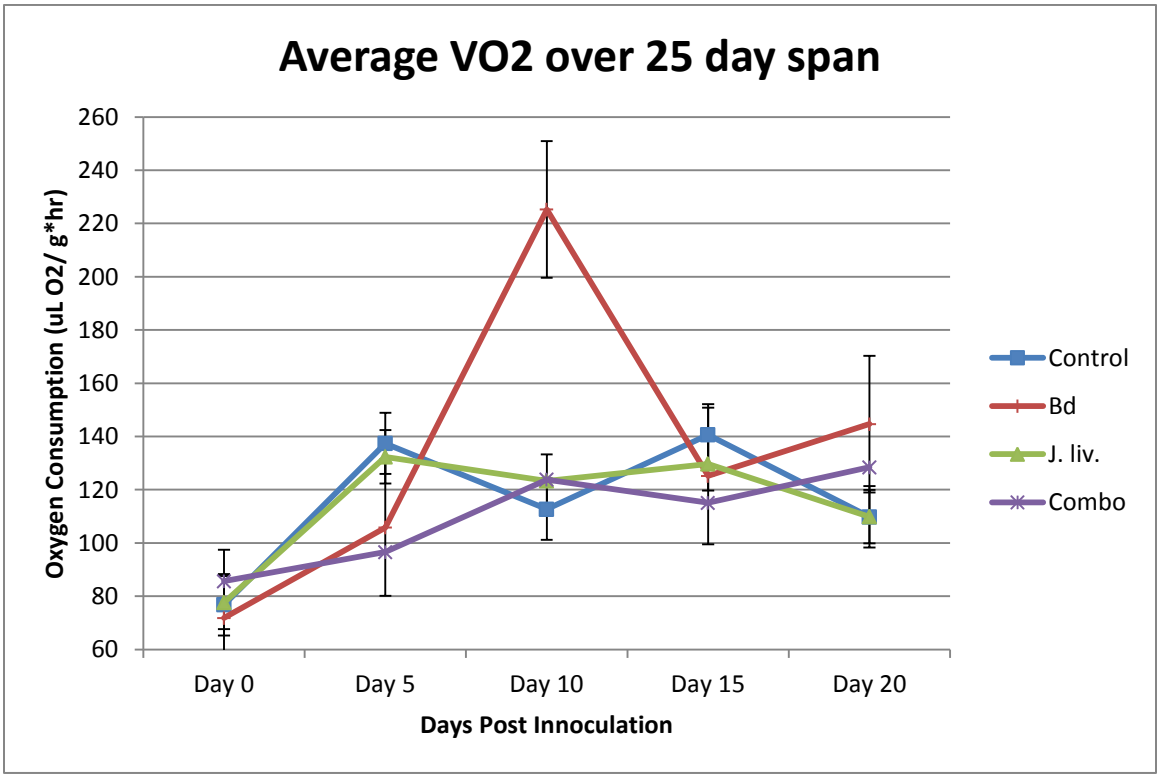

Figure 2. Plot of average VO_2 measurements for each group temporally with standard error bars. Measurements were made on day of inoculation and days post inoculation. Oxygen consumption is recorded in microliters of O_2 per gram*hour. Please note that different colors and shapes represent the different experimental groups.

The second metabolic parameter was mean carbon dioxide production, VCO_2 measured in microliters per hour (Table 6). The *Bd* group displayed the highest mean carbon dioxide production, but also had the largest standard error in the experiment. The *Bd/J. liv.*, *J. liv.*, and control groups followed in carbon dioxide production, respectively. Figure 3 visually demonstrates the changes in VCO_2 measurements over the 25 day span. Figure 4 visually summarizes the average metabolic rates for both VO_2 and VCO_2 for the entire 25 day span.

Table 6. Summary of mean metabolic carbon dioxide production for each group over the entire 25 day span of the experiment.

Group	VCO2 (µL/hr)	std error
Control	92.50359	10.33446
J. liv.	89.96192	13.74496
Bd	112.0944	32.41634
Bd/J. liv.	94.9258	12.85698

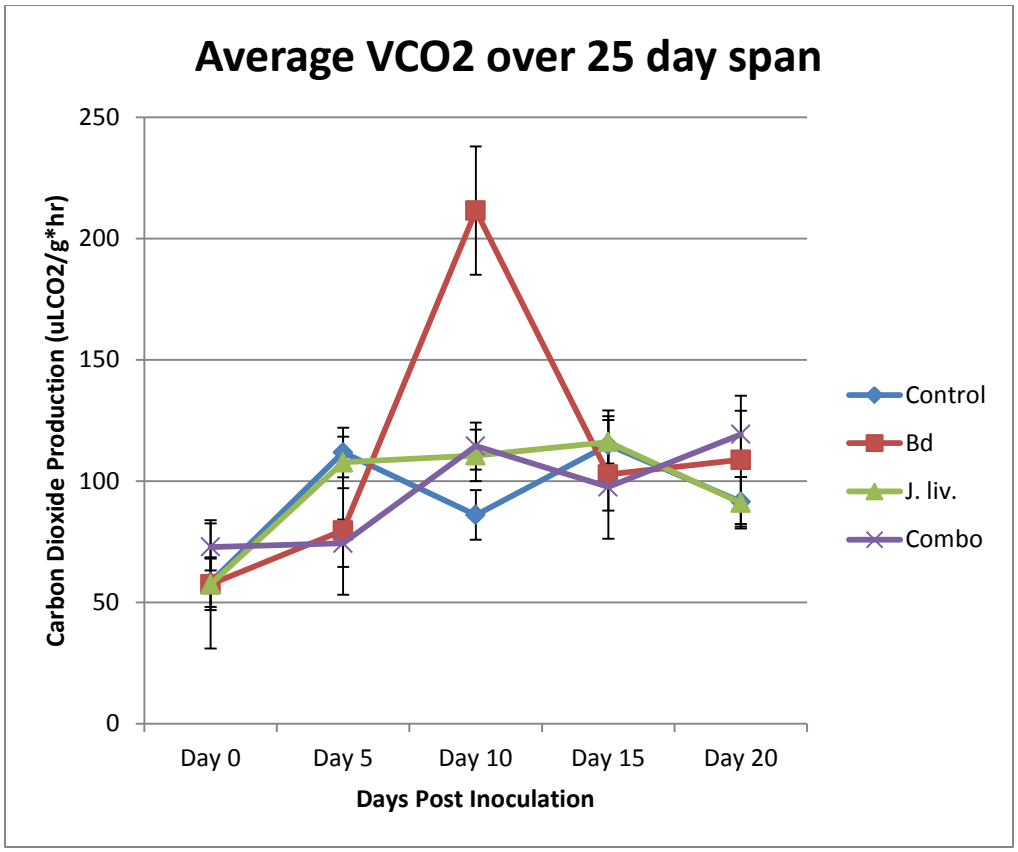

Figure 3. Plot of average VCO_2 for each group temporally with standard error bars. Carbon Dioxide production is recorded in microliters of CO_2 per gram*hour.

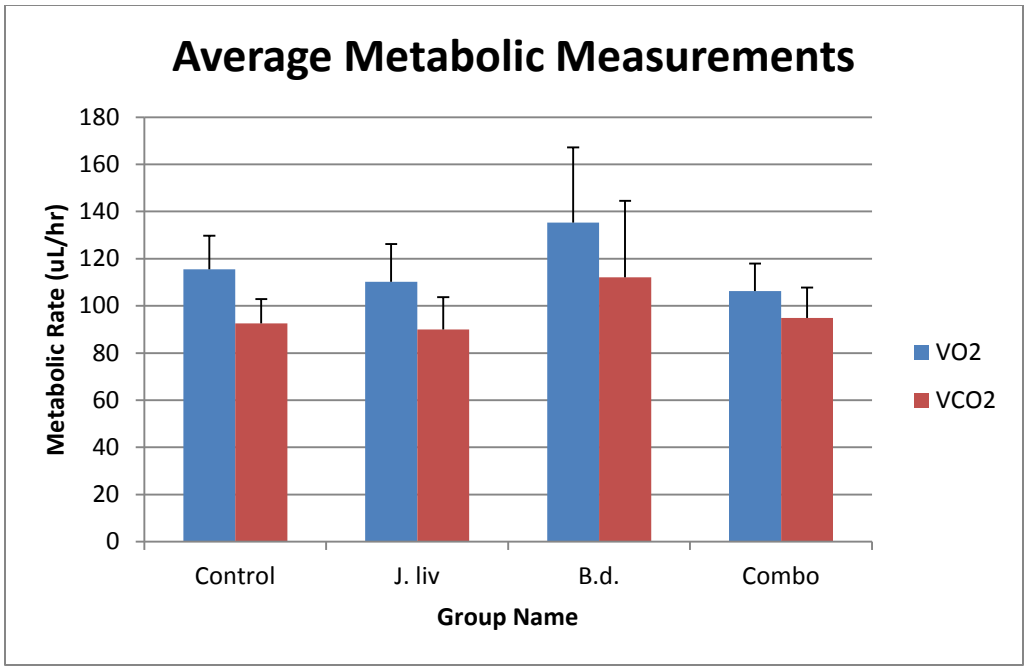

Figure 4. Average metabolic measurements of VO_2 and VCO_2 were calculated over the entire 25 day span. Metabolic rates were measured in microliters of gas per hour.

Table 7 Summarizes the important results of this study.

Table 7. Summary of important results. The numbers "1-4" are used to indicate placement; 1 being the largest. The final row summarizes possible alleviation of *Bd* infection with n/a (not applicable) and yes/no responses.

	Control	***Bd***	***J. liv.***	***Bd/J. liv.***
Largest Mean Oxygen Consumption (VO_2)	2	1	3	4
Largest Mean Weight Change	2	1	3	4
Indication of clearing of *Bd* infection?	n/a	yes	n/a	yes

Discussion

The objectives of this study were to 1) analyze the change in mass of Red-backed Salamanders as they are infected with *Bd*, inoculated with the probiotic *J. liv.*, and inoculated with both, and compare all masses to the control group, 2) quantify infection and probiotic load on the skin of each salamander using real-time PCR techniques, and 3) successfully record metabolic measurements over a 25 day span for each salamander and compare mean metabolic results to possible changes in mass and infection load.

This study supports the results of Becker and Harris (2010) in that salamanders infected with *Bd* demonstrate greater mean weight loss, and cutaneous bacteria on *P. cinereus* may be important for protecting weight loss, a non-lethal symptom of chytridiomycosis. However, this study fails to confirm these results on a statistically significant level. Body mass of *Bd*-infected salamanders decreases over time when compared with *Bd/J. Liv.* and control groups (Becker and Harris, 2010). Given the data obtained, the *Bd* group visually demonstrated the largest mean net loss of weight when compared with the other groups. However, the ANOVA test results suggest that there is no statistical difference among the *Bd* group and others. The lack of mortality and loss of infection is likely due to components of the innate defense system other than anti-*Bd* bacteria (*J. Liv.*), such as antimicrobial peptide secretions and behavioral changes as suggested by Becker and Harris (2010). A number of species possess antimicrobial peptides and can inhibit *Bd in vitro* (Rollins-Smith and Conlon, 2005).

Analysis of *Bd* rt-PCR results suggests that genomic equivalence of *Bd* zoospores were detected on *Bd* and *Bd/J. Liv.* groups. These data suggest that these two groups were

successfully infected with *Bd*, but their genomic equivalence of zoospores detected over the 25 day span decreased over time. The low number of zoospore equivalents found on salamanders suggests that the infection may have subsided before the salamanders' skins were assayed on day 15. Therefore, as determined by real-time Taqman PCR, salamanders infected with *Bd* may have been able to clear all infections by day 20. In a comparable infection experiment with *P. cinereus*, infection intensities with low zoospore equivalents were found at day 14, suggesting that the pathogen is quickly cleared from the skin in this species of salamander (Harris et al., 2009). Further research should consider longer inoculation times with a higher concentration *Bd* per mL to severely slow clearing of infection. Further research should also acknowledge the possibility of PCR inhibitors that inhibit the detection of *Bd* in rt-PCR and use current laboratory techniques to minimize these effects to ensure adequate quantitative detection (Kosch and Summers, 2013).

Analysis of *J. Liv.* rt-PCR results suggests that genomic equivalence of *J. Liv.* were successfully detected on *J. Liv* and *Bd/J. Liv.* groups. Results also suggest contamination of genomic product in *Bd* wells (Table 4). If this experiment is to be repeated, one should be careful to avoid contamination of genomic product in wells while preparing for rt-PCR. The most genomic product was detected on salamanders in the *J. liv.* and *Bd*/*J. liv.* groups. This indicates that these two groups were probably bioaugmented with *J. lividium* bacteria successfully. Interestingly, the combo group possessed no deaths of its six salamanders. However, the *J. liv.* group had one mortality—salamander 12 at day 25. Becker et al. (2009) suggests that *J. liv.* possesses anti-*Bd* microbiotas that protect *P. cinereus* salamanders from disease symptoms and are

an important component of the innate immune system. However, this thesis cannot make any further assumptions regarding the effect of *J. liv.* bioaugmentation until this experiment has been performed again with data from all critical days past inoculation.

Field Metabolic System results indicate that the largest overall mean VO_2 consumption with the greatest amount of variance (standard error) belonged to the *Bd* group, but I fail to reject the claim that this result is statistically significant. Thus, despite the visually apparent increase in oxygen consumption, the mean oxygen consumption of the *Bd* group is not statistically significant compared to the rest of the groups. As cited from Becker et al. (2009), successful chytridiomycosis infection in *P. cinereus* may generate an innate immune response, thus the salamander may increase the rate of buccal pumping and metabolic consumption to adjust for this stress.

In regards to overall mean VCO_2 production, salamanders in the *Bd* group also produced the highest amount of carbon dioxide along with the greatest amount of variance. This analysis of carbon dioxide production is comparable to that of studies cited in the previous paragraph.

Interestingly, the temporal figures that plot VO_2 and VCO_2 at five day time intervals over the 25 day span (Figures 2 and 3 respectively)—especially at day 10. For the time between day 5 and day 10 of the PCR genomic equivalence graph of *Bd*, the amount of DNA detected for *Bd* appeared to decrease, but was still detected. At day 10 when the metabolic measurement took place for the *Bd* group, the VO_2 and VCO_2 recordings were calculated to be the highest rates of metabolic gas exchange compared to the other groups (VO_2 = 225.2 µL/g*hr and VCO_2 = 211.5 µL/g*hr, respectively). The

subsequent *Bd* recordings then proceeded to level with the rest of the groups. The question that arises is: when *Bd* has colonized on the skin of *P. cinereus*, is there a response that is responsible for elevated gas exchange rates? Also, *Bd* genomic equivalence was detected on the combo group between days 5 and 15 with the highest genomic equivalence detected at day 10 (Table 4). But, figures 2 and 3 demonstrate no spike to the combo group at day 10 of metabolic measurement as it did for the *Bd* group. Thus, does the addition of *J. liv.* to the salamander alleviate a metabolic spike that may have been caused by *Bd* infection? Future research must be conducted to properly calibrate the experimental set up so that these questions may be answered in the future.

For future reference, several laboratory changes need to be made to the Field Metabolic System construct. Because the very small deflections in respiratory gasses were being measured (0.001%), the determination of *P. cinereus* metabolic rates may have been affected by the presence of breathing from researchers in the same room as the FMS system. Future runs should be performed in an isolated environment with no other sources of respiration. Moreover, the metabolic measurement run time for each salamander should also be increased to two hours to ensure further accurate data acquisition. Also, future experiments should consider using a larger species of lungless salamander susceptible to *Bd*; this may allow easier metabolic consumption measurements that would not be as easily affected by oxygen fluctuations caused by the external environment (e.g., researchers breathing in the same room as the FMS).

The original hypothesis stated that chytridiomycosis will decrease overall rate of respiratory gas exchange in *P. cinereus*, and bioaugmentation with the anti-fungal bacterium *J. liv.* will correct this decreased metabolic rate. The null hypothesis being that

there is no difference in overall metabolic rate when salamanders are infected with *Bd* and/or bioaugmented with *J. liv.* The results observed in this study fail to reject the null hypothesis on a statistically significant level. Further research should adjust the original hypothesis to state: It is hypothesized that chytridiomycosis will increase rate of respiratory gas exchange because of the possible glycogen/glucose metabolism. Once this has been performed with proper laboratory adjustments, the next step may add the effect of bioaugmentation with bacteria in the examination of metabolic rate and chytridiomycosis.

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References

Austin R (2000). Cutaneous microbial flora and antibiosis in *Plethodon ventralis*. In: *The biology of plethodontid salamanders*, Bruce, R, Jaeger, R, Houck, L, eds. New York: Kluwer Academic/Plenum Publishers. pp 451-462.

Andre SE, Parker J, and Briggs CJ (2008). Effect of Temperature on Host Response to *Bactrachochytrium dendrobatidis* Infection in the Mountain Yellow-legged Frog (*Rana muscosa*). Journal of Wildlife Diseases 44 3: 716-720.

Becker M, Brucker R, Schwantes C, Harris R, and Minbiole K (2009). The Bacterially Produced Metabolite Violacein Is Associated with Survival of Amphibians Infected with a Lethal Fungus. American Society for Microbiology 21 75: 6635-6638.

Becker M and Harris R (2010). Cutaneous Bacteria of the Redback Salamander Prevent Morbidity Associated with a Lethal Disease. Public Library of Science One 5.

Belden L and Harris R (2007). Infectious disease in wildlife: the community ecology context. Frontiers in Ecology Environment 5 10: 533-539.

Berger L and Speare R (1998). Chytridiomycosis—a new disease of amphibians. Australian & New Zealand Council for the Care of Animals in Research and Teaching News 11: 1-3.

Berger L, Speare R, and Hyatt AD (1999). Chytrid fungi and amphibian declines: overview, implications and future directions. In: Campbell A (ed) Declines and disappearances of Australian frogs. Environment Australia, Canberra, p 23-33.

Blaustein A, Gervasi S, Johnson P, Hoverman J, Belden L, Bradley P, and Xie G (2012). Ecophysiology meets conservation: understanding the role of disease in amphibian population declines. Philosophical Transactions of the Royal Society B 367: 1688-1707.

Bosch J, Carrascal LM, Duran L, Walker S, and Fisher MC (2007). Climate change and outbreaks of amphibian chytridiomycosis in a montane area of Central Spain; is there a link? Proceedings of the Royal Society B 274: 253-260.

Boyle DG, Boyle DB, Olsen V, Morgan JAT, Hyatt AD (2004). Rapid quantitative detection of chytridiomycosis (Batrachochytrium dendrobatidis) in amphibian samples using real-time Taqman PCR assay. Diseases of Aquatic Organisms 60: 141-148.

Collins, J (2010). Amphibian decline and extinction: What we know and need to learn. Disease of Aquatic Organisms 92: 93-99.

Collins J and Storfer A (2003). Global amphibian declines: Sorting the hypotheses. Diversity and Distributions 9: 89-98.

Crawford A, Lips K, and Bermingham E (2010). Epidemic disease decimates amphibian abundance, species diversity, and evolutionary history in the highlands of central Panama. Proceedings of the National Academy of Sciences of the United States of America 107: 13777-13782.

Feder, M (1976). Lunglessness, body size, and metabolic rate in salamanders. Physiological Zoology 49 4: 398-406.

Harris R, Lauer A, Simon M, Banning J, and Alford R (2009). Addition of antifungal skin bacteria to amphibians ameliorates the effects of chytridiomycosis. Diseases of Aquatic Organisms 83: 11-16.

Kriger K and Hero JM (2007). The chytrid fungus *Batrachochytrium dendrobatidis* is non-randomly distributed across amphibian breeding habitats. Diversity and Distributions 13: 781-788.

Kosch TA and Summers K (2013). Techniques for minimizing the effects of PCR inhibitors in the chytridiomycosis assay. Molecular Ecology Resources 13 2: 230-236.

Lips K, Brem F, Brenes R, Reeve J, Alford R, Voyles J, Carey C, *et al*. (2006). Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. Proceedings of the National Academy of Science of the United States of America 103 9: 3165-3170.

Lips K, Diffendorfer J, Mendelson III JR, and Sears MW (2008). Riding the Wave: Reconciling the Roles of Disease and Climate Change in Amphibian Declines. Public Library of Science 6 3: 441-454.

Muletz C, Myers M, Domangue R, Herrick J, and Harris R (2012). Soil bioaugmentation with amphibian cutaneous bacteria protects amphibian hosts from infection by *Batrachochytrium dendrobatidis*. Biological Conservation 152: 119-126.

Ouellet M, Mikaelian I, Pauli B, Rodrigue J, and Green D (2005). Historical Evidence of Widespread Chytrid Infection in North American Amphibian Populations. Conservation Biology 19 5: 1431-1440.

Peterson, J (2012). Physiological effects of chytridiomycosis, a cause of amphibian population declines. PhD dissertation. Auburn University, Auburn Al.

Pessier AP, Nichols DK, Longcore JE, and Fuller MS (1999). Cutaneous chytridiomycosis in poison dart frogs (*Dendrobates spp.*) and White's tree frogs (*Litoria caerulea*). Journal Veterinary Diagnostic Investigation 11: 194-199.

Piotrowski J, Annis S, and Longcore J (2004). Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. Mycologia 96: 9-15.

Pullen KD, Best A, and Ware J (2010). Amphibian pathogen *Batrachochytrium dendrobatidis* prevalence is correlated with season and not urbanization in central Virginia. Diseases of Aquatic Organisms 91: 9-16.

Rachowicz LJ, Knapp RA, Morgan JAT, Stice MJ, Vredenburg VT, Parker JM, and Briggs CJ (2006). Emerging infectious disease as a proximate cause of amphibian mass mortality. Ecology 87: 1671-1683.

Rollins-Smith LA, Carey C, Longcore J, Doersam JK, Boutte A, Bruzgal JE, *et al*. (2002). Activity of antimicrobial skin peptides from ranid frogs against Batrachochytrium dendrobatidis, the chytrid fungus associated with global amphibian declines. Developmental & Comparative Immunology 26: 471-479.

Rollins-Smith LA and Conlon JM (2005). Antimicrobial peptide defenses against chytridiomycosis, an emerging infectious disease of amphibian populations. Developmental & Comparative Immunology 29: 589-598.

Sheafor E, Wood S, and Tattersall G (2000). The effect of graded hypoxia on the metabolic rate and buccal activity of a lungless salamander (*Desmognathus fuscus*). The Journal of Experimental Biology 203: 3785-3793.

Vazquez VM, Rothermel BB, and Pessier AP (2009). Experimental infection of North American plethodontid salamanders with the fungus *Batrachochytrium dendrobatidis*. Diseases of Aquatic Organisms 84: 1-7.

Voyles J, Young S, Berger L, Campbell C, Voyles WF, Dinudom A, Cook D, *et al*. (2009). Pathogenesis of Chytridiomycosis, a Cause of Catastrophic Amphibian Declines. Science 326: 582-585.

Wake D and Vrendenburg V (2008). Are we in the midst of the sixth mass extinction? A view from the world of amphibians. PNAS 105: 11466-11473.

Withers, PC (1977). Measurement of VO_2, VCO_2, and evaporative water loss with a flow-through mask. Journal of Applied Physiology: Respiratory, Environmental, and Exercise Physiology. 42: 120-123.