**The influence of antimicrobial peptides, cutaneous microbial communities and water quality on the susceptibility of Columbia spotted frogs (*Lithobates luteiventris*) to Chytridiomycosis**

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**Abstract:**

Chytridiomycosis is a potentially deadly skin disease found in amphibian populations worldwide. The disease is caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*) which grows on the skin of amphibians. The fungus can disrupt the homeostatic functions of amphibian skin and can lead to death. An amphibian's antimicrobial skin secretions, cutaneous microbial communities and the environmental water quality have the potential to influence resistance to *Bd* infection. However, few studies have examined how these factors interact to impart resistance. Columbia spotted frogs (*Lithobates luteiventris*) are native to western Montana and can be affected by chytridiomycosis. Three lakes known to be positive for *Bd* were used as collection sites over the course of the summer of 2018. Each frog was swabbed for bacterial and fungal DNA and the frogs were placed in a solution to collect antimicrobial peptides (AMPs) secreted from the skin. Water samples were collected at each site and analyses were performed to assess various environmental parameters. Early results suggest that AMPs may affect the relative abundance of an important anti-fungal bacteria (*Rhizobacter*) which influences *Bd* status. The goal of the ongoing research is to better understand how interactions between skin secretions, cutaneous microbial communities and environmental parameters allow Columbia spotted frogs to resistant chytridiomycosis, and to use this information to better predict and treat future outbreaks.

**Introduction:**

Chytridiomycosis is a deadly amphibian skin panzootic that has caused a significant loss of amphibian biodiversity (Scheele et al., 2019). According to Science Magazine, this disease has caused approximately 90 amphibian species to become extinct

and many others to be placed at high risk (Scheele et al., 2019). Chytridiomycosis is caused by the fungus, *Batrachochytrium dendrobatidis* (*Bd*) or *Batrachochytrium salamandrivorans* (*Bs*). It has been hypothesized that *Bd* originated in Asia about 10,000-40,000 years ago – an exact date or area is still unknown (Collins, 2013). It was not until 1998 that *Bd* was recognized as an infectious pathogen (Scheele et al., 2019).

*Bd* thrives in cool, moist environments in areas of higher elevation and has been successful in its population destruction due to its broad host range and high transmission rate (Norris, 2007). The fungus travels through an aquatic habitat and will then replicate in the microbial communities on the skin of the amphibian. It slowly begins to inhibit the organism's ability to interact with its environment by causing a disruption in the homeostatic functions of the amphibian skin, ultimately leading to death (Rollins-Smith, 2009). Despite the devastating consequences to biodiversity all over the world, there is still hope for resistance. Recent studies suggest that some species' populations have recovered and increased post *Bd* infection due to potential resistance (Scheele et al., 2019). However, the biological mechanism that provides this resistance to *Bd* remains unclear.

It has been estimated that chytridiomycosis is associated with the decline of at least 501 amphibian species (Scheele et al., 2019). This estimate only includes species that are well known and whose population size can be quantified throughout their geographical distribution. The majority of declines have been documented in tropical areas including Australia, Mesoamerica and South America, but there are also notable declines in Asia, Africa, Europe and North America (Scheele et al., 2019). Along with

chytridiomycosis, amphibian population declines have been intensified by habitat loss, changes in the climate and other invasive species (Norris, 2007).

The loss of any component of an environment can cause a shift in the function of the ecosystem. When amphibians disappear from an environment, some ecological effects include uncontrolled algae growth, alterations in nitrogen levels, and shifts in the food web (Norris, 2007). Due to the expansion of chytridiomycosis across the globe, drastic steps need to be taken in order to have any hope of changing the current fate of amphibian populations. Conservation estimates made for all vertebrate species by the Alliance for Zero Extinction show that on average, the cost to save a species is $1.3 million (Conde, 2015). Hypothesized conservation efforts often include preserving a species in captivity for population insurance (Norris, 2007). However, keeping a species from going extinct in captivity does not inherently coincide with saving a species overall. For a species to thrive, they must also survive in the wild (Conde, 2015). To save amphibians from mass extinction and for the populations to grow, a process of enhancing resistance to chytridiomycosis needs to be discovered.

Amphibians such as Columbia spotted frogs are a key component in the habitats of western Montana. The frogs are distinguishable by bright reddish pigment on the ventral side of their legs and they inhabit wetlands near forest openings or at the tree line (Montana Natural Heritage, 2019). They primarily feed on insects and are preyed upon by fish, larger frogs, and birds (Montana Natural Heritage, 2019). Although Columbia spotted frog populations have not seen substantial declines due to chytridiomycosis, a sudden change in the habitat or climate could increase the probability of infection along with mortality rates (Norris, 2007). Even though Columbia spotted frog populations are

affected by chytridiomycosis, they are currently not at risk of extinction and even demonstrate some resistance to the disease (Sheafor, 2017). This makes them an excellent model organism to survey when researching how to prevent chytridiomycosis extinction in other species (Sheafor, 2017). Insights gained from these studies can be applied to other amphibian populations that are in danger.

The aquatic habitat of the frogs was analyzed by examining nutrient load conditions and physical parameters measured on site. Since amphibians rely on their skin for gas exchange and environmental interaction, the health the environment inhabited by amphibians will affect the health of the amphibian population. Amphibian populations have declined due to chytridiomycosis, but also because of global warming and habitat loss (Conde, 2015). Climate change can increase the likelihood of *Bd* lethality and hence, amphibian death (Norris, 2007). It is important to track any changes seasonally and annually in the habitat conditions of Columbia spotted frogs due the potential declines that may result. While environmental disruption has been linked to increases in *Bd* infection, it has also been shown to affect the microbiota living in an amphibian's cutaneous microbial community (Loudon et al., 2014).

An amphibian's microbial skin community has a symbiotic relationship with the organism. The cutaneous microbial community is made up of bacteria that have been able to live on the amphibian. In a study done on red-backed salamanders (*Plethodon cinereus*), it was discovered that "good" skin bacterium can defend amphibians against chytrid (Loudon et al., 2014). The microbiota identified by Loudon et al. was found to produce antifungal metabolites which may have prevented *Bd* infection in the population surveyed. The core community of bacterium living on the salamander's skin was found to

be independently determined when compared to the bacterial community of the environment the salamander inhabited (Loudon et al., 2014). From this information, it can be inferred that the microbial community of amphibians, including Columbia spotted frogs, is selected for, possibly by the organism's antimicrobial skin secretions.

Antimicrobial peptides (AMPs) are excreted from the skin of Columbia spotted frogs. Not all amphibians secrete AMPs, but this variable may significantly affect the resistance of some amphibian species to chytridiomycosis. AMPs are defensive chemical produced by cutaneous granular glands that are released to initiate immunological protection (Rollins-Smith, 2009). With the knowledge that AMPs are a potential line of defense against *Bd,* Woodhams et al. 2006 studied multiple frog species in Australia that were not declining due to chytridiomycosis and identified the minimal inhibitory concentration (MIC) of AMPs required to kill off an infection of *Bd.* The study showed that, though AMPs do not guarantee protection from infection, they do influence the host-pathogen interactions. Studies performed on in vitro *Bd* infections showed that AMPs effectively combat chytridiomycosis in Columbia spotted frogs (Rollins-Smith, 2009).

In this study, Columbia spotted frog resistance to *Bd* was measured by comparing the water nutrient load conditions, antimicrobial peptide secretions, and microbial communities to quantified *Bd* infections. Since multiple variables were examined, a multi-regression will be performed to look at potential inhibitors and stimulators of *Bd* infection. The primary goal of this research is to identify correlations between water nutrient load and physical parameters, antimicrobial peptide secretions (AMPs), microbial communities, and the *Bd* infection load present on Columbia spotted frogs (*Lithobates luteiventris*) in western Montana. Over the course of a summer, samples were

collected from three pre-determined sites known to be positive for *Bd* infection. At each location, Columbia spotted frogs were sampled for their metabolites, microbial community DNA, and antimicrobial peptides. Water samples were also collected at each location. The relationship between an amphibian's antimicrobial skin secretions, cutaneous microbial communities and environmental water quality were hypothesized to affect its resistance and susceptibility to infection (Sheafor, 2017).

## <u>Methods:</u>

### Field Collection

<u>Location</u>

Over the course of three months (May, June, and July) in 2018, collections were made at each of the three lakes in Western Montana that were pre-selected for sampling. These lakes included Park Lake near Clancy, Gipsy Lake located in the Big Belt Mountains, and Jones Pond located in the Lubrecht Research Forest (Figure 1). Each of these lakes had been confirmed to be positive for *Bd* and were also known habitats for Columbia spotted frogs. Sampling excursions were typically performed in consecutive days at the end of each month.

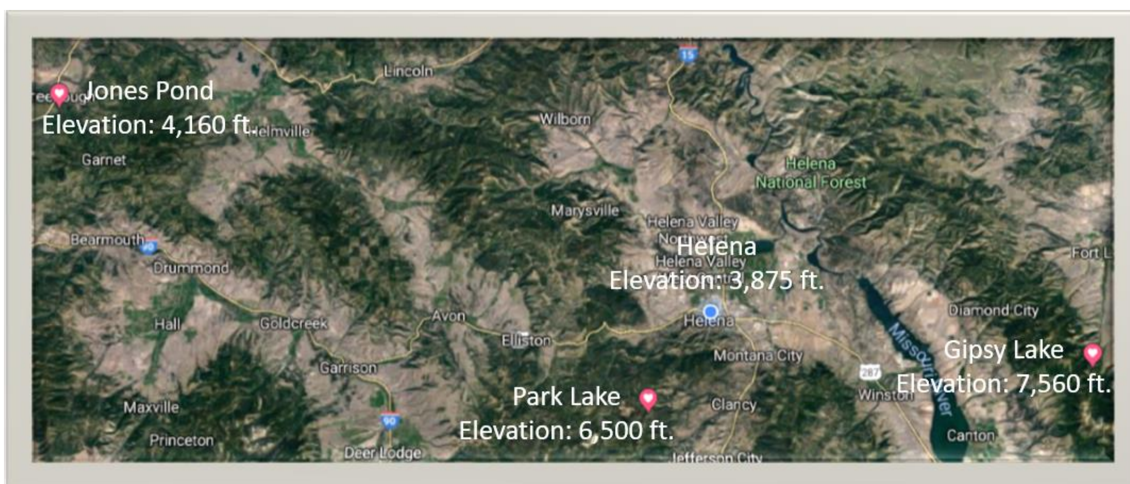

**Figure 1:** Map showing areas of collection sites in Western Montana designated by the red pins. Locations included Park Lake (elevation: 6,500 feet) near Clancy, MT, Gipsy Lake (elevation 7,560 feet) in the Big Belt Mountain range, and Jones Pond (elevation 4,160 feet) located in the Lubrecht Research Forest.

Water Collection

At each lake, three water samples were collected from multiple areas around the lake. Using a 50 cc syringe, 50 mL of water was extracted from three different locations at each lakes' edge. The collected water was then passed through a 0.2 µm filter to collect bacteria from the water. The samples each had 3.62 µL of nitric acid added to balance any elements in the samples from falling out of the solution. The samples were stored on ice until placed in a 4˚C refrigerator to await lab analyses. The filtered water was sealed in an acid washed 50 mL falcon tube which was placed on ice to prevent degradation of the sample for later elemental analysis in the lab. Water parameters were also recorded at every collection site for each lake. These parameters included pH, conductivity (mg/L), temperature (˚C), and dissolved oxygen (mg/L). Each parameter was quantified using a YSI-55 portable DO meter that was capable of measuring each of the required parameters.

Amphibian Capture

Upon arrival at each lake, ten Columbia spotted frogs were captured by researchers wearing nitrile gloves to avoid contamination. Frogs were frequently found either basking by the water's edge or sitting in the water within emergent vegetation. If the frog was muddy or covered in grasses, the hind legs were firmly grasped so the frog could be rinsed off in the lake water to avoid environmental contamination. The frog was

then placed in a sterile container until sampling could be completed. Each frog was weighed prior to swabbing in order to determine the mass (grams) of the specimen.

Swabbing

Frogs were first swabbed for bacterial metabolites using swabs prepared in the lab following the procedure outlined by Umile et al. (2014). The ventral side (either right or left) that was initially swabbed on each frog was determined by the random chance of a flipped coin with heads being right and tails being left. The swabbing procedure consisted of firmly dragging the swab over each section of the frogs, ten times for a full collection. The swabbed sections of the frog include: hand, forelimb, foot (and on the webbing between the phalanges), hind limb, and the abdominal area. After the metabolite swabbing was completed, the tip of the swab was stored in a 2 mL microcentrifuge tube on dry ice to preserve the sample until was placed in the -70˚C freezer in the lab.

The frog was then rinsed with sterile Provasoli medium, a pH neutral solution designed to wash off the transient bacteria attached to the frog. A sterile DNA swab was used to collect DNA from the microbial communities of the frog which would later be analyzed in the lab. The same procedure for swabbing described for metabolite swabbing was used, but the opposite side of the frog from the metabolite swab was sampled. The DNA swab was stored in a sterile container on ice until it was placed in a -20˚C freezer in the lab.

Peptide Collection

Following the swabbing procedures, frogs were placed in 50 mL of collection buffer, that contained 50 mM NaCl and 25 mM sodium acetate (pH = 7.0). To the collection buffer, 500 μL of 20 mM norepinephrine was added in order to stimulate

release of antimicrobial skin secretions. Each frog was left in the solution for fifteen minutes, at which time the solution was collected using a 50 cc syringe with tubing attached so that the tip could reach into the container without releasing the frog. The 50 mL of solution containing peptides was placed into a 50 mL falcon tube and 500 µL of trifluoroacetic acid (TFA) was added to it to charge the peptides. The charged peptide solution was pushed through an activated C18 SepPak to collect the peptides so that they could be transported back to the lab. Before storing the SepPaks on ice, they were washed with 10 mL of 0.1% TFA solution to ensure preservation of the peptides.

**Lab Analyses**

Water Analyses

Water samples collected in the field were preserved and prepped for analyses by the addition of trace metal grade nitric acid ($HNO_3$) to produce a 1% concentration of $HNO_3$ in solution. From each water sample, 25 mL was separately measured in a 25 mL volumetric flask and 362 µL of 70% $HNO_3$ was added to the volumetric flask. This 1% $HNO_3$ sample solution was then analyzed using inductively coupled plasma mass spectrometry (ICP). Each sample was analyzed for the concentration of Fe, Mg, Mn, Na, Ca and K in ppm using a Perkin Elmer Optima 2000DV atomic emission spectrometer following the techniques described by the manufacturer's instructions.

DNA Extraction

DNA from the cutaneous microbial communities was isolated from swabs that were sampled in the field. To extract the DNA from the swabs, an OMEGA bio-tek E.Z.N.A. soil DNA kit was used, following manufacturer's instructions. After an

extraction was performed, samples were tested using a Thermo Scientific Nano Drop Lite to confirm the presence of DNA following manufacturer's instructions.

*Bd* Quantification

After each DNA sample was extracted, *Bd* DNA quantification was performed using a Bio Rad iQ5 for quantitative polymerase chain reaction (qPCR). Procedures by Annis et al. (2004) were used to determine the copy number of *Bd* present in each sample. This allowed for the determination of whether or not each frog was infected and, if they were, to what degree. With this technique, the levels of *Bd* copy number were determined and monitored over the course of the summer.

Peptide Quantification

Peptides collected from each frog were extracted using the methodology of Sheafor et al. (2008). These procedures included eluting the peptides collected in the field by pushing 10 mL of a 70% acetonitrile and 0.1% TFA solution, through each SepPak. The process of pushing 0.1% TFA solution, peptide solution, and the acetonitrile solution through the SepPak was repeated to obtain a total of 20 mL of collected peptide solution. Each solution had to be pushed through the SepPaks at approximately 2 mL per minute and was accomplished using syringe pump.

The 20 mL of extracted peptides from each frog was then concentrated down to approximately 2 mL using an Acid-Resistant CentriVap Concentrator that was attached to a -50˚C CentriVap Cold Trap. The cold trap prevented any acid in solution that was being evaporated off in the CentriVap from damaging the pump. The peptides collected from each individual frog were initially in two separate 10 mL falcon tubes to fit into the CentriVap rotor, but eventually combined. Depending on the peptide solution

concentration, it would take approximately four hours to spin the 20 mL of peptide solution down to 2 mL. The concentrated peptide solution of each frog was stored in a 2 mL Axygen microtube to prevent the proteins from sticking to the microtube, and in turn remain in solution.

The protein concentration of each frogs' peptide solution was quantified using a Micro BCA protein assay. A Bradykinin standard series was produced by Emma Esposito (2018). From the kit instructions the microplate procedure was used that used 50 μL of working reagent and 50 μL of peptide sample added to each well of a 96 micro-well plate in replicates of 3. The prepared 96 micro-well pates were then measured at the absorbance of 562 nm using a microplate reader. The quantified protein concentrations were used when growth inhibition assays were performed.

Chytrid Bioassays

The strain of chytrid (PTHO2) was originally collected from British Columbia by Dr. Joyce Longcore and has been repeatedly cultured at Carroll College for testing the potency of peptides produced by Columbia spotted frogs. The strain was re-cultured every week in 20 mL of 1% tryptone solution. Agarose plates were made to form cultures from which zoospores were collected for testing. Spread plating was used to place the chytrid onto the agarose for development. At the point in development when zoospores were most prevalent, the plates were flooded with 2% tryptone and left to sit for ten minutes whiles the zoospores collected in the concentrated medium. After the ten minute period the plate was tilted on its side so the broth could collect at the bottom. After three minutes, the broth was pipetted out and placed into a 15 mL falcon tube. The zoospores

were counted on a hemocytometer and diluted to between $5 * 10^4$ and $5 * 10^5$ zoospores/mL.

A dilution series was made from each peptide collection, with concentrations of: 900 µg/mL, 800 µg/mL, 700 µg/mL, 600 µg/mL, 500 µg/mL, 400 µg/mL, 300 µg/mL, 200 µg/mL, and 100 µg/mL. Fifty microliters of these dilutions were plated in a 96 well plate in replicates of three and combined with 50 µL of zoospore solution. The prepared microplates were measured at an absorbance of 492 nm in a microplate reader. Procedures from Sheafor et al. (2008) for chytrid growth inhibition assays were used to determine the minimum peptide concentration required for the frog to be able to fight off a *Bd* infection (MIC).

Outsourced Analyses

Bacterial communities were analyzed via Amplicon sequencing by Dr. Andrew Loundon of the Cleveland Metroparks Zoo in Ohio. Bacterial metabolite analyses were performed using HPLC/UV-Vis techniques by Dr. Kevin Minbiole and Dr. Tom Umile of Villanova University.

**Statistical Analyses**

Microsoft Excel was used to perform all statistical analyses. Averages were calculated for all sampled variables in each lake and for each month. These variables included: frog weight, water parameters (pH, conductivity, temperature, and dissolved oxygen), water nutrient concentrations, *Bd* load, and protein concentrations. The regression analyses were performed with the independent variable as either a water parameter, water nutrient concentration, or protein concentration and the dependent variable was the corresponding *Bd* load. Significance between any correlation that was

found was determined by the x-variable's p-value calculated by the regression analysis. Significance between the amount of *Bd* load in each lake seasonally was determined by a two-sample t-test assuming unequal variances. MIC calculations and analyses were performed following the procedure outlined by Esposito (2018) and Woodham et al. (2006). Calculations and figures concerning microbial bacterial communities were completed by Dr. Andrew Loudon using methods from Loudon et al., 2014. Figures and tables were developed from the data analyses performed in Microsoft Excel.

## Results:

### Water Analyses:

Water parameter averages were calculated from samples taken out in the field for each month of the season and in each lake that was studied (Table 1). Each water parameter average was run in a regression analysis with the *Bd* load for each corresponding month and lake. There was not any significance found between the water parameters and the *Bd* load. Lack of significance was determined by p-values. The p-values calculated were 0.081 for temperature, 0.375 for dissolved oxygen, 0.119 for pH, and 0.886 for conductivity. Each p-value was greater than 0.05 and hence was not significant with a confidence level of 95%. Each p-value was rounded to the closest thousandths place.

**Table 1:** Calculated water mean parameters (temperature, dissolved oxygen, pH, and conductivity) for Park Lake, Gipsy Lake and Jones Pond over the course of the summer for the months of May, June and July. Calculations were performed using Microsoft Excel. Positive and negative data differentiation was calculated using the standard error of the data.

| Time | Water ID | Water Temp (˚C) | DO (mg/L) | pH | Conductivity (mg/L) |
|---|---|---|---|---|---|
| May | Park Lake | 12.00±0.60 | 8.05±0.70 | 6.09±0.06 | 64.97±12.32 |
| | Gipsy Lake | 8.47±1.28 | 8.10±0.93 | 6.94±0.29 | 235.60±37.07 |
| | Jones Pond | 20.77±0.03 | 10.55±0.39 | 7.73±0.54 | 114.17±6.12 |
| June | Park Lake | 12.10±0.21 | 72.00±31.61 | 6.36±0.06 | 73.50±12.95 |
| | Gipsy Lake | 11.67±2.64 | 106.27±10.83 | 6.81±0.08 | 245.30±30.35 |
| | Jones Pond | 22.03±0.43 | 209.33±0.39 | 10.34±0.29 | 134.43±1.09 |
| July | Park Lake | 12.43±1.95 | 88.27±1.13 | 7.05±0.27 | 125.20±27.87 |
| | Gipsy Lake | 12.67±2.62 | 86.67±23.02 | 6.90±0.09 | 250.10±21.80 |
| | Jones Pond | 24.00±0.50 | 135.63±17.54 | 9.67±0.26 | 139.53±0.43 |

The average elemental concentrations of each water sample were found using ICP spectral analysis and were compared to each corresponding *Bd* load (Figure 2). Regression analyses were performed for each water nutrient (Fe, Mg, Mn, Na, Ca, and K) vs. *Bd* load to assess significance between *Bd* load and water elemental concentrations. Significance was not found between *Bd* load and Fe (p-value: 0.530), Mg (p-value: 0.735), Mn (p-value: 0.999), Ca (p-value: 0.734), or K (p-value: 0.083). A positive A significant positive correlation between the *Bd* load of sodium (Na) throughout the collection season in each of the lakes. The sodium regression had a p-value of 0.007 and was the only elemental concentration to have a significant correlation with a confidence level of 95%.

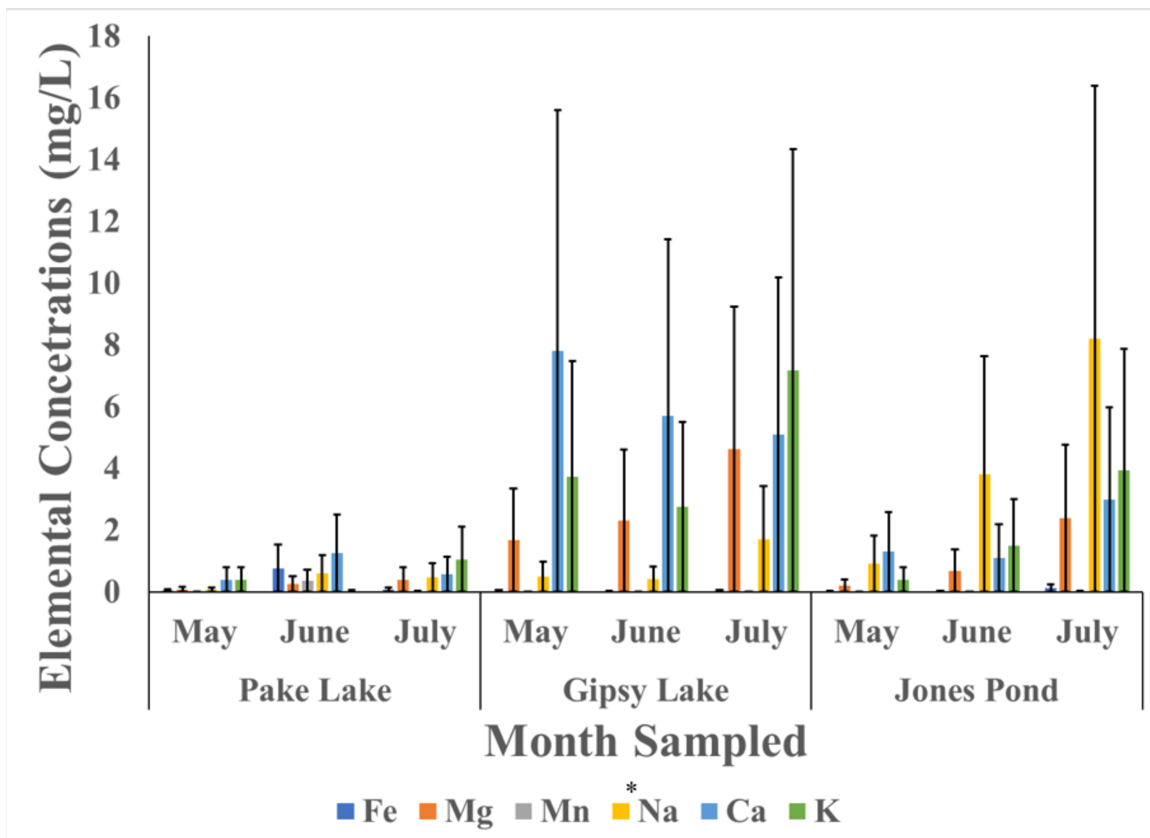


**Figure 2:** Mean element concentrations (mg/L) for each lake during each collection period over the course of summer 2018. Error bars were created using standard error of the data. *Indicates a significant correlation with *Bd* copy number identified by a regression analysis (p-value of 0.007 with 95% confidence level).

<u>*Bd* qPCR Analysis:</u>

Average *Bd* infection load was determined for each lake over the course of the season using qPCR analysis. The procured data was used to calculate *Bd* load by multiplying the quantified amount by the total amount of DNA utilized (50 µL) from the DNA extraction that was performed. The average was calculated for the now known *Bd* load for each lake in May, June, and July (Figure 3). *Bd* load increased in Jones Pond and Gipsy Lake over the summer of 2018, but *Bd* load did not increase continually over the summer of 2018 in Park Lake. A two-sample t-test assuming unequal variances was

performed between the lakes in each collection month to identify significant growth between the different lakes. There was no significance found between the any of the lakes in the months of May or June. The only significance found between the *Bd* loads of Gipsy Lake and Park Lake in the month of July. The p-value was less than 0.05 at the quantity of 0.038 with a confidence level of 95% indicating significance between the data points.

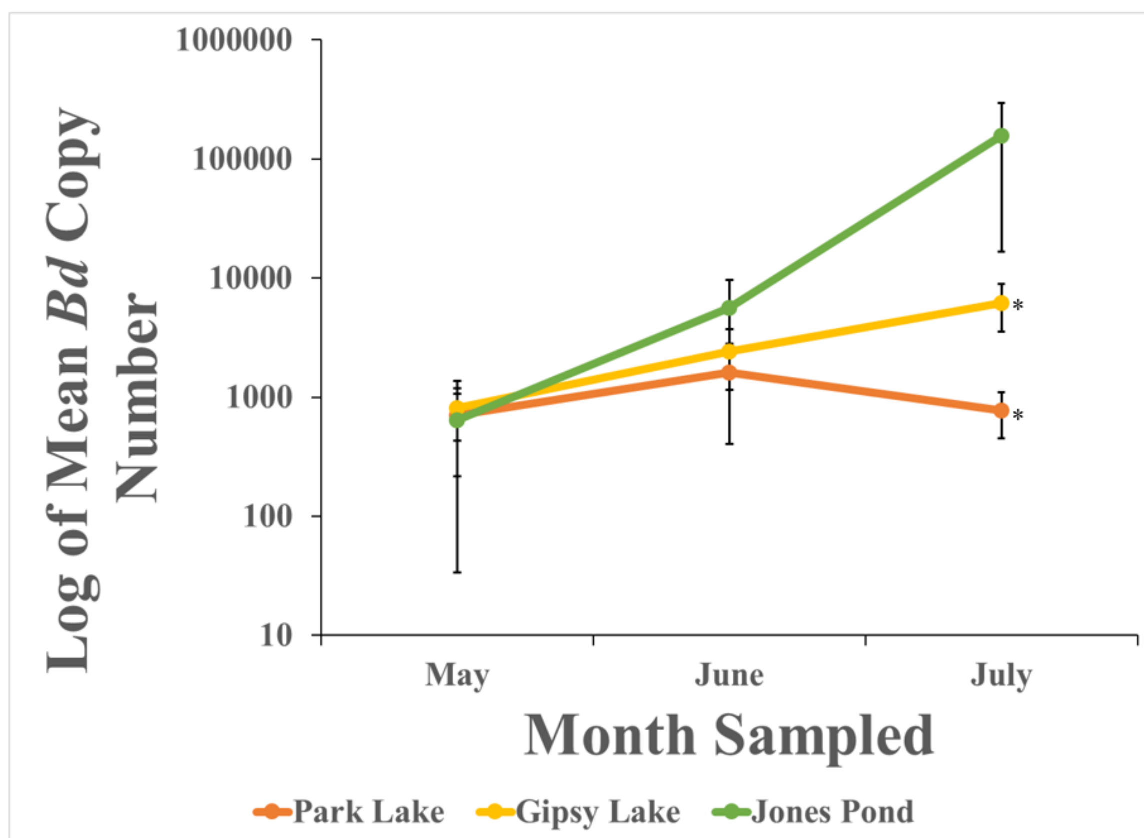


**Figure 3:** Mean *Bd* copy number in each lake over the course of the summer of 2018. *Bd* copy number is graphed on a log 10 scale to incorporate the large-scale differences in the last month's data. Error bars were created using standard error of the data. *Indicates a significant difference between Park Lake and Gipsy Lake *Bd* copy numbers in the month of July using a t-test: two-sample assuming unequal variances with a p-value of 0.038.

The percentage of individuals infected was determined in each lake over the course of the season (Figure 4). In the month of May, none of the lakes had a 100% infection rate in the sampled frogs. In May, Park Lake had an infection rate of 70%, Gipsy Lake had an infection rate of 80%, and Jones Pond had an infection rate of 60%. In the month of June, Gipsy Lake and Jones Pond had 100% infection rate and Park Lake had a 90% infection rate. In the month of July, Park Lake had an infection rate of 80%, Gipsy Lake and Jones Pond both had infection rates of 100%.

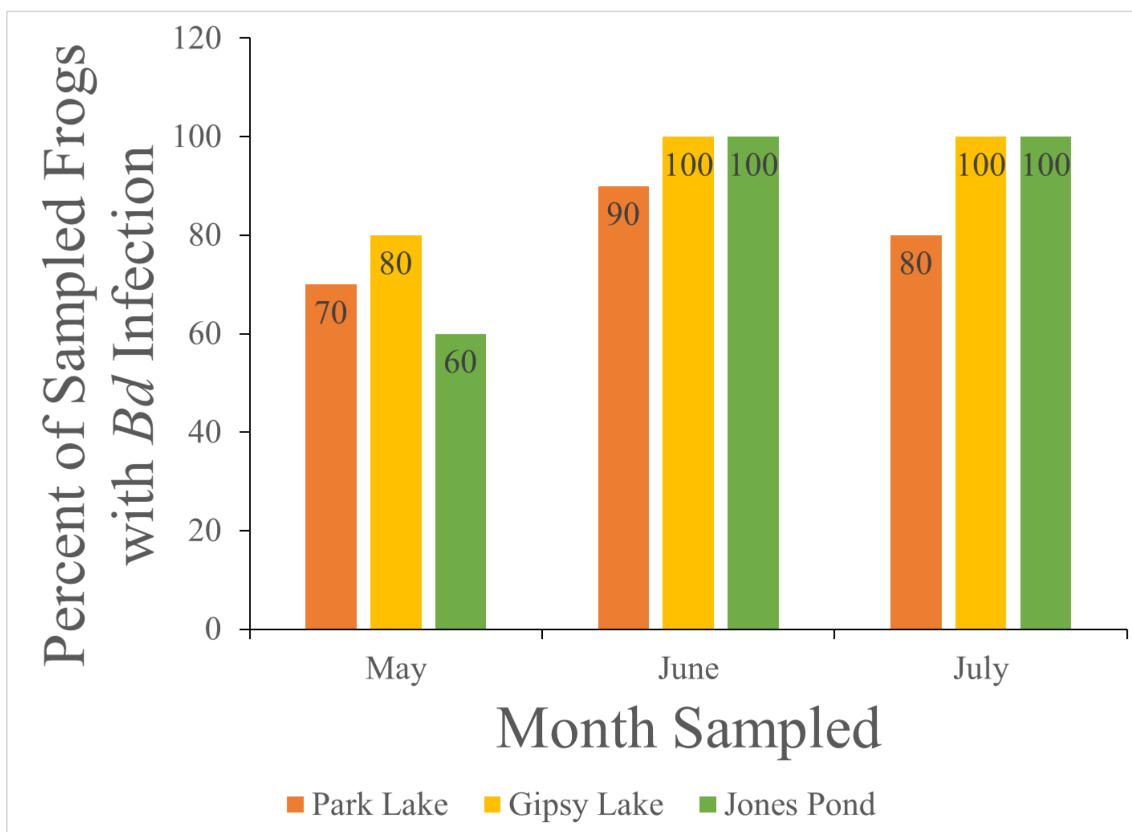


**Figure 4:** The percentage of infected Columbia spotted frogs over the course of the summer of 2018. The percentage was calculated out of the ten samples collected at each collection site during each month.

Protein Analysis:

The concentration of the cutaneous peptides collected from each frog sampled in the summer of 2018 was quantified using a Micro BCA protein assay. These quantifications were then averaged in each lake for each month over the summer (Figure 5). The month of May had the highest concentrations while the months of June and July had lower yet inconsistent concentrations across the lakes. When the protein concentrations were run in a regression analysis with the *Bd* load of individual frogs there were three instances that had a significant correlation. In the month of May, Park Lake and Jones Pond had significant correlation between the individual frog's AMPs concentration and *Bd* load, with p-values of $4.48 * 10^{-9}$ and 0.003 respectively. In the month of July, Park Lake had a significant correlation between the individual frog's AMPs concentration and *Bd* load with the p-value of $1.02 * 10^{-4}$. Each of the p-values had a confidence level of 95%.

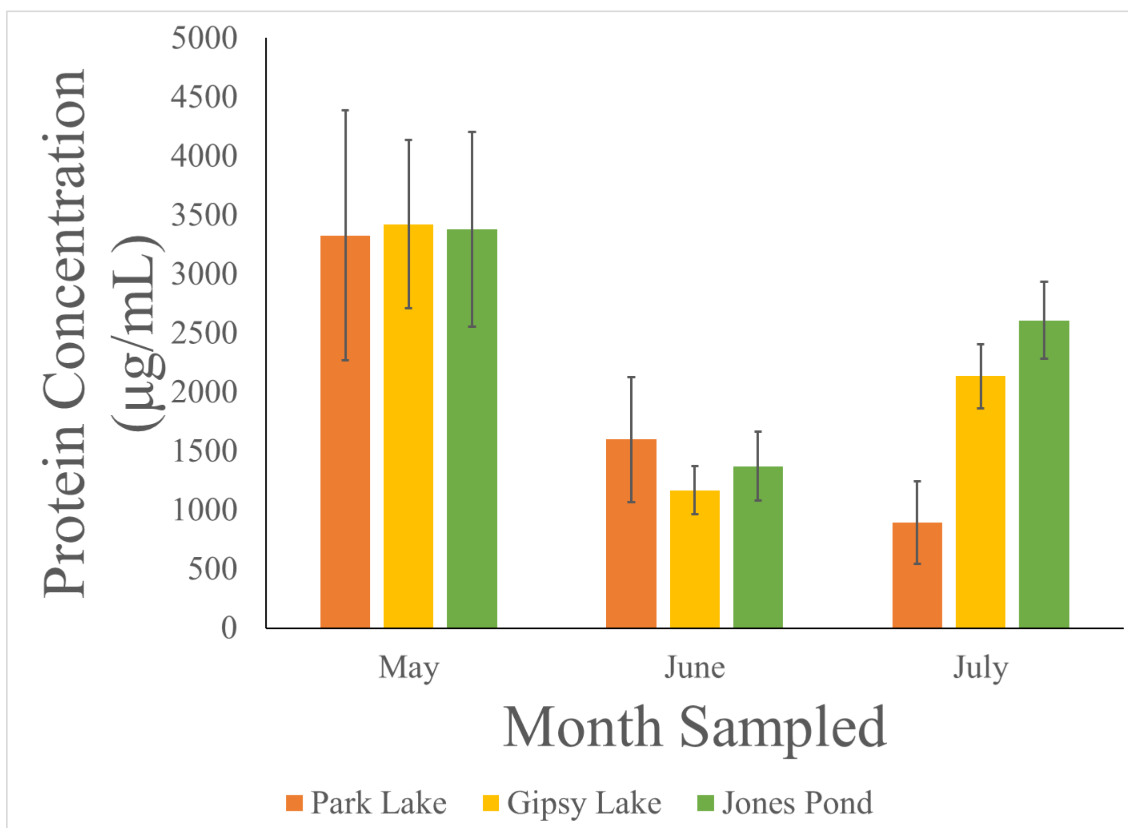

**Figure 5:** Mean protein concentration for the antimicrobial peptides of the Columbia spotted frogs sampled over the summer of 2018. Error bars were calculated from the standard error of the data.

<u>Microbial Communities:</u>

The microbial communities were identified and quantified by Dr. Andrew Loudon using amplicon sequencing techniques. From the analyses performed, there was a significant difference between the cutaneous microbiota found in the water compared to that found on the Columbia spotted frogs (Figure 6, A). However, there was no significant difference found between the cutaneous microbial communities living on the frogs of the different lakes (Figure 6, B). Five main microbia Genera were identified to inhabit the microbial communities of the Columbia spotted frogs in each lake, but those genura were extremely rare in the water analyzed (Figure 7). The main bacterial genura found on Columbia spotted frogs include *Rhizobacter* which has antifungal properties, and *Chryseobacterium* which had a positive correlation with *Bd* abundance (citation). Known antifungal microbiota, including *Rhizobacter*, were graphed against the abundance of *Bd* found on the frogs and a significant negative correlation was observed (Figure 8).

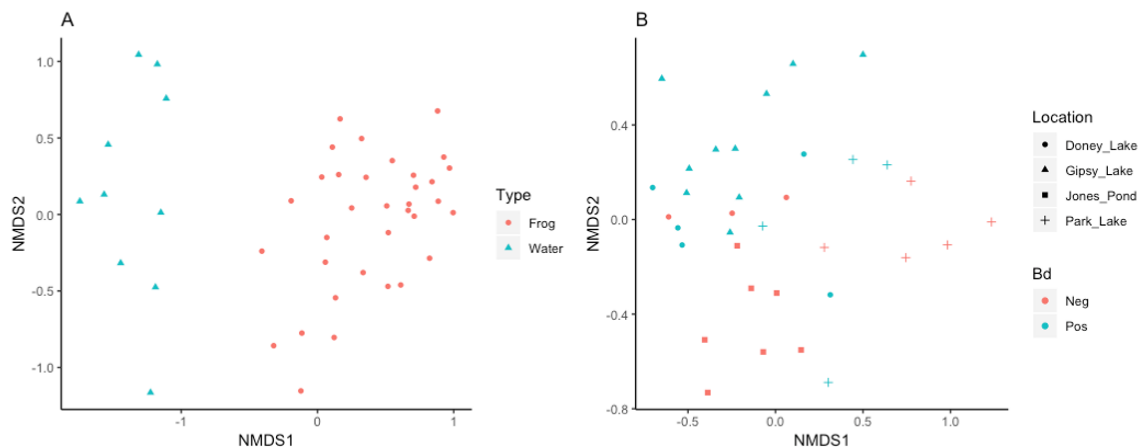

**Figure 6:** Bacterial community composition differed between Columbia spotted frogs and water. Compositions differed between location of sampling, but not based on *Bd* status. *Bd* intensity affected composition when location is not accounted for in the model. (Loudon)

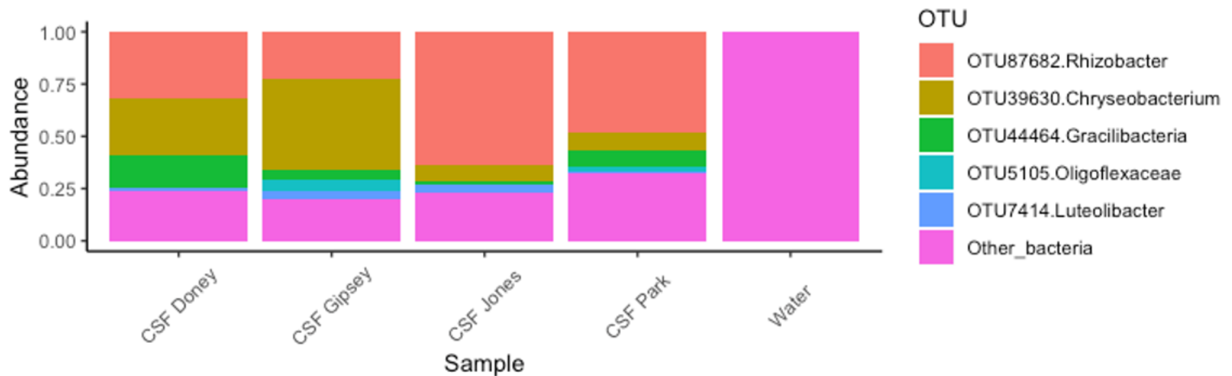


**Figure 7:** The core bacteria (bacteria that are significantly different than water and >1% of all bacteria found on >5% of frogs) of Columbia spotted frogs make up a large portion of bacteria found on Columbia spotted frogs. The relative abundance of the core bacteria, in addition to whether the individual was *Bd* positive or negative. The y-axis is not fixed. (Loudon)

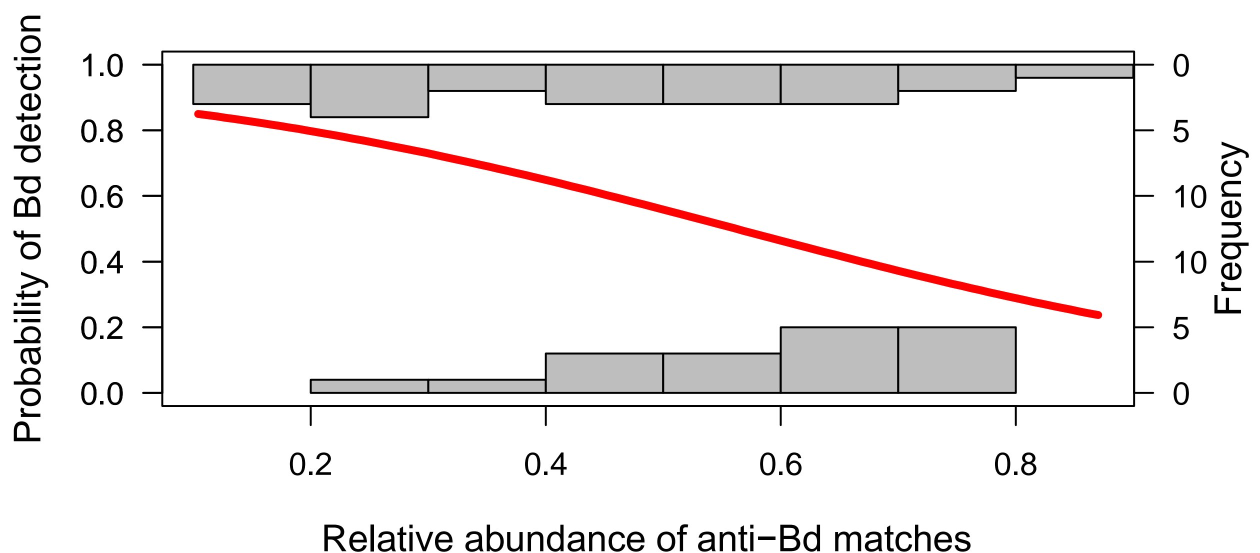

**Figure 8:** Graph of the negative correlation found between the probability and frequency of *Bd* detection in comparison to the relative abundance of antifungal microbiota on Columbia spotted Frogs. (Loudon)

**Discussion:**

The water parameters measured at field sites were compared to the *Bd* concentrations found on the skin of the frogs using regression analyses. No significance was found between the *Bd* concentrations or any of the water parameters collected in the field (temperature, dissolved oxygen, conductivity and pH). From these analyses it can be hypothesized that these parameters do not have a substantial influence on the infection rate of Columbia spotted frogs. However, when water nutrients were compared to the *Bd* concentrations in regression analyses, a positive correlation was found between *Bd* load and the concentration of sodium in the water for all three of the lakes. The other nutrients analyzed in this study (magnesium, manganese, iron, calcium and potassium) did not have a significant correlation with the *Bd* concentrations. The positive correlation between sodium and *Bd* load could be due to sodium having a potential inhibitory effect on the frogs' abilities to produce antimicrobial skin secretions. Sodium could also be an inhibitor or activator to bacteria in the microbial communities of the frogs that influenced the abundance of *Bd*. Sodium could also be a potential activator for *Bd*. However, it could be that Gipsy Lake and Jones Pond, which had 100% *Bd* infection, happened to be high in sodium and, therefore, produced this correlation. Further experimentation is required to make any strong conclusions. This might require alterations to the conditions of lab chytrid to be tested. This could include adding sodium to the medium in which *Bd* is

cultured test for any alteration in growth. These hypotheses will require further analyses to reject or support them.

The qPCR analysis of the swabbed DNA determined the *Bd* load of each Columbia spotted frog. The *Bd* load was graphed between the different lakes over the course of the summer (Figure 3). From these analyses, a significant difference was found between Park Lake and Gipsy Lake in the month of July. This was likely due to the steady growth of *Bd* in Gipsy Lake over the course of the summer while Park Lake had a loss of *Bd* growth between the months of June and July. Over the course of the summer, the mean *Bd* infection load in Jones Pond increased exponentially. Gipsy lake increased at a steady rate, and Park lake initially increased then decreased between June and July. Jones Pond might have had the highest rate of infection due to it being the lowest elevation lake and having a more ideal environment for the growth of *Bd*. Further analysis in the coming years will allow for this hypothesis to be investigated and be accepted or rejected.

Skin peptide concentrations were individually quantified, averages were then calculated and graphed between lakes (Figure 5). Regression analyses between the independent *Bd* load of each frog and the coinciding protein concentration of each frog were performed. Significance was only found between Park Lake in the months of May and July, and Gipsy Lake in the month of May. Due to the inconsistent correlation between the *Bd* load and the protein concentration in each frog, these two variables can not be strongly linked.

MIC data has not been statistically analyzed from the summer of 2018. However, analyzed data from past years lead to the conclusion that MIC is not a strong indicator for

susceptibility in Columbia spotted frogs to chytridiomycosis (Esposito, 2018). Once the data has been successfully analyzed for the summer of 2018 and eventually the summer of 2019, this conclusion can be supported or discarded.

Microbial communities were analyzed via amplicon sequencing by Dr. Andrew Loudon of the Cleveland Metroparks Zoo. The importance of the bacterial richness of the frogs' microbial communities is apparent when examining statistical analyses (need to find out what stats were used). Five main bacteria were identified to inhabit the cutaneous microbial communities of the frogs. These included members of the genus *Rhizobacter* and *Chryseobacterium*. *Rhizobacter* had a negative correlation with *Bd* load, likely due to the antifungal properties that the bacteria was known to have (Woodhams et al., 2006). In contrast, *Chryseobacterium* has a positive correlation with *Bd* load. Additionally, when *Rhizobacter* has a greater abundance, the abundance of *Chryseobacterium* is lower and vice versa. This leads to the hypothesis that the two bacteria are competing within the cutaneous microbial communities of the frogs.

The bacteria living in the microbial communities of the frogs and in the water are significantly different (Figure 6 and 5). From this information, it can be surmised that Columbia spotted frogs can affect what is living in their microbial community. It is hypothesized that this is related to antimicrobial skin secretions, but it will require further testing to confirm or deny this concept. Previously collected microbial community data is still undergoing analysis at Cleveland Metroparks Zoo by Dr. Andrew Loudon and may give new insights to these hypotheses.

Bacterial metabolite analyses are still being processed at Villanova University by Dr. Kevin Minbiole and Dr. Tom Umile. These results could provide further insights to

the ability of Columbia spotted frogs to cultivate resistance to *Bd* infection. Regression statistical analyses will be performed by Dr. Eric Sullivan of Carroll College upon the completion of data analyses to determine any statistically significant correlations between the multiple variables. Future research will continue to strengthen or disprove current hypotheses from initial analyses that have been completed. Hopefully, this information will allow biologists to predict and treat outbreaks of chytridiomycosis in the future and serve to protect amphibian populations that are ask risk.

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