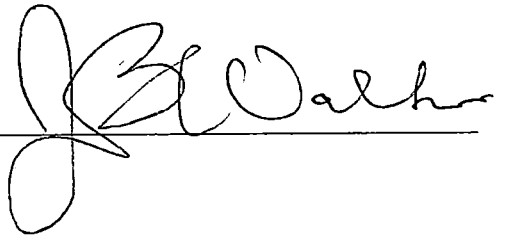
Relationships between the amphibian skin microbiome and the fungal pathogen *Batrachochytrium dendrobatidis* in northern Idaho

Philip Campos

Dr. Jenifer Walke

Faculty advisor signature 

Statement of Purpose

The fungal pathogen *Batrachochytrium dendrobatidis* (Bd) poses a great threat against the biodiversity of amphibian species. My goal is to investigate correlations between Bd presence and skin microbiome composition from Columbia spotted frogs (*Rana luteiventris*) sampled in northern Idaho surveys during 2013 and 2018 to identify skin microbes that potentially provide protection against Bd.

Background and Significance

Emerging infectious diseases threaten global biodiversity by contributing to population declines and extinctions, having been associated with declines in a large range of organisms, including amphibians, birds, lions, invertebrates, and plants (1, 2). Chytridiomycosis, the disease caused by Bd, has resulted in the decline or extinction of up to 200 frog species globally (3-6). The disease spreads in epidermal cells, which is problematic because amphibian skin is crucial in regulating the exchange of respiratory gases, electrolytes, and water (7). Chytridiomycosis is a cause for concern for amphibian populations in northern Idaho. In a large scale 2013 study, Bd was detected on Columbian spotted frogs in 80% of 153 sampled locations (Figure 1) (8). In these sampled sites, it is not yet known how the skin microbiome influences susceptibility to infection.

Previous research shows the amphibian skin microbiome plays a role in defense against Bd. For example, the bacteria *Janthinobacterium lividum* was found to produce the metabolite violacein, which inhibits the development of Bd (9). When Harris et al. applied *J. lividum* to skins of *Rana muscosa* frogs, 100% of Bd exposed frogs survived after 20 weeks, compared to approximately 20% of frogs when *J. lividum* was not applied. These findings were consistent with data showing that violacein was strongly associated with frog survival. Another example of

an antifungal metabolite produced by skin bacteria is 2, 4-diacetylphloroglucinol, found on red-backed salamanders (*Plethodon cinereus*) (10). Antifungal metabolites have also been found as a byproduct of interactions between two bacteria. *Bacillus sp.* and *Chitinophaga arvensicola* from red-backed salamanders produce the Bd-inhibiting tryptophol when cultured together, but not in isolation (11). An Antifungal Isolates Database has been produced to compile a list of amphibian skin bacteria isolated from around the world, their 16S rRNA gene sequences, and their Bd-inhibiting properties (12). The database shows that *Pseudomonas* is a genus commonly found on the skin microbiome with anti-Bd properties, however, a variety of genera have been found with Bd-inhibition.

These studies demonstrate the importance of investigating amphibian skin microbiome composition in relation to disease. By characterizing the skin microbial communities in Columbia spotted frog samples taken from northern Idaho in 2013 and 2018, we can discover if the microbe community differs in infected and uninfected frogs. Differences in the community may indicate whether certain microbes provide an antifungal effect. This knowledge could lead to future studies which test the effectiveness of applying probiotics containing antifungal microbes. Questions remain over how conservation should be managed for disease-threatened amphibian wildlife. Since probiotic bacteria will continue to maintain themselves on the skin microbiome, probiotics are potentially an effective solution to protecting individuals over their entire lifetimes, making probiotics more than a temporary solution.

Objectives

First, I will detect presence of Bd in 2018 skin samples of northern Idaho Columbia spotted frogs. In both 2013 and 2018 samples, I will characterize the skin microbial communities. Next, I will investigate correlations between Bd presence and the microbial

community composition to identify microbes with potential anti-Bd effects. I hypothesize that microbe community composition in frogs infected by Bd will differ from composition in frogs uninfected by Bd in both sets of samples. I expect to find a greater proportion of bacteria with antifungal properties, such as but not limited to *Pseudomonas*, in frogs uninfected by Bd.

Procedures and Rationale

The Idaho Department of Fish and Game sampled *Rana luteiventris* (Columbian spotted frog) during the periods of May-September 2013 (N=396) and 2018 (N=163) (Table 1). The study area consisted of most of the Idaho Panhandle, the northernmost region of Idaho encompassing 10 counties (see Figure 1). Skin swab samples were collected using sterile buccal swabs to swab the underside of the frog back and forth 15 times (30 total swipes) as described in the Multi-species Baseline Initiative project report (8). Ends of swabs are currently stored in tubes containing RNAlater to preserve DNA. For samples from 2013, DNA isolation and screening for Bd infection have previously been completed.

***Rana luteiventris* Skin Swab Samples: # Samples (# Sites)**		
2013	2018	Sites Sampled In Both Years
396 (153)	163 (68)	53 Sites

Table 1. Number of skin swab samples and sites sampled from the years 2013 and 2018. Sampling effort was lower in 2018. Data from Idaho Department of Fish and Game.

For all 2018 samples, I will isolate DNA from these samples using the Qiagen DNeasy Blood & Tissue Kit for DNA purification. After isolation, I will detect presence of Bd using polymerase chain reaction (PCR) with primers Bd1a and Bd2a (13). For a subset of both 2013 and 2018 sample sets, I will characterize the microbial community composition by taking a sample barcoding approach during PCR using amplicon sequencing of the V4 region of the 16S

rRNA gene with primers 515F and barcoded 806R (14). I will use agarose gel electrophoresis and GelRed DNA stain to separate the mixture of DNA by molecular size. The samples will be pooled at equimolar concentrations and sent for sequencing on the Illumina MiSeq platform at the University of Idaho's IBEST Genomics Resources Core.

Bioinformatics will be performed on the Quantitative Insights Into Microbial Ecology (QIIME) platform (15). Using the platform, I will visualize data and compare differences between microbial communities in each sample. I will use a weighted UniFrac distance matrix, a technique developed for comparison of microbial communities in a phylogenetic context (16). A Non-Metric Multidimensional Scaling (NMDS) ordination will be used to visualize the differences of microbial communities in infected samples compared to the communities of uninfected samples. To analyze the statistical significance of these differences, I will use a Permutational multivariate analysis of variance (PERMANOVA). PERMANOVA is a semiparametric method that allows for analysis of multivariate data which may be non-normal (17).

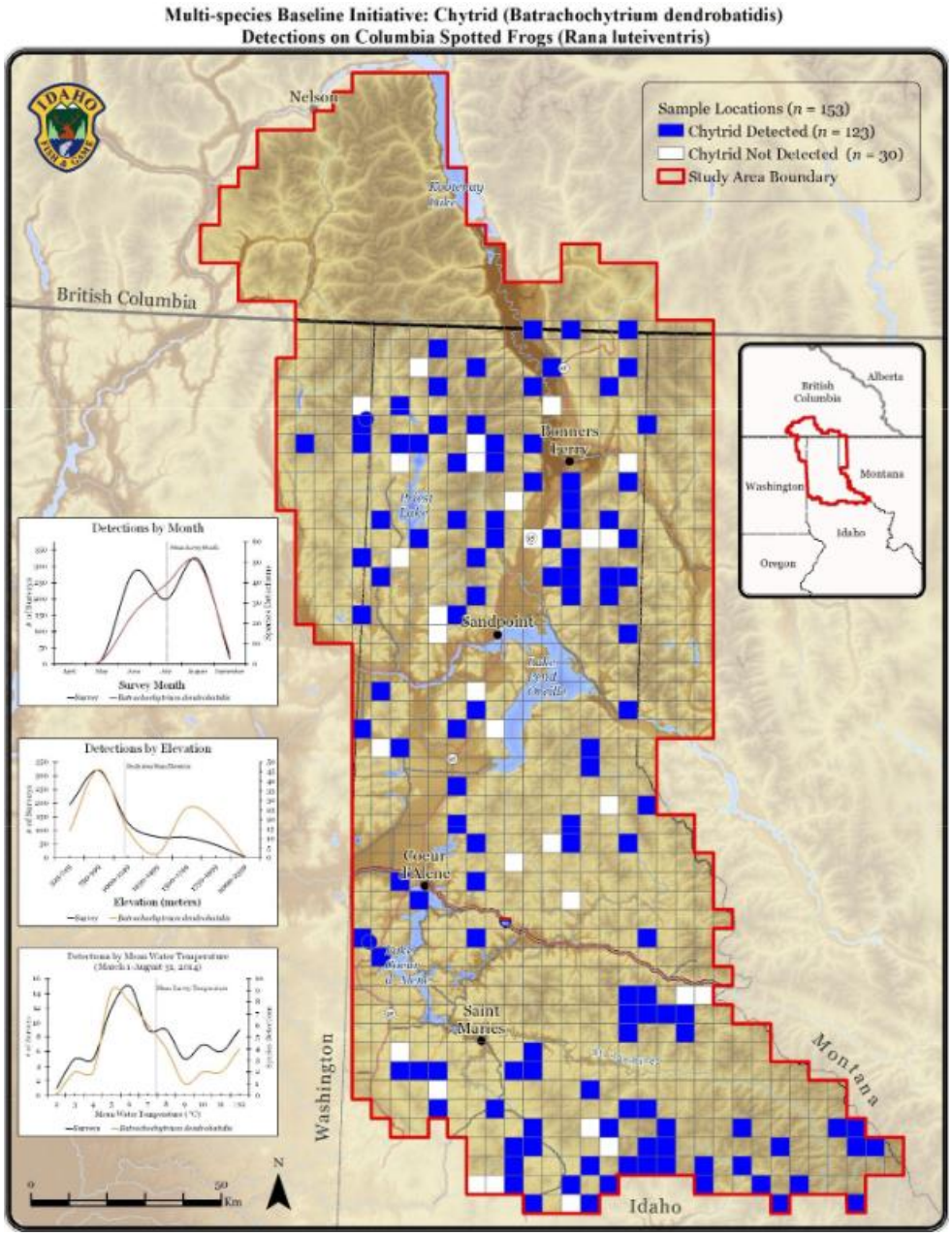


Figure 1. Map of northern Idaho study area by Idaho Fish and Game. Bd was detected on Columbia spotted frogs in 80% of sampled locations (N = 123 out of total N = 153).

Budget Justification

Item	**Cost**	**Quantity**	**Total Cost (+ shipping)**
Qiagen DNeasy Blood & Tissue Kit (50 samples)	*$176.04*	*3*	*$588.12*
QuantaBio HotMaster Mix	*$74.55*	*2*	*$149.10*
DNA primers	In lab	N/A	$0.00
Agarose BioReagent	In lab	1	$0.00
GelRed® Nucleic Acid Gel Stain 3X in water	In lab	1	$0.00
DNA sequencing at University of Idaho (100 samples)	$2,000.00	1	$2,000.00
TOTAL BUDGET REQUESTED			***$737.22***

Timeline

Quarter	Winter 2019	Spring 2019	Fall 2019	Winter 2020	Spring 2020
Task	DNA extraction, PCR, species identification of Idaho samples.	Data analysis, Collect amphibian skin samples at Turnbull NWR.*	DNA extraction, PCR, species identification of Turnbull samples.*	Data analysis	Complete thesis

*Note: Part 2 of thesis project not included in this proposal.

References

1. Harvell C, Mitchell C, Ward J, Altizer S, Dobson A, Ostfeld R, et al. Ecology - Climate warming and disease risks for terrestrial and marine biota. *Science* (2002) **296**:2158-62.

2. Daszak P, Cunningham A, Hyatt A. Wildlife ecology - Emerging infectious diseases of wildlife - Threats to biodiversity and human health. *Science* (2000) **287**:443-9.

3. Skerratt LF, Berger L, Speare R, Cashins S, McDonald KR, Phillott AD, et al. Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *Ecohealth* (2007) **4**:125-34 doi: 10.1007/s10393-007-0093-5.

4. Berger L, Speare R, Daszak P, Green DE, Cunningham AA, Goggin CL, et al. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proc Natl Acad Sci U S A* (1998) **95**:9031-6.

5. Garner T, Walker S, Bosch J, Hyatt A, Cunningham A, Fisher M. Chytrid fungus in Europe. *Emerg Infect Dis* (2005) **11**:1639-41.

6. Ouellet M, Mikaelian I, Pauli B, Rodrigue J, Green D. Historical evidence of widespread chytrid infection in North American amphibian populations. *Conserv Biol* (2005) **19**:1431-40.

7. Rosenblum EB, Voyles J, Poorten TJ, Stajich JE. The deadly chytrid fungus: a story of an emerging pathogen. *PLoS Pathogens* (2010) **6**:e1000550.

8. Lucid M, Robinson L, Ehlers S. Multi-species Baseline Initiative Project Report: 2010-2014. *Idaho Department of Fish and Game, Coeur d'Alene* (2016).

9. Harris RN, Brucker RM, Walke JB, Becker MH, Schwantes CR, Flaherty DC, et al. Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. *Isme Journal* (2009) **3**:818-24.

10. Brucker RM, Baylor CM, Walters RL, Lauer A, Harris RN, Minbiole KP. The identification of 2, 4-diacetylphloroglucinol as an antifungal metabolite produced by cutaneous bacteria of the salamander Plethodon cinereus. *J Chem Ecol* (2008) **34**:39-43.

11. Loudon AH, Holland JA, Umile TP, Burzynski EA, Minbiole KP, Harris RN. Interactions between amphibians' symbiotic bacteria cause the production of emergent anti-fungal metabolites. *Frontiers in microbiology* (2014) **5**:441.

12. Woodhams DC, Alford RA, Antwis RE, Archer H, Becker MH, Belden LK, et al. Antifungal isolates database of amphibian skin-associated bacteria and function against emerging fungal pathogens: Ecological Archives E096-059. *Ecology* (2015) **96**:595.

13. Annis SL, Dastoor FP, Ziel H, Daszak P, Longcore JE. A DNA-based assay identifies Batrachochytrium dendrobatidis in amphibians. *J Wildl Dis* (2004) **40**:420-8.

14. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, et al. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME journal* (2012) **6**:1621.

15. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. *Nature methods* (2010) **7**:335.

16. Lozupone C, Hamady M, Knight R. UniFrac–an online tool for comparing microbial community diversity in a phylogenetic context. *BMC Bioinformatics* (2006) **7**:371.

17. Anderson MJ. Permutational multivariate analysis of variance (PERMANOVA). *Wiley StatsRef: Statistics Reference Online* (2014) 1-15.