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An Overview of Batrachochytrium dendrobatidis in Utah, With a Focus on Boreal Toads and Their Changing Conservation Status

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AN OVERVIEW OF *BATRACHOCHYTRIUM DENDROBATIDIS* IN UTAH, WITH A FOCUS ON BOREAL TOADS AND THEIR CHANGING CONSERVATION STATUS

by

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ABSTRACT

An overview of *Batrachochytrium dendrobatidis* in Utah, with a focus on Boreal Toads and their changing conservation status

by

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The Boreal Toad (*Anaxyrus boreas*) has disappeared from a large portion of its range in southern Utah and it has been questioned whether *Batrachochytrium dendrobatidis* (Bd, also known as chytrid) has played a role in its disappearance. The role of chytrid in range contraction of Boreal Toads and other amphibians in Utah is unknown. The primary objective of this project is to determine if any historic Boreal Toad specimens have chytrid to determine its arrival in Utah. If any old specimens test positive, the secondary goal of this study is to determine if there is a relationship between the presence of chytrid and Boreal Toad disappearance. Previously collected Boreal Toad specimens from the Natural History Museum of Utah and the Monte L. Bean Life Science Museum were swabbed for chytrid. Sampling preserved specimens for chytrid is a fundamental step for a complete understanding of Boreal Toad disappearance in Utah. PCR and histological methods were the primary techniques for analyzing chytrid presence. The resulting genetic analysis detected chytrid in specimens dating back to the early 2000s as well as a specimen from 1961, but further skin analyses could not confirm chytrid presence in the skin of the specimen from 1961. As a result, this research project was not able to provide enough data for a thorough understanding of chytrid in historical Boreal Toad populations. However, this

study examines the effectiveness of swabbing preserved specimens for chytrid and concludes that further research is needed to make progress in understanding the history of chytrid in Boreal Toads throughout the state and over time.

ACKNOWLEDGEMENTS

This project received support from several institutions and was conducted in close collaboration with the Utah Museum of Natural History, The Monte L. Bean Life Science Museum, Pisces Molecular, and the Utah Veterinary Diagnostic Lab. Dr. Karen Beard served as my thesis advisor and has served as an excellent research mentor. She has provided numerous opportunities for academic growth and professional development, and has provided valuable feedback and support on my project. Dr. Eugene Schupp has also provided additional support as my departmental honors thesis advisor. I would also like to thank my funding sources, which have contributed largely to the success of this project. I received support from the Utah State University Undergraduate Research and Creative Opportunities Grant, the Quinney College of Natural Resources, and the University Honors Program. I would also like to thank Dr. Jack Sites, who allowed me to swab his collection of Boreal Toads and collect skin samples at the Monte L. Bean Life Science Museum. Dr. Eric Rickart also allowed me to swab the Boreal Toad specimens from the Utah Museum of Natural History. I would also like to thank Dr. John Wood, for running PCR analyses on the chytrid swabs and Dr. Arnaud VanWettere, who prepared slides for histological analysis and demonstrated how to detect chytrid in microscopic skin samples. Special thanks are also extended to Neal Hengge, who aided in the swabbing process of the museum specimens. I am thankful for the support of the University and Departmental Honors Program in helping me complete this project.

CONTENTS

INTRODUCTION

Batrachochytrium dendrobatidis (Bd or, hereafter chytrid) was first identified in 1998 by a team of scientists interested in explaining the reason for amphibian die-offs across the globe (Muths et al. 2000). Chytrid can be described as an aquatic fungal pathogen that infects the skin of amphibians (Gahl et al. 2011). Over the years, chytrid has led to the decline of many amphibian species globally, particularly in tropical areas, but also in high elevations in the western continental United States (Ouellet et al. 2005). For example, there have been documented massive declines of yellow-legged frogs (*Rana muscosa)* in the Sierra Nevada Mountains (Fig. 1; Ouellet et al. 2005). There have also been other western species affected by the disease, including the disappearance of the Wyoming Toad (*Anaxyrus baxteri*). Historically, the role of chytrid in these populations was unknown, but recent studies suggest chytrid played a significant role in their disappearance (Geraud et al. 2004). Furthermore, Boreal toads have been declining throughout a large part of their range, and in many states including Colorado, chytrid has been implicated in the declines (Muth et al. 2000).

Boreal Toads have also disappeared from a major portion of their range in Utah, mostly in the southern and central part of the state. There has been some debate in Utah regarding whether or not chytrid has played a role in their declines. There are currently populations in Utah that have chytrid present, but the populations appear to be surviving. This is not unexpected because as noted by Pillod et al. (2010), there are often individuals in infected populations that test positive and negative for chytrid. However, those individuals that test positive have a lower probability of survival than unaffected individuals in the same population, and thus there are questions about why some individuals are more susceptible and whether populations can become

resistant. Furthermore, we have no information about whether or not past populations have disappeared from Utah because they had chytrid and were not able to overcome the disease.

Currently the Boreal Toad is listed as endangered in Colorado and New Mexico. In Utah, the Boreal Toad was listed as a candidate species in 1995, meaning that it is warranted but precluded for federal listing (Hogrefe et al. 2005). This listing is set to be reviewed in 2017. At this time, the U.S. Fish and Wildlife Service will decide whether the Boreal Toad will be protected under the Endangered Species Act in Utah, Southern Idaho, and Nevada. Whether or not Boreal Toads in Utah are threatened by chytrid will play a major role in its listing status. One of the major reasons the species is listed in Colorado and New Mexico is because chytrid was implicated in their decline (Muths et al. 2000). The primary objective of this project is to determine if any historic Boreal Toad specimens have chytrid to determine its arrival in Utah. If any old specimens test positive, the secondary goal will be to determine if there is a relationship between presence of chytrid and Boreal Toad disappearance.

METHODS

To conduct this project, Boreal Toad specimens were sampled from Utah museums for chytrid to determine if chytrid was present in previous populations. Specimens were sampled from the Utah Museum of Natural History in Salt Lake City, UT, and the Monte L. Bean Life Science Museum in Provo, UT. Combined, the two museums house over 110 historic Boreal Toad specimens. Additional Boreal Toads collected in Utah have been identified as part of collections at the California Academy of Sciences and the Museum of Vertebrate Zoology. While these additional toads were not tested for chytrid, they serve as potential opportunities for accessing more Boreal Toad specimens that were originally collected in Utah.

Approximately 80 preserved Boreal Toad specimens were swabbed using specific protocols for museum specimens provided by Pisces Molecular in Boulder, CO. As demonstrated by Cheng et al. (2011), sampling modifications can be applied for genetic analysis of chytrid in older specimens (Cheng 2011). Specimens were swabbed using Mediwire (M100) swabs that were left dry and placed in a corresponding sleeve. Each individual was swabbed individually a total of 20 times along its ventral surface, ensuring that the underside, legs and feet were swabbed for each toad. Prior to swabbing, each specimen was rinsed with 70% ethanol to remove any debris from the surface of the specimen. Each specimen was recorded on an Excel spreadsheet along with the date and location of where each individual was collected. Using a figure by Thompson et al. (2005), the location of Utah Boreal Toad museum specimens is depicted on a map according to where they were collected (Figure 1).

Additionally, both museums organized their Boreal Toad specimens by placing individuals from the same county into the same jar. Therefore, there were individuals from varying years in the same jar for a particular county. There were many specimens represented from the same year and county in some jars. In cases where there were many replicates for the same location and year, only 1/3 of the specimens from the replicate were swabbed to have the opportunity to swab individuals from other locales. Individual swabs from the same location and date were pooled together at full volume for PCR analysis. These samples were combined to reduce the number of PCR analyses. Single individuals from a separate location and date were not pooled and were processed individually. The total number of Boreal Toads after pooling amounted to 40 total samples analyzed by Pisces Molecular.

PCR Analysis

Samples were shipped to Pisces Molecular in Boulder, CO, for genetic analyses, which analyzed our samples for the presence of chytrid DNA. PCR was the primary method of analysis, modifying the procedure to elute the DNA from the spin column in a smaller volume of buffer (Cheng 2011). Individuals from the same location and date were pooled into one sample at full volume, while single individuals from their own location and date were run individually.

Histology

Following genetic analysis, an additional histology was conducted to determine whether a specific individual from 1961 was actually positive for chytrid. Small skin samples were taken from the ventral surface, pelvis, medial thighs and feet of the individual. Histological slides were then prepared by Dr. Arnaud VanWettere at the Utah Veterinary Diagnostic lab for further analysis (Figure 2). Skin samples were cut into strips and dyed to make the surface of the skin visible under a microscope. This method allowed the veterinary pathologist to examine the epidermis of the skin. Individuals that are positive for chytrid have specific zoosporangia embedded in the subsurface of the skin (Figure 3) (Berger et al., 1999). Without chytrid present in the skin, the epidermis appears thick and continuous (Figure 4).

RESULTS

Following a thorough PCR analysis by Pisces Molecular, there were two samples positive for chytrid out of 40 analyzed samples (Table 1 and Table 2). One sample was comprised of a single specimen dating back to 1961 (Individual 22142) and the other sample was a pooled sample of five specimens (Individuals 5046 and 5861-5864). The pooled sample included one specimen from 1999 and four specimens from 2002. Both of the positive samples were collected

in Wasatch County and all of the individuals were stored in the same jar at the museum. However, only a few of the specimens were collected on the same date. Individual 22142 was collected in 1961 near the Upper Falls of the Provo River. Individual 5046 was collected at Bryant's Fork in 1999 and individuals 5861-5864 were collected at Strawberry Reservoir in 2002. The individuals from 1999 and 2002 (5046; 5851-5864) were pooled for sampling because they were both collected from close sites during the same time frame. The 1961 specimen (22142) was submitted individually because it was collected during an earlier time frame. While all of these specimens tested positive for chytrid, they were stored in the same jar, indicating a high probability that a positive individual contaminated the other specimens. While the chytrid positive individual(s) was unknown, it is more likely it originated from a more recently deceased specimen because the DNA in more recent specimens is often less degraded than in older individuals. It is also common for chytrid to be prevalent around Strawberry Reservoir. This area contains many individuals that have tested positive for chytrid in more recent years. Discovering the presence of chytrid in Strawberry Reservoir would not have been a significant find.

However, the discovery of chytrid in an individual dating back to 1961 would be a significant find. There have been few discoveries of chytrid in Boreal Toads at such an early date. If the specimen was indeed positive, it would have been one of the oldest specimens to test positive for chytrid in the Rocky Mountains. To confirm the findings of the genetic analysis, the PCR analyses were supplemented with histology of skin samples from the 1961 specimen. The resulting skin samples were taken to the Utah Veterinary Diagnostic Lab for analysis to determine whether the 1961 individual had Bd or was likely contaminated from other specimens in the jar.

After analyzing the several skin samples from the 1961 specimen, there appeared to be no presence of the disease within the skin. This result was confirmed by Dr. David Green, USGS (pers. comm.). While this specimen was genetically positive, it is likely that a more recent individual came in contact with the specimen and made it appear as if the individual was positive for chytrid. It cannot be ruled out that the individual never had the disease, but lack of evidence of the disease throughout the skin is a likely indicator that it did not have the disease.

DISCUSSION

Our first goal was to determine if there was chytrid present in any historic samples from Utah. Our research could not confirm chytrid in any specimens collected earlier than the early 2000's. There remain historic specimens from Utah in museums in California that should also be tested for chytrid. Because we did not find any historic samples with chytrid, we could not address our second goal of determining whether there is a relationship between the presence of chytrid in populations and historic declines.

Further analysis of chytrid prevalence in Boreal Toad specimens has indicated a need for improved preservation of museum specimens for research. Many of the specimens from the Utah Natural History Museum were previously formalin-fixed before being placed in ethanol. Formalin fixation often crosslinks with nucleic acids and proteins that compose DNA. As a result, the quality of DNA decreases and it becomes difficult to detect presence of the disease through genetic analysis (Skage and Schander, 2007).

Additionally, all of the Boreal Toad specimens from the same county were stored together in the same jar, despite having been collected in different years. If an individual is positive for chytrid, this is likely to impact the genetic results of all of the other specimens

sharing the jar. It would not be accurate to assume that any of the individuals stored together are positive individually without further analysis. Thus, a histological method was used to gain a more accurate understanding of whether an older specimen was actually positive for the disease.

While most of the older specimens tested negative for chytrid, it is possible that their DNA was too degraded for sampling and does not necessarily indicate that the specimens were negative for chytrid. While this project was largely contingent on funding, it would be interesting to examine the DNA of the swabbed specimens to determine whether individuals were negative for chytrid or whether the DNA was too degraded for sampling. This additional analysis could provide valuable information regarding the quality of preserved specimens for research. Accordingly, histology of the amphibian specimens was an effective tool to aid in determining infection and may prove to be a valuable method in the future.

There are 120 preserved Boreal Toad specimens collected in Utah that are available for research at the California Academy of Sciences. These individuals were not included in the study, but may provide additional information for a more thorough analysis of chytrid in Utah's Boreal Toad populations over time. The next step for the project would be to continue sampling these preserved specimens with the intent to uncover more information about the historical distribution of chytrid in Utah's Boreal Toad specimens.

REFLECTIVE WRITING

The process of completing my capstone was definitely a success. I contribute the majority of my success largely to guidance from my undergraduate research mentor. One of the biggest challenges I faced with my capstone was finding a research project that I was passionate about. I began several projects before I began researching chytrid in Boreal Toads. After several attempts to get started on other projects, I realized I was not excited about any of the topics, and decided to find a professor who would fit my research interests. I contacted Dr. Karen Beard after learning about her interest in amphibians and conservation biology. She suggested a project I would be interested in and I was excited to get started. Once I had found a research project I was passionate about, it became a lot easier to write my thesis.

This project required a lot of permission from outside sources and was done cooperatively with other institutions. It was difficult coordinating with people and traveling to sample the Boreal Toad specimens, but the museum curators were flexible and eager to assist with my project. This project would not have been successful without the permission of the museum curators. They showed genuine interest in my research project and asked me to share my results with them.

One of the project's biggest successes was detecting chytrid through the PCR analyses. I followed a detailed protocol for swabbing, but was unsure whether we would find chytrid in the preserved specimens. The positive results provided by Pisces Molecular reinforced my confidence in our procedure and demonstrated that swabbing of preserved individuals was an effective method for detecting chytrid, at least in more recent specimens and potentially in older ones.

Perhaps one of the most challenging aspects of the project was determining the success of the swabbing technique for older preserved specimens. It was difficult to determine whether this was because the DNA was too degraded for PCR analysis or because the specimens were negative for the disease. After a 1961 individual was genetically identified as positive for chytrid, I was excited about the possibility that this could be one of the oldest individuals positive for chytrid in the Rocky Mountains. After an additional histology demonstrated otherwise, the results of the study were not as profound as previously thought. There were not enough data to determine the historical distribution of chytrid in Boreal Toads. Determining how to evaluate the success of the project was challenging. More extensive research is needed to evaluate the role of chytrid historically in Boreal Toad populations. Ultimately, this project was a good experiment for evaluating the swabbing technique of preserved specimens and served as a basis for further research.

Students that are beginning the capstone process should focus on finding a project they are passionate about. It is incredibly difficult to write a thesis on a topic that is not motivating to the student. Once I found a good project, I applied for research funding. I was supported by the Undergraduate Research and Creative Opportunities Grant, the College of Natural Resources, and the University Honors Program. Without this funding, my project would not have been possible. Funding can make a big difference in the success of a capstone project.

I would also recommend starting the capstone process early and begin writing at each stage of the process. Starting the capstone process early produces a more comprehensive and thorough paper. Writing at each stage of the process is a good way to document the project more effectively without trying to remember every detail at the last minute. I also kept a notebook with

all of the components of my project for reference. Overall, I was pleased with the outcome of my project and attribute its success to good organization and documentation.

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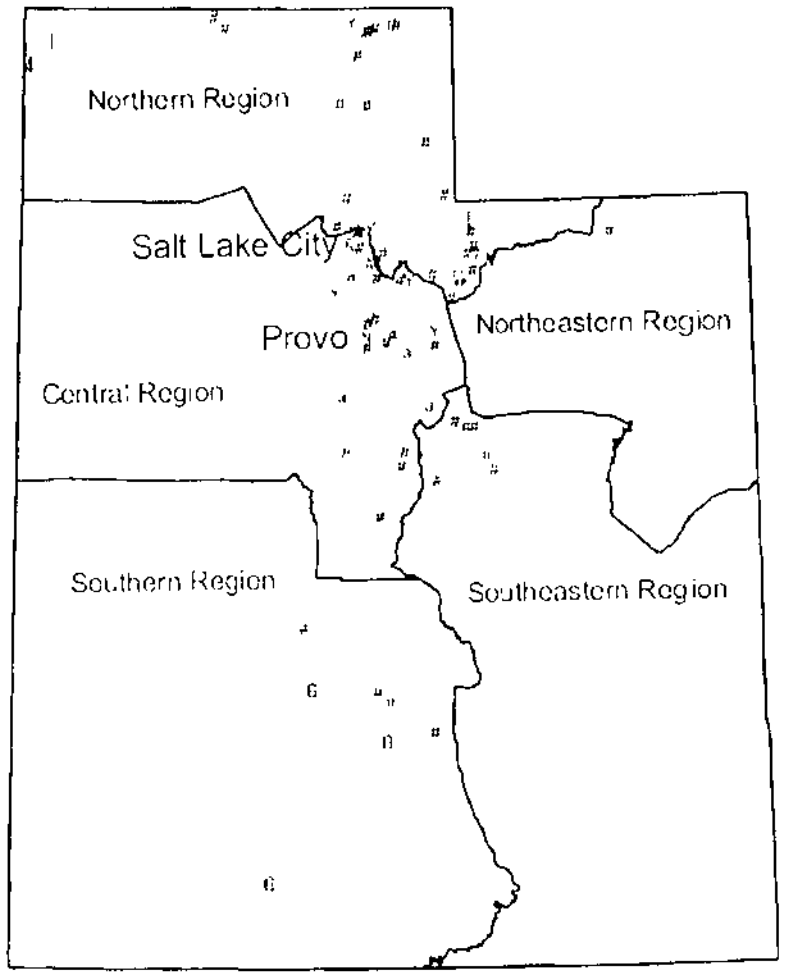
TABLES AND FIGURES



- Museum records (N = 51)
- Pre-1971 reports within reported elevation limits (N = 3)
- Tadpoles present 1992-1993 (N = 3)
- Tadpoles present 1971-1991, not present 1992-1993 (N = 6)
- New record exterior to historic range (N = 3)
- Adults present 1971-1991 (N = 4)

Figure 1-Depicts the historical distribution of Boreal Toads in Utah (Thompson et al. (2005). *Boreal Toad Conservation Plan)*

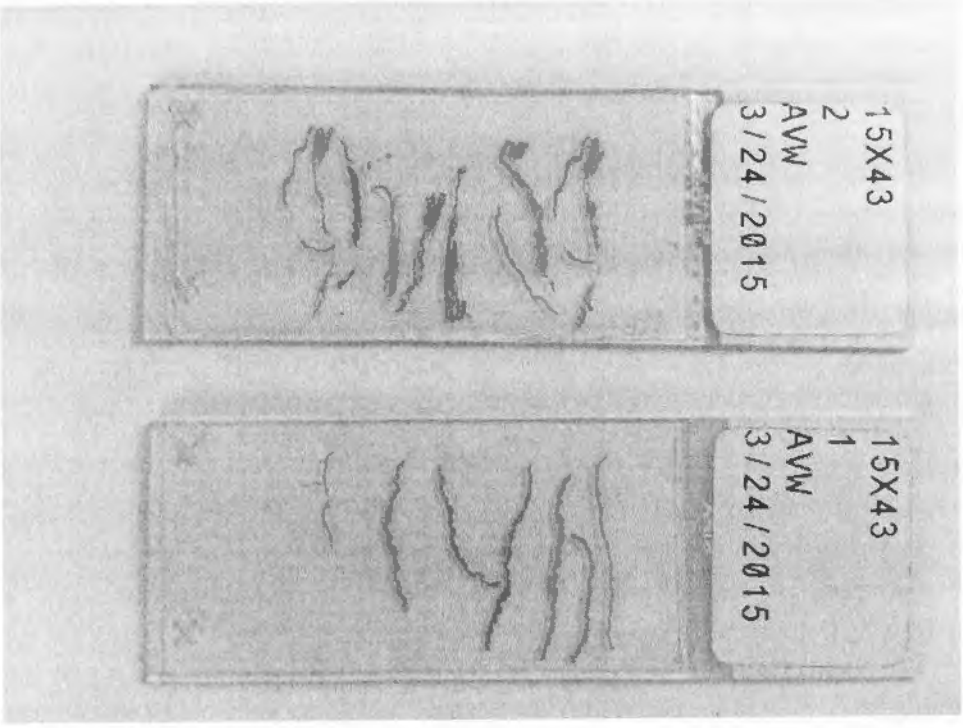


Figure 2-Slides prepared by the Utah Veterinary Diagnostic Lab for histological analysis of Boreal Toad skin samples

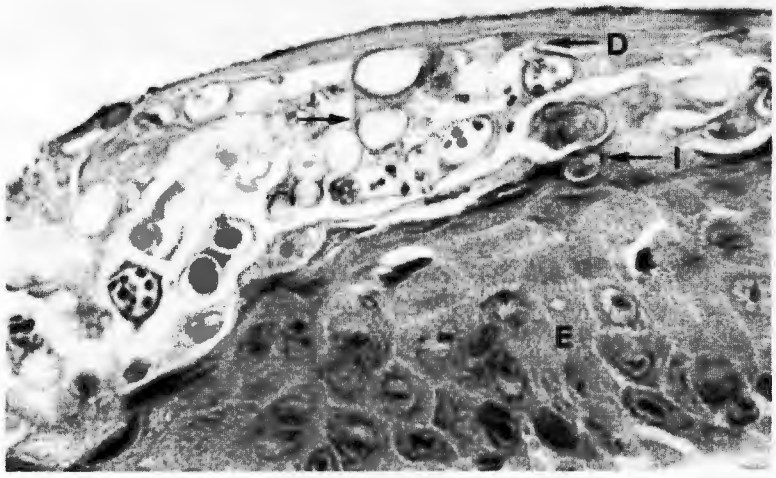


Figure 3- A microscopic skin sample of an adult Australian Green Tree Frog that is heavily infected with chytrid (Berger et al., 1999).

- I- homogenous immature stage
- D- zoosporangium with discharge papillae containing zoospores and empty zoo sporangium
- E- epidermis

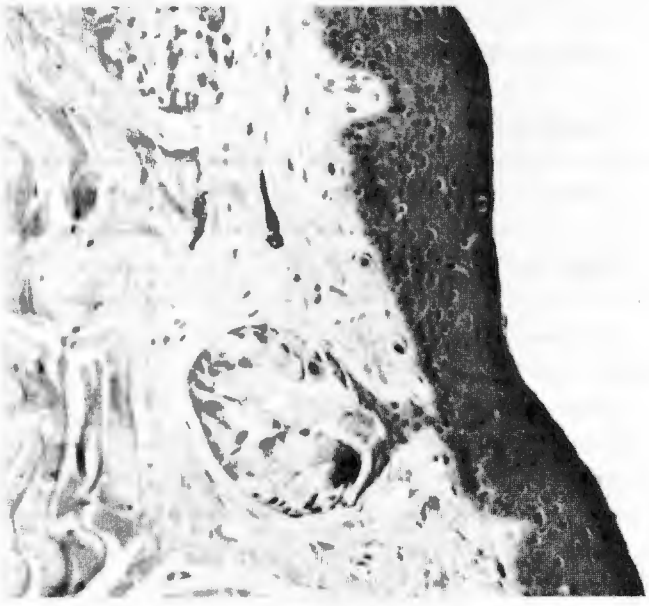


Figure 4-Microscopic view of a skin sample negative for chytrid from the histology of an individual collected in 1961.

Source ID #	Source Information	Tested For	Total Bd Target Copies in Original Sample
2265	BYU-H 1939 (on tube)	B. dendrobatidis	0.00E+00
2852	BYU-H 1939 (on tube)	B. dendrobatidis	0.00E+00
636(A)	BYU-H 5/2/1926 (on tube)	B. dendrobatidis	0.00E+00
539-810, 540-811, 546-63	BYU-H 6/14/1926 (on tubes)	B. dendrobatidis	0.00E+00
10250, 10251, 10175, 10176	BYU-H 6/17/1950 (on tubes)	B. dendrobatidis	0.00E+00
516	BYU-H 6/18/1937 (on tube)	B. dendrobatidis	0.00E+00
1627	BYU-H 6/18/1937 (on tube)	B. dendrobatidis	0.00E+00
547	BYU-H 6/19/1937 (on tube)	B. dendrobatidis	0.00E+00
49996	BYU-H 6/28/11 (on tube)	B dendrobatidis	0.00E+00
764(71)	BYU-H 6/6/1927 (on tube)	B. dendrobatidis	0.00E+00
46480	BYU-H 6/7/1995 (on tube)	B. dendrobatidis	0.00E+00
1302, 1304, 1311, 2187(59)	BYU-H 7/1928, 9/1931 (on tubes)	B. dendrobatidis	0.00E+00
43705	BYU-H 7/2/1960 (on tube)	B. dendrobatidis	0.00E+00
43706	BYU-H 7/2/60 (on tube)	B. dendrobatidis	0.00E+00
41833	BYU-H 8/12/1952 (on tube)	B. dendrobatidis	0.00E+00
43516	BYU-H 8/7/1958 (on tube)	B. dendrobatidis	0.00E+00
961	BYU-H Aug. 1927 (on tube)	B. dendrobatidis	0.00E+00
638-81-6, 640-815, 642-66	BYU-H June 1926 (on tubes)	B. dendrobatidis	0.00E+00
1040(57)	BYU-H June 1928 (on tube)	B. dendrobatidis	0.00E+00
1629	BYU-H June 1937 (on tube)	B. dendrobatidis	0.00E+00
22142	BYU-H May 20, 1961 (on tube)	B. dendrobatidis	**9.43E+02**
5046, 5861 - 5864	BYU-H May 2003; 2004/2005 (on tubes)	B. dendrobatidis	**2.25E+02**

Table 1- The PCR results for each of the BYU samples processed by Pisces for chytrid

Source ID #	Source Information	Tested For	Total Bd Target Copies in Original Sample
19813	UMNH 4/12/1961 (on tube)	B. dendrobatidis	0.00E+00
20296	UMNH 4/22/1961 (on tube)	B. dendrobatidis	0.00E+00
19678	UMNH 5/19/1972 (on tube)	B. dendrobatidis	0.00E+00
19679	UMNH 5/19/1972 (on tube)	B. dendrobatidis	0.00E+00
20297	UMNH 5/27/1961 (on tube)	B. dendrobatidis	0.00E+00
19810 - 19812	UMNH 5/27/1961, 4/22/1961 (on tubes)	B. dendrobatidis	0.00E+00
19817	UMNH 5/30/1972 (on tube)	B. dendrobatidis	0.00E+00
20477 - 20480	UMNH 6/15/1960 (on tubes)	B. dendrobatidis	0.00E+00
21349	UMNH 6/25/1988 (on tube)	B. dendrobatidis	0.00E+00
19777	UMNH 6/3/1967 (on tube)	B. dendrobatidis	0.00E+00
3356 - 3359	UMNH 7/12/1959 (on tubes)	B. dendrobatidis	0.00E+00
377, 8013 - 8016	UMNH 7/15/1934 (on tubes)	B. dendrobatidis	0.00E+00
2685	UMNH 7/18/1941 (on tube)	B. dendrobatidis	0.00E+00
20298	UMNH 7/22/1947 (on tube)	B. dendrobatidis	0.00E+00
12211 - 12213	UMNH 7/25/1964 (on tubes)	B. dendrobatidis	0.00E+00
1869 - 1874	UMNH 8/17/1932 (on tubes)	B. dendrobatidis	0.00E+00
1850 - 1856	UMNH Autumn 1932 (on tubes)	B. dendrobatidis	0.00E+00
1813	UMNH June 17 1927 (on tube)	B. dendrobatidis	0.00E+00
1814	UMNH June 17, 1927 (on tube)	B. dendrobatidis	0.00E+00

Table 2- The PCR results for each of the UMNH samples processed by Pisces for chytrid

AUTHOR BIOGRAPHY

Samantha A. Beirne is a senior from Castle Rock, CO. She will be graduating from Utah State University in Spring 2015 with a Bachelor of Science in Wildlife Science and a minor in Biology. During her undergraduate career, Samantha worked as an Academic Advising Assistant for the Quinney College of Natural Resources and has participated in undergraduate research under the mentorship of Dr. Karen Beard. Samantha has also worked as a lab assistant for Drs. Diane Alston and Michelle Baker in the Biology department. Samantha was also involved in the Quinney College of Natural Resources Student Council and the University Honors Student Council as a service director. Samantha has received several scholarships throughout her career, including the USU Dean's Scholarship, the Nat E. and Kacky Bailey Frazer Scholarship, and the Seely-Hinckley Scholarship. Samantha has spent time working at the Willow Park Zoo in Logan, UT and spent last summer in Houston, TX as a Toad Conservation Intern for the Houston Zoo. Samantha frequently volunteers at Global Village Fair Trade Gifts and enjoys rock climbing. Samantha is looking forward to a career as a zookeeper and would also like to pursue conservation biology. She has taken an interest in the husbandry and research of primates, amphibians, and other wildlife species. Samantha will be working as a Keeper Assistant at the Cheyenne Mountain Zoo in Colorado Springs, CO this coming May.