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PROMOTING AMPHIBIAN CONSERVATION THROUGH THE COLLEGE CLASSROOM: DETECTION OF *BATRACHOCHYTRIUM DENDROBATIDIS* AMONG LOCAL AMPHIBIANS

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Abstract.—Many global amphibian declines have been linked to the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*). The knowledge on *Bd* distribution provides a fundamental basis for amphibian conservation planning. Yet, such *Bd* distribution information is currently insufficient, in particular at a regional scale. The college classroom provides an excellent opportunity to expand the knowledge of *Bd* distribution. Here we provide an example of such research projects to detect *Bd* prevalence among local amphibians in a college course setting and present the results of work conducted in central Pennsylvania, USA. We collected toe clips and conducted PCR assays of six species, *Plethodon cinereus*, *Desmognathus fuscus*, *Notophthalmus viridescens*, *Lithobates catesbeianus*, *L. clamitans*, and *L. sylvaticus* (59 individuals). Four groups of students independently conducted entire projects, orally presented their findings, and submitted manuscripts to the professor at the end of the semester. This example demonstrates that it is feasible for an undergraduate class to complete a *Bd*-detection project within a single semester. Such a project not only contributes to *Bd* research but also promotes conservation education among students through hands-on research experiences. We found *Bd* infection in only one sample of *N. viridescens*, but no sign of infection in the rest of the samples. As a relatively high prevalence of *Bd* has been reported in surrounding areas, our results suggest spatial heterogeneity in *Bd* occurrence at a regional scale and thus, the need for continued efforts to monitor *Bd* prevalence.

Key Words.—amphibian conservation; *Batrachochytrium dendrobatidis*; chytridiomycosis; college classroom; environmental education; Pennsylvania

INTRODUCTION

A large proportion of global amphibian populations are disappearing or under threat of extinction (Stuart et al. 2004). As a result, amphibians are the most imperiled vertebrate taxa (Hoffmann et al. 2010). Many recent studies have linked this global decline with the parasitic fungus *Batrachochytrium dendrobatidis*, or *Bd*, that causes the disease amphibian chytridiomycosis (Berger et al. 1998; Daszak et al. 1999; Stuart et al. 2004; Lips et al. 2006; Skerratt et al. 2007). While researchers seek tools and mechanisms by which global amphibians may resist, tolerate, or coexist with *Bd* (Griffiths and Pavajeau 2008; Harris et al. 2009; Buck et al. 2011; Savage and Zamudio 2011; Searle et al. 2011), understanding dynamic spatial patterns of *Bd* provides a fundamental basis for conservation efforts. Detection of *Bd* requires specific skills and knowledge, appropriate facilities, and funding; thus, unlike frog calling surveys it is difficult for laypersons (e.g., volunteers) to conduct

such assays. On the other hand, such projects often lack scientific novelty and may not be rewarding or appealing to many university researchers. These reasons, at least in part, likely explain the lack of knowledge of *Bd*'s spatial distribution in many areas of the world, including the United States (e.g., www.Bd-maps.net).

One way to promote the expansion of *Bd* distribution data is through the use of college classrooms. Integrating *Bd*-detection projects into courses such as Herpetology or Amphibian Biology could provide valuable information to the scientific community and also an excellent educational opportunity for undergraduate students. Students learn scientific processes by doing science and develop a vivid understanding and appreciation of the conservation issues surrounding amphibians.

As students in a junior/senior-level Amphibian Biology course at Bucknell University (Lewisburg, Pennsylvania, USA), we investigated the prevalence of *Bd* in six local amphibian species: *Notophthalmus*

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TABLE 1. An example of single-semester laboratory schedule to which chytrid fungus (*Batrachochytrium dendrobatidis*) detection project is integrated. A semester consists of 14 weeks and a single laboratory session (3 hr) is given to each week. “Half class” indicates that the class is divided into two groups, each of which is assigned to a different activity within a laboratory session.

Week	Activities
1	Introduction to global amphibian decline and <i>Bd</i> Field trip arrangement
2	Field Trip I to streams
3	Field Trip II to ponds
4	Field Trip III to forests
5	Field Trip IV to all habitats
6	DNA extraction I (half class) Anuran identification (half class) Research paper introduction due
7	DNA extraction II (half class) Anuran identification (half class)
8	PCR I (half class) Anuran identification (half class)
9	PCR II (half class) Caudata identification (half class)
10	Electrophoresis I (half class) Caudata identification (half class)
11	Electrophoresis II (half class) Caudata identification (half class)
12	Review
13	Lab practical (species identification)
14	Research paper due Research project presentations

viridescens (Eastern Newt), *Plethodon cinereus* (Red-backed Salamander), *Desmognathus fuscus* (Northern Dusky Salamander), *Lithobates catesbeianus* (American Bullfrog), *L. clamitans* (Green Frog), and *L. sylvaticus* (Wood Frog). *Batrachochytrium dendrobatidis* prevalence data are lacking from central Pennsylvania where the University is located. To our knowledge, only one species, *N. viridescens*, had been examined for *Bd* occurrence in our region (Raffel et al. 2010).

Batrachochytrium dendrobatidis requires a constant aquatic environment for survival (Johnson et al. 2003) and certain amphibian species can carry the pathogen without experiencing morbidity or mortality (Daszak et al. 2004; Hanselmann et al. 2004; Weldon et al. 2004; Garner et al. 2006). Species such as *D. fuscus*, *L. catesbeianus*, and *L. clamitans* that breed in permanent streams and ponds are more likely to come into contact with *Bd* than species that are terrestrial throughout their life histories, such as *P. cinereus* (Kriger and Hero 2007). *Lithobates catesbeianus* has been indicated as a

key vector and reservoir species for the global spread of *Bd* as it does not generally show morbidity or mortality from *Bd* infections (Daszak et al. 2004; Hanselmann et al. 2004; Weldon et al. 2004; Garner et al. 2006; Schloegel et al. 2010). *Notophthalmus viridescens* often occupies the same habitat as *L. catesbeianus*, and may experience an increased risk of infection as a result. Therefore, we predicted that *Bd* would be more likely detected in water-dwelling species over terrestrial species.

Raffel et al. (2010) sampled 16 *N. viridescens* populations in Centre County, an adjacent county to our sampling sites, and found *Bd* in 12 populations. Groner and Relyea (2010) sampled adult *N. viridescens* and juvenile *L. clamitans* in northwestern Pennsylvania and found a high occurrence of *Bd* in the former and low occurrence in the latter. These studies suggest that *Bd* may be common among amphibians in central Pennsylvania. However, *Bd* is known to display high levels of heterogeneity in its occurrence depending on regions, habitat types, and host species (Peterson et al. 2007, Glenney et al. 2010; Groner and Relyea 2010; Raffel et al. 2010). Thus, it is important to gain the higher regional resolution of *Bd*'s spatial distribution to better understand the prevalence of *Bd* in Pennsylvania and the eastern United States. The aims of the current paper are twofold; first, to promote the accumulation of data on *Bd* distribution via the college classroom by providing a course framework, and second, to provide our findings on *Bd* prevalence among amphibian populations in central Pennsylvania, USA, where the information about *Bd* occurrence is limited.

MATERIALS AND METHODS

Our research was performed by 12 undergraduate students and one graduate student during a laboratory portion of an Amphibian Biology course offered at Bucknell University in the fall of 2011. Research was supervised by the professor (MKT) but the students performed all of the work themselves. Students were divided into four groups (three groups of three and one group of four). Each group chose a specific habitat type: forest, stream, or pond, from which each group collected toe or webbing clips of amphibians. Because there were more pond-breeding than stream or forest-dwelling amphibians in our region, we created two pond groups. Each group conducted preliminary research on amphibian species they were likely to encounter in their designated habitat (Week 1 in Table 1). During the following four weeks, the entire class conducted several field sampling trips to forest, pond, and stream habitats (Fig. 1) located within 45 min driving distance from the campus (40°57'22.3"N, 76°52'58.7"W). We caught all animals by hand, except for *N. viridescens*, which we caught using dip nets. We collected tissues samples

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FIGURE 1. Field sample collection by students in an Amphibian Biology course at Bucknell University in (A) a pond, (B) a stream, and (C) a terrestrial habitat. D: A field sample collection station in which students organized the collected tissue samples and recorded data. (Photographed by Mizuki K. Takahashi)

instead of skin swabs because recent studies suggest tissue samples may provide higher sensitivity in detection of *Bd* than cotton-tipped swabs (Davidson and Chambers 2011; Gratwicke et al. 2011). Whenever possible, we collected toe webblings from frogs because toe-webblings provide reliable tissue samples for *Bd* detection (Longcore et al. 2007). We also believed that removing toe webblings would be less invasive than removing toes. All toe clips and webblings were removed from animals by the professor of the class or the graduate student (MJW), who were issued the collecting permit by the Pennsylvania Fish and Boat Commission.

We collected 18 *N. viridescens*, 15 *D. fuscus*, 15 *P. cinereus*, eight *L. clamitans*, two *L. catesbeianus*, and one *L. sylvaticus* specimen from Columbia, Northumberland, and Union counties, Pennsylvania, during September and October 2011. The terrestrial habitat group analyzed *P. cinereus* samples collected

from two sites (40°54'19.6"N, 76°54'27.5"W, 239 m in elevation and 41°1'39.5"N, 76°56'34.6"W, 169 m in elevation). The stream group analyzed *D. fuscus* samples collected from two sites (40°55'3.6"N, 76°53'20.3"W, 175 m in elevation and 41°1'39.5"N, 76°56'34.6"W, 169 m in elevation). One pond group investigated three frog species: *L. catesbeianus* from two sites (41°1'39.5"N, 76°56'34.6"W, 169 m in elevation and 40°50'13.5"N, 76°21'21.4"W, 345 m in elevation), *L. clamitans* from one site (40°50'13.5"N, 76°21'21.4"W, 345 m in elevation), and *L. sylvaticus* from one site (40°50'13.5"N, 76°21'21.4"W, 345 m in elevation). Finally, the second pond group analyzed *N. viridescens* samples collected from three sites (41°1'39.5"N, 76°56'34.6"W, 169 m in elevation; 40°50'13.5"N, 76°21'21.4"W, 345 m in elevation; and 40°54'40.4"N, 77°17'0.9"W, 456 m in elevation). All specimens were live upon discovery and none showed any visible signs of infection such as red feet and groin or sloughing skin. After

TABLE 2. *Batrachochytrium dendrobatidis* prevalence among amphibians in central Pennsylvania, USA surveyed in the autumn of 2011 by students in an Amphibian Biology course at Bucknell University. One group of students sampled a terrestrial (*Plethodon cinereus*) habitat, a second group a stream (*Desmognathus fuscus*) habitat, and two groups surveyed a pond (*Lithobates catesbeianus*, *Lithobates clamitans*, *Lithobates sylvaticus*, *Notophthalmus viridescens*) habitat for local amphibians. Two pond groups were used because of the higher species diversity of pond-associated amphibians.

Species	Number of <i>Bd</i> positives with original DNA concentration /Number tested	Number of <i>Bd</i> positives with diluted DNA concentration /Number tested
<i>Desmonagthus fuscus</i>	0/15	0/15
<i>Lithobates catesbeianus</i>	0/2	0/2
<i>Lithobates clamitans</i>	0/8	0/8
<i>Lithobates sylvaticus</i>	0/1	0/1
<i>Notophthalmus viridescens</i>	0/18	1/18
<i>Plethodon cinereus</i>	0/15	0/15
Total	0/59	1/59

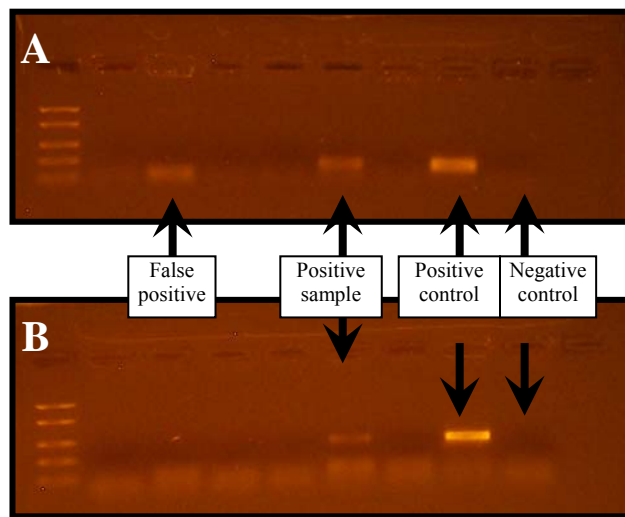


FIGURE 2. Gel electrophoresis images after PCR with (A) primer set from Boyle et al. (2004) and (B) primer set from Annis et al. (2004). The same six diluted DNA samples of *N. viridescens* as well as positive and negative controls are shown in both gel images. Samples were loaded and run from top to bottom. DNA ladders to the left indicate 50, 200, 400, 800 and 1500 bps from the bottom to the top. (Photographed by Mizuki K. Takahashi)

collecting toe clips and webbings, we released amphibians at the site of capture. We wore nitrile gloves when collecting amphibians and changed them between samplings to prevent cross contamination and spread of *Bd*. We rinsed all tools used for tissue collection in a 50%

bleach solution and a 50% ethanol solution between samples. Collected samples were stored at -20 °C until we conducted PCR assays.

During weeks six through 11, we conducted DNA extraction, PCR, and electrophoresis (Table 1). During this period, the class was divided into two groups; one half worked on molecular analyses while the other half studied amphibian specimen identification (Table 1). This class division allowed the professor to closely train and monitor a small group of students (i.e., six to seven) working on molecular analyses.

To extract DNA, 40.0 µL of Prepman Ultra was added to each toe clip vial. We prepared a 1:10 diluted DNA sample by adding 5.0 µL of the extracted DNA to 45.0 µL of double distilled water. We prepared a PCR master mix containing 0.4 µM of forward and reverse primers, 50 mM KCl, 0.5 units of Taq polymerase, 0.8 mM dNTPs, 10 mM Tris at a pH of 8.4, and ultrapure reagent water. To test for presence of *Bd* DNA, we added 1 µL of each extracted DNA sample to 11.5 µL of master mix for PCR. We used ultrapure reagent water as a negative control while we used known *Bd* DNA as a positive control. In total, we prepared 118 PCR reactions (each of 59 samples with original and diluted DNA concentrations).

There are two different sets of primers available for PCR assays (Annis et al. 2004; Boyle et al. 2004). Because our preliminary tests suggested the primer set described in Boyle et al. (2004) may detect *Bd* DNA with a slightly wider concentration range, we used these primers for the first round. When we detected positive results through gel electrophoresis, we retested samples using the primers reported in Annis et al. (2004). We double checked positive samples because some of the positive results detected by the primers reported in Boyle et al. (2004) showed a slightly shorter fragment size (between 50 and 100 bps) than the expected length of 146 bps which the positive control correctly showed (Fig. 2A). The PCR cycle that we used follows that described in Annis et al. (2004) and Davidson and Chambers (2011). We ran each sample through agarose gel electrophoresis at 120 v for 16 min and examined them under UV light.

RESULTS

Using the primers reported in Boyle et al. (2004), we found 10 positive reactions (four *P. cinereus*, three *N. viridescens*, and three *D. fuscus*). However, only one (a diluted *N. viridescens* sample) expressed a band at the expected length of ca. 146 bps, while the other nine had visibly shorter fragments (50–100 bps, Fig. 2A). Accordingly, we tested these samples with an alternate set of primers as reported by Annis et al. (2004). With this primer set, the sample expressing the 146 bps

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fragment size in the first round was again positive, while the other samples were negative (Fig. 2B). Thus, our analyses suggest that there was only one positive reaction out of 118 PCR reactions of the 59 samples tested (Table 2).

DISCUSSION

This laboratory project of *Bd* detection among local amphibians demonstrates that it is feasible for an undergraduate class to complete such a project within a 14-week semester with the exception of the preparation of this manuscript submitted to *Herpetological Conservation and Biology* (HCB hereafter). Upon the completion of the project, each group prepared a manuscript following the format of HCB and submitted it to the professor. Each group gave a formal presentation to the rest of the class at the end of the semester. One student (JLW) synthesized a single manuscript for submission to HCB by combining the manuscripts submitted by each of the four groups. Another student (NML) presented the findings at a campus-wide research symposium in March 2012.

The maximum class size to conduct a project such as this would be 16 students (four groups of four students). Groups larger than four students would not allow each student to gain sufficient research experience, while it may be difficult for an instructor to manage more than four groups. Our sample size of 59 amphibians may not be ideal for a scientific purpose; yet, a sample size around 60 is a realistic goal for an undergraduate class to accomplish within a semester. Indeed, we had to organize occasional meetings and lab work outside of scheduled laboratory to complete the project. To increase sample size and sample diversity included in a project, continuous monitoring of *Bd* prevalence through the college classroom is essential.

Our results indicate that only one specimen out of 18 *N. viridescens* (5.6%) examined, and only one specimen out of the 59 total specimens (1.7%) tested, was positive for *Bd*, suggesting low *Bd* prevalence among our sampled amphibians in central Pennsylvania. Other Pennsylvania studies have reported higher occurrences of *Bd*. For example, Groner and Relyea (2010) reported an infection rate of 34.8% (16 of 46 individuals tested) in *N. viridescens* populations of northwest Pennsylvania and Raffel et al. (2010) found an infection rate of 39.2% (60 of 153 individuals tested) in this species in an adjacent county to our sampling sites. However, another study of *N. viridescens* populations in eastern Pennsylvania along the Delaware Water Gap National Recreation Area found no infected individuals with 41 tested for *Bd* (Glenny et al. 2010). A similar case of spatial heterogeneity in this pathogen has been reported in Wise and Warren counties, Virginia, USA. When

only comparing salamander species surveyed in both studies, the prevalence in Wise County was 14.8% (4/27; Davidson and Chambers 2011), while it was only 0.6% (1/171) in Warren County (Gratwicke et al. 2011). These variations in infection rates exemplify our limited understanding of *Bd* spatial heterogeneity and highlight the need for an increased resolution of the pathogen's distribution.

It is possible that the low *Bd* occurrence found in our study is explained by the sampling season. *Batrachochytrium dendrobatidis* prefers cold-water environments under 25 °C (Berger et al. 2005) and we sampled during September and October following warm summer months, which might have reduced *Bd* prevalence. Groner and Relyea (2010) sampled in April/May and Raffel et al. (2010) sampled in May/June and both studies found *Bd* positive specimens. Seasonal fluctuations in water temperature and hydrology (e.g., drying of ephemeral ponds) would be expected to impact infection rates of an aquatic fungus that grows best in cooler aquatic habitats. Our sample size for some species was small and thus, we suggest future classroom projects should test more individuals. In particular, it will be beneficial to examine seasonal changes in *Bd* infection rates within populations.

Improving our understanding of the spatial and temporal heterogeneity in *Bd* distribution is one area that would greatly benefit from future research. Multiple factors such as habitat, pond-substrate configuration, water temperature, and shading influence the distribution and prevalence of this pathogen (Kriger and Hero 2007; Raffel et al. 2010). A more accurate map of *Bd* distribution would allow for improved predictive models of its occurrence. This study is an example of one method for improving our knowledge of the distribution of *Bd*, as it was carried out as part of an undergraduate course. Students gained hands-on experience with *Bd* and amphibian conservation as well as general scientific techniques, development of a research project, field and laboratory work, and writing for submission to a scientific journal. Projects such as *Batrachochytrium dendrobatidis* detection foster passion, excitement, and engagement among undergraduate students, while making a significant contribution to the scientific community.

The primers described by Boyle et al. (2004) provided false positives in our study that occurred with shorter fragment lengths (50–100 bps) than the expected length of 146 bps. Although Boyle et al. (2004) designed the primers specifically for quantitative PCR, such primers should, in principle, work in a traditional PCR as well. Indeed, these primers consistently detected our positive control at the expected fragment length. However, because of potential false short-length positives, we suggest that future studies using traditional PCR should

use the primer set that Annis et al. (2004) developed specifically for traditional PCR.

Amphibians are the most imperiled taxa on earth (Hoffmann et al. 2010) and amphibian chytridiomycosis has contributed significantly to this decline (Skerratt et al. 2007). Our results, and those of other studies, indicate that the distribution of *Bd* is heterogeneous throughout the eastern United States (Glenney et al. 2010; Groner and Relyea 2010; Raffel et al. 2010). Because *Bd* displays regional heterogeneity in its prevalence, high resolution spatial data are crucial for understanding and predicting the spatial and temporal patterns of *Bd* prevalence (Peterson et al. 2007, Glenney et al. 2010; Groner and Relyea 2010; Raffel et al. 2010). It is important to understand the external factors influencing the resistance and tolerance of species and populations to *Bd* (Hayes et al. 2010). It appears that amphibians in the eastern US have not experienced mass die-off associated with *Bd*. This may be because the *Bd* strain in the eastern US is endemic and does not exhibit high virulence (Farrer et al. 2011). Yet, a hyper virulent *Bd* strain, which has likely emerged through the recent recombination of isolated *Bd* populations, has been expanding its distribution range (James et al. 2009; Farrer et al. 2011). If such a hyper virulent *Bd* emerges in areas with healthy amphibian populations, the expanded knowledge of *Bd* distribution may help conservation biologists determine how conservation efforts should be prioritized. Understanding how, why, and where *Bd* has spread is fundamental to protecting amphibian diversity and undergraduate students may be the key to cataloguing this information.

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Herpetological Conservation and Biology



JULIE L. WUNDER is currently an undergraduate student at Bucknell University in Lewisburg, Pennsylvania working towards a B.S. in Biology. She is most interested in animals, particularly mammals and aquatic species, on an organismal and ecological level. (Photographed by Jordan Van Horn)



NOEL M. LAMPAZZI is currently an undergraduate student studying Environmental Science with a concentration in conservation at Bucknell University in Lewisburg, Pennsylvania. Noel is interested in invasive species remediation, marsh ecology, and amphibians and will be researching invasive species under Mizuki Takahashi in 2012. Originally from Houston, Texas, Noel currently plans to attend graduate school to study marsh restoration or species conservation and to one day return to the Gulf Coast as a salt-water marsh ecologist and/or conservationist. (Photographed by Tom Beaman)



TAMARA F. MILTON (left) is a Bucknell University senior student graduating in May 2012. Tamara is double majoring in Environmental Studies and Biology. She will enter the Peace Corps upon graduation, participating in the environmental education and awareness program in Latin America.

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