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Boreal Toad Research Report

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Preface

The Boreal Toad Recovery Team was formed in 1994, in response to reports of significant declines in boreal toad distributions in the Southern Rocky Mountains. These apparent declines resulted in an "Endangered" listing by the states of Colorado and New Mexico, and a "Status 1" protected species designation in Wyoming. The boreal toad is currently classified as a candidate species that is "warranted but precluded" for federal listing under the Endangered Species Act. The first Boreal Toad Recovery Plan was completed in 1994 under the direction of John Goettl; the Recovery Plan and Conservation Agreement have since been combined into one working document (Loeffler 2001). Currently, the Boreal Toad Recovery Team is coordinated by Tina Jungwirth, Aquatic/Herptile Coordinator for the Colorado Division of Wildlife (CDOW).

This report represents the CDOW sponsored boreal toad research done in 2003 by several researchers, and has been consolidated into a single document to make this information available to members of the Boreal Toad Recovery Team and other interested parties. The report covers research on a variety of topics, including:

- Repatriation of boreal toads *Bufo boreas* on the Grand Mesa, Colorado.
- Evaluation of techniques for detecting *Batrachochytrium dendrobatidis* from amphibians for PCR testing.
- Survey of *Bufo boreas* and other Southern Rocky Mountain amphibians for *Batrachochytrium dendrobatidis*.
- Use of sentinel tadpoles and toadlets to detect *Batrachochytrium dendrobatidis*.
- Genetic analyses of *Bufo boreas*: analyses of toads from Buck and Chall Creeks, Wyoming.
- Preliminary look at aquatic macroinvertebrates as reservoirs of *Batrachochytrium dentrobatidis* infection
- Smile widths as an alternative to measuring snout-vent lengths

Loeffler, C. 2001. Conservation plan and agreement for the management and recovery of the southern Rocky Mountain population of the boreal toad (*Bufo boreas boreas)*. Boreal Toad Recovery Team. 76 pp.

Funding for boreal toad research and recovery efforts in Colorado is provided through Great Outdoors Colorado.

Repatriation of boreal toads *Bufo boreas* on the Grand Mesa, Colorado

By Kevin B. Rogers[1] and Tanya Banulis[2]

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Introduction

Populations of boreal toads *Bufo boreas boreas* have experienced a dramatic decline in Colorado over the last 25 years (Carey 1993, Loeffler 2001), leading to its listing as a state endangered species and the formation of the Boreal Toad Recovery Team. In an effort to reverse this discouraging trend, this team has identified experimental repatriation as a high priority (Loeffler 2001). In fact, repatriation is probably necessary to meet recovery criteria (Holland 2002). Unfortunately, efforts to date have been largely unsuccessful (Muths et al. 2001, Livo and Loeffler 2003). Chytrid fungus *Batrachochytrium dendrobatidis* (BD) has been implicated in the demise of several populations of boreal toads in Colorado (Holland 2002, Livo and Loeffler 2003, Muths et al. 2003), and presumably has played a role in limiting the success of repatriation attempts to date. New attempts to reestablish populations of boreal toads should focus on suitable habitats where chytrid fungus is not present. This study explores the efficacy of introducing various boreal toad life stages for establishing new populations in what was thought to be a BD negative site on the Grand Mesa, Colorado. Replication of this effort will occur at additional sites as viable candidate sites are identified.

Study site

The proposed study site lies in the Kannah Creek drainage on the south side of the Grand Mesa in Mesa County, Colorado (Figure 1). This location falls in the Grand Mesa National Forest within 5 km of sites occupied historically in the Island Lakes and Bull Creek Reservoir area. The site is comprised of half a dozen small ponds in close succession that provide a number of potentially suitable breeding areas with excellent breeding shallows. Willow (*Salix* sp.) and large boulders surround the immediate area. The Grand Mesa was intensively surveyed from 1997 through 1999, but no boreal toads were documented (Mark Jones, Colorado Division of Wildlife, personal communication).

Three adult boreal toads and a group of unidentified tadpoles were discovered in 2002 in the Buzzard Creek drainage of the Grand Mesa (approximately 25 miles from the proposed Kannah Creek study site). Two adults were detected in 2003, but additional surveys failed to locate a breeding site. These toads did test positive for BD with the polymerase chain reaction (PCR) test (Annis et al. 2004), making them less suitable as a source of eggs for the repatriation effort.

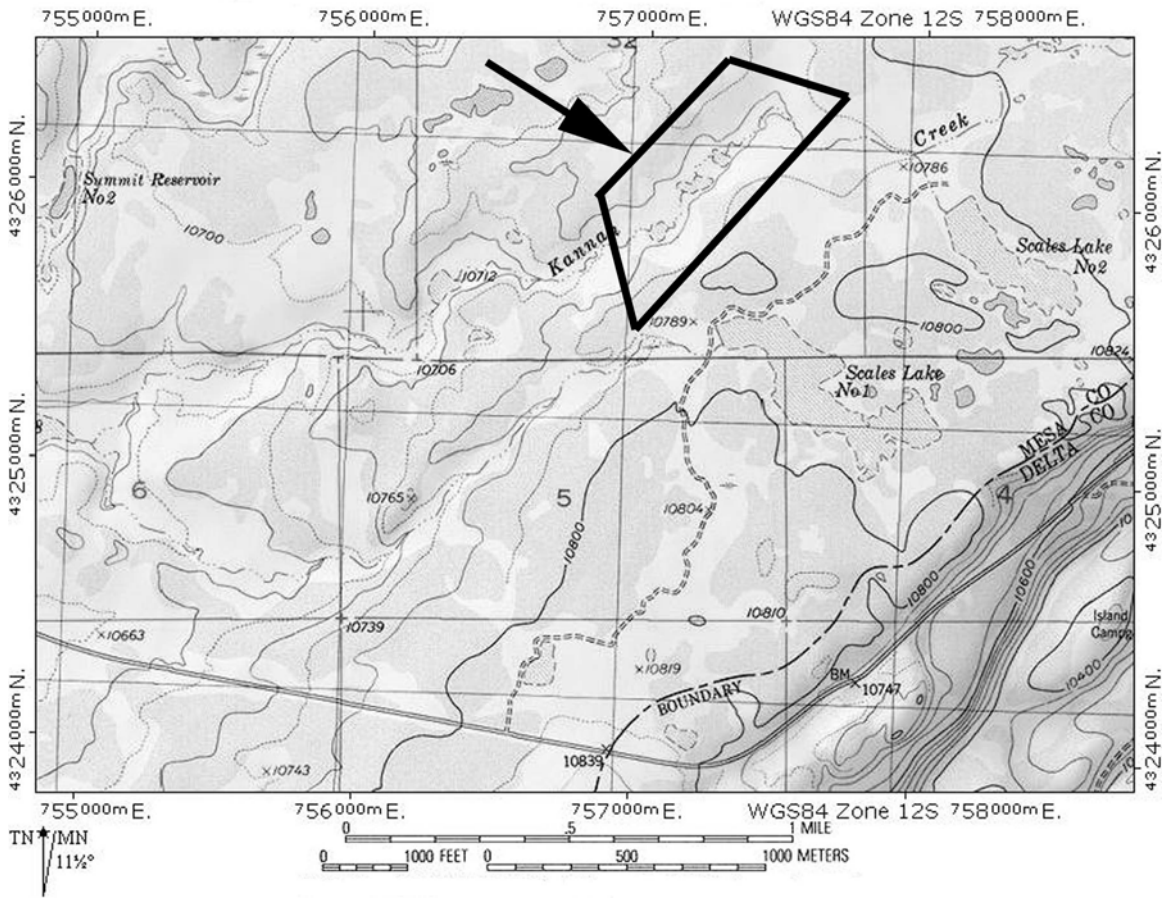

FIGURE 1: Proposed study site is outlined and indicated by the arrow. The site lies in the Kannah Creek drainage on the Grand Mesa National Forest.

Methods

Release:

Given difficulties transporting live eggs, and the prohibitive cost of raising toadlets past 4 weeks, this effort is focused on life stages that are realistic in terms of their ability to be implemented on a broad scale for recovery efforts. The Chaffee County boreal toad metapopulation was selected as a founding source for this effort because they represent the nearest known breeding population, are BD negative, and the large population is capable of producing surplus eggs that can be taken in to the Native Aquatic Species Restoration Facility (NASRF) in Alamosa, Colorado and raised to the life stages needed without negatively effecting the source population. In addition, captive breeding stock from Chaffee County at NASRF is coming of age, and can potentially reduce the need to harvest of eggs from the wild. Breeding of these individuals in 2003 was unsuccessful, presumably due to their lack of maturity. It is hoped that in subsequent years these animals will also provide eggs suitable for use in the repatriation effort. As the hatchery stock of Chaffee County boreal toads at the NASRF are derived from only 3 females however, they will only contribute a portion of the eggs to be used in this study.

Approximately 20,000 eggs from 14 clutches were harvested from the Chaffee County boreal toad metapopulation on May 26, 2003. These eggs represented four clutches from the Collegiate sub-population, three from the Middle Cottonwood sub-population, and seven from the South Cottonwood sub-population. Eggs were brought to NASRF, and reared to Gosner stage 25 tadpoles (Gosner 1960). Although the bulk of the egg stock was mixed after hatching, several dozen eggs from each clutch were raised separately in buckets to establish a bank of genetic material. These tadpoles were sacrificed, and their tails preserved in 70% ethanol. Over 12,000 (TAD) were released unmarked around the margins of three ponds at the study site on June 25th, 2003 (Table 1). An additional 1,200 were divided among six pens (two per pond) on that same day. These pens were similar to those used by Scherff-Norris (1999) but used fiberglass insect screening stapled to a wood frame, reinforced with silicone (Figure 2). Each pen was fitted with a locking lid that enclosed a wetted surface area of 1.11 square meters. These tadpoles comprised the PEN group and were raised to metamorphosis, toe clipped for future identification (see below), and released. Two thousand tadpoles were retained at NASRF and reared for three weeks post metamorphosis. Eight hundred were then given a finger clip and divided among the same three ponds on August 29th, 2003. Original plans called for a group of tadpoles raised at NASRF to be released as metamorphs in addition to the three-week old toadlets. Production problems prevented the release of such a group in 2003, though this group will not be reinstated in 2004 even if animals are available. Metamorphosis occurs across such a broad time frame even among siblings held at identical conditions that a discrete release of metamorphs that had not yet been trained on live feed would not be feasible.

FIGURE 2: Tadpoles (PEN group) were reared on site in 0.6 x 1.8 m wooden pens enclosed with a fiberglass insect screen

TABLE 1: Number of boreal toads released in the Kannah Creek drainage study site including their respective life stages.

Life stage	Code	Number	Clip
Tadpole	TAD	12,638	Unmarked
Tadpole	PEN	1,200	Right hand finger
Toadlet	HAT	816	Left hand finger

Marking:

Origin of introduced animals will be identified with toe clip batch marks following Martof's (1953) numbering scheme. If a viable alternative marking scheme is produced during the study, it may be implemented in future years. The thumb and first finger on the front foot were not cut, as they are important in feeding and mating (Ferner 1979, Honegger 1979). All toads were clipped with sterilized clippers, with the entire finger being removed and the open wound treated with Bactine®. All hatchery toadlets (HAT) received a left hand finger clip (finger 400 in 2003). Metamorphs reared in pens on site from tadpoles (PEN) received a right hand clip (finger 3200 in 2003). Since the remaining experimental group was released as tadpoles (TAD), they were unmarked.

Rearing and Monitoring:

Following release, captive tadpoles (PEN) were fed a combination of diets three times weekly that matched the feeding regime of their siblings raised at the hatchery (HAT). Gosner stage, mass (g) and total length (mm) of growing tadpoles was recorded weekly in each pen as well as for individuals captured in the wild. Water temperatures were monitored at four-hour intervals with temperature loggers (Onset Computer Corp., Pocasset, MA) over the course of the study. Pen raised toadlets were clipped and released once metamorphosis was complete.

The reintroduction site was surveyed weekly with a modified line transect protocol. Randomly placed transects radiated perpendicularly from the perimeter of each pond and extend for 100 m (Figure 3). Monitoring procedures followed those outlined by Loeffler (2001). Vegetation was moved aside to aid in detection, but no destruction or permanent modification of habitat occurred. Surveys were conducted between mid-morning and mid-afternoon, with emphasis on sunny days. Surveys were initiated one week following the release of tadpoles, and conducted weekly thereafter until no more toadlets were found. When toadlets were recaptured, the location (distance from line), substrate, snout-vent length (mm), mass (g), Gosner stage, and presence and condition of toe clips was evaluated. In addition, condition was also monitored continuously to evaluate discrepancies between treatment groups.

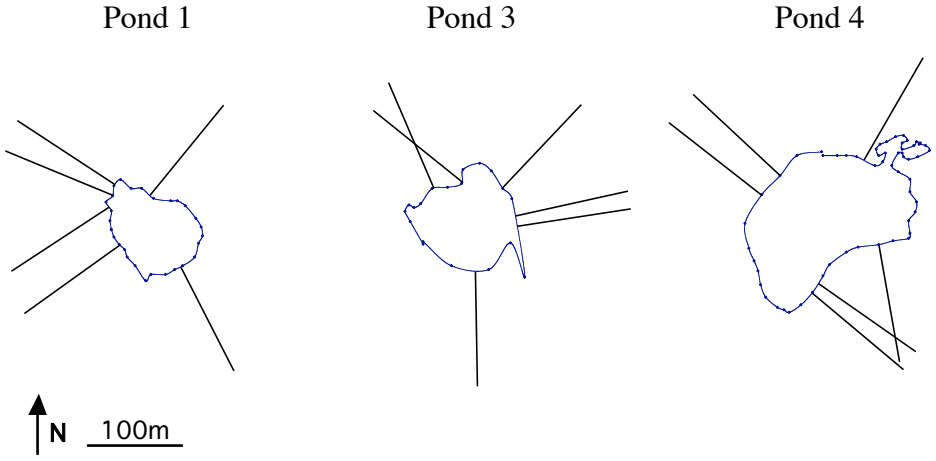

FIGURE 3 – Distribution of random established 100 m line transects around the three Kannah Creek release ponds on the Grand Mesa, Colorado.

BD testing:

All amphibians encountered during monitoring were tested for BD infection by PCR (Annis et al. 2004; Livo and Loeffler 2003). Amphibians were captured with a gloved hand, inverted, and their ventral regions scraped with a wooden applicator stick cut at an angle as per Livo et al. 2004). Samples were then stored in 70% ethanol and sent to Pisces Molecular in Boulder, Colorado for analysis. Each skin scrape sample was mixed then spun at approximately 16,000 G for 3 minutes. The supernatant was drawn

off and discarded, while any pellet was resuspended with vortexing and the addition of tissue lysis buffer. Total DNA was extracted from all samples using a spin-column DNA purification procedure. All sample DNA preparations were assayed for the presence of the BD ribosomal RNA Intervening Transcribed Sequence (ITS) region by 45 cycle single-round PCR amplification with appropriate controls.

Simulation experiments were used to determine the sample sizes required to comfortably detect the presence of BD assuming variable prevalence of infection. Boreal toads in 1000 simulated populations of 500 boreal toads each were randomly assigned BD infection based on a prevalence of 5, 10, and 20%. Increasing numbers of toads were then sampled from each population with replacement and detection of the fungus was calculated (Figure 4). Even if only 10% of a population were infected, one could be 90% confident that it would be detected in a sample of 20 toads. Two key assumptions of this model are that BD can be detected in all animals that are infected, and that infected animals are randomly distributed in the population. Since prevalence of BD in infected wild adult boreal toad populations has not yet been documented below 33% (L. J. Livo, personal communication), a sample of 20 animals appears to be conservative even considering the assumptions of the model. As such, we strived to attain sample sizes of twenty individuals. In addition, twenty toadlets to be released from NASRF were also subject to skin scrapes and tested for the presence of BD using PCR (Livo and Loeffler 2003; Livo et al. 2004).

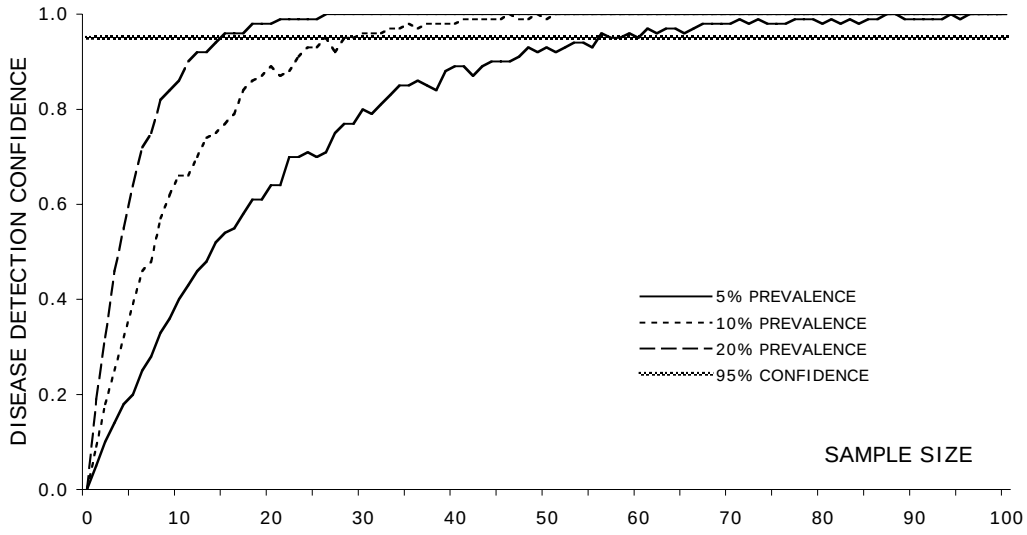

FIGURE 4: Detection of BD was simulated in 1000 populations of boreal toads containing 500 individuals each. Individuals were randomly assigned BD at a prevalence of 5, 10, or 20%. Samples of individuals from each simulated population were then tested for BD to determine how many were needed in a sample to detect the disease with 95% confidence.

Results and Discussion

Rearing and Monitoring:

We had excellent success raising tadpoles to metamorphosis in the pens, with all but one pen having better than 80% survival to metamorphosis (Table 2). Of the mortalities, most were directly attributable to desiccation rather than inadequate husbandry practices. Metamorphosis was initiated the last week in July, and was completed by mid-August. Average water temperature in the pens over that time (release date to metamorphosis) was 17.4 C. Record dry conditions on the Grand Mesa in the summer of 2003 resulted in the disappearance of Pond 1 midway through the study, despite efforts to maintain water levels with a mechanical pump. Many of the TAD group from this pond as well as half of the PEN group from Pen 2 succumbed to desiccation. The remainder was transported to an adjacent Pond 2 on July 19th, where they metamorphosed. The first PEN metamorph appeared in Pond 3 on July 17th (Table 2) while metamorphosis was complete for all pens by August 28th. Peak emergence was late July for Pond 1 and Pond 3, and early August for Pond 4 (Figure 5). It was thought that the smaller volumes of water contained in Ponds 1 and 3 would translate to higher water temperatures allowing for more rapid development. While peak afternoon temperatures were slightly higher in Pond 1 and 3, the mean values were not different (Figure 6) as smaller volumes resulted in greater diel fluctuations in water temperature. It is possible that more rapid maturation was facilitated by those increased peak temperatures, but interestingly, the metamorphs from Pond 4 in taking longer to mature, were 45% heavier than their counterparts in Pond 1 and 3 (Figure 7). Alvarez and Nicieza (2002) have suggested that tadpoles can reduce the minimum size of metamorphosis to reduce the likelihood of desiccation in a receding breeding pond. While temperature and food quality were similar in these trials, perhaps boreal toad tadpoles can sense pending desiccation of a breeding pond and accelerate the developmental process, as Pond 4 was the only pond that retained water until winter.

TABLE 2: Number of boreal toad tadpoles surviving to metamorphosis in each pen at the Kannah Creek site. The date of first and last metamorphosis as well as the peak of metamorphosis is given.

Pond	Pen	Survival (%)	First	Peak	Last
1	1	86	July 20	July 29	August 22
1	2	47*	July 25	July 29	August 14
3	1	82	July 19	August 1	August 17
3	2	92	July 17	July 29	August 17
4	1	97	July 25	August 4	August 28
4	2	93	July 25	August 7	August 20

*many in this pen lost to desiccation

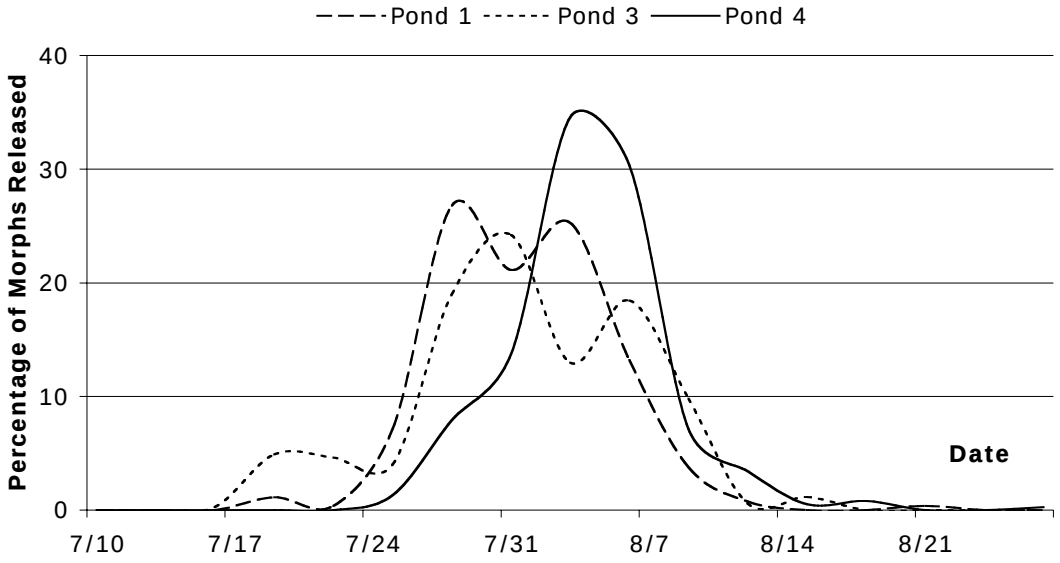

FIGURE 5: Date of boreal toad (PEN) metamorphosis as a percentage of the total surviving to metamorphosis at the Kannah Creek site on the Grand Mesa, CO.

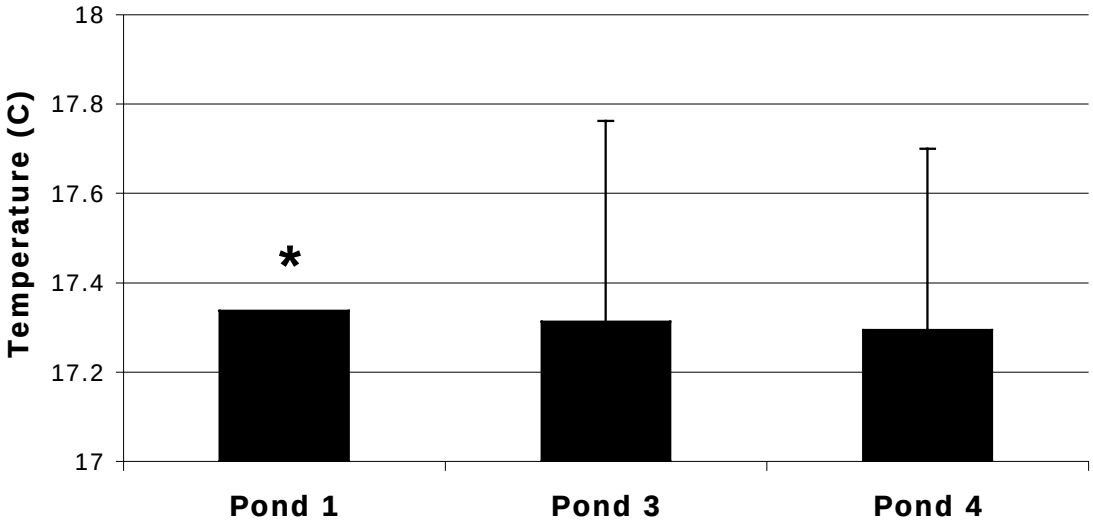

FIGURE 6: Mean water temperature from June 26th through August 16th for each pond measured at four-hour increments with associated 95% confidence intervals. No

interval was calculated for Pond 1 (*) as the temperature monitor failed. The mean temperature value was generated by regressing hand-held readings for all ponds against those obtained from the temperature monitors, then extrapolating a value for Pond 1.

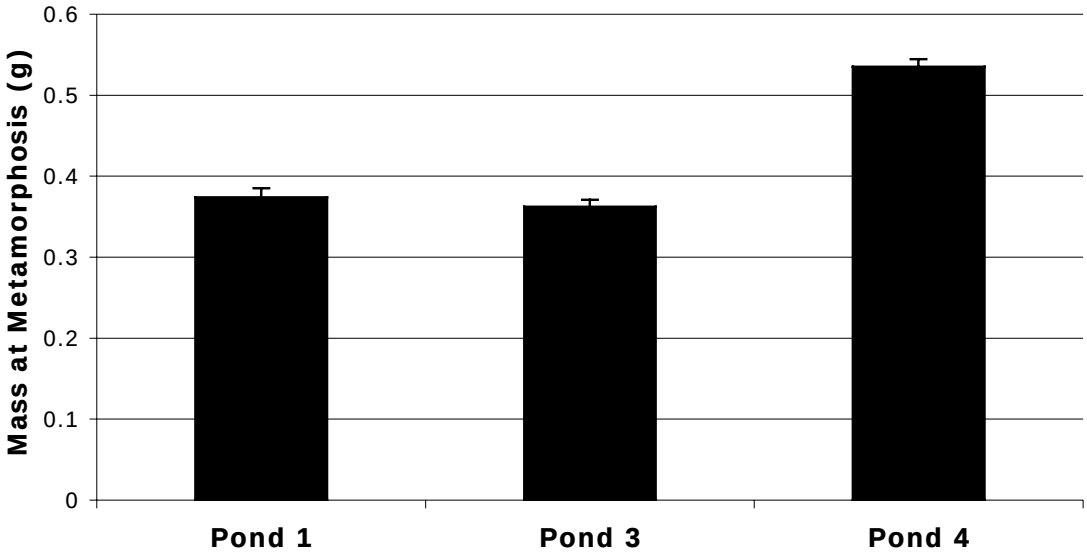

FIGURE 7: Mass at metamorphosis for PEN tadpoles raised in three ponds with associated 95% confidence intervals.

The reintroduction site was monitored weekly with a modified line-transect survey protocol. Randomly placed transects radiated perpendicularly from the perimeter of each pond and extended for 100 m. When toadlets were observed, the location (distance from line), substrate, snout-vent length (mm), mass (g), and presence and condition of toe clips was evaluated. Although the origin of any young boreal toads found this coming field season will be used to evaluate which life stage is best for repatriation efforts, we did notice that pen raised toadlets recaptured in late August were significantly shorter (t = -3.202; P = 0.003) and only half as heavy (t = -3.905; $P <$ 0.001) as those tadpoles released directly into the wild (TAD; Figure 8). The ability to behaviorally thermoregulate apparently was critical for maximizing growth, as their counterparts in the pens were fed *ad libitum*. If larger size going in to winter results in increased survival, then this group should do well. Subsequent years will confirm if this does confer a fitness advantage.

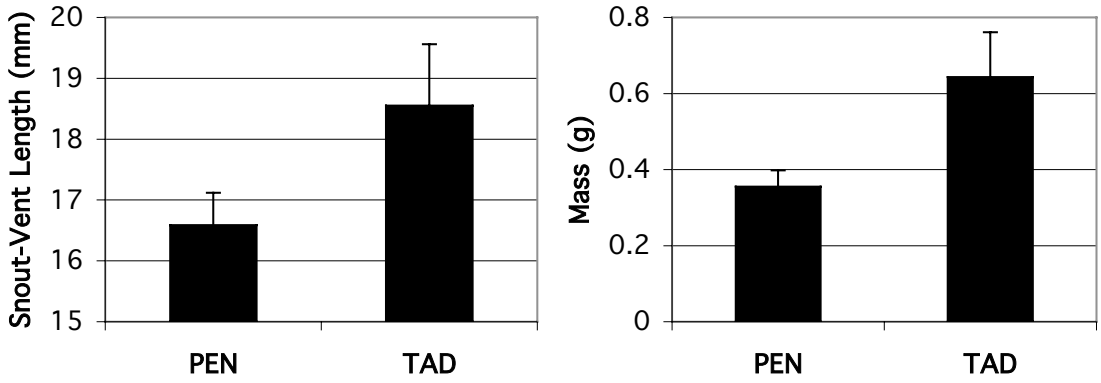

FIGURE 8: Comparison of length and mass in pen (PEN) and wild raised (TAD) boreal toad tadpoles released on the Grand Mesa, CO with associated 95% confidence intervals.

Unfortunately, the line-transect methodology did not appear to be a viable approach for quantitative estimation of population size or emigration, as we were not able to identify all toadlets on the survey line. This combined with the very low number of individuals spotted on each transect, and the risk of squashing those individuals on the line that were not detected, caused us to suspend this monitoring approach midway through the study. It should be noted that the habitat surrounding the ponds was not complex. Future translocations in more complex habitats will likely have even more difficulty implementing a line-transect monitoring scheme.

BD testing:

Amphibians encountered during monitoring were tested for BD infection with PCR. A third of the 39 chorus frogs *Pseudacris triseriata* collected in June tested positive for the chytrid fungus. These results were very disappointing, especially after samples collected in 2002 showed no sign of BD infection. By the end of the summer it became apparent that tiger salamanders and juvenile chorus frogs are not good sentinel animals for evaluating presence of BD (L. J. Livo, personal communication). As such, our subsequent testing focused on adult chorus frogs well as released boreal toads. It appeared that infection in adult chorus frogs did diminish over the course of the summer (Figure 9), giving renewed optimism toward the persistence of the newly introduced boreal toad population. In addition, over 100 skin scrapes from introduced boreal toads acquired over the course of the summer also tested negative for the disease. Infection levels in the resident chorus frog population will be monitored closely in subsequent years to examine if the infusion of large numbers of susceptible boreal toads can aggravate infection in the chorus frog population. Since the chytrid fungus has been detected at the Kannah Creek study site, a more rigorous inspection of toadlets from all treatment groups will be conducted to evaluate the relative vulnerability of the treatments to infection.

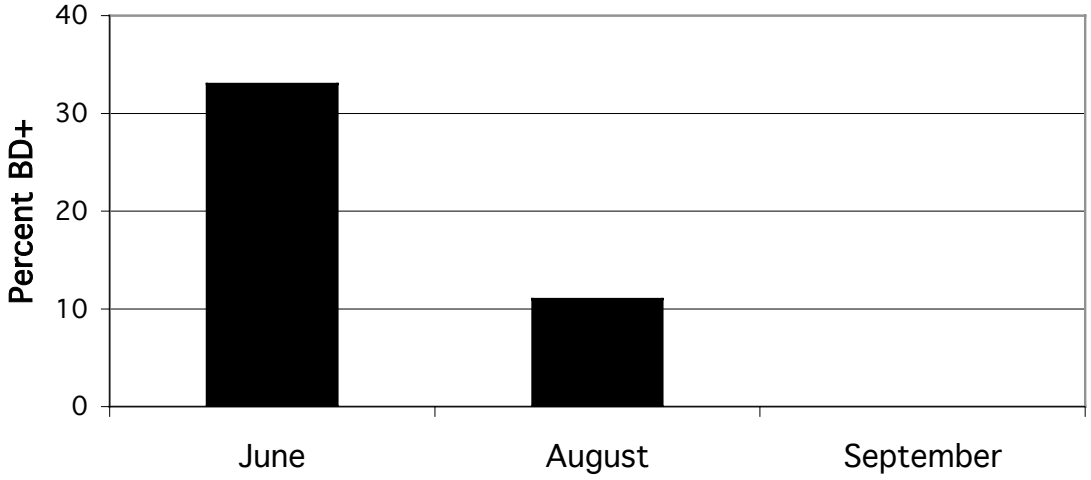

FIGURE 9: Percent of chorus frogs infected with BD by month at the Kannah Creek site as determined by PCR.

Acknowledgements

Our gratitude is extended to Craig Fetkavich, Jenn Logan and the NASRF team for raising the boreal toads used in this repatriation effort. We thank Tina Jungwirth, Tom Holland, Mark Jones, Dan Kowalski, Chuck Hassler, Raquel Wertsbaugh, and other folks who assisted with fieldwork associated with this project. Drying of Pond 1 was delayed in spite of drought conditions by the persistent pumping efforts of Scott and Kim Frost and their neighbors. Anna Goebel, Sherman Hebein, Terry Ireland, Lauren Livo, Erin Muths, and Alan Pessier provided insightful comments during the development of this proposed repatriation effort.

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Evaluation of techniques for detecting *Batrachochytrium dendrobatidis* from amphibians for PCR testing

By Lauren J. Livo[1], John Wood[2], Seanna Annis[3], Cynthia Carey[1], Janet Epp[2], and Mark S. Jones[4]

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Introduction

Many recent declines of amphibian populations in widely separated geographic areas have been associated with the presence of the pathogenic chytrid fungus, *Batrachochytrium dendrobatidis* (referred to here as BD). Areas with infected amphibians include Australia, New Zealand, Europe, Africa, Central and South America, and North America (Berger et al., 1998; Lips, 1999; Bishop, 2000; Carey, 2000; Ron and Merino, 2000; Bosch et al., 2001; Green and Kagarise Sherman, 2001; Bradley et al., 2002; Weldon, 2002; Bonaccorso et al., 2003; Collins and Storfer, 2003; Daszak et al., 2003; Hopkins and Channing, 2003; Kosoff et al., 2003; Muths et al., 2003). Recent studies of BD suggest that this pathogen is recently evolved (Morehouse et al., 2003). This pathogen fits the criteria of an emerging infectious disease which has probably recently increased in incidence and geographic range and may be moving into new host populations (Daszak et al., 2001).

At present, the only reliable method to detect this pathogen at a locality is on an infected resident amphibian. To date, most researchers have used microscopic and histological techniques to detect BD in amphibian skin tissues (Berger et al., 1998; Berger et al., 1999; Pessier et al., 1999; Berger et al., 2002; Briggs and Burgin, 2003; Van Ells et al., 2003). Although skin scrapes and skin smears can be obtained from living amphibians, in general microscopic and histological techniques used to diagnose BD have relied upon *post mortem* skin samples. In addition, these techniques are time-consuming, often require specialized training and experience, and they may not detect low levels of BD infection.

Recently a polymerase chain reaction (PCR) assay was developed that is specific for BD and enables detection of BD from samples obtained from living amphibians (Annis et al. 2004). This PCR test is for a fragment of the ITS region (Intervening

Transcribed Sequence) of the ribosomal RNA locus in BD; this DNA sequence of the BD gene fragment and the PCR primers were developed by Annis et al. (Annis et al. 2004).

The purpose of this study was to assess which sampling methods yield the most reliable detection rates of BD from living amphibians using the PCR assay.

Methods

Experiment 1:

As part of a more extensive survey effort, three sampling methods were performed on each of 99 field-caught amphibians: skin scrapes, toe clips, and water baths. Species sampled were 75 *Bufo boreas,* 11 *Bufo cognatus,* 1 *Pseudacris triseriata,* 11 *Rana* pipiens, and 1 *Spea bombifrons.* The amphibians came from 11 localities in 7 counties in Colorado and were released at the collection site immediately after sampling. Localities ranged between 2295 and 3505 m (7530 to 11500 ft) in elevation. Field equipment such as boots and nets were cleaned thoroughly, treated with a 10 percent bleach solution, and allowed to dry between localities to prevent inadvertent spread of BD or other pathogens (Livo and Jones, 2000).

The skin scrape method involved gently but firmly stroking the pointed end of a wooden applicator (Puritan 2mm-diameter wooden applicator cut to a 3-cm length with one end having a 45 degree angle) 25 times against the ventral surface of animals < 20mm snout-vent (SV) length or, for animals ≥ 20 mm SV, 20 times against the ventral surface and 5 times against the rear feet and webbing.

Toe clips were obtained by using fine scissors to amputate the tip of the rightmost rear toe and then sealing the wound with VetBond or a similar compound. The wood sticks and toe clips were placed in individual screw cap cryogenic tubes (VWR catalog # 20170-217) containing 1 ml of 0.25M EDTA pH 8 saturated with NaCl.

Water baths were performed by placing the animal in an individual 236-ml plastic container (Ziploc brand) with a perforated lid with 10 ml of distilled water. After 2 hours, the water was decanted into a 15-ml tube containing 1 ml of 0.1M Tris, 0.1M NaCl, 0.1M EDTA, and 10% lauryl sarcosine, pH 7.5.

For 64 of the amphibians (Experiment 1a), all three methods (skin scrapes, toe clips, and water baths) were analyzed for BD using the PCR assay. For the remaining 35 amphibians (Experiment 1b), only the skin scrapes and toe clips were tested by PCR analysis for BD. All PCR analyses were performed at Pisces Molecular LLC (Boulder, Colorado). Test results for each sample were scored visually based on the strength of the band as follows: negative = 0, weak or very weak positive signal = 1, positive signal = 2, strong positive signal = 3, and very strong positive signal = 4.

Experiment 2:

The skin scrape method was compared with a skin swab method by obtaining both samples from each of 15 laboratory-exposed *Bufo boreas*. All were known to be BD-positive from prior PCR testing of skin scrapes and skin swabs. Exposure was to BD strain JEL#275, a chytrid strain isolated by Joyce Longcore from *Bufo boreas* in Clear Creek County, Colorado. Sticks used were as in Experiment 1; swabs used were Puritan

Cotton-tipped Applicators on 2mm-diameter wood without adhesive, cut to 3-cm lengths. Both a skin scrape and a skin swab were obtained from each animal. For each animal, a random assignment was made as to whether the skin scrape or skin swab was performed first. In addition, a random assignment was made on whether the skin scrape was performed on either the left or right side of the animal, with the swab being taken from the opposite side. All animals were > 20 mm, so both the ventral surface and foot webbings were sampled. Skin scrapes were taken as described in Experiment 1, except that all strokes were made on one side of the ventral surface. Skin swabs were obtained by stroking the opposite side of the animal on its ventral surface 20 times and its foot webbing 5 times. The wood sticks and swabs were placed in individual screw cap cryogenic tubes containing 1 ml of 70 percent ethanol. PCR analysis was performed by Pisces Molecular LLC and sample scoring was as for Experiment 1.

***Statistical Analysis**:*

Comparisons between methods were performed with Fischer's 2 sided exact test in StatXact-5 (Cytel Software Corporation, 2001). Scores for intensity were analyzed using Wilcoxon paired-sample test (Zar, 1999).

Results

***Experiment 1**:*

Of the 64 amphibians with skin scrape, toe clip, and water bath samples (1 *Ambystoma tigrinum*, 1 *Pseudacris triseriata*, and 62 *Bufo boreas*), 11 amphibians (1 *Pseudacris triseriata* and 10 *Bufo boreas*) from 3 localities had at least one of the three samples yield positive BD test results.

In experiment 1a, the water bath samples did not detect BD as well as the skin scrape and toe clip methods because only 7 of the 11 of these samples tested positive (compared to 10 of 11 for the skin scrapes and 11 of 11 for the toe clips). There was a significant difference in the toe clip vs. water bath (Fischer's Exact Test, 2-sided, $P < 0.05$) in detecting BD. There was no significant difference in detection rates of skin scrapes and toe clips (Fischer's Exact Test, 2-sided, $P = 0.3219$) or between the skin scrape vs. water bath (Fischer's Exact Test, 2-sided, $P = 0.1416$).

The mean band strength score for water bath samples was 1.3 (compared to 2.5 for skin scrapes and 2.1 for toe clips). In no instance did the water bath detect BD when one or both of the other methods did not. When scores were compared using the Wilcoxon paired-sample test (Zar, 1999), scores for the skin scrapes were significantly higher than those for water bath samples in detecting BD ($T=11.5$, $T'=36.5$, $n = 11$, critical value $T_{0.05(2)11}=13$). There were no significant differences in all other pairwise comparisons of the scores from the sampling methods (water bath vs. toe clip: $T=14$, $T'=34.5$, $n = 11$, critical value $T_{0.05(2)11}=13$; skin scrape vs. toe clip: $T=42$, $T'=18$, $n = 11$, critical value $T_{0.05(2)11}=13$).

Because of the lower sensitivity of BD detection using water samples relative to the toe clip and skin scrape samples, only skin scrape and toe clip samples were used for analysis of the remaining 35 individuals from experiment 1 for experiment 1b (see Table

1). Of these, four additional animals (all *Bufo cognatus*) tested positive using the toe clip method, while testing negative using the skin scrape method. There was a significantly higher detection rate for toe clips versus skin scrapes when these 4 samples were pooled with the initial 11 samples (Fischer's Exact Test, 2-sided, $P < 0.05$). When comparing the PCR scores of toe clips from all positive animals with skin scrapes from all positive animals using Wilcoxon paired-sample test, the toe clip method had a significantly higher score ($T=50$, $T'=20$, $n = 15$, critical value $T_{0.05(2)15}=25$).

TABLE 1. Results from Experiment 1.

Method	Number of animals	Number of animals positive	Average sample band strength score
Experiment 1a			
Water bath	64	7	1.3
Skin scrape	64	10	2.5
Toe clip	64	11	2.1
Experiment 1b			
Skin scrape	35	0	0
Toe clip	35	4	1.7

Experiment 2:

Although all the animals tested were known to be BD-positive, the PCR assay did not detect BD in 3 of the skin scrape and 2 of the skin swab samples respectively. This resulted in false negative rates of 20 percent and 13.3 percent for the skin scrape and skin swab methods, respectively (see Table 2). In only one case were both tests negative. There was no significant difference in the methods in their ability to detect BD (Fischer's Exact Test, 2-sided, $P = 0.5795$). However, using the Wilcoxon paired-sample test, the skin swab method had a significantly higher score than the skin scrape method ($T=49$, $T'=15$, $n = 15$, critical value $T_{0.05(2)15}=25$).

TABLE 2. Results from Experiment 2.

Method	Animals	Positive	Average sample band strength score
Skin scape	15	12	3.1
Skin swab	15	13	3.3

Discussion

Toe clips appear to be a more reliable method of detecting BD in wild amphibians compared to skin scrapes. However, it is possible that the reliability of these two sample collection techniques may vary by species. In experiment 1a, 10 of the 11 positive samples were for *Bufo boreas*. For this species, the rate of detection for skin scrapes and toe clips is equivalent. In the second group of samples (experiment 1b), *Bufo cognatus*

contained positive toe clip samples but negative skin scrape samples. When all data from Experiment 1 are combined, the toe clip method had a significantly higher detection rate than the skin scrape method. The lower ventral surface and pelvic patch region of frogs is often found to be heavily infected by BD (Pessier et al., 1999). Unlike *Bufo boreas*, which has a visually distinct pelvic patch region on the lower ventral surface, the ventral surface of *Bufo cognatus* appears uniform in texture and pattern. This difference may result in an altered ability to detect BD by the skin scrape method.

Skin scrape samples for the first group of animals (with 11 positive animals) had stronger PCR scores than those of the toe clip samples. Both skin scrapes and toe clips were superior to water bath samples for detecting BD infection.

An examination of skin scrapes versus skin swabs demonstrated that skin swabs tended to have stronger positive scores than skin scrapes, although the ability to detect BD-positive animals did not differ significantly between the methods. Skin swabs do appear to be a gentler sample collection technique, as some discoloration of the skin could be observed several days post-sampling on animals in areas where skin scrapes had been performed, while skin swabs did not produce similar discolorations.

The ability to detect BD in a sample is not the only concern. Methods that decrease the possibility of cross-contamination between samples or between individuals are the most desirable. If scissors used to clip toes are not properly cleaned (e.g., blades wiped clean with ethanol and then placed in flames to destroy residual DNA), the samples may yield false positive results due to contamination. Sticks and swabs, on the other hand, are single-use items and if other procedures are followed to prevent contamination, are less likely to produce false positive results. Further, it is feasible to sample the same animal repeatedly with sticks or swabs, while multiple toe clip samples are expected to be increasingly deleterious to the animal (Clarke, 1972; Reaser and Dexter, 1996).

Recommendations. Collection of samples for PCR analysis by skin swabs is the recommended method for studies involving *Bufo boreas* in Colorado. Although this collection technique may be generally applicable to amphibians, further investigations of species-specific sample collection techniques may be warranted. Skin swab samples can be collected in the field by technicians with a minimal amount of equipment and after a nominal amount of training.

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Survey of *Bufo boreas* and other Southern Rocky Mountain amphibians for *Batrachochytrium dendrobatidis*

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Introduction

Population declines, beginning by the early 1970s, have been observed in *Bufo boreas* throughout the Southern Rocky Mountains (Corn et al., 1989; Carey, 1993; Corn, 2000). As a consequence, *Bufo boreas* was listed as an endangered amphibian in Colorado in 1993 (Livo and Loeffler, 2003). Recently, a pathogenic chytrid fungus, *Batrachochytrium dendrobatidis* (referred to here as BD), has been associated with *Bufo boreas* population declines at monitored sites in Colorado (Milius, 1999; Livo, 2000; Jones, 2000; Muths et al., 2003). This pathogen has been found to infect a growing number of amphibian species and has been found nearly worldwide, often associated with amphibian population declines (Berger et al., 1998; Speare and Berger, 2000; Carey et al., 2003).

Previous pathology reports from *Bufo boreas* populations detected BD-positive boreal toads from localities in Clear Creek, Larimer, and Pitkin counties, Colorado (Jones et al., 2001). Little is known about the geographic distribution and prevalence within amphibian populations of this pathogen. Recently a polymerase chain reaction (PCR) assay was developed that is specific for BD and enables detection of BD from samples obtained from living amphibians (Annis et al. 2004).

The purpose of the survey work during the 2000-2001 field seasons was to use the PCR assay to determine the geographic distribution of BD and its prevalence in Colorado amphibians, especially *Bufo boreas*. In 2003, an effort was made to visit each known *Bufo boreas* breeding site in Colorado at least once to further refine prevalence rates for populations of this endangered species.

Methods

General practices:

For all field work, boots, nets, and other equipment were cleaned thoroughly and decontaminated with a 10 percent bleach solution between localities using Declining Amphibian Populations Task Force Guidelines:

www.mpm.edu/collect/vertzo/herp/Daptf/fcode_e.html

Fresh disposable gloves were used to capture and handle amphibians. After capture, each amphibian was placed in an individual plastic container (ZipLoc brand, 236 ml or larger depending on amphibian size) with a perforated lid. Mass for each amphibian was obtained using an Acculab Pocket-Pro (PP-250B) digital scale. The snout-vent length for each animal was measured with digital calipers. Samples were obtained from the amphibians at the field site, and then the amphibians were released. The number of amphibians sampled at any one time depended in part on the number of plastic containers that could be transported to the locality. Consequently, a sample size of 12 might be obtained from a particular locality even though 13 or more amphibians were observed. Names used for boreal toad breeding localities are those used by the Boreal Toad Recovery Team (Livo and Loeffler, 2003); other locality names are informal and usually based on nearby named geographic features.

***2000-2001 surveys**:*

In total, 538 amphibians from 75 localities were sampled. Most samples came from localities throughout Colorado, but 23 chorus frogs (*Pseudacris triseriata*) were sampled from 3 sites in southern Wyoming. Emphasis was on obtaining samples from boreal toad populations, of which 213 samples were collected from 34 sites.

Three sample types were obtained from captured amphibians: water bath, skin scrape, and toe clip. See "Evaluation of techniques for detecting *Batrachochytrium dendrobatidis* from amphibians for PCR testing" by Livo et al. elsewhere in this report for a description of sampling techniques. Water samples from water baths were decanted into 15-ml tubes containing 1 ml 0.1M Tris, 0.1M NaCl, 0.1M EDTA, and 10% lauryl sarcosine, pH 7.5. Skin scrapes and toe clips were placed in individual 2-ml screw cap cryogenic tubes (VWR catalog # 20170-217) containing 1 ml of 0.25M EDTA pH 8 saturated with NaCl.

All PCR analyses were performed at Pisces Molecular LLC (Boulder, Colorado). For samples from 64 of the amphibians, each sample type (water bath, skin scrape, and toe clip) was analyzed separately; the amphibian was counted as BD-positive if any of the samples resulted in a positive PCR analysis. For an additional 35 amphibians, the skin scrape and toe clip samples were analyzed separately; the amphibian was counted as BD-positive if either of the samples resulted in a positive PCR analysis. Analysis of samples from these 99 amphibians indicated that skin scrapes and toe clips were more sensitive in detecting BD than water baths. Because both the skin scrape method and toe clip method sometimes resulted in a false negative when the other method resulted in a BD-positive result, both samples were combined for all other amphibians sampled in these surveys. An amphibian was counted as BD-positive if this combined sample resulted in a positive PCR analysis.

***2003 field season**:*

Of 64 known boreal toad breeding localities surveyed, one or more boreal toads were found at 46 localities. In addition, two boreal toads were found at a locality where breeding has not been reported, resulting in a total of 417 boreal toads sampled. Boreal toads were categorized as males, females, juveniles (individuals that overwintered at least one time but are not yet sexually mature), or young of the year (metamorphs). At 3 boreal

toad breeding sites, small samples (ca. 5-cm lengths of egg strands) were collected from 7 boreal toad egg masses and placed in 50-ml bottles containing 70 percent ethanol for PCR analysis of this life history stage.

Opportunistic surveys were made at 9 additional localities, resulting in samples from an additional 189 amphibians of 7 species. From live amphibians, I collected skin scrape samples. For animals found dead, I collected toe clip samples or a small piece of skin from the pelvic patch. All samples were placed in individual 2-ml screw cap cryogenic tubes (VWR catalog # 20170-217) containing 1 ml of 70 percent ethanol. All PCR analyses were performed at Pisces Molecular LLC (Boulder, Colorado). An amphibian was counted as BD-positive if the sample resulted in a positive PCR analysis.

Results

During the 2000-2001 field season, one or more BD-positive PCR tests were obtained from *Bufo boreas* in the following populations: Torso Creek (Routt County); Twin Lake (Larimer County); Kettle Tarn (Larimer County); Pole Creek (Grand County); Urad/Henderson (Clear Creek County); Upper North Fork of the Snake River (Summit County); Peru Creek (Summit County); and Conundrum (Pitkin County). Based on the 2000-2001 sampling, no Chaffee County populations appear to be affected by BD (Figure 1).

When boreal toads at BD-positive localities are considered, 25 of 49 adult and juvenile toads tested BD-positive, yielding a prevalence rate of 51 percent (Figure 2).

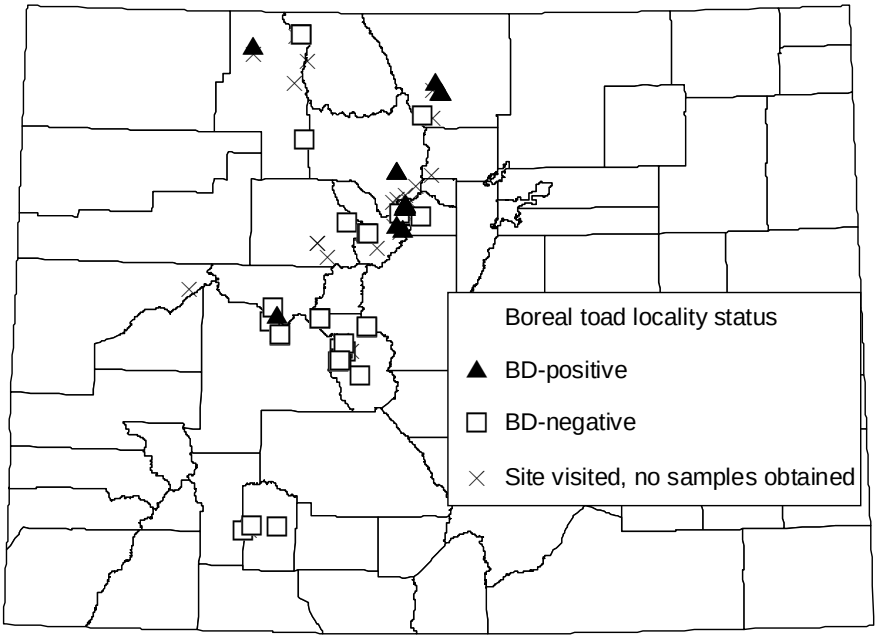

FIGURE 1. Survey results from *Bufo boreas* localities, 2000-2001 (BD-positive: black triangles; BD-negative: open squares; site visited but no samples obtained: X).

Eight of 10 other amphibian species sampled in Colorado had at least one BD positive individual (Table 1). BD-positive species include: tiger salamander (*Ambystoma tigrinum*), Woodhouse's toad (*B. woodhousii*), Great Plains toad (*B. cognatus*), chorus frog (*Pseudacris triseriata*), bullfrog (*Rana catesbeiana*), wood frog (*R. sylvatica*), northern leopard frog (*R. pipiens*), and Plains leopard frog (*R. blairi*). BD-negative species include: Plains spadefoot (*Spea bombifrons*; 21 samples from 5 localities) and canyon treefrog (*Hyla arenicolor*; 1 sample). A group of 9 hybrids between northern leopard frogs and Plains leopard frogs from 1 locality also yielded BD-negative results. BD was geographically widespread in the sampled populations (Figure 3).

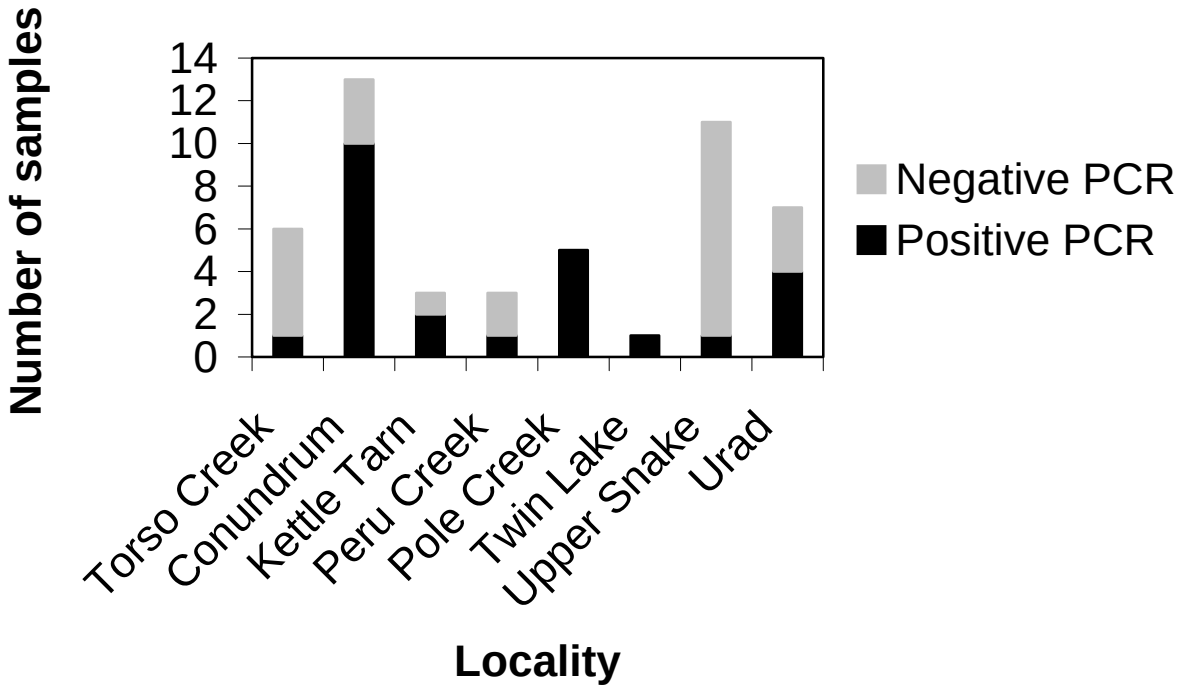

FIGURE 2. Prevalence of BD-positive *Bufo boreas* (adults and juveniles) at BD-positive localities in 2000-2001.

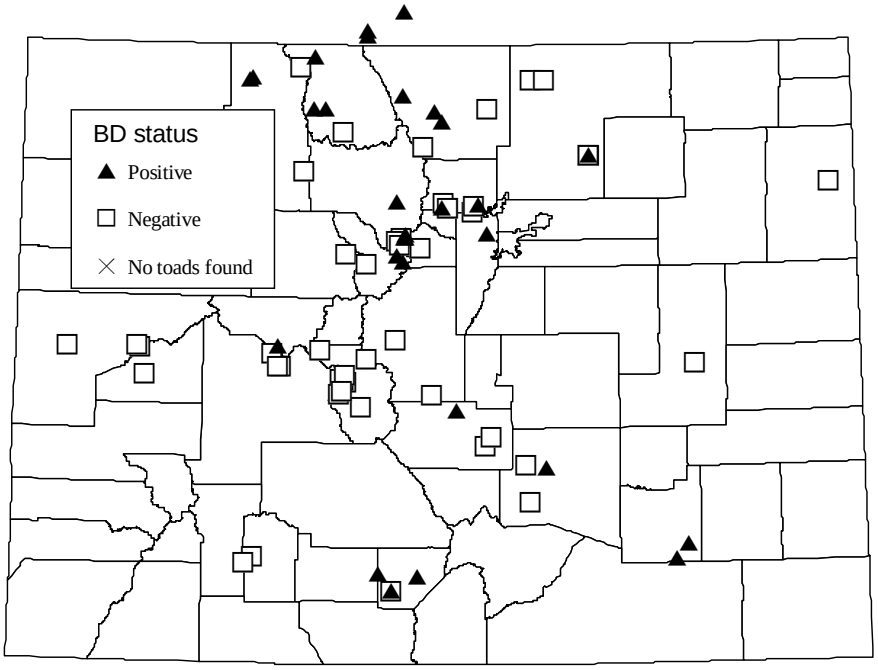

FIGURE 3. Results of PCR analyses by locality for all surveyed sites (2000-2001) in Colorado and southern Wyoming with one or more sampled amphibians (BD-positive: black triangles; BD-negative: open squares).

TABLE 1. Summary of PCR analyses of amphibian samples obtained during 2000-2001 in Colorado and southern Wyoming.

Species	Number of samples	Positive samples	Negative samples	Overall prevalence
Bufo boreas	213	25	188	12%
Bufo cognatus	24	4	20	17%
Bufo woodhousii	22	2	20	9%
Hyla arenicolor	1	0	1	0%
Pseudacris triseriata	48	25	23	52%
Rana blairi	6	3	3	50%
Rana catesbeiana	28	18	10	64%
Rana pipiens	94	18	76	19%
R. pipiens x blairi	9	0	9	0%
Rana sylvatica	15	5	10	33%
Spea bombifrons	21	0	21	0%
Ambystoma tigrinum	57	4	53	7%
TOTAL	538	104	434	

In 2003, 64 boreal toad localities were visited and samples obtained from a total of 417 boreal toads at 46 of these sites (Figure 4). In addition, 189 samples were obtained from 7 additional amphibian species (Table 2).

TABLE 2. Summary of PCR analyses of amphibian samples obtained during 2003 in Colorado.

Species	Number of samples	Positive samples	Negative samples	Overall prevalence
Bufo boreas	417	33	384	8%
Bufo cognatus	10	0	10	0%
Bufo woodhousii	10	0	10	0%
Pseudacris triseriata	127	27	100	21%
Rana pipiens	9	3	6	33%
Rana sylvatica	3	0	3	0%
Spea bombifrons	5	0	5	0%
Ambystoma tigrinum	25	0	25	0%
TOTAL	606	63	543	

Thirty-three adult and juvenile boreal toads from 8 localities tested positive via PCR for BD in 2003. At the 9 localities considered BD-positive, adults and juveniles have generally high rates of BD-infection: 33 of 46 adult or juvenile toads were positive for this pathogen, yielding a prevalence rate of 72 percent (Figure 5). However, in 2003, all boreal toad metamorphs sampled, even at known BD-positive sites, were BD-negative around the time of metamorphosis. For example, in 2003, 20 metamorphs at Torso Creek were BD-negative, despite 5 of 5 boreal toad adults having been BD-positive earlier in the season at the same site. Metamorphs at Kettle Tarn (n=20) and Pole Creek (n=15) were also BD-negative when sampled in 2003.

None of the samples from the 7 boreal toad egg masses tested BD-positive, although 5 of these samples were obtained in the BD-positive Urad Valley.

Thirty-three localities had samples from boreal toads in 2000-2001 and then additional samples from boreal toads or other amphibians in 2003. Of these, no boreal toad breeding localities considered BD-negative in 2000-2001 had any amphibians test BD-positive in 2003. However, chorus frogs and tiger salamanders at a boreal toad translocation site on Grand Mesa tested BD-negative in 2000-2001, but yielded BD-positive chorus frog samples in 2003.

Three sites had boreal toads that tested BD-positive in 2000-2001 with only BD-negative samples obtained from amphibians in 2003. Three toads were sampled at Kettle Tarn in 2000; a male tested BD-negative, while a female and juvenile tested BD-positive. In 2003, the 20 metamorphs sampled from Kettle Tarn all tested BD-negative. At the Upper North Fork of the Snake River locality, 11 juveniles were sampled in 2001, one of which tested BD-positive. In 2003, the 3 juveniles sampled all tested BD-negative. A juvenile boreal toad and a chorus frog both tested BD-positive in 2001 at the Twin Lake locality, while in 2003, 32 chorus frogs (16 sampled on each of two dates) all tested BD-negative.

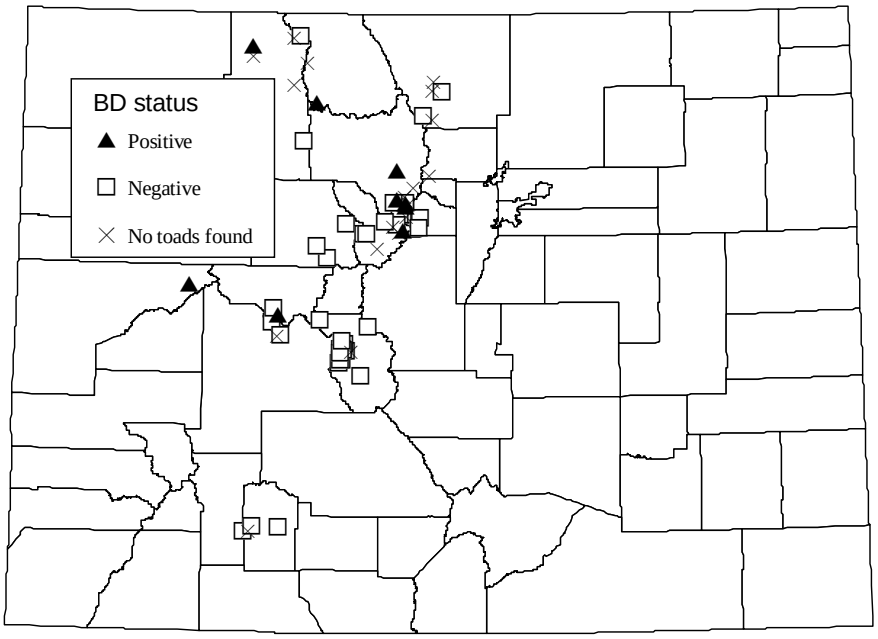

FIGURE 4. Survey results from *Bufo boreas* localities, 2003 (BD-positive: black triangles; BD-negative: open squares; site visited but no samples obtained: X).

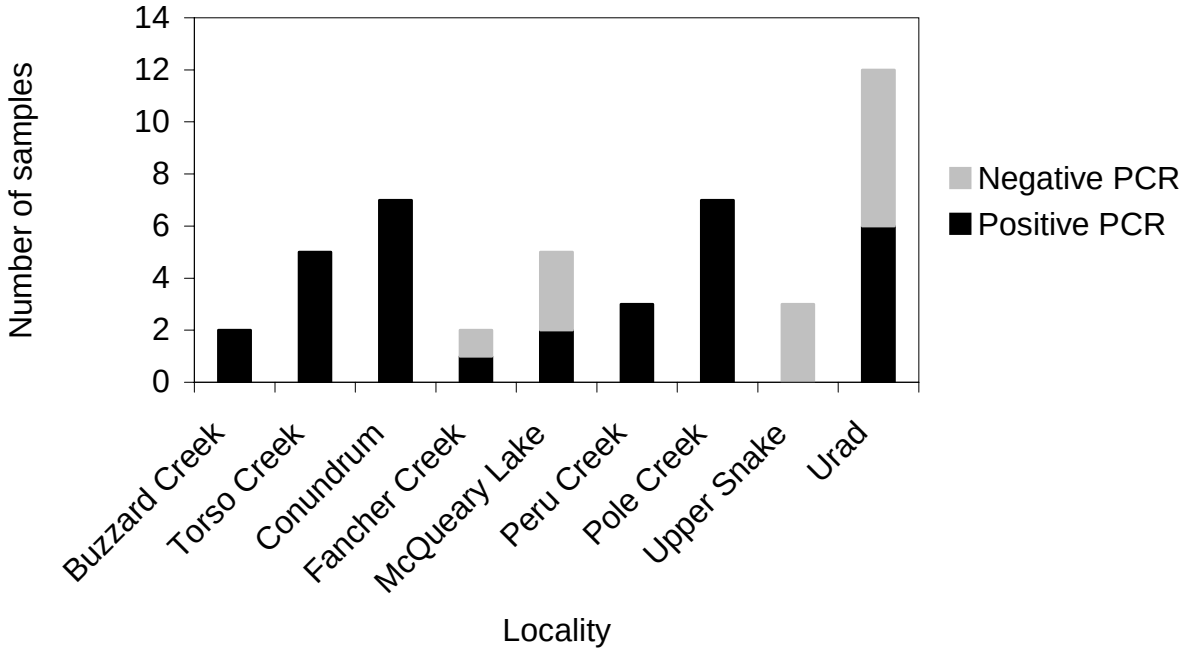

FIGURE 5. Prevalence of BD-positive *Bufo boreas* (adults and juveniles) at BD-positive sites in 2003.

Apparently BD-negative boreal toad localities sometimes were in some degree of proximity to BD-positive localities. Mt. Bethel, a BD-negative locality, is a linear distance of 6.5 km from BD-positive localities in the Urad Valley, but is located in a drainage separated by a 3645m pass. Similarly, the BD-positive Conundrum locality is separated from two BD-negative localities, each in separate drainages: East Maroon Creek (linear distance of 6.2 km in a drainage separated by a 3840 m ridge) and Triangle Pass (linear distance of 6.6 km in a drainage separated by a 3920 m pass).

Of the amphibian sites visited in 2000-2001, the most proximate BD-negative/BD-positive locality pair was in Boulder County, where a linear distance of 3.2 km separates the BD-positive bullfrogs at the Cherryvale Pond site from BD-negative northern leopard frogs at a South Boulder Creek site further up the drainage.

In Grand County, a linear distance of 2.9 km separates the BD-positive McQueary locality from the BD-negative Upper Williams Fork locality, where limited samples from toads obtained in 2003 were all BD-negative. At this site, the Upper Williams Fork locality is downstream of the McQueary locality.

Of 127 chorus frogs sampled from 9 localities in 2003, 27 frogs were BD-positive, yielding an overall prevalence rate of 21 percent. In comparison, the overall prevalence rate for chorus frogs sampled during 2000-2001 was 52 percent. Evaluation of seasonality in prevalence rates for chorus frogs is problematic, because different numbers of frogs from different localities were sampled at different times. However, prevalence rates during both sampling periods were highest during June (Figure 6). July is omitted from this figure because fewer than 5 chorus frogs were sampled during both sampling periods. During both the 2000-2001 and 2003 sampling periods, chorus frogs sampled during August were all BD-negative, yielding a prevalence rate of zero.

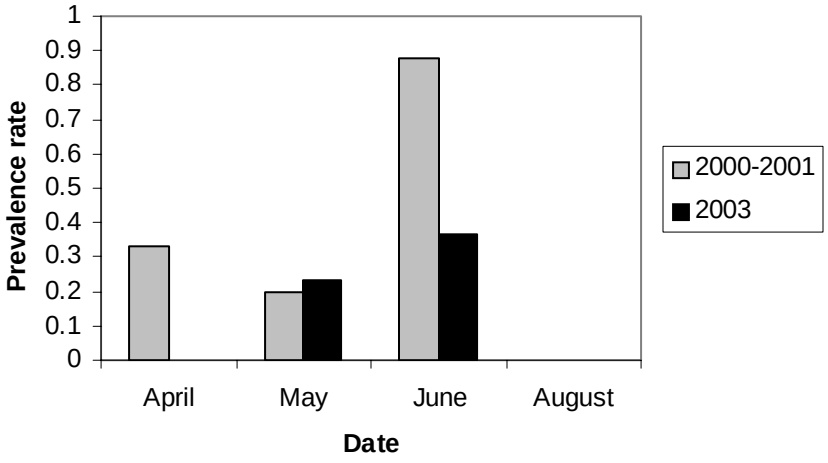

FIGURE 6. Prevalence of BD-positive *Pseudacris triseriata* by month.

Discussion

Overall prevalence (total number of BD-positive animals divided by total number of animals sampled) probably overestimates BD prevalence rates for boreal toads: while all available animals may be sampled at some localities (especially small, BD-positive ones), at other localities (especially BD-negative localities with robust populations), many more toads may be present than can be sampled with the equipment available. On the other hand, the prevalence rate at BD-positive localities may be an underestimate: skin scrapes alone may have false negative rates as high as 20 percent (see "Evaluation of techniques for detecting *Batrachochytrium dendrobatidis* from amphibians for PCR testing" by Livo et al. elsewhere in this report).

There appears to be variability among species regarding life stage at which BD infection is detectible using the PCR assay. For example, *Bufo boreas* metamorphs appear to reach metamorphosis with no BD infection or a BD infection beneath the threshold of detection using the skin scrape method to obtain PCR samples. Metamorphs of other species, such as *Rana catesbeiana* and *R. pipiens*, did have detectable levels of BD infection when sampled, although there are too few data to compare metamorph infection rates with infection rates of other life history stages for those species.

It is possible to encounter BD-positive boreal toad metamorphs: 8 of 11 metamorphs at Pole Creek were BD-positive in 2002 (Livo, unpublished data), perhaps after being able to move around in the terrestrial habitat for a longer time prior to sampling. However, in the absence of sampling of adults and juveniles, sampling of early life history stages (eggs, tadpoles, and metamorphs) in general does not appear to provide reliable information about the BD status of a locality. Boreal toad tadpoles and metamorphs seek the warmest temperatures available, so through their thermoregulatory behavior may minimize the opportunity for BD growth around the time of metamorphosis. In laboratory experiments, for example, high environmental temperatures eliminated BD infections in some Australian frogs (Woodhams et al., 2003).

At any scale, *Batrachochytrium* is geographically widespread and capable of infecting taxonomically diverse amphibians. This pathogen has been reported from all continents except for Asia. In North America, *Batrachochytrium* has been reported from 17 states and two Canadian provinces (Carey et al., 2003; Speare and Berger, 2000; USGS National Wildlife Health Center, 2000; USGS National Wildlife Health Center, 2001; USGS National Wildlife Health Center, 2002).

Most reports of infected amphibians involve sick or dead amphibians submitted for necropsy, resulting in little data regarding prevalence. Frogs collected from sites experiencing mortality events were collected from 8 Arizona localities, confirming the presence of *Batrachochytrium* at the time of the die-offs (Bradley et al., 2002). Prevalence rates were obtained in Maine, where road-killed amphibians were sampled for *Batrachochytrium.* Seven of nine species encountered had BD-positive individuals with prevalence rates ranging from 15 to 50 percent (Longcore et al., 2003). In this study, positive BD samples were obtained from 9 of 11 amphibian species with prevalence rates that varied both among species and between sampling periods.

At BD-positive localities, prevalence rates in adult and juvenile boreal toads tended to be high (51 percent in 2000-2001 and 72 percent in 2003). This compares to rates observed in Wyoming, which also used the skin scrape method to obtain samples.

At the National Elk Refuge in Jackson Hole, Wyoming, 25 of 37 (68 percent) adult *Bufo boreas* tested postive for BD during the 2003 field season, as did 2 of 4 *Rana luteiventris* (Patla, 2004).

In some areas, BD-positive amphibian populations were present within a relatively short distance of an apparently BD-negative population. Where these situations exist, the BD-negative sites are either higher in the drainage or separated by a barrier such as a ridge. The exception to this is the apparently BD-negative Upper Williams Fork locality and BD-positive McQueary locality. Here, the Upper Williams Fork locality is downstream of the McQueary locality. However, only two female toads and 10 metamorphs were sampled from the Upper Williams Fork locality, so it is possible that BD is present in the population but was not detected in the samples obtained.

There were no confirmed observations of boreal toads on the Grand Mesa for more than 20 years. In 2002, members of survey parties documented boreal toads along Buzzard Creek at the eastern portion of the Grand Mesa. In 2003, my survey efforts resulted in BD samples from two males found at one of the sites documented in 2002. Unfortunately, both individuals proved to be BD-positive, casting a pall on the long-term prospects of boreal toads in this area.

To date, BD has not been confirmed in Chaffee County, the last area in the Southern Rocky Mountains with robust boreal toad populations. A U.S. Geological Survey/Biological Resources Division study involving skin scrape sampling at the Denny Creek locality, Chaffee County, resulted in 2 apparent BD-positive samples out of 30 (Erin Muths, personal communication). However, when I conducted sampling at a later date (July 17, 2003) of 14 boreal toads (12 adults and 2 juveniles) at Denny Creek, and 14 additional samples from other sites in the same drainage (for a total of 28 samples), none of the samples resulted in a BD-positive test result. The USGS/BRD sampling had been conduced without the use of disposable gloves, so the apparent BD-positive samples may have been a result of contamination, although the source of this contamination is not obvious.

Recommendations*:*

Sampling amphibians, and especially boreal toads, for *Batrachochytrium* should continue. Field crews responsible for monitoring breeding sites should be equipped with BD sampling equipment and trained to obtain samples as part of their monitoring responsibilities. Over time, this data will not only provide information on the fate of BD-positive boreal toad populations, but information about the conversion rate of localities from BD-negative to BD-positive (and possibly also from BD-positive to BD-negative). Three boreal toad breeding localities (Upper North Fork of the Snake River and Kettle Tarn) with BD-positive samples in 2000-2001 had only BD-negative samples in 2003, although this might be attributed to the life history stage sampled (juveniles and metamorphs) versus actual conversion of the site to BD-negative status. At a third locality (Twin Lake), BD-positive samples of a juvenile boreal toad and a chorus frog in the 2000-2001 survey were followed by BD-negative samples from numerous chorus frogs in 2003. Continued sampling of amphibians from this site is warranted to determine whether BD is now absent from this site or simply not observed in the 2003 samples.

In addition to sampling boreal toads at known breeding sites, it would be valuable to sample any boreal toads encountered during surveys and other opportunistic situations.

Consequently, field crews should be provided with additional sampling equipment for use in these circumstances.

The effects of seasonality on prevalence rates should also be investigated. The lack of BD-positive samples from boreal toad metamorphs at localities where BD-positive individuals had been recorded previously indicates that sampling of this life history stage may not be an effective method for detecting BD at a site.

Because BD-negative and BD-positive amphibian populations may exist in proximity to one another, decontamination and survey policies may need to be revised. At present, sites lower in a drainage usually are surveyed first, followed by surveys of sites higher in the same drainage. However, within a drainage, it may be prudent to survey the uppermost sites first, and move down the drainage to conduct subsequent surveys. This modification of survey procedures may help limit the likelihood of surveyors bringing *Batrachochytrium* to new sites.

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Use of sentinel tadpoles and toadlets to detect *Batrachochytrium dendrobatidis*

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Introduction

Chytridiomycosis is an amphibian disease caused by the chytrid fungus *Batrachochytrium dendrobatidis* (referred to here as BD) (Berger et al., 1998; Longcore et al., 1999). In adult frogs, this pathogen infects keratinocytes in the skin, often causing excessive shedding and mortality (Pessier et al., 1999). In tadpoles, keratinized tissue is limited to the mouthparts, and BD has been detected in these structures (Berger et al., 1999). At localities with amphibian populations affected by chytridiomycosis, tadpole mouthparts may show various levels of malformations attributed to BD infection, although fungal infection has not always been confirmed (Fellers et al., 2001; Lips, 1998; Lips, 1999; Vredenburg and Summers, 2001). Although BD may be able to survive in the environment in the absence of an amphibian host (Longcore et al., 1999), at present the only way to detect BD at a locality is by obtaining samples from amphibians.

The boreal toad (*Bufo boreas*) is an endangered species in Colorado, and translocation efforts may be necessary to reach recovery goals (Livo and Loeffler, 2003). BD has caused mass mortality events in Colorado populations of *Bufo boreas* (Livo and Jones, 2000; Milius, 1999; Muths et al., 2003), making it important to develop techniques to determine the BD status of potential translocation sites. When amphibian populations are present, it is possible to sample resident amphibians to detect BD. However, at potential translocation sites that lack resident amphibians, no current technique exists to detect BD.

Sentinel animals are employed to detect a variety of human and wildlife pathogens, including chickens and other birds for the West Nile Virus (Blackmore et al., 2003; Centers for Disease Control and Prevention, 2000; Ludwig et al., 2002), rodents for hantavirus (Vetcha et al., 2002; Yee et al., 2003), and salmonids for detection of whirling disease in fish (Horsch, 1987; Thompson et al., 1999; Sandell et al., 2001). Tadpole sentinel animals have been used in ecotoxicological studies (Demichelis et al., 2001; Ralph and Petras, 1997).

The Colorado Division of Wildlife operates the Native Aquatic Species Restoration Facility (NASRF), which among other species, houses *Bufo boreas* derived from a number of populations throughout the mountains of Colorado. This hatchery

provides the opportunity to obtain BD-free tadpoles. This paper describes using tadpoles and toadlets from NASRF to determine whether they would be suitable sentinel animals and enable detection of BD at sites where this pathogen would otherwise be unavailable for detection.

Methods

In 2001 and 2002, groups of sentinel tadpoles were placed at several sites to determine whether they could be used to detect BD in the environment (Figure 1). During this time, tadpoles were placed at known BD-positive sites in the Urad Valley (Clear Creek County). In addition, tadpoles were placed at Lost Lake (Boulder County; a former translocation site); along Clear Creek (Clear Creek County) at a BD- negative site; on the Grand Mesa (Mesa and Delta counties) for evaluation of potential translocation sites; and at the Henderson Mill Site (Grand County).

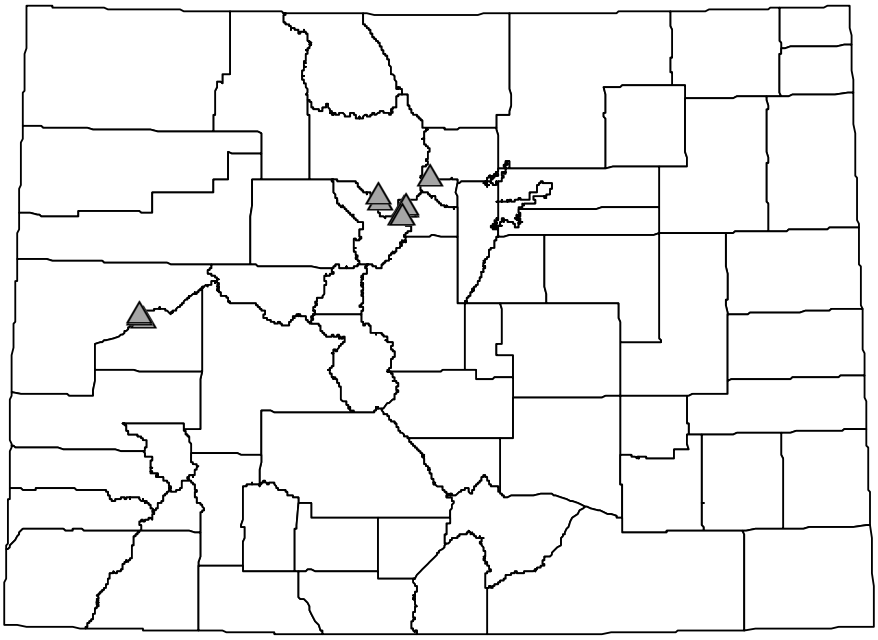

FIGURE 1. Locations of sentinel tadpole and toadlet placement, 2001-2002.

In 2002, batches of 30 *Bufo boreas* tadpoles were placed in cages with mesh sides and bottoms (Figure 2) and remained at the assigned site for 2 or 4 week periods starting July 2 and continuing until September 19. Tadpoles were obtained from NASRF on a regular basis to stock the sentinel cages. Late in the season tadpoles became toadlets, so cages were moved so that at least part of the mesh bottom extended out of the water and onto the shore.

Several samples were lost due to rapid drops in water levels in 2001. Consequently, floating buckets with mesh bottoms were employed in 2002, placing tadpoles in deeper water with less likelihood of desiccation (Figure 3). In 2002 availability of tadpoles varied; while we strived to place 15 tadpoles per bucket, on 15 occasions we stocked between 9 and 12 per bucket and on 5 occasions we stocked 20 tadpoles per bucket (overall mean for 74 stocking events = 14.4 ± 0.29 S.E.). Batches of tadpoles remained at the assigned site for 1 or 2 weeks starting June 1 and continuing until August 1. At two sites, including one known to be BD-positive, 10 to 15 wild tadpoles were collected periodically to determine whether free-swimming tadpoles would have a different likelihood of becoming infected with BD. In addition, 6 groups of tadpoles were sampled without being placed in the field to ensure that the tadpole stock remained free of BD.

After euthanization, the specimens and samples were stored in 70 percent ethanol. Each tadpole sample submitted for PCR testing consisted of mouthparts pooled from five individuals, while each toadlet sample consisted of small pieces of the pelvic patches pooled from five individuals. All samples were placed in 2-ml microcentrifuge tubes containing 1 ml of 70 percent ethanol. Over the two field seasons, a total of 138 samples were obtained before the PCR test became available. Approximately half of these samples were submitted for PCR testing, including three-quarters of the samples from the known BD-positive sites. All PCR samples were analyzed by Pisces Molecular.

FIGURE 2. Mesh cages used in 2001.

FIGURE 3. Floating buckets used in 2002.

Results

Of 68 samples submitted (19 pooled pelvic patch samples and 49 pooled mouthpart samples), only two (one a tadpole sample, the other a toadlet sample) tested positive for BD, despite 42 samples deriving from sites in the Urad Valley, a known BD-positive area. Table 1 lists the areas at which sentinel tadpoles or toadlets were placed in both 2001 and 2002, and the PCR test results for the samples submitted for testing.

TABLE 1. Sample sites for sentinel tadpoles and toadlets.

Site	County	2001 samples	2002 samples	PCR test results	
				BD positive	BD negative
Lost Lake	Boulder	X		0	5
Urad Valley	Clear Creek	X	X	2	40
Clear Creek	Clear Creek		X	0	16
Henderson Mill	Grand		X	0	1
Grand Mesa	Mesa & Delta	X	X	0	2
NASRF hatchery			X	0	2
Total:				2	66

Overall, sentinel animals in this study had a BD detection rate of 2.9 percent. Even when samples considered are limited to those from a known BD-positive site, the BD detection rate for sentinel animals was only 4.76 percent.

Each of the samples with BD-positive PCR test results had been in place for 14 days (tadpoles from the interval of July 4 to July 18, 2001, and toadlets from the interval of August 1 to August 15, 2001). Both samples were collected at the Hesbo site in the Urad Valley. Samples from 7 other groups of tadpoles or toadlets were collected from overlapping times at the same site but yielded BD-negative results. Table 2 shows the timing of both the samples that tested negative and those that tested positive. (Note: Hesbo was not available for sampling in 2002 as it dried up early in the field season.)

TABLE 2. Coverage of sentinel animals at the Hesbo Site, Urad Valley, 2001.

Time interval for sentinel animals					
6/19/01-7/04/01	7/04/01-7/18/01	7/18/01-8/01/01	8/01/01-8/15/01	8/15/01-8/29/01	8/29/01-9/13/01
Negative Tadpoles	**Positive tadpoles**				
Negative Tadpoles					
			Positive Toadlets	Negative Toadlets	Negative Toadlets
			Negative Toadlets	Negative Toadlets	Negative Toadlets

In 2002, 3 groups of tadpoles were tested that came from wild tadpoles at BD-positive sites. None of these samples tested positive for BD.

Mouthpart abnormalities were noted in some of the sentinel animals. However, missing toothrows and other apparent malformations occurred both at known BD-positive and BD-negative sites, so the importance of the observed abnormalities is not clear.

Discussion

The results of this study indicate that *Bufo boreas* tadpoles and newly metamorphosed toadlets do not offer a reliable method for detecting BD at localities, even where BD is known to be present. PCR testing did not detect BD in wild tadpoles caught at BD-positive sites. As these wild tadpoles could be expected to have maximum opportunity to come into contact with BD in the environment, their failure to test BD-positive further strengthens the lack of sensitivity that *Bufo boreas* tadpoles offer in functioning as sentinel animals. Even attempts to infect *Bufo boreas* tadpoles in laboratory exposure experiments have met with very limited success (Cindy Carey, pers. comm.; LJL personal observation).

It is possible that, while *Bufo boreas* tadpoles are not an appropriate organism to use as sentinel animals for BD, tadpoles of some other anuran species might be. One potential area for future research should include evaluation of other tadpoles species, for example, *Pseudacris triseriata*, as potential sentinel animals. However, until an alternate species can have demonstrated sensitivity to BD, other means of detecting BD in the environment should be investigated.

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Genetic analyses of Bufo boreas: analyses of toads from Buck and Chall Creeks, Wyoming

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Introduction

Previous Analyses:

Analyses of mitochondrial DNA (mtDNA) control region sequences of *Bufo boreas* across western U.S. identified substantial and previously unrecognized genetic diversity (Goebel 1996). Three major mtDNA clades were identified. One (identified as the northwest clade) was distributed from central California north to Alaska and east through northern Nevada, Idaho, Montana, and the northwest corner of Wyoming (Yellowstone National Park). A second (identified as the southwest clade) was distributed from central California south to San Diego and east into southern Nevada. This clade included both species, *Bufo nelsoni* and *Bufo exsul*, as well as a number of *B. boreas*. A third highly differentiated clade (eastern clade) was distributed across the southeastern portion of the toads distribution including southeast Idaho, Utah, south central Wyoming (Albany County) and Colorado. A single population in Kane County Utah was identified as a fourth highly divergent lineage. While the distributions of the northwest and southwest clades overlapped in central California, regions of clade overlap were not identified for the eastern clade.

Further subdivision was identified within the eastern mtDNA clade (Goebel 1998). Northwest Utah (Box Elder County) and southeast Idaho (Caribou County) had divergent and unique lineages. *Bufo boreas* from central Utah (Piute County), although geographically isolated from toads in Colorado, were very closely related to the Southern Rocky Mountain group (SRM) in Colorado and Albany County, Wyoming.

More extensive analyses of mtDNA within the Southern Rocky Mountains (Goebel 1997, 1998) resulted in two major conclusions. First, all specimens from the Southern Rocky Mountains (SRM) fell into a cluster of closely related mtDNA haplotypes. No additional highly divergent populations (equivalent to *B. nelsoni, B. exsul*, or toads in Kane Co., UT) were identified. Second, the three largest populations in Colorado (Rocky Mountain National Park, the Clear Creek Drainage, and Chaffee County) all had unique mtDNA haplotypes and significantly different frequencies of shared mtDNA haplotypes. The latter suggests that even though the SRM groups

comprises a set of closely related toads, there is still substantial mtDNA variation within and between populations.

Nuclear DNA variation was examined from the same specimens identified as the mtDNA eastern clade (Goebel 2000a, Goebel 2000b). Like mtDNA, unique lineages were identified in northwest Utah (Box Elder County), southeast Idaho (Caribou County) and southern Utah (Kane Co.). Unlike mtDNA, nuclear DNA identified central Utah (Piute County) and the Southern Rocky Mountains (SRM) as distinct groups. Although nuclear data was collected from a few samples from the northwest and southwest mtDNA clades for use as outgroups, these samples were from Oregon and southern California and were not geographically close to the eastern mtDNA clade. More extensive examination of nuclear DNA within the SRM group (Goebel 2000a) identified substantial diversity within the group. There was a significant correlation between distance and geographic divergence. Within the SRM group nuclear diversity appeared clinal; toads from the northern portions of Colorado (and Albany Co., WY) were more closely related to toads in southeast Idaho (Caribou Co., ID) while all toads from the southern portions of Colorado were less so.

In 2001, three samples (tadpoles or toadlets) from Sawmill Creek, Sublette Co., WY, were analyzed. Both mtDNA and nuclear DNA identified these as belonging to the SRM group. However, these toads were more closely related to toads in Caribou Co., Idaho (the sister taxon to the SRM group) than most toads in the SRM group, continuing the clinal pattern described above.

Importance of Analyzing Bufo boreas in Wyoming:

Due to the findings above, toads in west and central Wyoming are of particular interest to managers of *Bufo boreas*. Toads in the SRMs have been listed as endangered by the state of Colorado and while federal listing is currently "warranted but precluded", the status of the toad will be reconsidered shortly (Terry Ireland, Boreal Toad Meeting, Alamosa CO, November 2002). Federal listing and conservation management programs in Colorado may be less urgent if the SRM group extends well into Wyoming and the toad populations are more stable in numbers than the Colorado toads.

Although all toads in the SRM group (including the three toads from Sublette Co., WY) are very closely related, geographic and genetic boundaries to all surrounding regions have not been identified. Geographic barriers and genetic divergence are both critical to identifying species and thus setting priorities for conservation. To the west, the SRM group is geographically isolated by the desert regions of Utah. In addition, the nuclear DNA profiles of the SRM group and toads in Utah are distinct, even though the two groups share some mtDNA haplotypes. The SRM group is at the eastern edge of the toad's distribution, and it may be at the southeastern edge also, as the toad is thought to be extinct from New Mexico. To the north of the SRM group, geographic barriers do not seem to parallel genetic differentiation. The dry deserts of western Wyoming appear to be a geographic barrier between toads in the SRM and those is Sublette Co., Wyoming, but toads in these regions are not genetically distinct. Perhaps toads have been able to migrate from the Wind River Range (Sublette Co.) east to the Laramie Range thereby connecting to the SRM group in Albany Co. In addition, toads from Yellowstone National Park in northwest Wyoming are highly divergent in their mtDNA (nuclear

DNAs were not examined from Yellowstone) yet geographic barriers between Sublette and Yellowstone Counties are not apparent.

Methods

Specimens Analyzed:

Nuclear amplified fragment length polymorphisms (AFLP) and mitochondrial control region sequences were collected from eight new samples recently collected from Buck and Chall Creeks, Wyoming (Table 1). AFLP data from five samples, previously collected from Wyoming (Table 1), as well as representative samples from southeast Idaho and Colorado, were collected a second time to ensure consistency among AFLP runs.

DNA Extraction and Data Collection:

DNA was extracted from muscle tissue from the thigh of juveniles, whole leg of metamorphs, or tails of tadpoles. Total DNA was extracted using a DNA extraction kit (DNeasy Tissue Kit, Cat. No. 69504, Qiagen, 28159 Avenue Stanford, Valencia CA 91355).

About 1040 bases of the mtDNA control region were amplified and sequenced using previously described methods (Goebel et al. 1999) and an ABI 310 Genetic Analyzer. A repeated fragment (21 base pairs) that varied in number among specimens was not included in the analysis.

AFLP analyses followed the methods detailed in Vos et al. (1995), with minor modifications from Rosendahl and Taylor (1997), Lin and Kuo (1995), Life Technologies AFLP Instruction Manual (Anonymous 1997), and Janssen et al. (1996). Methods were outlined in detail elsewhere Goebel (2000a). Briefly, DNA was digested (cut) with two restriction enzymes, MseI and EcoRI. Short DNA fragments (oligos) were ligated (pasted) to the restriction enzyme cutting sites. These oligos served as exact matches for a first round of PCR amplification. A second round of amplification was performed four times with four different primer pairs, one of each primer pair being fluorescently labeled. These primer pairs matched not only the preamplification primers from the first round of amplification, but extended a few bases into the amplified fragment. Thus the selective primers amplified only a subset of the fragments amplified with preamplification primers, each subset depending on the additional bases of the four sets of selective primers. The amplified fragments were then separated by size using a 310 Genetic Analyzer from Applied Biosystems. The sizes of the fragments were estimated using a size standard run with each sample. The presence and absence of fragments from multiple samples was identified using the computer program Genotyper (also from Applied Biosystems). This method provided about 1000 fragments ranging in size from 100 to 600 base pairs. Although the gene or DNA regions amplified in AFLP analyses are not identified, AFLP fragments are believed to represent random portions of the whole genome (Vos et al.1995; Anonymous 1997).

Phylogenetic Analyses of Sequence Data:

Newly collected mitochondrial DNA sequence data were analyzed with control region sequence data from over 200 *Bufo boreas* and closely related species in the *Bufo boreas* species group. These were collected throughout the toad's distribution across western U.S. and Canada (Figure 1).

Phylogenetic analyses of mtDNA sequence data were performed using parsimony (PAUP 4, Swofford 2002) with the heuristic search option. All characters were weighted equally, because sequence divergences among even the most divergent lineages were small (<3%). Nine single-base gaps in the control region were scored as present or absent. To identify a set of most parsimonious trees, 1000 random addition sequence replicate searches were run, using the options of TBR swapping and steepest descent, and saving five trees per replicate. A strict consensus tree of all trees of minimum length was calculated. Even though the total number of most-parsimonious trees was not known, the strict consensus tree described above was considered to reflect the strict consensus tree of all most-parsimonious trees accurately, because further replicates did not result in the collapse of any additional nodes in the strict consensus tree. Bootstrap (Felsenstein 1985, 1988) values were estimated in order to compare degree of support for discovered clades. Final bootstrap values were estimated with 1000 bootstraps, using five replicates per bootstrap, a random tree to start each replicate, and saving only the five most parsimonious trees per replicate. Bootstrap values >90% in this analysis were considered to support clades strongly (Hillis and Bull 1993; Cummings et al. 1995).

Comparison of New AFLP Data to Previously Collected AFLP Data:

Much previously collected AFLP data was collected by manual methods; radioactivity was used to identify fragment sizes, presence and absence states were identified visually from autoradiography. All recently collected data (2000-2003) was collected with automated methods; fragments were tagged with fluorsecent primers, scanned by a Genetic Analyzer, and presence and absence states were identified from electropherograms. Currently, the manual data and the data collected by automated methods is being combined, but this process is not yet complete. Previously collected AFLP data were collected from outgroup samples from California, Oregon, and southwest Nevada (N=7), multiple localities throughout Colorado and Wyoming (N=86), Utah (N=28), northeast Nevada (N=2), and southeast Idaho (N=7). Analyses using parsimony (Goebel 2000a, and described above for mtDNA analyses), neighbor joining methods (Saitou and Nei 1987; Goebel 2000a), and principal components (Rohlf 1992; Goebel 2000a) identified the same groups found with mtDNA (northwest Utah, Kane Co., UT, central UT, southeast ID, and the SRM group (Figure 2; Goebel 2000a, b). Relationships among groups varied between mtDNA and nuclear DNA analyses, but did not vary among nuclear DNA analyses (parsimony and neighbor joining).

In the current analysis, all new samples from Wyoming were run simultaneously with selected toads from the SRM group and all previously collected toads from Sawmill Creek and Albany Co., WY. Toads from the southeast Idaho group were run also, because these were the closest related group to the SRM group. All newly collected AFLP profiles were then compared with each other and to all previously collected AFLP profiles from automated methods (about 50 samples from the SRM group, southeast Idaho, and outgroups from Oregon and California).

Results

The mtDNA control region sequences from all eight samples from Buck and Chall Creeks were identical. Parsimony analysis of the control region sequences identified these toads as belonging to the northwest mtDNA clade (see Figure 1). In contrast, toads from Sawmill Creek, Sublette Co., WY and Albany Co.,WY were in the SRM group of the eastern mtDNA clade.

The profile of nuclear AFLP fragments collected from Buck and Chall Creeks, WY, was nearly identical to the profile of fragments from Sawmill Creek, WY (Figure 2). The profile was very similar to a number of samples from the SRM group, and was easily distinguishable from toads from southeastern Idaho (Caribou, Co), the sister group to the SRMs.

Unlike the toads from Sawmill Creek which had both mtDNA and nuclear DNA profiles closely related to the SRM group, the mtDNA and nuclear profiles from toads from Buck and Chall Creeks differed and each showed a close relationship to different divergent groups. The mtDNA inferred a relationship to the northwest mtDNA group, but the nuclear data inferred a close relationship to the SRM group. However, nuclear AFLP data from the northwest mtDNA clade is still lacking so the interpretation of relationships from nuclear data is limited.

Discussion

West central Wyoming is geographically located between two divergent mitochondrial DNA clades (the northwest and eastern clades) which may represent different species (Goebel 2000a, Goebel 2000b). Although toads from the northwest and southwest mtDNA clades occur together in central California, a region of overlap between the northwest and eastern mtDNA clades has not been identified. The new data did not identify overlap between the mtDNA clades, although the geographic proximity of Sawmill Creek (with mtDNA from the SRM group) and Buck and Chall Creeks (with mtDNA from the northwest group) suggest that overlap may occur in the region. The new data did identify individual animals with conflicting mtDNA and nuclear DNA group identity. This can occur through hybridization and gene introgression. Due to the limited data, it is not clear whether the distribution of two divergent lineages might overlap in a small geographic range (e.g., a small region in west central Wyoming) or whether the divergent lineages overlap in a broad range (e.g. much of west central Wyoming and parts of Montana and Idaho).

Two kinds of data are needed. First, both mitochondrial and nuclear DNA need to be examined from toads in western and central Wyoming in order to identify the pattern of genetic divergence in this region. Second, nuclear DNA needs to be examined from toads in Yellowstone and surrounding regions to the north in order to determine if nuclear divergence parallels the high mtDNA divergence.

Identifying the occurrence and extent of gene introgression is important to understanding species boundaries. For example, if introgression is limited to a small geographic region (i. e., a hybrid zone), then species boundaries are still recognized despite the occurrence of viable hybrids. Such is the case with *B. hemiophrys* and *B.*

americanus in Manitoba, Canada, which hybridize in a three-mile wide zone (Green 1983).

Toads are notorious for their ability to hybridize, and seem to do so with other bufonids where distributions overlap. Highly divergent species of bufonids hybridize, yet offspring are sterile and there is no gene introgression (Blair 1972). However, all identified species in the *B. boreas* group are very closely related, and even though the divergent lineages identified may be species, they are very closely related species. There is no specific genetic divergence at which hybrids are known to be sterile, but the more closely related species are, the lower the probability that barriers to hybridization have developed. It is highly likely that all species and divergent lineages in the *B. boreas* group can interbreed and produce viable offspring. However, the lack of isolating mechanisms does not preclude independent species identification as shown by the species *B. hemiophrys* and *B. americanus* (described above, Green 1983).

There is little comparative data to suggest a correlation between the degree of genetic divergence and speciation. However, the mtDNA divergences between the three major mtDNA clades (northwest, southwest and eastern) of the *B. boreas* group are greater than the mtDNA divergences between the closely related species *B. hemiophrys* and *B. americanus* (Goebel 1996) which are accepted species despite a known hybrid zone (Green 1983).

Acknowledgements

I thank Mark Jones for continued funding for genetic analyses of the Boreal toad. I thank the very large number of people that collected samples for this analysis, including the as yet unidentified people that collected samples from Buck and Chall Creeks, Wyoming. This work was completed in the Biotechnology Department of the Community College of Aurora (Todd Bergren, Biotechnology Department Chair).

TABLE 1. *Bufo boreas* samples collected from Wyoming

Accession Number	Date	Locality/Comments
747	9/21/01	Buck Creek, tributary to South Beaver Creek, Green River Drainage, WY 12T EO548331 N4759342 (juvenile)
748	9/25/01	Chall Creek, tributary to Beaver Creek, Green River Drainage, WY 12T 0548588 N 4763527 (juvenile)
749-52	9/24/01	South Fork Chall Creek, tributary to Chall Creek, Green River Drainage, WY 0547842 4763453 (metamorphs)
753	9/21/01	Buck Creek, tributary to South Beaver Creek, Green River Drainage, WY 12T EO548331 N4759342 (metamorph)
754	9/21/01	Buck Creek, tributary to South Beaver Creek, Green River Drainage, WY 12T EO548239 N4759364 (metamorph)
710-12	7/25/00	Between South and Middle fork of Sawmill Creek, Sublette Co., WY 20NE 12T 4693725N 55117E 2450M These 3 tadpoles probably represent at least two egg masses, they were collected from a single pond 15x30 yards. They were collected at three localities at least 10 yards apart (Erin Muths).
392	9/25/94	Albany Co, WY Blood only, from juvenile (Steve Corn).
331	7/13/94	South West Medicine Bow National Park, rear Albany. End of 305B, ponds on the west side. Blood only from female SVL 6.5cm (Anna Goebel/Steve Corn).

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A look at aquatic macroinvertebrates as reservoirs of *Batrachochytrium dendrobatidis* infection

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Introduction

In an effort to reverse the declines of boreal toad *Bufo boreas boreas* populations in Colorado, the Boreal Toad Recovery team has identified experimental repatriation as a high priority (Loeffler 2001). Potential introduction sites should be free of the chytrid fungus *Batrachochytrium dendrobatidis* (BD) as it has been implicated in the declines of boreal toad populations (Livo 2000, Muths et al. 2003). Our current ability to detect BD however, requires that resident amphibians be available at the site for testing. Unfortunately, many of the sites deemed most suitable do not harbor any amphibians, even though many supported historic populations of boreal toads prior to 1980. BD may persist at a location even in the absence of amphibian species (Longcore et al. 1999), so simply failing to document amphibians at a site does not guarantee that BD is not present. This also suggests that amphibians may not be the primary host for BD. Infection of a site may be maintained in other organisms. If these organisms can be identified, then we would be able to evaluate the suitability of many more sites than we are currently able. This effort represents a very cursory initial look at potential reservoirs of BD in environments capable of supporting boreal toads. Chytrid fungi degrade chitin which aquatic insects contain large amounts of. It is reasonable to assume therefore that aquatic macroinvertebrates may also harbor chytrids, and provide a good place to start looking.

Methods

Aquatic macroinvertebrate were collected from two known BD-positive sites in Colorado (Table 1). Samples were collected from Hesbo Pond on the Henderson Mine and Ranch Pond #4 on the Pole Creek Golf Course in early August of 2003. These samples included a variety of families such as *Notonectidae*, (backswimmers), *Coenagrionidae* (damselflies), *Dytiscidae* (diving beetles), and *Hydrophilidae* (water

scavenger beetles). Insects were preserved in individual 20 mL scintillation vials containing in 70% ethanol prior to analysis.

Table 1: Source locations and taxonomic descriptions of insects collected at two known Bd positive sites in August 2003.

Location	Samples	Family	*Genus*
Henderson (Hesbo)	10	Dytiscidae	*Acilius*
Henderson (Hesbo)	6	Dytiscidae	*Acilius*
Pole Creek (Ranch4)	20	Coenagrionidae	*Enallagma*
Pole Creek (Ranch4)	5	Dytiscidae	*Acilius*
Pole Creek (Ranch4)	4	Dytiscidae	*Agabinus*
Pole Creek (Ranch4)	6	Hydrophilidae	*Tropisternus*
Pole Creek (Ranch4)	20	Notonectidae	*Notonecta*

The standard sample digestion and DNA extraction procedure was modified slightly to accommodate these sometimes relatively large, and hard chitinous shelled insects. We found that the detergent and proteinase based tissue lysis buffer did not break down the chitin exoskeleton, even after an extended incubation time. However, physically breaking apart or puncturing the insect exoskeleton did allow the lysis buffer to digest the softer internal tissues and release the cellular contents, and would have presumably released chytrid DNA on the surface of the exoskeleton as well. The remainder of the procedure followed the standard spin column DNA extraction protocol. We checked the quantity and quality of DNA extracted from all the different types of insects, and all yielded desirable high molecular weight DNA. All sample DNA preparations were assayed for the presence of the BD ribosomal RNA intervening transcribed sequence region by 45 cycle single-round polymerase chain reaction (PCR) amplification (Annis et al. 2004) that was modified for greater specificity and sensitivity at Pisces Molecular.

Results and Discussion

No evidence of BD was detected in DNA samples obtained from these insects. An aliquot of DNA from one sample of each macroinvertebrate genus was spiked with known BD positive DNA (JEL270), and tested by PCR to make certain that the extracted insect DNA samples did not contain PCR inhibitors, which could cause false negatives. All spiked samples were deemed BD-positive by PCR, indicating that the original unspiked DNA samples were truly BD-negative.

While no evidence of BD was detected by PCR on these samples, this effort was admittedly very cursory. One should not conclude that aquatic insects are not involved in perpetuating BD in the environment. To be certain that macroinvertebrates are inadequate sentinel organisms for detecting BD in aquatic systems, more rigorous study

will be required. Additional efforts will be made in the following field season to evaluate the ability of aquatic organisms to harbor BD that can then be readily detected by the PCR test.

Acknowledgements

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Gape width: an alternative to snout-vent length

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Introduction

The snout-vent length (SVL) is the most frequently used morphometric to describe the size of anurans captured in the field (Hammerson 1999, Stebbins 1985). In addition to taxonomy and systematics, its use has been pervasive in a wide variety of studies including those on age (Senning 1940; Schroeder and Baskett 1968; Kellner and Green 1995; Wake and Castanet 1995), growth (Quinn and Mengden 1984; Ritke et al. 1991), demography (Miller 1977; Van Gelder and Rijsdijk 1987), fecundity (Hairston 1983; Quinn and Mengden 1984; Reading 1986; Tejedo 1992), mate selection (Olsen et al. 1986; Arak 1988; Reading 1991; Marco et al. 1998), and locomotion (Daugherty and Sheldon 1982; Navas et al. 1999). Despite the pervasive use of SVL, little has been published on the precision and accuracy of this metric (Blouin-Demers 2003). Users have recognized the limitations of SVL, but have continued to acquire this metric presumably because of its ease of use and lack of a robust alternative. Acquiring mass has become increasingly popular as a metric to describe size (Carey 1978; Alvarez and Nicieza 2002), but its properties have also not been well studied, and it is generally more difficult to obtain in the field. Inadequacies in SVL are a direct result of the inherent elasticity in the anurans being studied, as amphibian vertebral columns are somewhat flexible (Fellers et al. 1994). Unlike fish where measurements between researchers on the same individual yield functionally equivalent results (Anderson and Neumann 1996), measurements on frogs are highly variable. Variation in SVL was quantified in this study, and variability in alternative morphometrics thought to be more reproducible between researchers were also explored.

Methods

This effort was conducted with the developing brood stock of boreal toads *Bufo boreas boreas* housed at the Colorado Division of Wildlife's Native Aquatic Species Restoration Facility (NASRF) in Alamosa, Colorado. One hundred toads from nine lots representing three different year classes were selected. Six biologists were set up in assembly line fashion, and each measured the SVL, length of the tibiofibula (TF), width of the gape (GW; Figure 1), and mass for every toad. Lengths were measured in mm with digital calipers, while mass (g) was obtained with digital balances tared before each measurement. An ANOVA was used to evaluate differences between readers. The coefficient of variation (CV) between readers for each boreal toad was also calculated, and a mean CV for all 100 toads for each body metric was determined. Differences between mean CV's were also evaluated with an ANOVA followed by Bonferroni's multiple comparison test with simultaneous 95% confidence intervals.

FIGURE 1: Gape width measurements were taken with a digital caliper from the corners of each toad's smile

Results

There was considerable variation between readers ($P = 0.029$) in the average SVL calculated for all 100 toads measured (Figure 2). In fact, measurements varied by as much as 30% of the calculated mean SVL for a given toad, with a mean of 12% (95% CI from 11-13%). yet this was not a function of the sex of the biologist ($t = 1.964$, $P = 0.874$)

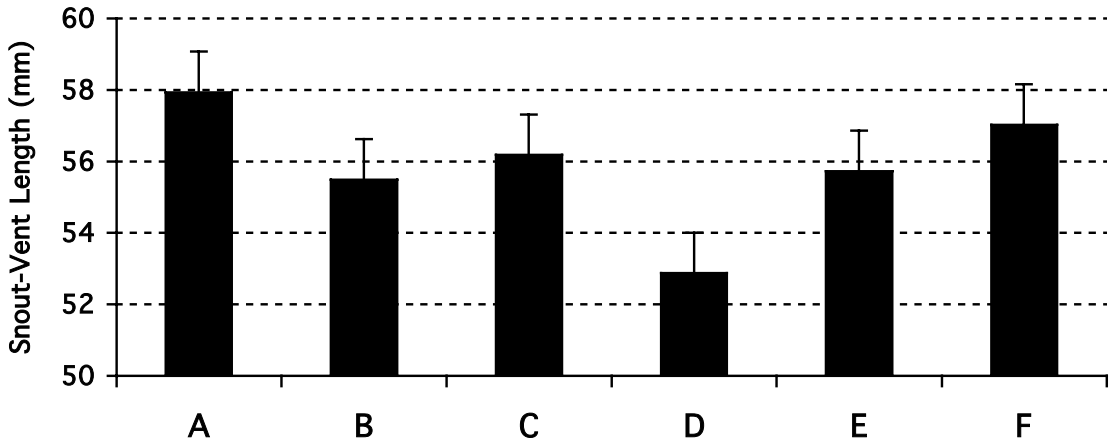

FIGURE 2: Average snout-vent length (mm) for all 100 boreal toads measured by each of six biologists (A-F) with associated standard errors.

Significant variation occurred between readers measuring TF as well ($P = 0.035$; Figure 3). Differences between biologists were not evident for GW ($P = 0.081$; Figure 4) or mass ($P > 0.999$; Figure 5).

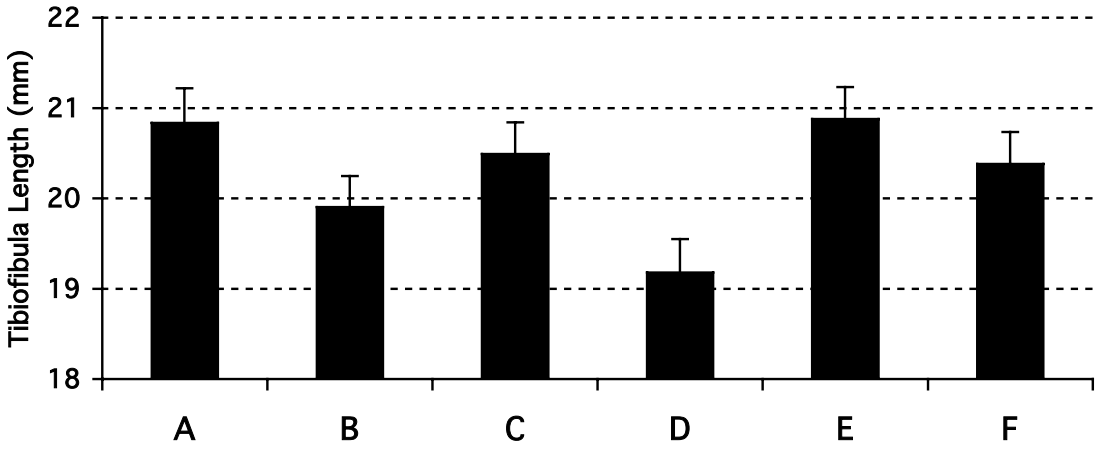

FIGURE 3: Average tibiofibular length (mm) for all 100 boreal toads measured by each of six biologists (A-F) with associated standard errors.

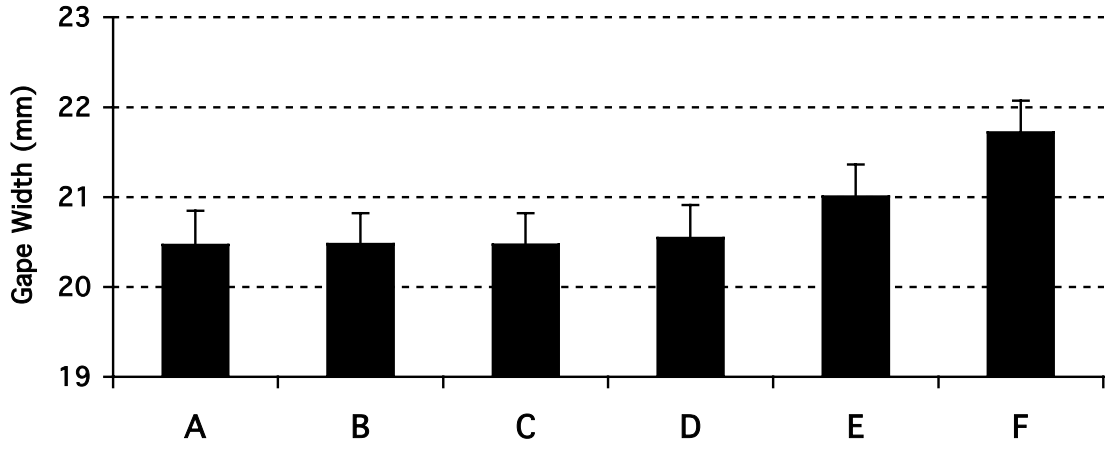

FIGURE 4: Average gape width (mm) for all 100 boreal toads measured by each of six biologists (A-F) with associated standard errors.

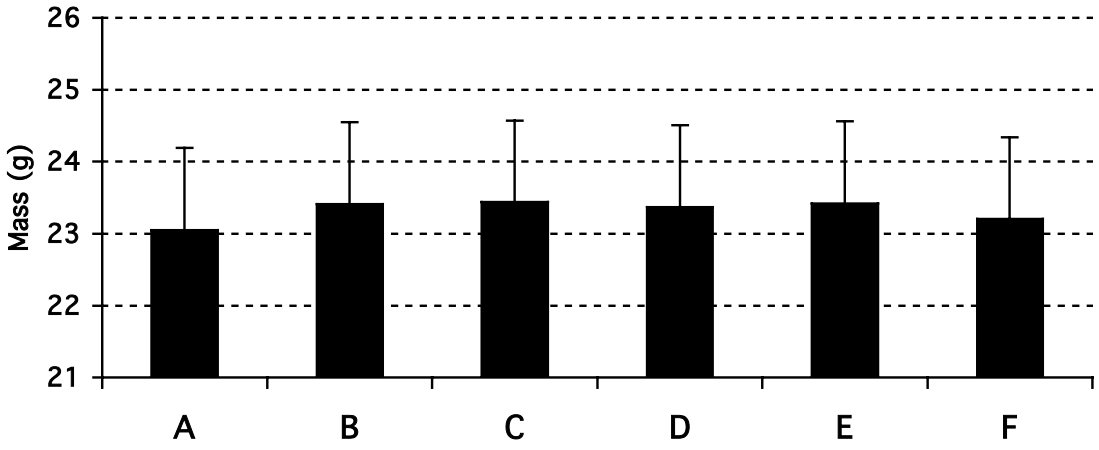

FIGURE 5: Average body mass (g) for all 100 boreal toads measured by each of six biologists (A-F) with associated standard errors.

Coefficients of variation were greatest for SVL and TF and least for mass (Figure 6). Gape width did provide significantly more precision than SVL and TF, but not mass.

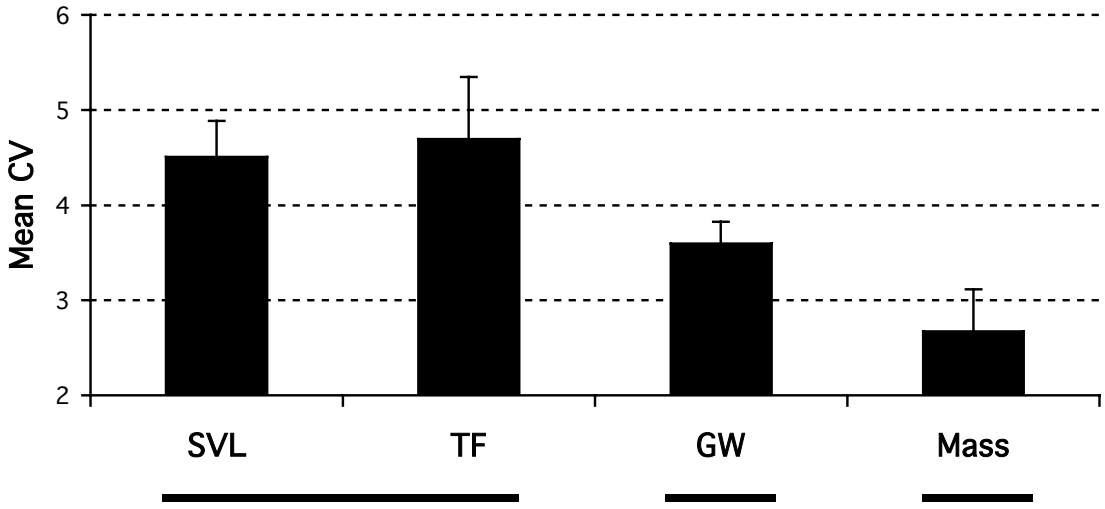

FIGURE 6: Mean coefficient of variation (CV) calculated from all 100 CVs generated across six biologists for snout-vent length (SVL), tibiofibular length (TF), gape width (GW), and mass with associated 95% confidence intervals. Bars sharing the same bold line below the x-axis are not significantly different ($\alpha = 0.05$).

As captive boreal toads used in this study were of known age, correlation of the various morphometrics with age was possible. Animal age ranged from 16-40 months. All morphometrics were positively correlated with age, with highly significant regressions ($P < 0.001$). However, mass was least predictive with an $r^2 = 0.76$. SVL and TF were intermediate, while GW showed the highest correlation with age ($r^2 = 0.85$; Figure 7)

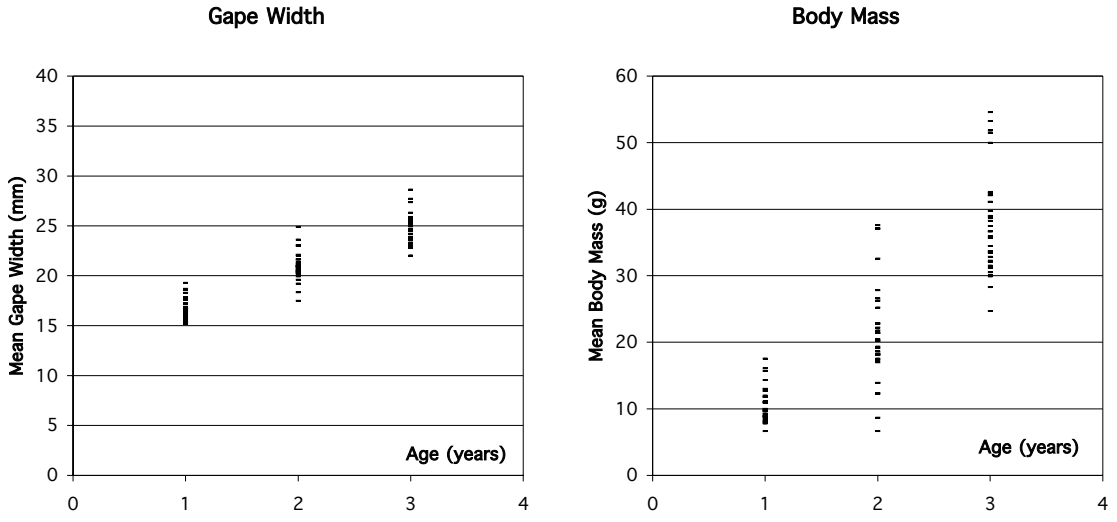

FIGURE 7: Mean values for gape width (mm) and mass (g) calculated across biologists as a function of age.

Discussion

Variation in mean SVL measurements was considerable, and no doubt a function of how aggressively boreal toads were handled during the measurement process, yet this was not related the sex of the biologist acquiring the measurement ($t = 1.964$, $P = 0.874$). Precision of SVL also varied over the range of boreal toad sizes measured in this study (39-77 mm), with larger individuals yielding more repeatable results ($P = 0.003$) but with little variability explained by the relationship ($r^2 = 0.082$). It was expected that measurements of body parts less elastic than the anuran vertebral column would be more reproducible between readers. I was surprised therefore that the TF measurement was no better than SVL for repeatability as the TF bone is rigid, and should have yielded readings that were more precise. The GW was significantly less variable between readers. In fact this assessment is probably conservative, as much of the variation observed was due to contributions by only one of six biologists (Figure 4). This was in part due to the fact that the corners of the smile are well defined, and easily located by all readers. Mass was the most repeatable measure used in this study, alluding to its value as repeatable metric for describing size of boreal toads.

Perhaps the most useful aspect of measuring the width of the smile is that in addition to being very repeatable between measurers, it gave the tightest correlation with age over the size ranges examined here, a function that mass was not able to do consistently. While condition or plumpness of an individual can greatly affect the mass of a boreal toad, they have little to do with age. The GW on the other hand is intimately tied to the animal's more rigid cranial dimensions. Though others have documented a poor relationship of SVL or mass to age in sexually mature amphibians (Halliday and Verrell 1988; Reading 1991; Wake and Castanet 1995), cranial dimensions in the first several years of a boreal toads life appear to more closely reflect age of the individual studied than mass.

Indeed for many analyses where only a rough index of size is required, SVL is adequate even with variation between readers. Certainly some of this variation can be mitigated if the same biologist acquires all of the readings. However, if precision does play a big role in the outcome of the study, or if a metric that is more closely correlated with age is required, then using GW is suggested.

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Methods for obtaining *Batrachochytrium dendrobatidis* (BD) samples for PCR testing

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General considerations

In terms of PCR samples, bleach and flame destroy DNA, while alcohol preserves DNA. Collection of samples for PCR testing requires that equipment used to collect samples not inhibit DNA detection while also not contaminating the current sample with DNA from a previous sample. In addition, equipment should be decontaminated so that it does not spread BD (or other pathogens) from one animal to another.

The PCR sample collection methods described here have been used for obtaining samples from Colorado amphibians for BD (*Batrachochytrium dendrobatidis*) detection. These methods should apply generally to collection of samples for BD testing from amphibians.

Steps for obtaining samples

Collect animals

Animals should be collected with clean, decontaminated equipment, individually handled with fresh disposable gloves, and placed in individual containers prior to obtaining the samples. Although using Purell or other hand decontamination solutions may prevent the spread of live BD from one animal to another, it is likely to allow contamination of samples with BD DNA (in other words, if you handle a BD-negative animal after handling a BD-positive animal, the PCR samples you obtain may both appear to be positive for BD).

Do not place multiple animals in the same container prior to sampling. In this situation, a single infected animal could infect others, and PCR tests could have inflated numbers of positive test results.

Equipment (such as individual containers for holding animals) can be cleaned and bleached so that they can be reused. However, this equipment must be rinsed well and allowed to dry prior to reuse so that there is no residual bleach (note that even parts per million bleach in/on/around a sample could possibly destroy all of the DNA in a sample over the course of a few weeks.)

Obtaining samples

Skin swabs or skin scrapes are the preferred methods of collecting samples from live individuals as the same individual can be tested repeatedly over time. Skin swabs appear to be more gentle to the animal than skin scrapes. Both skin swabs and skin scrapes have similar rates of false negative results when tested on known BD-positive individuals (2 of 15 skin swab and 3 of 15 skin scrape false negative rates in one experiment).

Obtain the PCR sample before doing other procedures with the animal (for example, before weighing, checking PIT tags, and so on). Samples require the following equipment:

- Swabs or pointed sticks:
    - Swabs: use cotton swabs on 2mm-diameter wood without adhesive (such as Puritan Cotton-Tipped Applicators, #VWR 10806-005, or equivalent) cut to lengths (ca. 3-cm) that fit into 2-ml tubes.
    - Skin scrapes: small (ca. 3-cm) lengths of wooden applicators cut so one end is pointed (6-inch long, 2mm-diameter wooden Puritan Applicators, cut into about 3-cm lengths, VWR catalog # 10805-018, or equivalent)
- 2-ml screw-cap tubes containing 1 ml of 70 percent ethanol (2.0 ml screw cap tube with cap/500 per bag, VWR catalog # 20170-217, or equivalent)

To obtain the sample, hold the animal (using fresh gloves) in one hand, and gently but firmly swab (with the cotton swab) or scrape (using the pointed end of the stick) the ventral surface 25 times; for large animals, you may swab or scrape the ventral surface 20 times and the feet and webbing 5 times.

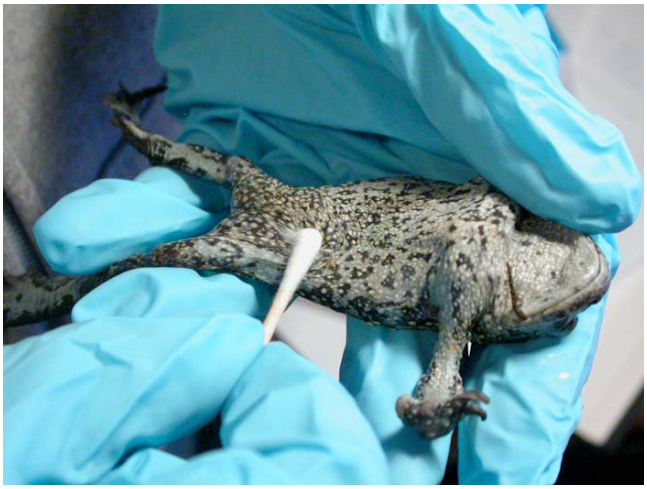

FIGURE 1. Swabbing ventral surface of amphibian.

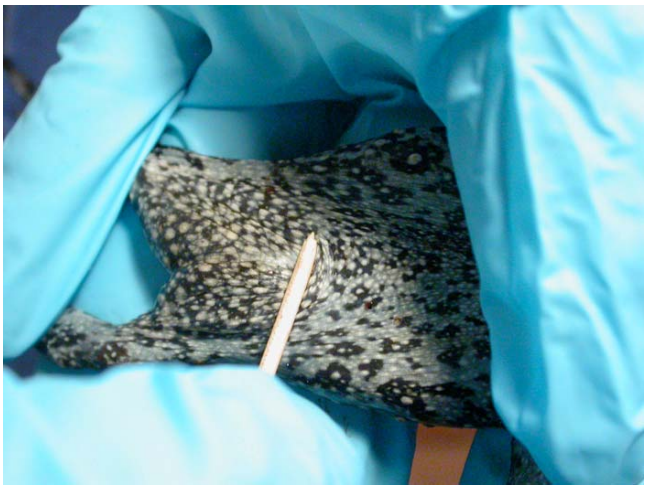

FIGURE 2. Scraping ventral surface of amphibian.

Place the swab (cotton side down) or stick (pointed end down) in the tube. Secure the lid and place in a rack or other container so that the tube remains upright. (Leakage from one tube with BD may get on other tubes and result in contamination of your samples.)

FIGURE 3. Insert swab or stick into tube with sample at bottom of tube.

Other skin tissues (such as toe clip samples or samples of ventral skin from dead animals) may also be collected for PCR testing. Use fine scissors to obtain the tissue. Between each sample, clean the scissors with an ethanol-soaked swab or tissue, and then hold the blades over an open flame to destroy any DNA from the previous sample. Place each sample in a 2 ml tube containing 1 ml of 70 percent ethanol.

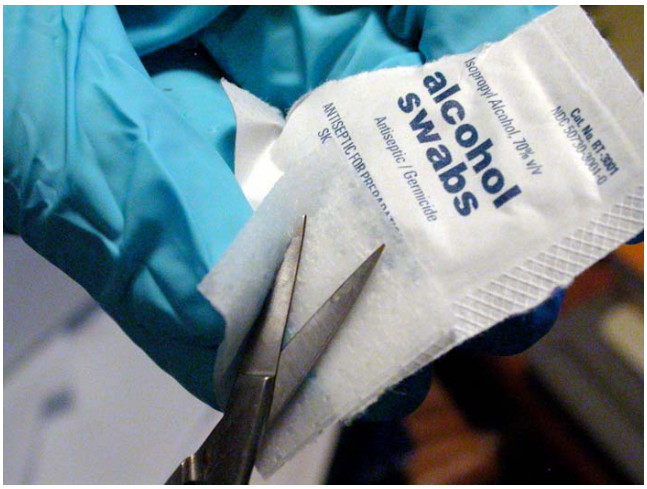

FIGURE 4. Cleaning scissor blades with alcohol.

FIGURE 5. Passing scissor blades through flame to destroy residual DNA.

Toe clipping: If you collect toe clips from live individuals, use fine scissors to amputate the toe tip. When selecting a toe to amputate, you should avoid especially important digits such as the thumb, to avoid having an undue effect on the ability of the animal to feed, reproduce, and so on. Toe clips have rates of false-negative results similar to skin scrapes, but may have more potential for false-positive results through contamination. To obtain a toe clip, cut off the toe tip with the scissors. If bone protrudes from the wound, trim the bone further back (preferably to a joint) so that skin covers the wound, then dab a drop of Vet-bond or other sealant on the wound. In my study, I selected the right rear toe, and continued to encounter individuals with this digit missing 2 to 3 years after the initial sampling.

Labeling samples

Label each tube with a unique sample number. Solution (for example, 70 percent ethanol) and sample type (for example, skin swab, skin scrape, pelvic patch sample, or toe clip) should also be associated with each sample:

FIGURE 6. Label on tube.

Do not place sample information inside the tube. (It can be difficult to extricate, may contaminate other samples through handling, and as paper may contain bleaching agents, may inhibit detection of the target DNA).

Designate a plastic bag for the disposal of gloves and other materials (for example, alcohol wipes) to minimize the possibility of contamination.

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