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Detecting *Batrachochytrium dendrobatidis* in the Wild When Amphibians Are Absent

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Once common in the southern Rocky Mountains of North America, sharp declines in Boreal Toad (*Anaxyrus boreas boreas*) populations precipitated their listing as a state endangered species in Colorado, USA (Loeffler 2001) and consideration for listing under the Endangered Species Act (U.S. Fish and Wildlife Service 2005). The amphibian chytrid fungus (*Batrachochytrium dendrobatidis,* hereafter *Bd*) has been implicated in these declines (Livo 2000; Muths et al. 2003; Scherer et al. 2005). Interest in reintroducing *A. b. boreas* into historical habitats (Loeffler 2001) has spurred the need to develop a test for the presence of *Bd*. Reintroduction efforts are time consuming and costly, and their success may hinge on the occurrence of *Bd* at a potential site. As such, it is imperative that disease status be considered when evaluating potential reintroduction efforts.

Currently our ability to detect *Bd* at a site relies on resident amphibians being present, yet they are not at many promising potential reintroduction locations. Since *Bd* can persist at a location even in the absence of amphibian species (Longcore et al. 1999; Rowley et al. 2007; Speare et al. 2001), we suspect that amphibians may not be the only host, and that infection can be maintained through other alternate hosts or environmental reservoirs. We hope that by testing these non-amphibian sources, the *Bd* status at potential reintroduction sites can be evaluated. Rowley et al. (2007) did not detect *Bd* in retreat sites of rain forest stream frogs, while Lips et al. (2006) did find *Bd* DNA on stream boulders but not in filtered water samples. Others have detected *Bd* in filtered water samples (Kirshtein et al. 2007; Walker et al. 2007), but their approaches do not always perform well in waters carrying high organic loads that rapidly clog filters (Cossel and Lindquist 2009) or cause PCR inhibition (Kirshtein et al. 2007). Our initial efforts toward finding alternative *Bd* hosts focused on insects, because they are readily available and chytrid fungi can degrade chitin, a component of aquatic insect exoskeletons (Johnson and Speare 2003; Powell 1993). These early surveys were unable to confirm the presence of *Bd* in samples of Dytiscidae, Coenagrionidae, Hydrophilidae, or Notonectidae from two ponds known to harbor the fungus (Rogers et al. 2004). Samples of Corixidae, algae, snails, and clams taken from a third pond with infected Boreal Chorus Frogs (*Pseudacris maculata*) were also negative for *Bd* DNA (Rogers and Wood 2005). In an effort to establish a more rigorous examination of potential alternate hosts, we initiated a study to explore the feasibility of using sentinel cages and fish following reports that *Bd* could be found on the scales of Fathead Minnows (*Pimephales promelas*) that were exposed to *Bd* in the laboratory (R. Retallick, pers. comm., GHD, Australia). Feathers and keratin were included

in this field study as well when others demonstrated the ability to culture *Bd* on sterile duck feathers (Johnson and Speare 2005) or 1% keratin agar (Piotrowski 2004) *in vitro*.

Methods.—Sentinel experiments were conducted in both a mid-elevation site in the town of Steamboat Springs, Colorado, and a high elevation site on the Grand Mesa, Colorado after the spring thaw in 2005 when prevalence of *Bd* infection was greatest (K. Rogers, unpubl. data). This occurred in May for the low elevation site in Steamboat Springs, and in July for the high elevation sites on the Grand Mesa. *Bd* presence at both sites was confirmed by swabbing resident *P. maculata* following Livo (2004). DNA was extracted from the samples using a standard spin column protocol. 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 (Annis et al. 2004) that was modified for greater specificity and sensitivity at Pisces Molecular, Boulder, Colorado.

Cages (0.125 m^3) were constructed of 4 × 4 cm pine boards and 3 mm mesh to house sentinel animals. Cages were deployed in water less than 60 cm deep, and secured to the bottom with metal stakes. A protective hardware cloth (25 mm mesh) was attached to the outside of each cage to protect them from predators. Sentinel fish were sampled for *Bd* at 1, 3, 7, and 14 days following introduction to the cages, and mortalities were noted. Fish were swabbed on their right flanks one day after exposure. After 3, 7 and 14 days of exposure, 10 fish of each species from each pen were euthanized with MS-222, then swabbed, scraped, and fin clipped. A cotton swab (Puritan cotton-tipped applicators, VWR International, West Chester, Pennsylvania), was stroked 20 times unidirectionally across the left flank of each sentinel fish, then preserved in 70% ethanol (Livo et al. 2004) for subsequent PCR screening. The skin scrapes followed a similar protocol but used a sharpened wooden dowel (Livo 2004). Paired and caudal fins were removed and preserved in 70% ethanol.

In addition to sampling sentinel fish, six mallard (*Anas platyrhynchos*) flank feathers were taped together at the stalk and suspended in the surface film with a string attached to the outside of the cage. A feather was collected on each sampling day by clipping the exposed end of the feather and placing the complete piece in a 2-mL microcentrifuge tube containing 70% ethanol, then processed with the same PCR procedure.

The first study was conducted in a small temporary spring-flooded pond (Trafalger Pond) next to the Yampa River within the city limits of Steamboats Springs, Colorado (2051 m elev.; 40.47445°N, 106.83017°W). Skin swabs from 20 resident adult *P. maculata* collected during the breeding season suggest this pond has harbored *Bd* since at least 2004 (30% prevalence, K. Rogers, unpubl. data). Thirty Rainbow Trout (*Oncorhynchus mykiss*), 30 *P. promelas,* and 30 Goldfish (*Carassius auratus*) were used as sentinel fish in each of four cages spread throughout the pond, in addition to six Mallard flank feathers suspended outside of each cage.

The second study was conducted in the Kannah Creek drainage on the Grand Mesa near Grand Junction, Colorado (3268 m elev.; 39.04420°N, 108.02992°W). Dozens of small ponds in this drainage are home to robust populations of *P. maculata*. Ponds with perennial water also support Tiger Salamanders (*Ambystoma tigrinum*). *Bd* was first detected in this drainage in 2003 in *P. maculata* collected from a 1.0 ha pond (Pond 4) used in this study (Rogers and Banulis 2004). One cage with 30 *P. promelas* was deployed in each of two additional 0.5 ha ponds, hereafter referred to as Lands End and Cow Camp. Pond 4 received two cages, each with 30 *P. promelas*. Six Mallard flank feathers were installed outside of each of the four cages. In addition, we explored baiting *Bd* with pure keratin (VWR International, West Chester, Pennsylvania). Keratin tea bags were constructed from paper coffee filters, cut in half and sewed together. Five bags were fastened to each cage with 3 g of keratin per bag. A bag was removed from each cage on every sampling occasion, and a portion of the contents preserved in 70% ethanol. Skin swabs from 20 adult *P. maculata* were collected from each of these three ponds the day after this 14-day experiment to evaluate the prevalence of *Bd* in 2005.

Results.—In an effort to reduce assay costs in the first study, only the feathers collected at 1, 3, 7, and 14 days following exposure along with fish skin swabs collected one day following exposure on *O. mykiss* and three days following exposure on *P. promelas* and *C. auratus* were submitted for analysis. None of these 16 feather samples or 120 fish samples suggested that *Bd* was present. Because the ability to use sentinel organisms at this site did not appear promising and processing samples was costly, the remaining samples were archived.

In the second study, despite a substantial number of *Bd*-positive samples from the *P. maculata* collected at the end of our experiment (prevalence of *Bd* ranging from 25–30% in all three ponds), only six of 350 fish swab, scrape, and fin samples were *Bd* positive. These included five swabs collected one day after exposure on *P. promelas* in Cow Camp and a single swab from Lands End, also collected one day after exposure that returned a very weak positive signal. None of the fish swabs, scrapes, or fin clips collected 3, 7, or 14 days after exposure yielded positive results. Feathers and raw keratin were equally ineffective, as all 32 samples failed to register any evidence of *Bd* over the course of the experiment.

Discussion.—Caged fish, feathers, and keratin were ineffective at sampling *Bd* in ponds known to have amphibians with the disease. Although swabbing the flanks of *P. promelas* exposed for one day in a *Bd*-positive environment yielded Bd-positive results, fish sampling was clearly much less sensitive than sampling *P. maculata* in the same environment. The fact that the majority of the positive results came from the single cage at Cow Camp, and that positives were found after only one day of exposure but not after a week or two weeks makes the use of *P. promelas* as sentinel organisms problematic. Rather than *Bd* actually infecting the host, this suggests that the cage was fortuitously deployed in an area with *Bd* zoospores, and that they simply adhered to the fish when sampled on that first day. Chytrid spores have a short-lived free-swimming stage that only lasts about 24 hrs before encysting (Piotrowski et al. 2004). Even with this 24 hr active period the spore can only swim 2 cm (Piotrowski et al. 2004). Thus we may have simply been lucky in our placement of the Cow Camp cage in particular. There does not appear to be any particular affinity by the zoospores toward *P. promelas*, as subsequent samples revealed no indication of *Bd* presence. Given the inconsistent nature of the results, it is doubtful that using sentinel *P. promelas* would be a viable approach for screening potential amphibian reintroduction sites for the presence of the fungus. Although sentinel fish have been used to test for pathogens like whirling disease (Koel et al.

2006; Thompson et al. 1999), the construction and deployment of cages remains labor intensive, particularly in sites that are difficult to access. This is an additional consideration, given the apparently low sensitivity of *P. promelas* as a sentinel to detect *Bd*.

Because the majority of positive samples came from the same cage on the same day, contamination of the samples is a concern. The frog samples used to confirm the presence of the fungus were collected following the experiment, and stored in a different location making them an unlikely source of contamination. Using latex gloves between samples, and sterilizing equipment with an open flame further minimized contamination risk. Contamination occurring in the original source stock of *P. promelas* was also ruled out, as most positive signals came from a single cage, and only early in the study. Samples collected after 3, 7, and 14 days were all *Bd*-negative.

Given that others have been successful growing chytrid fungi on a 1% keratin agar (Piotrowski et al. 2004) or on feathers (Johnson and Speare 2005) *in vitro*, we were surprised that none of the keratin or feather samples yielded a positive PCR result. It was suggested that perhaps the *Bd* fungus did not colonize the interior of the keratin bags, but rather just the outside, which was not sampled. Subsequent tests using agar-impregnated swabs rolled in raw keratin however also failed to bait in *Bd* following three days of exposure (K. Rogers, unpubl. data).

If *Bd* has a patchy distribution in a pond environment, it would be difficult to sample with fixed organisms or objects. A more effective approach would be to release amphibians targeted for a reintroduction effort, then subsequently collecting survivors the following year to sample for *Bd*. Using a non-tethered organism allows the target to move through the environment as it would following a repatriation effort, encountering pathogens along the way. Repatriation efforts require the ability to produce large numbers of offspring for subsequent release; a portion of this captive production could be used to assay potential field sites for *Bd*. If a captive broodstock is not available, fertilized eggs could be secured from the wild, washed to minimize risk of *Bd* transfer, then raised to the larval stage for release (Rogers and Banulis 2004). This approach requires that target amphibians be produced during pilot studies prior to implementing full repatriation efforts to determine the suitability of potential translocation sites.

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