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Survey of Ranavirus and the Fungus *Batrachochytrium dendrobatidis* in Frogs of Central Virginia, USA

The Global Amphibian Assessment found that 42% of amphibian populations are in decline, and 32% of species globally face extinction in the near future (IUCN 2008). Emerging infectious diseases, including the fungus *Batrachochytrium dendrobatidis* (*Bd*) and ranaviruses, have been responsible for mass die-offs and are considered major international threats (Daszak et al. 1999; Schloegel et al. 2010). Ranavirus has low host specificity; fish, reptiles and amphibians can be lethally or asymptomatically infected and can serve as reservoirs for other vulnerable species (Chinchar 2002; Schock et al. 2008). Surveillance of these pathogens is important for understanding their distribution and potential threat to amphibians and other animals. We used non-destructive sampling to survey for *Bd* and ranavirus in central Virginia, USA. No amphibian die-offs had been recorded in this area, although dedicated monitoring had not previously occurred.

On eleven trips from 1 April through 2 July 2010 we swabbed adult animals and collected toe clips to assess the presence of *Bd* and ranavirus, respectively, in four anuran species in three water bodies in Prince Edward County, Virginia: Briery Creek Lake in Briery Creek Wildlife Management Area (north end of the lake; 37.2005°N, 78.4497°W), and two ponds on the campus of Hampden-Sydney College (Chalgrove: 37.2428°N, 78.4639°W and Tadpole Hole; 37.2452°N, 78.4529°W). Chalgrove and Tadpole Hole are both approximately 1 ha and located 0.8 km apart. Briery Creek Lake is a 342-ha lake located 4.5 km south of the other ponds. We collected adult frogs by hand, typically between 1900–2300 h. Each frog was placed in a new plastic bag, and nitrile gloves were changed between catching individuals. While processing animals, we used a recommended protocol with two people to prevent contamination of samples (Brem et al. 2007). To sample for *Bd*, frogs were swabbed five times with sterile cotton-tipped applicators on both sides of the abdomen, ventral abdomen, ventral surface of thighs, and rear feet (Brem et al. 2007). To sample for ranavirus, the front-right toes of large species (*Lithobates catesbeianus, L. palustris, Anaxyrus fowleri*) or 1–2 hind-right toes of small frogs (*Pseudacris crucifer, Acris crepitans*) were collected using sterile surgical blades (St-Amour and Lesbarrères 2007). Both swab tips and tissue samples were preserved in 70% ethanol. All animals were released within 1–3 h at the original site of capture. To prevent cross-contamination between sites, all supplies and equipment were disinfected with 1% Nolvasan. Although Nolvalsan has not been tested against *Bd*, it is used as a fungicide, bactericide, and virucide, has

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been proven to inactivate ranavirus at levels that are not toxic to amphibians, and is less caustic to equipment than bleach (Bryan et al. 2009; Kennedy et al. 2000). All materials that directly contacted animals (gloves, bags, scalpel blades, swabs) were used only once and contacted no more than one individual.

Of the 140 frogs processed, 103 were tested for ranavirus and *Bd*. Only species within a site that had ≥14 individuals were tested, because of the likelihood of misclassifying as uninfected sites with small sample sizes and low prevalence (Greer and Collins 2007). Disease testing was performed by the Veterinary Diagnostic and Investigational Laboratory in the College of Veterinary Medicine at University of Georgia. In brief, genomic DNA was extracted from toes following the tissue method of a commercially available kit (DNeasy Blood and Tissue Kit, Qiagen Inc., Valencia, California, USA). Conventional PCR was then performed using the protocol and primer sets reported by Annis et al. (2004) for Bd and those found in Mao et al. (1996, 1997; primers MCP4 and MCP5) for ranavirus. The PCR products were resolved via electrophoresis on a 1.0% agarose gel. Controls for all PCR runs included two negative controls (water and tissue from a ranavirus-negative tadpole) and two positive controls (cultured ranavirus and tissue from an experimentally infected and confirmed ranavirus-positive tadpole). The PCR reactions were repeated once to confirm results.

Bd was found in three of the five species tested, and at each of the three water bodies (Table 1). Among species with positives in a site, prevalence of the pathogen ranged from 6–20% (Table 1). No obvious pathological signs or dead or moribund animals were observed during the course of this study.

While ranavirus has been detected in a number of amphibian species sampled in the southeastern United States, including species in the current study: Bullfrogs (*Lithobates catesbeianus*), Cricket Frogs (*Acris crepitans*), Pickerel Frogs (*L. palustris*), Spring Peepers (*Pseudacris crucifer*), and Wood Frogs (*L. sylvaticus*) (Gray et al. 2009), none of the toe clips from the 103 frogs screened tested positive for ranavirus. Ranavirus was, however, detected in syntopic aquatic turtles, with prevalence ranging from 5–31.6% at these three sites, in a companion study using similar sample sizes and distal tissues (Goodman et al., unpubl.), which raises the question of whether we missed infections in the anuran populations.

We are confident that we did not miss infections in the animals we screened. St-Amour and Lesbarrères (2007) found that toe tips were comparable to liver tissue samples in detecting ranavirus in Green Frogs (*L. clamitans*), and although Greer and Collins (2007) demonstrated that tail clips were less sensitive than pulverized whole body samples in detecting ranavirus in salamanders, this difference disappeared after the first week post exposure. Our sample sizes were small within species at each site. However, combining species yields samples of 34 individuals at Briery, 33 at Chalgrove, and 36 at Tadpole Hole, which are sample sizes that should (with 95% confidence) be able to detect ranavirus prevalence of 10% or greater in a site (Brem et al. 2007). Importantly, we did not sample all species in each site, nor did we sample all life stages for any species. Thus if ranavirus was present, it was at likely at low prevalence, in an alternate life stage, or in another host species. Expanded monitoring is needed to establish whether ranavirus infects frogs at these sites.

Bd occurred with low prevalence in *P. crucifer*, *L. palustris*, and *A. crepitans*, but was not detected in *L. catesbeianus* or *A. fowleri* (each collected in one site). Larger sample sizes would be needed to rule out the possibility of infection in these species, especially

TABLE 1. Prevalence (number positive/number tested) and associated 95% confidence intervals (CI) for *Batrachochytrium dendrobatidis* (*Bd*) occurrence in frogs from three water bodies in central Virginia. Dashes indicate that small sample sizes precluded tested for that species and location. The 95% confidence intervals with no continuity correction were calculated using a program by Lowry (2011) based on methods in Newcombe (1998) and Wilson (1927).

	Lithobates catesbeianus		*Pseudacris crucifer*		*Anaxyrus fowleri*		*Lithobates palustris*		*Acris crepitans*		Site total	
Study Site	Prev	CI	Prev	CI	Prev	CI	Prev	CI	Prev	CI	Prev	CI
Briery WMA	0% (0/20)	0.0–16.1%	14.3% (2/14)	4.0–40.0%	—	—	—	—	—	—	5.9% (2/34)	1.6–19.1%
Chalgrove	—	—	—	—	0% (0/15)	0.0 –20.4%	5.6% (1/18)	1.0 –25.8%	—	—	3.0% (1/33)	0.5–15.3%
Tadpole Hole	—	—	20.0% (3/15)	7.0–45.2%	—	—	—	—	9.5% (2/21)	2.7 –28.9%	13.9% (5/36)	6.1–28.7%
Species Total	0% (0/20)	0.0–16.1%	17.2% (5/29)	7.6–34.6%	0% (0/15)	0.0–20.4%	5.6% (1/18)	1.0 –25.8%	9.5% (2/21)	2.7–28.9%		

because of the low prevalence of the pathogen estimated for co-occurring species. *Lithobates catesbeianus* has been shown to carry *Bd* but experience low morbidity and mortality due to infection (Daszak et al. 2004) and so it is surprising that infections were not detected. *Anaxyrus* species are also susceptible to *Bd*, though some studies have found absence or low prevalence in sites where other species tested positive (Rothermel et al. 2008; Tupper et al. 2011; Venesky et al. 2011). The current study adds to a body of research showing presence of the fungus *Bd* in frog populations that are seemingly asymptomatic. However, dedicated surveillance would be necessary to determine the potential impacts of *Bd* on local amphibian health and fitness.

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