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Sudden Mass Die-off of a Large Population of Wood Frog (*Lithobates sylvaticus*) Tadpoles in Maine, USA, Likely Due to Ranavirus

Starting in early April 2013, a 0.23-ha spring-fed, fish-free pond in Brunswick, Maine, USA (43.919°N, 70.040°W) was informally surveyed at least every other day until the end of August. The first Wood Frogs (*Lithobates sylvaticus*) appeared at the pond on 11 April; three days later the adult frogs numbered several thousand, mostly males. On 16 April we counted 550 Wood Frog egg masses, with an average clutch size of ca. 600 eggs. All adults left the pond within several weeks. Based on counts from photographs of tadpoles swimming near the surface of the pond taken in late May, we estimated that there were more than 100,000 Wood Frog tadpoles. The main predator of Wood Frog eggs at this site, the freshwater leech *Macrobdella decora*, was notably scarce in 2013 compared to most years, which may have contributed to the unusually large tadpole densities in May and early June. At 1600 h on 14 June 2013, we noted huge densities of tadpoles. Twenty-one hours later, on the afternoon of 15 June, a few surviving tadpoles were observed swimming slowly near the pond surface, but none was found alive by the end of the day. Tadpole carcasses were present on the pond bottom (1–2 m depth) at a density roughly estimated at 1300/m^2 around the entire 210-m perimeter of the pond (2250 m^2 area), based on qualitative observations and detailed counts from photographs at several haphazardly selected sites (Fig. 1). Assuming that all of the Wood Frog

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FIG. 1. Dead Wood Frog tadpoles on the bottom of a pond in Brunswick, Maine showing the representative density (ca. 1300/m^2) along the entire 210 m-perimeter of the pond on 15 June 2013.

FIG. 2. Dead and dying Wood Frog tadpoles showing hemorrhages in their well-developed legs and around their throats, as well as skin shedding. Tadpoles were photographed on the afternoon of 15 June 2013.

mortality was restricted to within 1 m of the shore and using a more conservative estimate of 1000 dead tadpoles/m^2, more than 200,000 tadpoles died in less than one day. Dead tadpoles had hemorrhages in their well-developed legs and around their throats (Figs. 1, 2). Skin sloughing was also observed on both dying and freshly dead tadpoles (Fig. 2). Repeated sampling of the pond through early September revealed no Wood Frog tadpoles.

During the die-off, there were no obvious stressors other than possible overcrowding. During the second week of June, White Pine (*Pinus strobus*) pollen had covered much of the pond; Wood Frog tadpoles formed especially dense congregations in the few pollen-free areas. Otherwise, weather conditions over the two-day period were typical for the season, with daily highs 23–25°C. It was sunny and there was no precipitation.

No other species of amphibian or reptile appeared diseased or experienced noticeable mortality. In informal censuses through September, densities of adult and larval Green Frogs (*L. clamitans*), Grey Treefrogs (*Hyla versicolor*), Spring Peepers (*Pseudacris crucifer*), Spotted Salamanders (*Ambystoma maculata*), and Eastern Newts (*Notophthalmus viridescens*) appeared to be unaffected. Bullfrogs (*L. catesbeianus*), which were scarce before the Wood Frog die-off, continued to be present all summer. Adult Painted Turtles (*Chrysemys picta*) were also apparently unaffected. Considering the isolation of the pond and the fact that we found no carcasses of other amphibian and reptile species and their population densities appeared to be unaffected, chemical contaminants or other human-caused stressors seem unlikely causes of the sudden die-off of Wood Frog tadpoles. In April 2014, the density of Wood Frog egg masses was normal, and on 3 June 2014, almost one year after the die-off, tadpoles seemed abundant and healthy.

Four freshly dead tadpoles were haphazardly collected on 15 June, frozen at –20°C, and submitted to the University of Tennessee Center for Wildlife Health. Given that gross signs were indicative of ranaviral disease (Miller et al. 2011), we tested for infection by this pathogen using real-time quantitative polymerase chain reaction (qPCR). Portions of the liver and kidney were removed from each tadpole, gDNA was extracted and quantified, and real-time qPCR was performed on a tissue homogenate using identical procedures as Richter et al. (2013). We used an ABI 7900 Real-time PCR System, and amplified and tested for a 70-bp of the major capsid protein of ranavirus, because this pathogen is frequently associated with die-offs of wood frog tadpoles (Greer et al. 2005; Harp and Petranka 2006; Brunner et al. 2011; Miller et al. 2011). A standard curve developed from our PCR system was used to predict the quantity of virus (plaque-forming units, PFU) in 0.25 μ g of gDNA. Four controls also were run with samples: DNA grade water, negative and positive tadpoles (*Lithobates sylvaticus*), and an FV3-like virus grown in cell culture (GenBank accession no. EF101698; Miller et al. 2007). All four tadpoles tested positive for ranavirus infection. Virus concentrations in the liver-kidney homogenate were between 10 and 115 PFU per 0.25 μ g of gDNA (Table 2).

To our knowledge, this is only the second published account and location of ranavirus-associated mortality in Maine, and the biggest and most sudden mortality event reported in the region. The first report of ranavirus in Maine was in Acadia National Park (Gahl and Calhoun 2008; Gahl and Calhoun 2010). Die-offs from ranavirus have been reported from New Brunswick, Canada, and many states in the eastern United States (Miller et al. 2011). Assuming that this die-off was caused by the ranavirus we identified, this would be the largest and most rapid event reported in amphibians. Increasing surveillance for ranavirus and estimating population impacts are important conservation directions (Gray and Miller 2013).

TABLE 1. Results of real-time qPCR estimating the quantity of ranavirus from four Wood Frog tadpoles. Ct = qPCR cycle threshold values (mean of two test runs); Concentration = PFU (plaque-forming units) of ranavirus per 0.25 micrograms of gDNA.

Specimen number	Ct	Concentration
1	27.05	24.55
2	28.47	9.98
3	24.62	115.00
4	25.03	88.83

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Prevalence of the Amphibian Chytrid Fungus among Zoo and Pet Store Collections in the Northeastern United States

Recent studies suggest that the amphibian trade has promoted the global transmission of the amphibian chytrid fungus, *Batrachochytrium dendrobatidis* (*Bd*) (Fisher and Garner 2007; Goka et al. 2009; James et al. 2009; Farrer et al. 2011), which is a pathogen implicated as a cause of global amphibian declines (Berger et al. 1999; Daszak et al. 1999; Skerratt et al. 2007; Kilpatrick et al. 2010). In particular, pet shops and zoos constantly acquire and exchange animals depending on their needs and exhibitions, making amphibian collections vulnerable to harboring and transmitting *Bd*. For instance, one study determined 28% of amphibians sold in a number of Japanese pet stores were infected with the fungus (Goka et al. 2009) and several studies reported *Bd*-positive samples among zoo amphibians (Longcore et al. 1999; Miller et al. 2008; Jones et al. 2012; Churgin et al. 2013; Goeroff et al. 2013). Despite the role that pet stores and zoos may play in propagating the disease agent, there is a paucity of studies investigating such possibilities in the United States. Thus, the purpose of this study is to enhance the current knowledge about *Bd* infection among traded amphibians in the United States by examining the prevalence of *Bd* in a variety of pet store and zoo amphibian collections.

A large number of pet stores are widely distributed throughout the United States that internationally and domestically import and sell amphibians. For example, Petco Inc. and Petsmart Inc. are the two largest pet store chains, each of which owns over 1000 stores throughout the United States (Petco: available from http://www.petco.com/ [Accessed 19 August 2013]: Petsmart: available from http://www.petsmart.com/ [Accessed 19 August 2013]). These numbers translate into the distribution of on average 40 or more pet stores per state between these two companies alone. It is likely that some pet owners have disposed of dead or alive purchased amphibians into wild habitats, making it possible that alien *Bd* strains carried by pet amphibians (e.g., Goka et al. 2009) can be transmitted to native amphibian populations. Zoos also may serve as a pathway for bringing *Bd* into amphibian collections and possibly into the local amphibian community, as they receive amphibians from a variety of sources. However, many zoos and aquariums have an Association of Zoos and Aquariums (AZA) accreditation, which sets standard procedures for the acquisition and husbandry of animals which should counter the risk of spreading disease (Poole and Grow 2012). These standards should decrease the likelihood of both presence and transmission of *Bd*.

In November 2012, we collected swab samples of 20 adults of four amphibian species from five different pet stores throughout Pennsylvania (three stores) and Massachusetts (two stores). The pet stores from which we sampled animals included several privately owned and recognized chain stores. From pet stores, we sampled 14 *Bombina orientalis* (Oriental Fire-belied Toads, native to the northeastern Asia), two *Lithobates catesbeianus* (American Bullfrogs, native to the eastern USA), three *Hyla cinerea* (Green Treefrogs, native to the southeastern USA), and one *Ceratophrys ornata* (Bell's Horned Frog, native to South America). Additionally, 30 adult amphibians of nine species were tested for *Bd* from a zoo located in Pennsylvania with AZA accreditation in September 2012. From this zoo we sampled three *L. catesbeianus*, three *Pyxicephalus adspersus* (African Bullfrogs, native to the southern Africa), three *Phyllobates terribilis* (Golden Poison Frogs, native to South America), four *Dendrobates tinctorius* (Dyeing Poison Frogs, native to South

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