

Low-level *Batrachochytrium dendrobatidis* Detection Persists in Plethodontid Salamanders Following Timber Harvest in Kentucky, USA

Habitat degradation is a primary cause of global declines in amphibian populations over the past three decades (McCallum 2007). However, amphibian biodiversity declines are often the result of a number of interacting factors including habitat loss, overexploitation, and the spread of pathogens such as *Batrachochytrium dendrobatidis* (*Bd*), a pathogenic fungus which causes the potentially lethal skin disease chytridiomycosis (Longcore et al. 1999; Stuart et al. 2004). Aquatic diseases affecting amphibians, including *Bd*, have been documented widely in both degraded and undisturbed ecosystems (e.g., Skelly et al. 2006; Saenz et al. 2015). The potential for habitat degradation to amplify the effects of disease have been documented in several cases. When urbanization occurs, stream-dwelling plethodontid salamanders are often confronted with stressors such as siltation and elevated temperatures which may result in diminished body size and perhaps increased susceptibility to other pathogens and parasites, including ranaviruses (Skelly et al. 2006; Price et al. 2011; North et al. 2015; Murphy et al. 2016). Contrary to ranavirus, *Bd* infections may be less prevalent in degraded habitats due to abiotic and biotic shifts (Becker and Zamudio 2011). *Bd* is an aquatic-dependent fungus and *Bd* infection of amphibians can vary inversely with temperature (Berger et al. 2004; Woodhams and Alford 2005; Olson et al. 2013). Temperature is often elevated after land uses involving forest canopy removal or impervious surface installation (Moore et al. 2005; Anderson et al. 2007), and *Bd* prevalence has been negatively associated with canopy density (Becker et al. 2012). Assuming density-dependent transmission, decreased amphibian population densities, common after urbanization or timber harvest (Price et al. 2011; Maigret et al. 2014), might also slow disease transmission. In urbanized landscapes, for example, *Bd* prevalence has been found to be lower than in adjacent forested habitat, though more

work is needed to determine how much of this difference is due to temperature or density effects (Saenz et al. 2015). While *Bd* prevalence has been examined along an urbanization gradient, to our knowledge the potential interacting effects of timber harvest with *Bd* occurrence have not been investigated.

Globally, a majority of amphibians are forest-dependent (Olson and Burton 2014), and timber harvest is known to produce environmental effects similar to urbanization, such as siltation and elevated water and air temperatures, which may subsequently impact amphibian populations (Anderson et al. 2007; Murphy et al. 2016). These effects often result in population declines in both stream-dwelling and terrestrial species (Peterman and Semlitsch 2009; Maigret et al. 2014), and these effects might increase individual susceptibility to infectious diseases by stressing individual animals. Simultaneously, temperatures elevated by canopy removal from logging may decrease *Bd* infection of stream salamanders in harvested stream habitats. Herein, our goal was to test for differences in *Bd* infection prevalence of stream-dwelling and terrestrial plethodontid salamanders occurring in reference (unharvested) watersheds in comparison to adjacent watersheds where large-scale timber harvests were recently completed.

We conducted our sampling in the main block of University of Kentucky's Robinson Forest (RF), located in Breathitt and Knott counties, in southeastern Kentucky, USA (37.46062°N, 83.15742°W). RF is a 4450-ha relatively intact second-growth deciduous forest. Elevations range from 243–487 m (Overstreet 1984), and mixed-mesophytic forest predominates, characterized by roughly 30 co-dominant tree species (Braun 1950). The sampling for this study was conducted in 17 randomly selected first-order ephemeral streams in four adjacent watersheds of the 1545-ha Clemons Fork drainage. Timber harvesting had occurred on both sides of the stream at eight of these locations, and nine locations were located in reference (unharvested) watersheds (Fig. 1). Logging was conducted between June 2008 and March 2009, and consisted of two-age deferment ("shelterwood with reserves") harvests (Witt et al. 2013). This resulted in a two-age stand with a target basal area of 3.4 m² per ha of reserve trees (four dominant or co-dominant trees per ha) (Witt 2012). This treatment was applied across the harvested area, with the exception of landings, trails, and areas subject to streamside management zone (SMZ) regulations. SMZ characteristics included unharvested stream buffers of at least 7.6 m on both sides of the stream and improved crossings for heavy machinery.

We sampled salamanders across the study area between March and May 2013. Each sampling site consisted of an approximately 50-m reach of ephemeral stream. Each site was sampled once by turning over logs and rocks within 3 m of the stream bank, and leaf litter searches were conducted within 3-m of the stream bank. A minimum of two people were present during each sampling event; one individual was responsible for handling the salamanders, collecting DNA samples, and recording time and location data, while the

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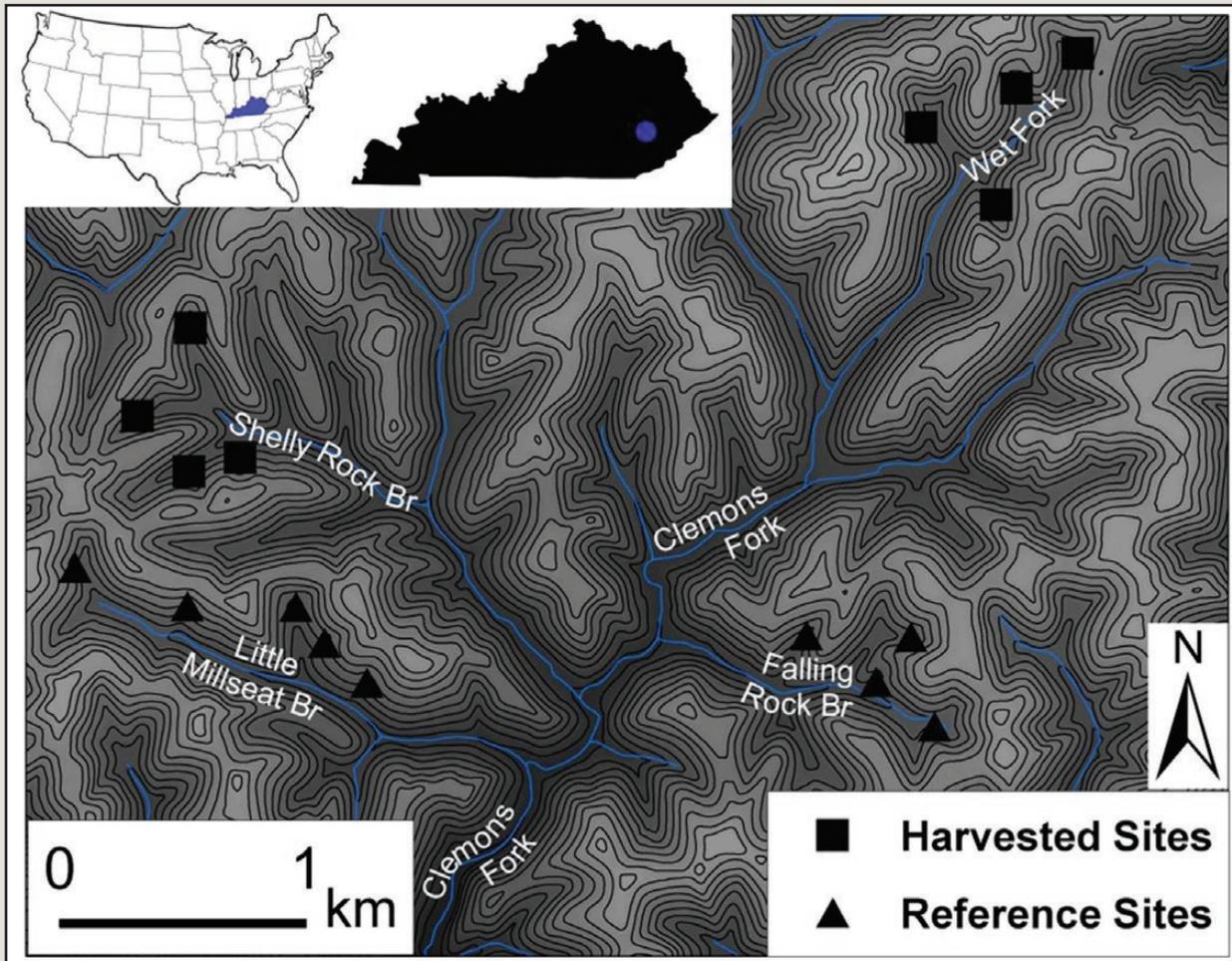


FIG. 1. Map of sampling sites for salamanders in eastern Kentucky, USA, to examine prevalence of *Batrachochytrium dendrobatidis* infection between watersheds with and without timber harvest. Each site represents one ephemeral stream from which salamanders were collected. Dark areas represent lower elevation intermittent and ephemeral stream networks, and lighter areas represent forested uplands. Blue lines represent perennial streams.

other(s) searched for and captured the salamanders. Disposable nitrile gloves were used when handling amphibians, and were replaced after each individual was captured and sampled. Boots and equipment were sterilized with 2% bleach after sampling was completed at each stream. Each captured amphibian was restrained in a fresh, clear plastic bag before being identified and examined for any gross abnormalities characteristic of *Bd* infection (e.g., lesions or sloughing skin). DNA samples were obtained by using a sterile, individually wrapped cotton-tipped swab to rub the limbs, in between the digits, and the ventral surface of the amphibian 20 times in accordance with Hyatt et al. (2007). Amphibians were returned to the location of their capture after sampling. Swabs were placed into individually labeled tubes filled with 1-mL 100% ethanol, and stored at 4°C until analyzed.

Quantitative polymerase chain reaction (qPCR) was used to determine the presence and quantity of *Bd* DNA in the samples. DNA was extracted from swabs using the Qiagen DNeasy kit eluted into 100 μ L of buffer. For the qPCR, 20 μ L reactions containing 1X Taqman Gene Expression Master Mix, 900 nmol 5.8SChytr Primer, 900 nmol ITS-1Chytr Primer, 250 nmol Chytr MGD Probe, DEPC water and 5 μ L of extracted DNA template were used (Boyle et al. 2004). Plates were run on a StepOne qPCR

machine (Life Technologies), and each plate included a negative control and standard curve from $1.573\text{--}1.573 \times 10^6$ molecules/ μ L, with an estimation of 7 DNA molecules per zoospore. Our standards were provided by Pisces Molecular (CO, USA), and were based on highly conserved genes verified across multiple strains of *Bd* (Boyle et al. 2004). Samples were first pooled for efficiency and run in triplicate, with DNA from five individuals per pool sample. Any pooled samples with > 2 replicates amplified with cycle threshold values < 40 were marked as positive. The positive pools were then run in a separate plate as individual samples, in triplicate. We generated 95% confidence intervals for *Bd* prevalence per treatment and for each species using Microsoft Excel.

We sampled 278 salamanders, 190 from reference streams and 88 from timber-harvested streams. These included 8 species, all plethodontids (Tables 1 and 2). None of the salamanders we sampled exhibited obvious clinical signs of chytridiomycosis. *Bd* was detected on 6 of 278 (2.2%) salamanders, and was found in all four watersheds. *Bd* was detected on 2 of 88 (2.3%) salamanders found in timber harvested streams and 4 of 190 (2.1%) salamanders found in reference streams. Species-specific detection rates were similar. We detected *Bd* on 2.7% of *Desmognathus fuscus*, 1.9% of *D. monticola*, 2.3% of *Eurycea*

TABLE 1. Salamanders sampled for *Batrachochytrium dendrobatidis* (*Bd*) from ephemeral streams in Kentucky, USA forests in watersheds that were either unharvested (reference streams) or recently timber harvested. The number of individuals on which *Bd* was detected is indicated in parentheses. The 95% confidence intervals (CI) are provided for the proportion infected per species, for totals among all species for each treatment, and for the overall total.

Species	Reference streams	Harvested streams	Total individuals captured (+ <i>Bd</i>)	Total proportion <i>Bd</i> infected	Total proportion 95% CI
<i>Desmognathus fuscus</i>	55 (1)	20 (1)	75 (2)	0.027	-0.0098–0.063
<i>Desmognathus monticola</i>	66 (1)	38 (1)	104 (2)	0.019	-0.0072–0.046
unidentified <i>Desmognathus</i>	6 (0)	—	6 (0)	0	—
<i>Eurycea cirrigera</i>	35 (1)	8 (0)	43 (1)	0.023	-0.022–0.068
<i>Gyrinophilus porphyriticus</i>	10 (0)	9 (0)	19 (0)	0	—
<i>Plethodon glutinosus</i>	—	3 (0)	3 (0)	0	—
<i>Plethodon richmondi</i>	16 (0)	10 (0)	26 (0)	0	—
<i>Pseudotriton ruber</i>	2 (1)	—	2 (1)	0.5	-0.019–1.19
All individuals	190 (4)	88 (2)	278 (6)	0.022	-0.0042–0.047
Total Proportions	0.021	0.023	0.022		
95% CI	0.00064–0.041	-0.0084–0.054	-0.0042–0.047		

TABLE 2. Salamanders sampled for *Batrachochytrium dendrobatidis* (*Bd*) from ephemeral streams in Kentucky, USA forests that were in unharvested watersheds (reference streams) or recently timber harvested watersheds. The number of individuals on which *Bd* was detected is indicated in parentheses. DEFU = *Desmognathus fuscus*, DEMO = *Desmognathus monticola*, UNDE = Unidentified *Desmognathus*, EUCI = *Eurycea cirrigera*, GYPO = *Gyrinophilus porphyriticus*, PLGL = *Plethodon glutinosus*, PLRI = *Plethodon richmondi*, PSRU = *Pseudotriton ruber*.

Watershed	Treatment	DEFU	DEMO	UNDE	EUCI	GYPO	PLGL	PLRI	PSRU	Total
Falling Rock	Reference	40 (1)	38 (1)	4	32 (1)	7	—	15	—	136 (3)
Little Millseat	Reference	15	28	2	3	3	—	1	2 (1)	54 (1)
Shelly Rock	Harvested	7	14 (1)	—	5	4	—	7	—	37 (1)
Wet Fork	Harvested	13 (1)	24	—	3	5	3	3	—	51 (1)

cirrigera, and on one of two *Pseudotriton ruber* individuals sampled. Confidence intervals for the proportion of individuals on which *Bd* was detected widely overlap when examined across treatments and species.

We detected *Bd* on four species of stream-dwelling plethodontid salamanders in southeastern Kentucky forests, the first documented occurrence in the Cumberland Plateau region of the state. Detection rates were generally low (< 3.0%), but were within the range of other studies conducted with the same species elsewhere when *Bd* was detected (Grant et al. 2008). We did not detect *Bd* on *Gyrinophilus porphyriticus*, and our low sample size (N = 19) likely precluded *Bd* detection if prevalence was low (Gray et al. 2017). Yet this is consistent with other studies, which have not detected *Bd* on *G. porphyriticus* (Hossack et al. 2010). The continued lack of infection in *G. porphyriticus* is especially interesting since highly aquatic species are often more prone to *Bd* infection (Chatfield et al. 2012), and increased exposure to *Bd* might be expected in *G. porphyriticus* due to their predation on species of salamanders we found to be infected (Bruce 1979). Reasons for this lack of infection remain unclear, but may be due to genetic expression or interactions among microbial skin fauna (Ramsey et al. 2010; Ellison et al. 2014). In terms of terrestrial plethodontids, we found no *Bd* present in *Plethodon richmondi* (N = 26), nor in *P. glutinosus* (N = 3), however both samples were small. Nevertheless, this may fit with a general narrative of lower *Bd* infection in terrestrial plethodontids of the eastern US, especially within the *Plethodon* genus (Muletz et al. 2014). Likely

causes of this difference may include bacteria present on the skin surface which may reduce infection prevalence in *Plethodon* spp. (Becker and Harris 2010), or the ability of some terrestrial plethodontids to shed infections of *Bd* (Weinstein 2009).

Unlike other habitat alteration studies which have focused on urbanization or agriculture, we tested for differences in *Bd* prevalence between harvested and unharvested forest habitats. We found no major differences in prevalence between streams in harvested and reference watersheds, with sample sizes similar to other studies that showed significant impacts of anthropogenic land uses on *Bd* infection of other amphibians (Saenz et al. 2015), and of the prevalence of other diseases such as ranaviruses (Gray et al. 2007). While our sample size was insufficient to detect small increases and our study was not able to disentangle potentially influential environmental variables, several factors might explain why stream-dwelling salamanders would not experience dramatically altered rates of infection after timber harvest. As previously mentioned, higher temperatures have been shown to reduce *Bd* infection (Woodhams et al. 2003; Chatfield and Richards-Zawacki 2011), and mean daily stream temperatures were found to be elevated by up to 0.56°C in our harvested streams in the years immediately after logging (Witt 2012). Alternatively, lower density of salamanders due to decreased abundances following harvests could reduce transmission rates, and indeed, lower abundances were found after logging occurred in our harvested streams (Maigret et al. 2014).

A simpler explanation could be that the ultimate cause of lower rates of *Bd* detection in stream salamanders compared to other amphibians such as ranid frogs might be totally independent of land use and more a reflection of the uneven prevalence of *Bd* across different habitats or species (Hossack et al. 2010). For example, many terrestrial taxa including *Plethodon* spp. show low rates of *Bd* infection across a wide range (Muletz et al. 2014), and studies have suggested *Bd* may be less lethal in ephemeral than perennial aquatic habitats (e.g., Kriger and Hero 2007). Ranids, many of which are pond dwellers, have larvae that can serve as optimal reservoirs for *Bd*, as higher larval densities allow for increased infection (Rachowicz and Vredenburg 2004; Woodhams and Alford 2005). Considering the low prevalence of *Bd* found throughout stream-dwelling salamanders of eastern North America, our results further suggest that either (a) low-order freshwater streams might be suboptimal habitats for *Bd* to thrive and may remain so regardless of land uses such as timber harvest, or (b) amphibian taxa inhabiting these streams might be resistant to *Bd* infection. Research examining the prevalence of *Bd* within the species we sampled under experimental settings may distinguish between these two possibilities. Further research into the impacts of extractive land uses on *Bd* prevalence might yield different results for sites where infection is higher across taxa, or for study organisms where *Bd* prevalence is higher. For example, higher *Bd* prevalence has been found in wooded wetlands, especially among ranid larvae and adults (e.g., Saenz et al. 2015). Thus while we found no effect of timber harvest on *Bd* prevalence in stream-dwelling or terrestrial salamanders, further research might be appropriate to design effective conservation plans for other amphibian taxa.

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First Confirmed Case of Ranavirus in Turtles from Shelby County, Western Tennessee, USA

Ranaviruses have been identified in fish, reptiles, and amphibians across the United States, including in central and eastern Tennessee (e.g., Gray et al. 2007; Gray et al. 2009; Hoverman et al. 2012). To date, ranavirus infections have not been detected in Tennessee west of the greater Nashville region (Green et al. 2002). Additionally, Hanlon et al. (2016) found no occurrence of ranavirus in eastern Arkansas in areas close to, and habitats similar to, those in western Tennessee (note that the Mississippi River may act as a barrier to disease spread; Sarkissian et al. 2015).

Our goal was to determine the prevalence of ranavirus in turtles in western Shelby County, which contains Memphis, Tennessee. We deployed six baited hoop nets (1-m diameter, 2.5-cm mesh) at seven sites within Shelby County over a total of 56 trap nights from 5 August to 30 September 2016. All traps

were checked the day following deployment. Due to high capture success of *Trachemys scripta elegans* (Red-eared Slider) we focused our surveillance on this species. Upon capture, we determined the sex, mass, and length of each individual, and checked for any clinical signs of ranavirus infection such as ocular discharge, dermatitis, or necrotic oral tissue (Allender et al. 2013). To assess ranavirus prevalence, we collected a tail clip (~ 1.0 cm) from each turtle with a sterile razor blade, placed it in a snap cap tube (Fisherbrand®, Cat. #02-681-272), immediately placed the tube on ice, and returned it to the lab within four hours of harvesting where it was stored at 4°C (Gray et al. 2012; Goodman et al. 2013). We used a new blade and gloves for each sample.

To extract DNA from tail clips, we used a DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, California, USA) according

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FIG. 1. County map of Tennessee, USA. The star represents the approximate location of the sampling sites.