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COMMUNICATION

Emerging Pathogens and a Current-Use Pesticide: Potential Impacts on Eastern Hellbenders

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Abstract

Populations of the eastern hellbender *Cryptobranchus alleganiensis alleganiensis* have been declining for decades, and emerging pathogens and pesticides are hypothesized to be contributing factors. However, few empirical studies have attempted to test the potential effects of these factors on hellbenders. We simultaneously exposed subadult hellbenders to environmentally relevant concentrations of either *Batrachochytrium dendrobatidis* (*Bd*) or a frog virus 3-like ranavirus (RV), a combination of the pathogens, or each pathogen following exposure to a glyphosate herbicide (Roundup). Additionally, we measured the ability of the skin mucosome to inactivate *Bd* and RV in growth assays. We found that mucosome significantly inactivated RV by an average of 40% but had no negative effects on *Bd* growth. All treatments that included RV exposure experienced reduced survival compared to controls, and the combination of RV and herbicide resulted in 100% mortality. Histopathology verified RV as the cause of mortality in all RV-exposed treatments. No animals were infected with *Bd* or died in the *Bd*-only treatment. Our results suggest that RV exposure may be a significant threat to the survival of subadult hellbenders and that Roundup exposure may potentially exacerbate this threat.

Amphibians are declining globally at an alarming rate (McCallum 2007; Alroy 2015). Conservation biologists and wildlife managers have been trying to understand and forestall the causes of amphibian decline for more than three decades (Semlitsch 2000; Collins and Crump 2009). Population declines have been attributed to a variety of stressors, including diseases and pesticides (Stuart et al. 2004; Wake and Vredenburg 2008; Brühl et al. 2011; Kolby 2018). The fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) is responsible for population declines in >500 amphibian species (Scheele et al. 2019). Ranaviruses (RV) occur on every continent where amphibians exist, and they are known to cause rapid mortality and population declines in some cases (Green et al. 2002; Muths et al. 2006; Price et al. 2014; Wheelwright et al. 2014; Cunningham et al. 2016; Miaud et al. 2016). Few studies have documented causal relationships between herbicide application and amphibian declines in the wild. However, glyphosate herbicides (such as Roundup; Monsanto, Saint Louis, Missouri) are used heavily worldwide and are known to cause amphibian mortality (e.g., Howe et al. 2004; Relyea 2005). They also have sublethal effects on growth, development, and behavior in amphibians (Wojtaszek et al. 2004; Cauble and Wagner 2005).

Despite the potential for any one factor to cause population decline in amphibians, populations likely experience complex combinations of stressors that happen over differing temporal scales, which makes predicting the influence of factors on populations difficult. For example, *Bd* can suppress the immune system by inhibiting proliferation and inducing apoptosis in lymphocytes (Fites et al. 2013), or it can suppress the secretion of skin defense peptides (Woodhams et al. 2012) such that subsequent exposure to other pathogens might result in greater mortality. In contrast, mucosal defenses (e.g., antimicrobial peptides and mucosal antibodies; Woodhams et al. 2014) may be critical in defense against *Bd* and RV, and antimicrobial properties of the skin can inhibit growth of *Bd* and RV (Chinchar et al. 2004; Rollins-Smith et al. 2011); thus, there is a possible interaction between immunosuppression by pathogens and immune defenses in an amphibian host.

Growing evidence indicates that exposure to pesticides (e.g., atrazine or carbaryl) can impair amphibian immune responses, thereby increasing host susceptibility to pathogens (Sifkarovski et al. 2014; Andino et al. 2017; Pochini and Hoverman 2017). However, other studies report herbicide exposure has little effect on infection rates and clinical effects from *Bd* (Gahl et al. 2011; Paetow et al. 2012; Jones et al. 2017), and in vitro assays suggest that some pesticides (e.g., Roundup) can inhibit *Bd* growth (Hanlon and Parris 2012). These examples illustrate the potential variability in host responses to concurrent stressors and justify the need for controlled experiments designed to test whether combined exposure results in differential risk from each factor alone, especially for species of conservation concern.

The hellbender *Cryptobranchus alleganiensis* has received conservation attention because populations of both subspecies (the eastern hellbender *C. a. alleganiensis* and the Ozark hellbender *C. a. bishopi*) have declined substantially in the past 30 years (Furniss et al. 2003; Mayasich et al. 2003; Wheeler et al. 2003; Foster et al. 2009; Burgmeier et al. 2011). Hellbenders are large, fully aquatic salamanders that commonly inhabit cool, highly oxygenated rivers and streams (Nickerson and Mays 1973; Bodinof et al. 2012). Hellbenders are generally long-lived species, living more than 20 years and not reaching sexual maturity until 5 to 7 years of age (Taber et al. 1975). This slow growth rate and delayed reproduction makes populations of this species particularly vulnerable to mortality events involving reproductively mature adults and reduced recruitment from larval to adult stages (Wheeler et al. 2003). Flooding and harvesting are among the documented impacts on hellbender populations (Nickerson and Briggler 2007), but the conversion of upland habitat for agriculture has received attention lately because of incidental impacts on nontarget organisms. For example, agricultural pesticides can enter stream systems via spray drift or runoff from agricultural fields (Schulz 2004; Schreiner et al. 2016). The pathogens *Bd* and RV have both been detected in amphibian species throughout the range of hellbenders (Rollins et al. 2013; Rothermel et al. 2013), and both have been detected in adult hellbenders in the USA, including occurrence of coinfection (Souza et al. 2012; Bales et al. 2015). Despite knowing that *Bd* and RV can infect hellbenders, we still know little about whether the infections lead to clinical disease. The addition of impaired water quality, such as through the introduction of an agricultural pesticide, further complicates the risk scenario and makes the effects hard to predict. Thus, our objectives were to estimate the susceptibility of hellbenders to RV and *Bd*, to measure the effects of these pathogens following exposure to a commonly used glyphosate herbicide, and to determine whether one facet of the hellbender innate immune system (the mucosome, or microecosystem of the mucus; Woodhams et al. 2014) was capable of inactivating *Bd* and RV.

METHODS

We designed a multiple-factor laboratory experiment to compare the potential effects of individual and combined exposures to RV, *Bd*, and an herbicide (H) in eastern hellbenders. All procedures followed approved University of Tennessee Institutional Animal Care and Use Committee Protocol 2140. Hellbender eggs were collected from the wild in eastern Tennessee (Tennessee Wildlife Resources Agency Collection Permit 1783) and raised in captivity at the Nashville Zoo. Eastern hellbenders are a species of high conservation concern, and as such, only a limited number of eggs could be collected for this study. Subadult hellbenders (approximately 9 months old, $n = 5$ per treatment plus three controls) were exposed to environmentally relevant concentrations of *Bd* (100,000 zoospores in 1 L water; Kirshtein et al. 2007; Walker et al. 2007), a frog virus 3 (FV3)-like RV (1,000 PFU/mL; Waltzek et al. 2014; Hall et al. 2016), a combination of *Bd* and RV (at equivalent concentrations to individual pathogen exposures), or each pathogen following sublethal exposure to a commonly used glyphosate-based herbicide (Roundup Super Concentrate; 4.2 mg acid equivalent per L; *Bd* + H and RV + H treatments). We monitored the hellbenders three times daily for gross signs of ranaviral disease (e.g., erythema, hemorrhage) or chytridiomycosis (e.g., lesions) for 33 days postexposure, and we recorded the number of days survived postexposure for each animal. After the experiment, we quantified each pathogen postmortem via quantitative PCR using DNA extracted from homogenized livers for RV (Miller et al. 2015) and homogenized toe clips for *Bd* (Boyle et al. 2004). A detailed experimental treatment timeline is given in Table 1. In addition, we applied a novel method to test whether the skin mucosome (containing all host and symbiotic skin defenses; Woodhams et al. 2014) inhibits the growth of *Bd* or RV. Briefly, mucosome isolates were incubated with either *Bd* or RV, then inactivation was measured using either optical density at 490 nm (for *Bd*) or via plaque assay (for RV). Detailed methods including information of strain origin, quantitative PCR pathogen detection, and pilot data justifying the choice of Roundup concentration, as well as statistical analyses, are included in the Supplementary Materials (available in the online version of this article).

RESULTS

Infection prevalence (i.e., the percentage of infected individuals in each treatment) for RV was high in all RV exposure treatments, and only one individual (in the

TABLE 1. Experimental timeline for pathogen and herbicide treatments of subadult eastern hellbenders. Exposure days are represented by X; animals were returned to flow-through aquaria on day 7 and remained there until the end of the experiment (END).

		Day							
Treatment	Exposure	1	2	3	4	5	6	…	33
Bd[a]	*Bd*					X	X	…	END
Bd + H	*Bd*					X	X	…	END
	Herbicide	X	X	X	X			…	END
Coinfection	RV					X	X	…	END
	Bd	X	X					…	END
RV[b]	RV					X	X	…	END
RV + H	RV					X	X	…	END
	Herbicide	X	X	X	X			…	END

[a]*Bd* = *Batrachochytrium dendrobatidis*.
[b]RV = frog virus 3-like ranavirus.

coinfection treatment) was negative for RV at the end of the experiment (qPCR critical threshold value = 34.7; Figure 1). In contrast, no animals tested positive for *Bd*, and no histopathological changes consistent with *Bd* or suggestive of herbicide exposure were noted. All animals positive for RV had histopathological changes consistent with ranaviral disease.

Survival differed among treatments ($\chi^2 = 17.88$, df = 5, $P < 0.005$). Survival was high in the *Bd*-only and *Bd* + H treatments (100% and 80%, respectively), whereas survival was substantially lower in the RV only, coinfection, and the RV + H treatments (20, 20, and 0%, respectively; Figure 2), with 100% mortality occurring in the RV + H treatment by 23 days postexposure to the pathogen. Sidak-adjusted post hoc comparisons suggested that mortality only differed between the RV + H treatment and the *Bd*-only treatment (Table 2). However, the lack of differences between other treatments (e.g., RV versus *Bd*) is likely an artifact of the small sample size. One control animal died during testing; however, pathological examination of the single control mortality did not yield a cause of mortality.

Mucosome samples significantly reduced concentrations of FV3 (mean reduction of 40%, $t = 13.02$, df = 12.091, $P < 0.001$; Figure 3) compared to the positive control (i.e., FV3 incubated with a synthetic ranatuerin-2P antibacterial peptide at 2μM; Chinchar et al. 2001). Mucosome samples did not reduce FV3 concentrations as effectively as the peptide control, however (mean reduction 95%, Figure 3). There was no correlation between FV3 inhibition by hellbender mucosome and RV infection status ($R^2 = 0.11$, $P = 0.23$), load ($R^2 = 0.01$, $P = 0.68$), or day of death ($R^2 = 0.05$, $P = 0.48$). Conversely, mucosome significantly increased growth of *Bd* compared to water-only controls (mean growth of 161%), while controls with media resulted in *Bd* growth of 124% ($t = 12.19$, df = 28, $P < 0.001$; Figure 3). Mean viability of *Bd* zoospores in mucosome was 98% (±0.39 SD).

DISCUSSION

Understanding the effects of co-occurring stressors like pathogens and contaminants is key to predicting amphibian population declines. We found that *Bd* was able to thrive in the skin mucosome of subadult hellbenders, and viability of *Bd* grown in mucosome isolates was not affected by the mucosome. This is contrary to other

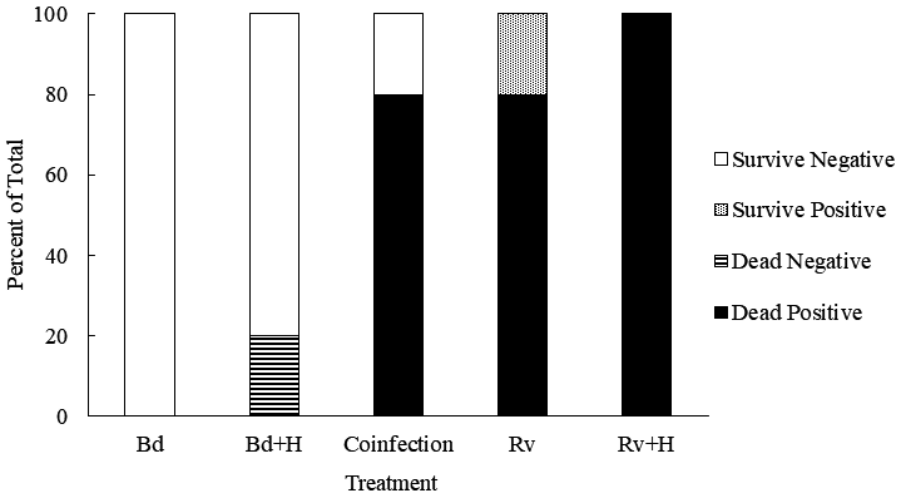

FIGURE 1. Ranavirus (Rv) infection prevalence (%) in subadult eastern hellbenders at day 33 of the experiment. Individuals in the coinfection, Rv, and Rv + H treatments were exposed to a frog virus 3 (FV3)-like ranavirus isolate at 100,000 PFU/mL Individuals in which no infection was detected via quantitative PCR are identified as negative, and individuals with detectable infection are identified as positive.

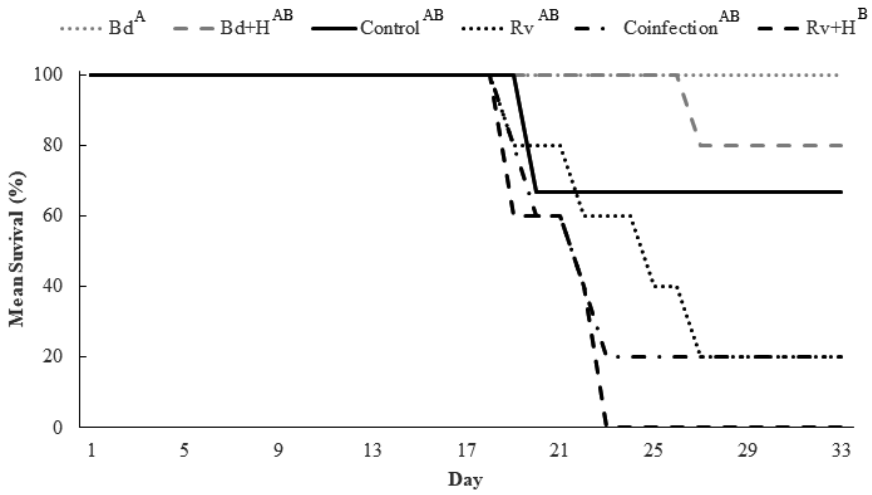

FIGURE 2. Mean percent survival of subadult eastern hellbenders following exposure to *Batrachochytrium dendrobatidis* (*Bd*), *Bd* and herbicide (*Bd* + H), a frog virus 3 (FV3)-like ranavirus (RV), *Bd* and RV (coinfection), and RV and herbicide (RV + H) as a function of time for each treatment. Unlike letters designate significant differences between treatments (Tukey's honestly significant difference; $P < 0.05$).

TABLE 2. Post hoc comparison *P*-values (with Sidak adjustment) for pathogen and herbicide treatments in the multi-factor experiment on eastern hellbenders. Values were compared to a significance value of $\alpha = 0.05$ (*$P < 0.05$).

	Comparison					
Treatment	Control	*Bd*	*Bd* + H	Coinfection	RV	RV + H
Control	—	0.94	1.00	0.88	0.99	0.28
Bd	0.94	—	1.00	0.14	0.38	0.02*
Bd + H	1.00	1.00	—	0.40	0.73	0.07
Coinfection	0.88	0.14	0.40	—	1.00	1.00
RV	0.99	0.38	0.73	1.00	—	1.00
RV + H	0.281	0.02*	0.07	1.00	1.00	—

studies that have found amphibian mucosa can inhibit growth of *Bd* (e.g., Woodhams et al. 2014), and does not explain the lack of *Bd* infection in the present study. It is important to note that the juvenile hellbenders used in the present study were raised in captivity. While some studies suggest there is no impact of captivity on skin bacterial communities (Flechas et al. 2017), it is generally believed that mucosal communities are environmentally dependent (Kueneman et al. 2016); thus, some components of mucosal defenses might have been absent, possibly allowing *Bd* to utilize keratin shed in the mucosome for growth.

The exposure concentration and strain of *Bd* used in this study may not have been sufficient to cause infection. Vredenburg et al. (2010) suggested that a threshold dose of 10,000 zoospores is sufficient for anuran species to become infected with *Bd*. However, infectious thresholds have not been investigated in salamander species. Moreover, other studies (e.g., Retallick and Miera 2007) suggest that susceptibility may differ between strains of *Bd*. Our study used the *Bd* strain JEL 197, which may not be as infectious as locally occurring *Bd* isolates. *Bd* also primarily infects keratinized skin, and subadult salamanders may have less keratin in their skin than adults (as observed in anurans; Voyles et al. 2011; McMahon and Rohr 2015) even though microscopically they look similar (Figure 4). Death due to chytridiomycosis has not been reported in wild adult hellbenders but has been occasionally seen in adult captive hellbenders (Dusick et al. 2017) and in hellbenders that are part of soft-release repatriation programs (E. M. Bunting, Cornell University, unpublished data). Importantly, naturally occurring die-offs would be obscured by rapid decomposition rates; thus, these anecdotal observations of susceptibility in adult hellbenders from other settings provide evidence that *Bd* may yet be a contributing factor in hellbender declines.

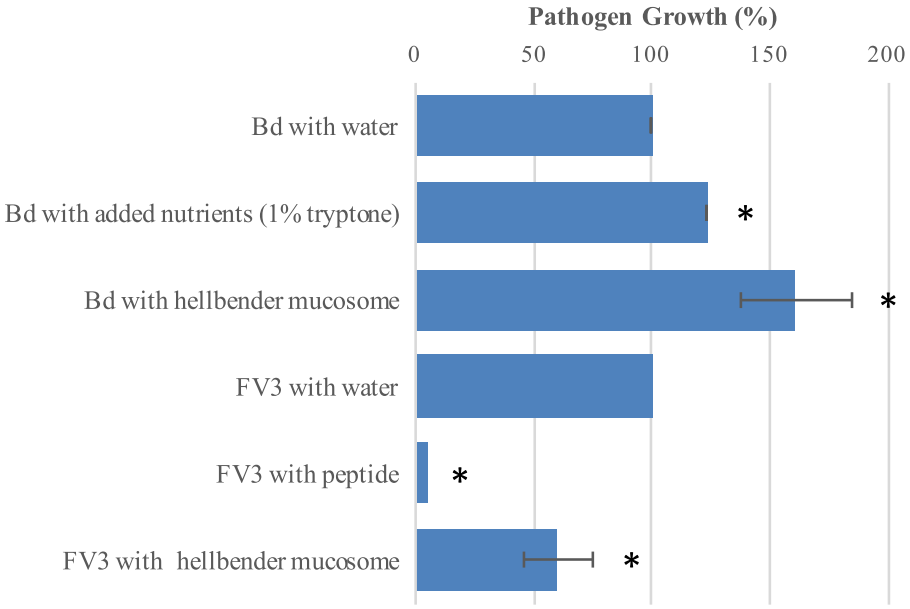

FIGURE 3. Percent growth of *Bd* in water, added nutrients (1% tryptone), or hellbender mucosome, and FV3 in water, R2P antibacterial peptide, or hellbender mucosome. Error bars represent one standard deviation. Asterisks represent samples that differed significantly from water-only controls. [Color figure can viewed at afsjournals.org.]

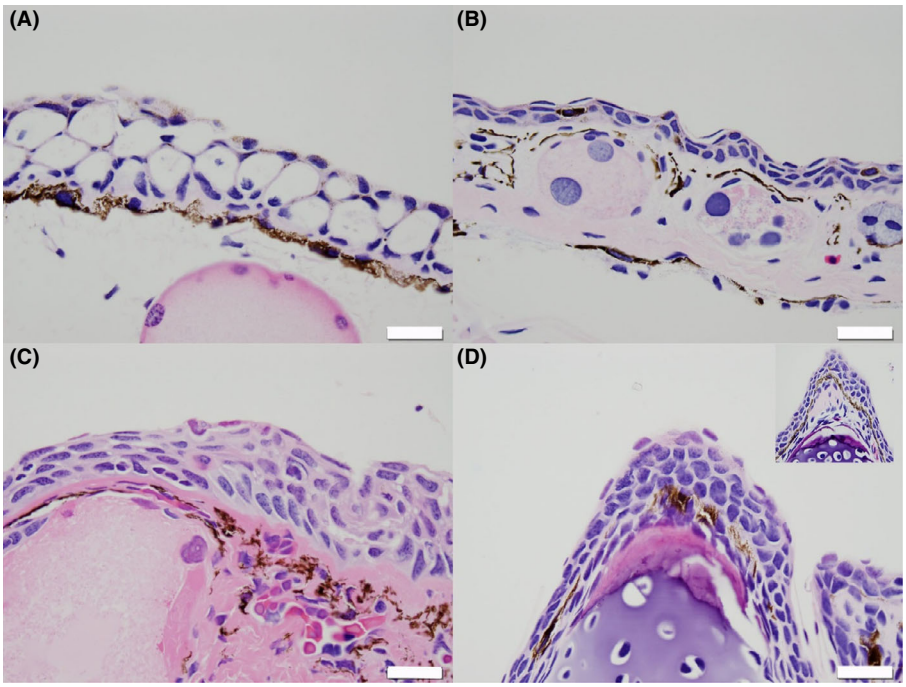

FIGURE 4. Although keratinized skin may be present at the different hellbender life stages, the quantity present might differ. Shown here are photomicrographs of skin (stained with hematoxylin and eosin) from the trunk (near the gill region) of **(A)** larval, **(B)** subadult, and **(C)** captive adult eastern hellbenders, showing the similarity in subadult and adult skin. In contrast, **(D)** the skin from a distal digit of a juvenile looks similar to that of the larval hellbender digit (inset), both of which are similar to adult skin. Bar = 50 μm. [Color figure can viewed at afsjournals.org.]

Reduced recruitment from juvenile to adult has been implicated as a cause of observed hellbender declines (Wheeler et al. 2003), and the rapid mortality of subadult hellbenders in our study is cause for concern. Subadult hellbenders were highly susceptible to RV, experiencing 80–100% mortality in all RV-associated treatments. The

lack of correlation between RV inactivation and infection status, load, or day of death suggests that despite the ability of hellbender mucosome to partially inactivate RV, animals became infected and died. This suggests that there were other significant routes of transmission associated with epithelial cells located in multiple organ systems (e.g., gill membranes).

Perhaps more concerning is the increased mortality from RV following exposure to Roundup; prior herbicide exposure and subsequent RV exposure caused 100% mortality. Other studies suggest that there may be synergies between chemical exposure and disease in amphibians, particularly when experienced simultaneously. For example, exposure to environmentally relevant concentrations of atrazine increased susceptibility of eastern tiger salamanders *Ambystoma tigrinum* (Forson and Storfer 2006; Kerby and Storfer 2009) and African clawed frogs *Xenopus laevis* (Sifkarovski et al. 2014) to the ATV ranavirus. Our data suggest that exposure to Roundup is not necessary to cause high mortality from exposure to RV; however, short-term, pulsed exposures to an herbicide may increase the impact of RV on juvenile hellbenders.

The strain of FV3-like RV we used was isolated from Missouri and is unlikely to interact with eastern hellbenders in Tennessee; however, it is possible that eastern and Ozark hellbenders in Missouri and possibly Arkansas could become exposed to it, and other strains of FV3-like RV are present in Tennessee where hellbenders occur (Souza et al. 2012). Given the rate of ranaviral disease progression we documented here (<1 month) and the lotic systems they inhabit, detection of diseased animals in the wild will remain a challenge (Gray et al. 2015). Despite the potential impacts of RV on subadult hellbender survival, it is important to consider that hellbenders generally inhabit flowing aquatic systems, which may reduce the likelihood that hellbenders are exposed to RV at an environmental concentration sufficient to cause infection and disease (Hall et al. 2016). Ultimately, more research is needed to definitively document interactions between glyphosate herbicides and pathogens in hellbenders, and more intensive investigations of *Bd* susceptibility are needed to make definitive conclusions about these threats to hellbender populations.

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SUPPORTING INFORMATION

Additional supplemental material may be found online in the Supporting Information section at the end of the article.