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State-wide Survey of Amphibian Pathogens in Green Frog (*Lithobates clamitans melanota*) Reveals High Chytrid Infection Intensities in Constructed Wetlands

James T. Julian[1,4], Robert P. Brooks[2], Gavin W. Glenney[3], and John A. Coll[3]

[1]Division of Mathematics and Natural Science, Pennsylvania State University-Altoona College, 3000 Ivyside Park, Altoona, Pennsylvania 16601, USA
[2]Department of Geography, Pennsylvania State University-University Park, University Park, Pennsylvania 16801, USA
[3]Northeast Fishery Center-Fish Health Center, U.S. Fish and Wildlife Service, Lamar, Pennsylvania 16848, USA
[4]Corresponding author e-mail: jtj2@psu.edu

***Abstract.*—We screened populations of larval Green Frogs (*Lithobates clamitans melanota*) for *Ranavirus* spp. and *Batrachochytrium dendrobatidis* (*Bd*) in naturally occurring reference wetlands (n = 12) and wetlands constructed for loss of habitat elsewhere (n = 12) throughout Pennsylvania, USA. We detected the ranavirus, *Frog Virus 3*, in one natural and one constructed wetland, and Bd occurred in 11 natural wetlands and 10 constructed wetlands. Infection intensities of *Bd* (zoospores per infected individual) were significantly higher in constructed (median = 771, mean ± SE = 1514 ± 450) than reference wetlands (median = 242, mean = 487 ± 216), and constructed wetlands contained four of the six highest *Bd* prevalence values. Our results suggest that infection intensity decreased with increasing forest cover, but only among wetlands with few anthropogenic disturbances and that best management practices for forested riparian zones may differ along gradients of human disturbance. If constructed wetlands are to replace and mitigate the loss of the ecological functions of natural wetlands, wetland-dependent wildlife need to withstand stressors like disease. To achieve this, resource managers should understand the role constructed wetlands play in the persistence and spread of emergent infectious disease.**

Key Words.—anthropogenic; anuran; *Batrachochytrium dendrobatidis*; constructed wetland; disease; ranavirus; riparian

Introduction

Wetland mitigation projects use excavation and vegetation planting/seeding to construct wetland habitats in terrestrial environments to replace the ecological services and functions of natural wetlands destroyed or degraded by human activities (Kentula et al. 1992; Brooks 1993; Brooks and Gebo 2013; Mitsch and Gosselink 2015). One benefit constructed wetlands can provide is habitat for healthy populations of wetland-dependent wildlife. While constructed wetlands can ameliorate habitat loss for pond-breeding amphibians, few studies have compared the occurrence or prevalence of amphibian pathogens between natural and constructed wetlands. Two important disease-causing pathogens in amphibians are ranaviruses in the family Iridoviridae and the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*). Ranaviruses occur in over 100 species of amphibian worldwide and contributed to mass mortality events of 50 North American species (Miller et al. 2011; Duffus et al. 2015). Ranavirus infects adult and larval amphibians and may cause death in both life stages via internal hemorrhaging (Green et al. 2010). *Batrachochytrium dendrobatidis* may cause the disease chytridiomycosis (Berger et al. 1998), in which *Bd* fungal spores overpopulate the skin and result in excessive keratinization and osmotic imbalance, followed by cardiac arrest (Voyles et al. 2009). Chytridiomycosis occurs globally in over 500 amphibian species (Olson et al. 2013) and contributed to population declines in more than 200 of them (Daszak et al. 2003; Wake and Vredenberg 2008; Forzán et al. 2010).

The health of amphibian populations could differ between natural wetlands and constructed wetlands because anthropogenic disturbances at constructed wetlands may lower ecosystem functions (Gebo and Brooks 2012; Moreno-Mateos et al. 2012) and the prevalence of *Bd* in amphibian communities can increase along a gradient of human impact (Adams et al. 2010). For example, pathogen infection prevalence can be higher for amphibian populations in wetlands affected by anthropogenic disturbances such as human development, livestock access, exposure to pesticides, and elevated levels of aluminum and ammonia (Forson and Storfer 2006; Gray et al. 2007; St-Amour et al. 2008; Gahl et al. 2010). Contaminants like fungicides, however, may lower the intensity of *Bd* infections by inhibiting fungal growth (Berger et al. 2010; Brannelly et al. 2012; Hanlon et al. 2012). Tornabene et al. (2018) found that livestock-influenced disturbances and water chemistry were not as important in explaining pathogen

occurrence as wetland proximity to infected ponds, high amphibian densities, and the abundance of key amphibian species.

The design of a constructed wetland could also influence the establishment of amphibian pathogens, especially if the wetland is created with suitable habitats for species that serve as pathogen reservoirs. While the U.S. has lost over 50% of its historic wetland acreage (Tiner 1984; Dahl 1990), the net loss of wetland acreage in the U.S. has drastically declined due, in part, to the construction of wetlands (Dahl 2011). Deep-water, permanently flooded wetlands have been a common design for constructed wetlands (Kentula et al. 1992) and permanently flooded ponds in the U.S. have recently increased in acreage while most other wetland types have decreased (Dahl 2011). These sparsely vegetated, permanently flooded ponds suit the habitat requirements of both American Bullfrog (*Lithobates catesbeianus*) and Green Frog (Hulse et al. 2001). These two species contract ranaviruses (Duffus et al. 2015) and are regarded as important reservoirs that maintain and spread *Bd* (Greenspan et al. 2012; Forzán et al. 2010). Fish and turtle species that inhabit deep-water ponds are included in the over 50 vertebrate taxa that contract ranaviruses (Williams et al. 2005; Duffus et al. 2015), including ranavirus lineages like Frog-virus 3 (FV3) that are detected in the majority of ranaviriosis mortality events in North America (Price et al. 2017).

Constructed wetlands can be colonized by pathogen-infected animals migrating over land as well as through the stream channels, ditches, and culverts that connect constructed wetlands to other water bodies. Furthermore, wetlands with these hydrologic connections could receive pathogen-contaminated water from upstream amphibian communities as both *Bd* (Rachowicz and Vredenburg 2004) and ranaviruses (Harp and Petranka 2006) can be spread in contaminated water. The loss of historic wetland acreage in Pennsylvania, USA, is roughly equivalent to the national average (Mitsch and Gosselink, 2015), and the wetlands research group Riparia of Pennsylvania State University (https://riparia.psu.edu/) has monitored 100 constructed wetlands that were created to offset some of the recent wetland losses in Pennsylvania. In addition, Riparia has conducted studies to compare the ecological functions of those constructed wetlands to over 200 naturally occurring wetlands.

The goal of our study was to compare the occurrence, prevalence, and infection intensity of ranavirus and *Bd* in populations of larval Green Frogs (*Lithobates clamitans melanota*) between natural and constructed wetlands in Pennsylvania. We expected infection parameters for both pathogens to be higher in constructed wetlands than natural ones. We sampled from these sets of wetlands to compare infection parameters in tadpoles of the Green Frog between constructed and natural wetlands across Pennsylvania. Our sampling included wetlands from each U.S. Environmental Protection Agency Level III ecoregion (ftp://newftp.epa.gov/EPADataCommons/ORD/Ecoregions/reg3/reg3_eco.pdf) within the state to determine whether pathogen parameters differed by geographic area. We also examined whether variation in infection parameters was best predicted by within-wetland variables like wetland size and stream connections, or forested land cover and anthropogenic disturbances that surrounded wetlands. Distinguishing between intrinsic aquatic and extrinsic terrestrial factors could be important in planning, designing, and maintaining constructed wetlands because wetlands that harbor pathogens could act as sink habitats for amphibian populations as well as intermediate linking habitats that infected animals disperse through on their way to landscapes previously free of disease.

Materials and Methods

Wetland selection and attributes.—Riparia has created a database of habitat assessments conducted at 322 wetlands throughout Pennsylvania. The database of Riparia includes naturally occurring reference wetlands (n = 222) that were randomly selected from federal wetland databases (Brooks et al. 2013), as well as constructed wetlands (n = 100) that were created 10–24 y before the initiation of this study (Gebo and Brooks 2012). Investigators visited each wetland in the database of Riparia during previous studies and scored them with a habitat suitability index for the American Bullfrog (Brooks and Prosser 1995). We generated a ranked list of wetlands based on American Bullfrog habitat suitability scores to identify wetlands with a high probability of containing Green Frog tadpoles because these two species share similar habitat requirements and often co-inhabit wetlands in Pennsylvania (Hulse et al. 2001). We searched for Green Frog tadpoles first from highly ranked wetlands and proceeded down this list attempting to achieve roughly equal representation of EPA Level III ecoregions in Pennsylvania, as well as constructed and reference wetlands. We considered ecoregions to be a more meaningful descriptor of geographic variation than north-south/east-west ordination because ecoregions are spatial classifications of land areas with similar geology, physiography, vegetation, climate, soils, land use, wildlife, and hydrology (for ecoregion descriptions see Omernik 1995).

From spring 2013 to summer 2014, we searched 56 wetlands from the database of Riparia for tadpoles, and successfully collected tadpoles from 20 of them. We collected tadpoles from five natural wetlands that were not part of this database to obtain an adequate number of sites. We identified these wetlands from federal wetland

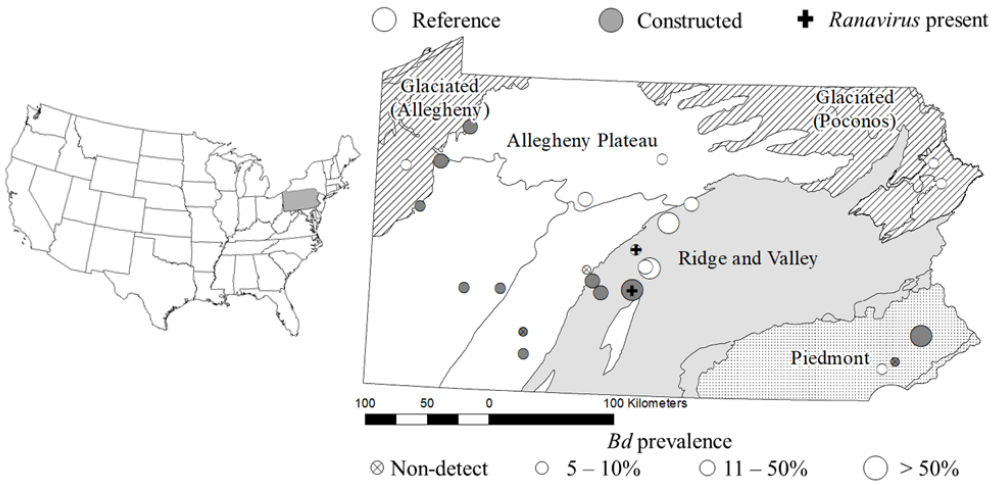

Figure 1. Location of ecoregions in the state of Pennsylvania, USA; chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) prevalence and ranavirus presence in constructed and natural reference wetlands. Open circles = reference wetlands, filled circles = constructed wetlands, circle size proportional to *Bd* prevalence value, and "+" = Ranavirus present.

databases and chose them based on their proximity to reference wetlands from the database of Riparia. We used tadpole collections to determine: 1) pathogen presence in a population for *Ranavirus* and *Bd*; 2) pathogen prevalence for *Ranavirus* and *Bd* (the percent of individuals infected in a population); and 3) infection intensity for *Bd* (the number of zoospore equivalents per infected individual). We collected tadpoles from 13 reference wetlands and 12 constructed wetlands from five Pennsylvania ecoregions (Table 1), and both wetland types were sampled within each ecoregion (Fig. 1, 2).

At wetlands (each fewer than 2.0 ha), we mapped the perimeter with a hand-held GPS unit (GPSmap 62stc; Garmin, Olathe, Kansas, USA) to estimate wetland area, while we estimated the areas of larger wetlands using polygons from the National Wetland Inventory of the U.S. Fish and Wildlife Service. In the field, if there was evidence of significant channelized water flow into, or out of, the wetland, we characterized it as being stream connected, even if channels were dry at the time of inspection. Alternately, we characterized wetlands that lacked such channels as hydrologically isolated. We used a Stressor Checklist developed by Brooks et al. (2006) to conduct a survey of anthropogenic disturbances found in terrestrial habitats within 100 m of the perimeter of a wetland (i.e., riparian buffer zones). The Pennsylvania Department of Environmental Protection use these protocols to assess wetland condition (Pennsylvania Dept. Environmental Protection 2017), and we refer to the environmental stressors they identify as disturbances in this manuscript. This protocol identifies nominal disturbances within major disturbance categories, but does not assign either quantitative values, nor ordinal ranks, to individual disturbances. The three major categories of disturbances we found were: 1) hydrologic disturbances, 2) vegetation disturbances, and 3) sedimentation disturbances. We categorized a wetland as High-disturbance if it contained disturbances from two or three categories and Low-disturbance if it contained disturbance from one category or no disturbance.

Tadpole collecting and processing.—We collected tadpoles between 23 May and 14 July 2013, and between 14 May and 10 July 2014. A wetland was one contiguous body of water, and all wetlands in

Table 1. Infection parameters of *Batrachochytrium dendrobatidis* (*Bd*) and Ranavirus by Pennsylvania, USA, ecoregion (mean values of per wetland prevalence and mean intensity based on pathogen-present sites only). The sample size n = number of wetlands.

		Allegheny Plateau (n = 8)	Glaciated Allegheny (n = 4)	Glaciated Poconos (n = 2)	Piedmont (n = 3)	Ridge & Valley (n = 7)
Chytrid fungus (*Bd*)	Occurrence	75%	100%	100%	67%	100%
	Mean prevalence Range	8.3% 0–16.7%	12.8% 2.6–26.7%	8.9% 7.7–10.0%	50.0% 0–95.0%	38.6% 5.1–89.2%
	Mean ± SE zoospores per infected tadpole	386 ± 96	821 ± 492	312 ± 268	2,023 ± 1,665	1,461 ± 558
Ranavirus	Occurrence	0%	0%	0%	0%	28.6%
	Mean prevalence	-	-	-	-	5.0%

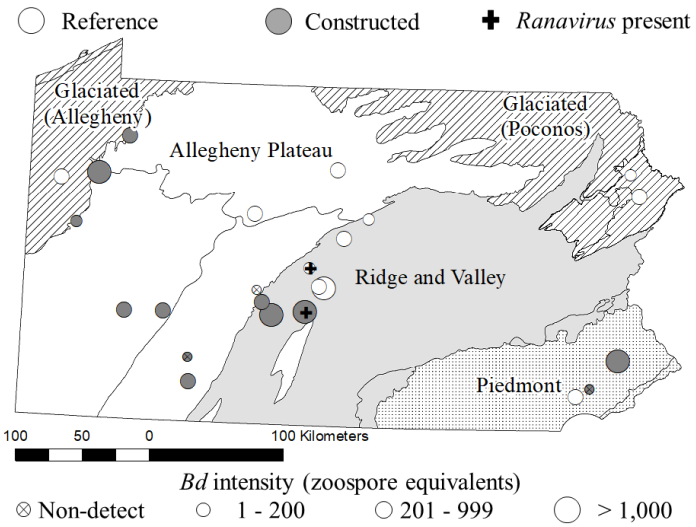

Figure 2. Map of *Batrachochytrium dendrobatidis* (*Bd*) intensity and ranavirus presence in constructed wetlands and natural reference wetlands. Open circles = reference wetlands, filled circles = constructed wetlands, circle size proportional to mean *Bd* intensity value, and "+" = *Ranavirus* present.

this study were entirely isolated from each other by terrestrial habitat. In a given year, we conducted only one sampling event at a wetland where we attempted to collect 60 tadpoles (mean = 36.5 tadpoles per event; Appendix Table 1). At each wetland, we collected tadpoles from shallow portions using standard D-frame nets (50 × 50 cm head with 5 mm mesh size) and placed them in the same plastic bucket with approximately 12 L of pond water. Between 2.0 and 9.75 h (mean = 5.4 h) passed from the time we collected tadpoles to the time we euthanized them in the laboratory at Penn State Altoona (Altoona, Pennsylvania, USA). We always housed tadpoles from different wetlands in separate buckets, so co-housed individuals from the same wetland may have cross-contaminated each other with pathogens. Cross-contamination would positively bias both prevalence and infection intensity estimates, but we suspect this bias was uniform amongst wetlands because we found no correlation between the amount of time co-housed and *Bd* prevalence ($r = 0.088$, $t = 0.342$, df = 26, $P = 0.756$), or *Bd* intensity ($r = 0.167$, $t = 0.656$, df = 26, $P = 0.553$). We decontaminated all sampling equipment and containers before and after visiting a site with a 10% bleach solution or a 2% chlorhexidine solution for 10 min, which exceed concentrations and contact times recommended by Bryan et al. (2009) and the Northeastern Partners in Amphibian and Reptile Conservation (NEPARC. 2014. Disinfection of field equipment to minimize risk of spread of chytridiomycosis and ranavirus. Available from http://northeastparc.org/our_products/ [Accessed 8 August 2018]).

In the laboratory, we euthanized tadpoles with a lethal dose of MS-222 (American Medical Veterinary Association 2013). We swabbed their mouthparts with a standard-sized sterile cotton swab (Puritan 25-806 2WC; Puritan Medical Products, Guilford, Maine, USA) with five rotations to collect epithelial DNA for *Bd* testing (Hyatt et al. 2007). We placed swabs in 1.5 ml polypropylene centrifuge tubes and stored them in a -16° C freezer. For ranavirus screening, we removed kidney tissue, liver tissue, and spleens from each tadpole. We dissected tadpoles in groupings of five individuals, with half of the tissue of each tadpole placed in a composite group for cell culture and stored in Hanks balanced salt solution (HBSS- 200 µg/ml Gentimicin). We stored the remainder of the tissue of each tadpole at -80° C before transporting them on ice to the Northeast Fisheries Center of the U.S. Fish and Wildlife Service in Lamar, Pennsylvania, USA, for cell culture, DNA extraction, and qPCR analysis.

Bd *testing with qPCR*.—We extracted sample DNA cotton swabs by following protocols outlined in the Omega Mag-Bind Tissue DNA KFA 96 kit (Omega Biotek Inc, Norcross, Georgia, USA) for DNA extraction using a Life Technologies KingFisher magnetic bead extraction machine (Thermo Fisher Scientific, Waltham, Massachusetts, USA). We performed extractions and quantitative polymerase chain reaction (qPCR) as described in Julian et al. (2016) with protocols and primer sets developed by Boyle et al. (2004). We used plasmid standards (supplied by Longcore, University of Maine, Orono, USA) of four known *Bd* concentrations (278, 2780, 27800, and 278000 zoospore equivalents) to create a calibration curve that estimated the number of *Bd* zoospore equivalents per sample, and each sample was run in duplicate to estimate an average number of

zoospore equivalents. We used eight internal positive controls (Applied Biosystems #4308323, TaqMan Exogenous Internal Positive Control [VIC™-Probe], Thermo Fisher Scientific, Waltham, Massachusetts, USA) per 96-sample qPCR plate in addition to one previous positive control and one negative control of water. To confirm qPCR *Bd* results, we chose up to three positive samples from each site for Sanger sequencing. We used conventional PCR (Annis et al. 2004), and amplicons were sequenced directly.

Ranavirus testing with cell culture and conventional PCR.—To reduce the number of cell cultures grown, as well as the number of conventional PCR reactions performed, we made composite tissue sample groupings of five individuals for cell cultures. When a composited culture displayed cytopathic effect (CPE), we ran separate PCR reactions for each individual in that composite group using their frozen tissue we reserved during our original dissections. We homogenized composited tissue samples and diluted them 1:10 in HBSS, then spun them for 20 min at 3,000 × g. We carried a sample of the supernatant in Eagles Minimal Essential Medium (1000 µg/ml Gentimicin) to a final dilution of 1:100 and inoculated preformed monolayers of Chinook Salmon Embryo (CHSE-214), Epithelioma Papulosum Cyprini (EPC), Bluegill Fry_2 (BF-2) and Fathead Minnow (FHM) cell lines. We grew positive control cultures with infectious pancreatic necrosis virus (a fish pathogen), in addition to growing negative control lines. We incubated cultures at 20° C for 28 d unless CPE was observed. To estimate ranavirus presence in individuals, we extracted DNA from each archived individual of any cell culture grouping exhibiting CPE. We used conventional PCR to determine the identity of viral pathogen via cloning and Sanger sequencing (Glenney et al. 2010).

Statistical analyses.—The experimental unit in our analyses was a wetland. For wetlands that were sampled in both years of our study (n = 5), we pooled pathogen test results from all individuals collected (during both years) so that each wetland constituted only one observation in our analyses. Dependent variables in our analyses included the prevalence of *Bd* among all individuals screened within a wetland and *Bd* infection intensity quantified as the average number of *Bd* zoospore equivalents per infected individual at a wetland. We used information from all 25 wetlands to generate maps of pathogen parameters, although we excluded data from one wetland (JAS031) from statistical analysis because we only collected four tadpoles from that wetland.

We used the categorical variable TYPE to identify a wetland as either a reference/natural wetland or a constructed wetland. Within-wetland explanatory variables included the $\log_{10}$ transformed surface area of the wetland (SIZE) and the categorical variable CONNECT that identified the wetland as being stream-connected or isolated. Riparian explanatory variables included the variable DIST that categorized a wetland as a high-disturbance or low-disturbance wetland and the estimated proportion of the 250 m buffer zone of a wetland classified as forested land use (FOREST250). We calculated FOREST250 using ArcMap 10.0 software (Esri, Redlands, California, USA) and a 2006 forested land cover data layer from the National Land Cover Database (Fry et al. 2011. Completion of the 2006 national land cover database for the conterminous united states, PE&RS. 77: 858–864 Published by Multi-Resolution Land Characteristics Consortium. Available from http://www.mrlc.gov/nlcd2006.php. [Accessed 24 October 2013]) that included all forest types (deciduous, mixed, and evergreen) when designating areas as forested. We chose a 250 m spatial scale because this distance is considered critical upland habitat for vernal pool breeding amphibians (Calhoun et al. 2005) and we believed the 50 × 50 m pixel size of our land cover layer was too coarse to accurately represent smaller spatial scales. We treated FOREST250 proportions with a $\sqrt{arcsin}$ transformation in all linear regression analyses so that data could conform to the assumptions of normality. We calculated an additional riparian variable, DISTxFOREST, to model an interaction effect among riparian stressor category and the proportion of forested land within 250 m.

When we detected *Bd*, we used three separate nonparametric Kruskal-Wallis tests ($\alpha = 0.05$) to compare *Bd* prevalence between ecoregions, between constructed vs reference wetlands, and between high-disturbance vs low-disturbance wetlands. Likewise, we used separate Kruskal-Wallis tests to compare *Bd* intensity for each of those three explanatory variables. Using observations from wetlands with *Bd*, we constructed a linear regression model (Fit Regression Model; MINITAB version 18, State College, Pennsylvania, USA) to determine the influence of wetland type on ranked *Bd* prevalence. We compared this wetland-type model to a model that also included within-wetland variables, as well as two models that included riparian-level variables. One riparian-level model examined the additive effects of riparian disturbances (TYPE+DIST+FOREST250), while the other included an interaction term (TYPE + DISTxFOREST). For every model, we converted its sum-squared error to Akaike's Information Criteria (AIC) values (Gordon 2015), and then corrected those values for small sample size (AICc). We concluded that models with AICc values at least 2.0 or lower were better fit models than the model to which they were compared (Burnham and Anderson 2002). We repeated this

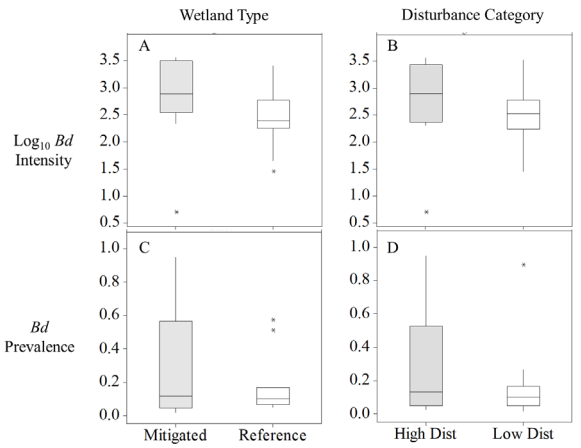

Figure 3. Boxplots comparing per wetland mean Log_{10}*Bd* intensity (A and B) and *Batrachochytrium dendrobatidis* (*Bd*) prevalence (C and D) between wetland types (A and C) and disturbance categories (B and D). Box height is interquartile range (IQR), horizontal line is the median value, width is proportional to sample size (n = 12), whiskers are maximum and minimum data points within 1.5 box heights, and * indicates outliers.

modeling procedure to create linear regression models using the response variable of ranked mean *Bd* intensity for the same set of wetlands where *Bd* was detected. We quantified correlations between FOREST250, *Bd* prevalence, and *Bd* intensity using Spearman's ranked correlation.

Results

Bd *and ranavirus sequencing*.—Sequenced amplicons (about 300bp - flanking internal transcribed spacer (ITS) regions ITS1 and ITS2 of 5.8S rRNA gene) from 41 *Bd* positive individuals from all positive sites shared nearly identical nucleotide sequence with reference sequence *Bd* (JQ582938). All sequenced amplicons differed from the reference sequence at nucleotide position 285 by having a cytosine instead of a thymine. Also, three sequences from CPA Lumber and Brandywine Slough contained four gaps when compared to the reference sequence from positions 160 to 167 and all other sequenced amplicons. Upon sequencing, amplicons (about 531bp-major capsid protein) from ranavirus positive individuals shared an identical nucleotide sequence with Frog virus 3 (FV3; AY548484).

Ecoregion comparisons of FV3 and* Bd *infections.—We detected FV3 in one reference wetland and one constructed wetland. While both of these wetlands were in the Ridge and Valley region (Fig. 1, 2) they are 32 km apart from each other and located in different watersheds (U.S. Geological Survey Hydrologic Unit Class 12) of the same sub-basin of the Susquehanna River. The reference wetland contained five (of 60) infected individuals, while only one of 60 from the constructed wetland was infected. No individuals that tested positive for FV3 exhibited gross signs of lethargy, an inability to right itself, swollen limbs, or internal hemorrhaging at the time of capture, nor upon examination in the laboratory. The only individual that tested positive for both FV3 and *Bd* came from the constructed wetland.

Table 2. Disease parameters of *Batrachochytrium dendrobatidis* (*Bd*) and Ranavirus among constructed (n = 12) and reference (n = 12) wetlands (mean values of per wetland prevalence and mean intensity were calculated using pathogen-present sites only) in the Pennsylvania, USA, sites.

		Constructed	Reference
Chytrid fungus (*Bd*)	Occurrence	83.3%	91.7%
	Mean prevalence Range	29.3% 0–95.0%	17.8% 0–57.1%
	Zoospores per infected tadpole Median / Mean ±SE	774/1,514 ± 450	242/487 ± 216
Ranavirus	Occurrence	8.3%	8.3%
	Prevalence	1.7%	8.5%

Unlike FV3, the occurrence of *Bd* was widespread in the wetlands we studied. We detected the presence of *Bd* in 88% of all wetlands we sampled, and *Bd* occurred in every county that we tested, with multiple wetlands from every ecoregion testing positive (Fig. 1, 2). The Allegheny Plateau and Piedmont were the only ecoregions that contained a wetland where we did not detect *Bd* (Table 1). Prevalence averaged 23.3% among all *Bd*-positive wetlands (range, 1.7–95.0%. The Piedmont region and the Ridge and Valley regions showed the highest mean prevalence of *Bd*, but low sample size in the Piedmont region precluded us from including it in statistical tests. Prevalence did not significantly vary between the Allegheny Plateau, Ridge and Valley region, and a combined Glaciated (Allegheny and Poconos) region ($H = 5.21$, df = 2, $P = 0.074$). The average *Bd* intensity per wetland differed by almost an order of magnitude between ecoregions (Table 1). Mean intensity was highest among wetlands in the Piedmont region and the Ridge and Valley region (Fig. 2), but there were no consistent differences between ecoregions ($H = 1.03$, df = 2, $P = 0.598$).

Wetland characteristics and* Bd *prevalence and intensity.—Prevalence did not differ between constructed and reference wetlands ($H < 0.01$, df = 1, $P = 0.944$), even though four out the highest six prevalence values were found in constructed wetlands (Table 2, Fig. 3C). Ignoring wetland type, we found no consistent differences between high disturbance vs low disturbance wetlands ($H = 0.84$, df = 17, $P =$

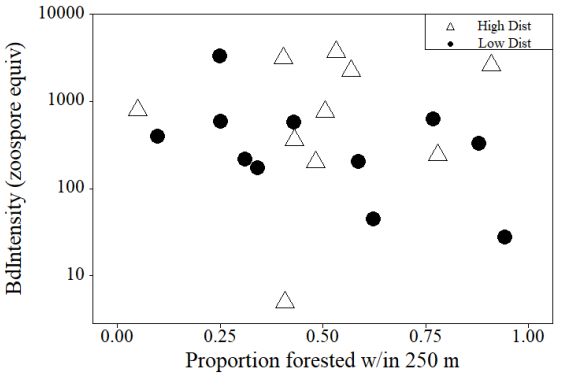

Figure 4. Interaction between stressor level and the proportion of a 250 m riparian zone around a wetland classified as forested land. Circles = low disturbance wetlands, triangles = high disturbance wetlands.

0.360; Fig. 3D), even though four out of the highest five prevalence values occurred in high-disturbance wetlands. None of our regression models for prevalence explained more than 10% of the variation in our data (Table 3). The most parsimonious model for prevalence considered only the effects of wetland type, but it had no explanatory power (r^2 < 1%). The addition of an interaction between forested cover and disturbance category produced an equally suitable model (ΔAICc < 2.0), although it still lacked explanatory power. While this interaction model explained less variation than the model for stream connection and wetland size model, as well as the model for the additive influences of forested cover and disturbance, its AICc score suggested it was a better fit than either of those two models.

Intensity values for *Bd* were significantly higher in constructed wetlands than reference wetlands (H = 4.46, df = 1, P = 0.035), with constructed wetlands having six out of the seven highest intensity values (Fig. 3A). Differences between high-disturbance and low-disturbance wetlands were not significant (H = 1.98, df = 1, P = 0.159), even though six of the seven highest mean intensities occurred in high-stress wetlands (Fig. 3B). Our model TYPE, along with the model TYPE+DISTxFOREST250, were more parsimonious than either of the two remaining models. The TYPE+DISTxFOREST250 model explained the most variation out of our competing models (r^2 = 29.5%; Table 3) even though one observation (from the Winegard population) had an undue influence on the model (Standardized residual = -2.73, Fit = 15.07). When this observation was removed, the model explained 52.2% of the variation in *Bd* intensity and its AICc score was even closer to the AICc score of the wetland type model (ΔAICc = 0.20). While not a significant correlation, *Bd* intensity tended to decrease as forest cover increased, but only in wetlands with relatively few riparian disturbances (ρ = -0.473, P = 0.142; Fig. 4). This trend was not evident among wetlands whose riparian buffers are influenced by two or more disturbance categories (ρ = 0.164, P = 0.651).

Discussion

We found *Bd* in 88% of larval Green Frog populations in Pennsylvania, which is slightly higher than occurrence rates (approximately 66%) among other Green Frog populations (Forzán 2010; Groner and Reylea 2010; Richards-Hrdlicka et al. 2013). Our surveys only detected FV3 in the Ridge and Valley ecoregion of Pennsylvania, while previous studies have detected it in Eastern Newts (*Notophthalamus virdescens viridescens*) from the Glaciated Poconos of Pennsylvania (Glenney et al. 2010), as well as Wood Frog (*Lithobates sylvaticus*) populations in the Glaciated Poconos, Ridge and Valley, and Allegheny Plateau of Pennsylvania (Crespi et al. 2015). We suspect the true occurrence of FV3 in Pennsylvania could be much higher than what we found because the pathogen is more easily detected (Hall et al. 2018), more infective (Brunner et al. 2007), and shed at a higher rate (Hall et al. 2016) later in the season. We sampled two-thirds of our wetlands in May and June because local cohorts of Green Frog tadpoles can complete metamorphosis in

Table 3. Comparison of linear regression models for ranked *Bd* intensity. The abbreviations SSE = error sum of squares and AICc = Akaike's Information Criteria corrected for small sample size.

Response Variable	Model Variables	r^2	SSE	AICc	ΔAICc
Bd intensity rank	TYPE	22.3%	598.2	32.70	0.00
	TYPE+DISTxFOREST250	29.5%	543.0	34.22	+1.52
	TYPE+DIST+FOREST250	27.9%	555.4	37.10	+4.40
	TYPE+CONNECT+SIZE	27.3%	559.5	37.17	+4.47
Bd prevalence rank	TYPE	0.1%	767.3	34.98	0.00
	TYPE+DISTxFOREST250	5.4%	725.9	36.87	+1.89
	TYPE+CONNECT+SIZE	10.0%	690.8	39.10	+4.12
	TYPE+DIST+FOREST250	8.79%	700.1	39.22	+4.24

late June. Our current studies estimate the likelihood of detecting FV3 environmental DNA increases more than eight-fold from May through July (Julian et al. 2019). Furthermore, we observed FV3-induced mortality events in 2015 and 2017 at several amphibian breeding ponds within 5 km of wetlands (Lake Perez and JAS 031) where we did not detect the pathogen.

Because amphibians in constructed wetlands were as likely to harbor *Bd* and FV3 as those in natural wetlands, resource managers should be aware that wetlands can be colonized by amphibian pathogens within a decade or two after construction (based on the ages of our constructed wetlands) and provide connecting habitats that could introduce pathogens into new landscapes and communities. We cannot rule out the possibility that created (or restored) wetlands can be colonized by pathogens sooner, even during construction stages before amphibian communities become established. Biosecurity protocols are a vital component of amphibian field studies (Greene et al. 2010; Gray et al. 2017), yet these protocols rarely address the decontamination of field vehicles and heavy equipment that are used in wetland restoration, maintenance and creation. To prevent human-assisted transmission of amphibian pathogens between wetlands, restoration/construction professionals might consider implementing vehicle clean-down procedures that are currently used to prevent the transmission of livestock pathogens (U.S. Dept. of Agriculture 2015), aquatic invasives (Gunderson and Kinnunen. 2010. Aquatic invasive species - hazard analysis and critical control point training curriculum. Available from http://www.habitat.noaa.gov/pdf/best_management_practices/Cleaning%20of%20Watercraft%20and%20Equipment.pdf [Accessed 11 November 2018]), and invasive seeds and plant material (DiVittorio et al. 2010) in addition to protocols for decontaminating personal wear and handheld equipment such as those listed by Partners in Amphibian and Reptile Conservation (parcplace.org/resources/herpetofaunal-disease-resources/) and The National Bsal Taskforce (salamanderfungus.org/resources/disinfection-procedures/).

Infection intensities of *Bd* were higher in constructed wetlands than reference wetlands, although infections were equally prevalent between these two wetland types. When we accounted for wetland type to explain *Bd* prevalence and intensity, the fit of regression models significantly worsened if we considered the additive influences of wetland size, stream connections, forest cover, and anthropogenic disturbances. Models that included an interaction term between forest cover and disturbance, however, fit our data as well as wetland type models. These results suggest that studies examining the influence of forest canopy cover on infection parameters should explicitly test whether this relationship is fundamentally different between wetlands with significant anthropogenic disturbances, compared to wetlands in landscapes with few disturbances. Studies that report a positive relationship between forested land/canopy cover and *Bd* infections propose that forested conditions help maintain cooler water temperatures that are conducive to *Bd* proliferation (Raffel et al. 2010; Becker and Zamudio 2011; Becker et al. 2012; Beyer et al. 2015; Scheele et al. 2015); however, these studies either fail to quantify anthropogenic riparian stressors, fail to examine vegetation cover at scales $\geq$ 30 m, or they do not model the interaction between riparian disturbances and forested land/canopy cover. Pauza et al. (2010) is a notable exception, and they found forested conditions increase the likelihood of *Bd* occurrence for wetlands in altered landscapes and those with gravel roads within 100 m, while forested conditions did not increase the likelihood of *Bd* in undisturbed areas.

The current literature on *Bd* infections and canopy cover could lead managers to the conclusion that thinning the forest canopy would reduce disease in amphibian populations. Not only could this fail to produce its intended effect, but it is in conflict with the vast body of literature that demonstrates the value of forested landscapes as a means to conserve amphibian communities (Semlitsch 2000; Skelly 2001; Semlitsch 2002; Gibbons 2003) and enhance amphibian diversity (Findlay and Houlahan 1997; Hecnar and M'Closky 1998; Knutson et al. 1999; Rubbo and Kiesecker 2005). Furthermore, the role of leaf litter composition could play a role in *Bd* proliferation, especially in the case of constructed wetland design. Stoler et al. (2016) demonstrated that leaf litter leachate from Red Maple (*Acer rubrum*), Sugar Maple (*Acer saccharum*), and White Oak (*Quercus alba*) can reduce densities of *Bd* zoospores and sporangia while species with the least inhibitory influence of *Bd* include commonly planted trees in constructed wetlands such as Green Ash (*Fraxinus pennsylvanica*), Eastern Cottonwood (*Populus deltoides*), American Elm (*Ulmus Americana*), and Sassafras (*Sassafras albidum*), as well as invasive grasses like Reed's Canary Grass (*Phalaris arundinacea*) and Common Reed (*Phragmites australis*).

While more research needs to be done on the effects of anthropogenic disturbances on amphibian disease dynamics, our study is supportive of work that suggests rapid changes in ecological integrity ensue once a landscape exceeds thresholds of anthropogenic disturbance. Brooks et al. (2006) developed a scaled index of human disturbance that factors forested land cover within 1 km, within-wetland disturbances, and 100 m buffer zone disturbances. Studies have shown once wetlands exceed the midpoint value of this index of human disturbance, there is a marked decrease in the ecological integrity of amphibian (Julian et

al. 2013), aquatic invertebrate (Yetter 2013), bird (O'Connell et al. 2013), and plant (Chamberlain et al. 2013) communities. Our study suggests that even forest-dominated landscapes are susceptible to intense pathogen infections if wetland riparian zones possess disturbances from multiple categories. If resource managers are concerned that forested riparian zones will create cool environments in constructed wetlands where *Bd* can proliferate, they could experiment with planting native species to further test the hypothesis that their leaf litter may inhibit *Bd* growth. We further suggest that managers periodically assess wetland riparian zones to identify anthropogenic disturbances that could be eliminated (or at least ameliorated) because six of the seven highest mean intensities of *Bd* infection occurred in high-stress wetlands.

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James T. Julian is an Assistant Professor of Biology and Environmental Studies at Pennsylvania State (Penn State) University's Altoona College, USA. He earned his Ph.D. in Ecology at Penn State in 2009 and has studied the distribution and seasonal infection rates of chytrid fungus and *Ranavirus* spp. in wetlands of Pennsylvania. He partners with scientists at the Northeast Fishery Center of the U.S. Fish and Wildlife Service on a number of amphibian disease projects, including citizen-scientist efforts to screen wetlands for amphibian pathogens using environmental DNA. (Photographed by Shannon Julian).

Robert P. Brooks is a Professor Emeritus of Geography and Ecology, and the Founder and Director of Riparia at the Pennsylvania State University, State College, USA. He is a practicing Wetland Scientist and Wildlife Biologist certified by the Society of Wetland Scientists and The Wildlife Society, respectively. His research and outreach emphasize assessments of wetlands and streams, habitat modeling for wetland-dependent wildlife, and restoration of aquatic ecosystems. He recently co-edited a book on Mid-Atlantic Freshwater Wetlands and was elected a Fellow of the Society of Wetland Scientists in 2017. (Photographed by Denice Wardrop).

Gavin W. Glenney has been a Fish Biologist with the U.S. Fish and Wildlife Service at the Lamar Fish Health Center in Lamar, Pennsylvania, USA, for the past 13 y. He graduated with a Ph.D. in 2005 from the College of Veterinary Medicine at Mississippi State University, Starkville, USA, studying innate immunity in Channel Catfish (*Ictalurus punctatus*). He is a certified Fish Health Inspector (American Fisheries Society), and routinely conducts fish health inspections and diagnostic investigations for Federal and State Fish Hatcheries in the northeastern U.S. He primarily conducts molecular assays (PCR, qPCR, and sequencing) for pathogen detection and confirmation. (Photographed by John Sweka).

John A. Coll is the Project Leader at the Fish Health Center of the U.S. Fish and Wildlife Service (USFWS) in Lamar, Pennsylvania, USA. After earning a B.S. in Biology in 1981 from the Pennsylvania State University, College Station, USA, and completing the Fish Health Management and Disease Diagnosis Long Course of the USFWS (1989), he has served as the Regional Fish Health Biologist / Lab Director for 22 y. He has published work on intensive culture of American Shad (*Alosa sapidissima*), diseases of wild trout in Pennsylvania and Virginia, USA, bacterial and viral pathogens of Atlantic Salmon (*Salmo salar*), and virus detection methods. (Photographed by John Sweka).

Appendix

Table 1. Characteristics of sampled wetlands by the disease status of its Green Frog (*Lithobates clamitans melanota*) population. An X indicates at least one riparian disturbance in category was present.

Wetland name	FV3 prevalence	*Bd* prevalence	Mean *Bd* Intensity	Tadpoles Tested	Year(s) Sampled	Wetland Type	Ecoregion	%Forest w/ in 250m	Disturbance Category	Hydrologic Disturbance	Vegetation Disturbance	Sediment Disturbance	Stream connection
Twin Ponds	0.085	0.051	27.6	59	2013	Reference	Ridge and Valley	94.1%	Low	X			Isolated
Huntingdon	0.008	0.892	3316.3	120	2013&14	Constructed	Ridge and Valley	24.7%	Low	X			Isolated
Gwynedd	0.000	0.950	3688.5	60	2014	Constructed	Piedmont	53.0%	High	X	X		Isolated
Wood Duck	0.000	0.571	202.3	42	2013	Reference	Ridge and Valley	48.1%	High	X	X		Isolated
JAS058	0.000	0.509	2565.3	55	2014	Reference	Ridge and Valley	90.8%	High	X	X		Connected
PSU Altoona	0.000	0.457	787.3	105	2013&14	Constructed	Ridge and Valley	4.8%	High	X	X		Isolated
Titusville	0.000	0.267	401.2	30	2014	Constructed	Glaciated Allegheny	9.5%	Low		X		Isolated
JAS031	0.000	0.250	377.4	4	2013	Reference	Ridge and Valley	60.5%	Low				Connected
Lockhaven	0.000	0.167	173.4	60	2013	Reference	Allegheny Plateau	33.8%	Low	X			Isolated
CPA Lumber	0.000	0.150	241.6	60	2013	Reference	Allegheny Plateau	77.9%	High	X	X		Connected
Polk	0.000	0.118	2246.4	34	2013	Constructed	Glaciated Allegheny	56.6%	High	X	X	X	Isolated
Perez	0.000	0.117	204.7	60	2013	Reference	Ridge and Valley	58.5%	Low	X			Isolated
Mowry AWC	0.000	0.108	3120.1	120	2013&14	Constructed	Ridge and Valley	40.2%	High	X	X		Isolated
Pecks Pond	0.000	0.100	580.1	40	2013	Reference	Glaciated Poconos	42.6%	Low	X			Connected
SGL 294 MF	0.000	0.100	632.1	60	2013	Reference	Glaciated Allegheny	76.7%	Low	X			Connected
Decker Pond	0.000	0.077	44.6	13	2013	Reference	Glaciated Poconos	62.1%	Low	X			Connected
Donut Hole	0.000	0.067	331.4	60	2013	Reference	Allegheny Plateau	87.9%	Low				Isolated
Env. Ed. Cntr.	0.000	0.050	760.5	60	2014	Constructed	Allegheny Plateau	50.5%	High	X	X		Connected
Pleasant Valley	0.000	0.050	215.9	60	2013	Constructed	Allegheny Plateau	30.8%	Low	X			Connected
PCH 4	0.000	0.050	357.7	60	2013	Reference	Piedmont	42.8%	High	X	X		Connected
Winegard	0.000	0.026	4.9	39	2013	Constructed	Glaciated Allegheny	40.6%	High	X	X		Isolated
Louie Beach	0.000	0.017	594.7	60	2013	Constructed	Allegheny Plateau	25.0%	Low	X			Isolated
Boswell	0.000	0.000	0.0	117	2013&14	Constructed	Allegheny Plateau	53.4%	High	X	X		Connected
Willisbrook	0.000	0.000	0.0	60	2013	Constructed	Piedmont	58.1%	High	X	X	X	Isolated
NWI50546	0.000	0.000	0.0	60	2014	Reference	Allegheny Plateau	81.9%	Low				Connected