Patterns of amphibian infection prevalence across wetlands on the Savannah River Site, South Carolina, USA

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ABSTRACT: Amphibian diseases, such as chytridiomycosis caused by *Batrachochytrium dendrobatidis* (*Bd*) and ranaviral disease caused by ranaviruses, are often linked to global amphibian population declines, yet the ecological dynamics of both pathogens are poorly understood. The goal of our study was to determine the baseline prevalence, pathogen loads, and co-infection rate of *Bd* and ranavirus across the Savannah River Site (SRS) in South Carolina, USA, a region with rich amphibian diversity and a history of amphibian-based research. We tested over 1000 individuals, encompassing 21 amphibian species from 11 wetlands for both *Bd* and ranavirus. The prevalence of *Bd* across individuals was 9.7%. Using wetland means, the mean (±SE) *Bd* prevalence was 7.9 ± 2.9%. Among toad species, *Anaxyrus terrestris* had 95 and 380% greater odds of being infected with *Bd* than *Scaphiopus holbrookii* and *Gastrophryne carolinensis*, respectively. Odds of *Bd* infection in adult *A. terrestris* and *Lithobates sphenocephalus* were 75 to 77% greater in metal-contaminated sites. The prevalence of ranavirus infections across all individuals was 37.4%. Mean wetland ranavirus prevalence was 29.8 ± 8.8% and was higher in post-metamorphic individuals than in aquatic larvae. *Ambystoma tigrinum* had 83 to 85% higher odds of ranavirus infection than *A. opacum* and *A. talpoideum*. We detected a 4.8% co-infection rate, with individuals positive for ranavirus having a 5% higher occurrence of *Bd*. In adult *Anaxyrus terrestris*, odds of *Bd* infection were 13% higher in ranavirus-positive animals and odds of co-infection were 23% higher in contaminated wetlands. Overall, we found the pathogen prevalence varied by wetland, species, and life stage.

KEY WORDS: *Batrachochytrium* · Chytrid · Metals · Ranavirus · Wetland

INTRODUCTION

At least 40% of all amphibian populations are experiencing declines or extinctions caused by both anthropogenic and natural stressors (Alford & Richards 1999, Voyles et al. 2009, Blaustein et al. 2011 and references therein). How these stressors interact and influence population declines is not well understood, although infectious disease is frequently associated with many of the documented amphibian declines. Our understanding of amphibian disease dynamics and amphibian population declines can be improved by investigating populations with known disease prevalence and varying degrees of disease sensitivity. Here, we investigated infection prevalence and co-infection rate of 2 amphibian pathogens, *Batrachochytrium dendrobatidis* (*Bd*; a fungus that can cause chytridiomycosis) and ranavirus, in

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the southeastern USA, where no mass mortality events have been documented (Daszak et al. 2005) but where both pathogens have been observed (Peterson et al. 2007, Hoverman et al. 2012a). Investigating 'cold spots' or regions where either the pathogen is absent or where it is present but not having negative impacts can provide important insights into disease dynamics (James et al. 2015).

The *Bd* fungus has been detected in at least 56 countries (Olson et al. 2013) and has been blamed for dramatic population declines worldwide (Berger et al. 1998, Daszak et al. 1999, Lips 1999). Additionally, ranavirus infections have been implicated in mass mortality events of amphibians across North America (Green et al. 2002), South America (Fox et al. 2006), Europe (Cunningham et al. 2007, Balseiro et al. 2009), Australia (Speare & Smith 1992, Laurance et al. 1996), and Asia (Une et al. 2009, Xu et al. 2010). These pathogens differ in modes of transmission and the disease symptoms they cause. The *Bd* fungus causes an amphibian-specific disease that is transmitted through physical contact or aquatic transmission. Ranavirus is found in amphibians, reptiles, and fish (Gray et al. 2009), and can be transmitted through ingestion or physical contact, as well as by mechanical, vertical, or aquatic transmission (Miller et al. 2011). For both *Bd* and ranaviruses, symptoms and transmissibility are correlated with higher loads of zoospores (Voyles et al. 2009, 2011) or virus (Brunner et al. 2005), respectively. Co-infection of these 2 pathogens is rarely investigated, although studies of other pathogens demonstrate that infection with one pathogen can alter susceptibility to another (e.g. Jolles et al. 2008, Munson et al. 2008).

The US Department of Energy's Savannah River Site (SRS; South Carolina, USA) offers an ideal opportunity to investigate amphibian disease dynamics across a variety of wetland types with diverse amphibian communities (Gibbons & Semlitsch 1991). The SRS has over 300 natural ephemeral wetlands, several constructed wetlands, and encompasses a broad range of habitat types typical across the southeastern USA. *Bd* has been present on the SRS at low frequency since at least the late 1970s, although it has not been linked to population declines (Daszak et al. 2005) and is known to be widespread in the southeastern USA. (Rothermel et al. 2008). Until now, ranaviruses have not been studied on the SRS, but they do occur in the southeastern USA, and have led to short-term population declines in North Carolina (Petranka et al. 2003). Furthermore, they are reported to be widespread in Tennessee (Hoverman et al. 2012a). The objectives of our study were to (1)

quantify a baseline prevalence of *Bd* and ranavirus across multiple species and wetlands of the SRS, (2) examine patterns of disease prevalence across life stages, (3) determine if disease prevalence differs between contaminated and reference wetlands, and (4) determine if *Bd* zoospore or ranavirus loads of infected individuals differ between contaminated and reference wetlands.

MATERIALS AND METHODS

Sample collection

From 25 February 2012 to 12 July 2013, we opportunistically sampled larval and adult amphibians from 11 wetlands in Aiken and Barnwell Counties on the US Department of Energy's SRS in South Carolina (Fig. 1). We typically defined a population of amphibians by the wetland boundary; however, multiple ponds at one site (wetland complex A-01, see below) were considered a single population due to close proximity (<300 m separation) and the likelihood of inter-pond movement. Eight of the sampled wetlands have no known history of contamination, including Castor Bay, Craig's Pond, Ellenton Bay, Fire Pond, Flamingo Bay, Risher Pond, Rainbow Bay, and Twin Bay (see Table 1 for geographic coordinates). Risher Pond and Fire Pond are historic farm ponds (permanent) that pre-date establishment of the SRS. The other wetlands are natural, isolated wetlands with a wide range of variation in hydroperiod, holding water from 4 to 5 mo yr^{-1} on average (Rainbow Bay) to nearly continuously holding water (Castor Bay). Although all wetlands contain some level of metals due to atmospheric deposition (mercury: Hg) or from background levels in the soil, we will refer to these 8 wetlands as our reference sites. Three wetlands—the A-01 wetland complex (Knox et al. 2006), the H-02 treatment system (Flynn et al. 2015), and Tim's Branch beaver ponds (Punshon et al. 2003)—are permanent ponds with known histories of metal contamination. Tim's Branch is a stream zone that historically received contaminated effluent from uranium (U) processing facilities on the SRS for several decades and has sediments with very high levels of U and nickel (Ni), as well as elevated levels of aluminum, chromium, iron, copper (Cu), zinc (Zn), Hg, and lead (Pb) (Knox et al. 2006). In 2010, Ni, U, and Hg levels in water samples from Tim's Branch ranged from 1.4×10^{-7} to 2.16×10^{-6}, 1.58×10^{-7} to 2.4×10^{-6}, and 7.35×10^{-8} (only 1 value provided for Hg) mg l^{-1}, respectively (Edwards et al. 2014). The H-02
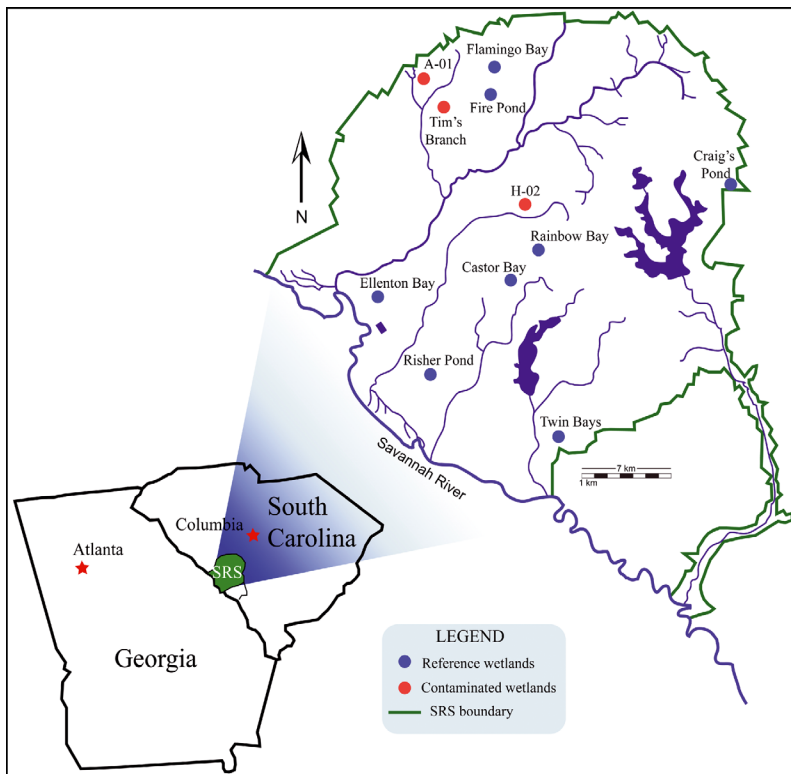
Fig. 1. Wetlands on the Savannah River Site (SRS) in Aiken and Barnwell counties (South Carolina, USA) sampled for chytrid and ranavirus prevalence and load during 2012 and 2013; 1004 individuals of 21 species were sampled at 3 metal-contaminated (red circle) and 8 reference (blue circle) wetlands

To sample amphibians for pathogen testing, we used a combination of minnow traps, active dip netting, and pitfall traps along drift fences. When more than 1 individual was caught in a single trap we only sampled 1 individual from each trap to reduce potential pathogen cross-contamination. We did not sanitize the dip net between dips or after an individual was caught due to the limited time an individual remained in the net and because each individual was primarily in contact with only the leaf litter found in the net. For tadpoles and salamander larvae, we collected a small (~4 mm long) tail clip and for adults we collected toe clips along with a swab. We used tail clips for ranaviral testing and swabs and tail clips for *Bd* testing. We did not directly sample the mouthparts of tadpoles. We stored all tissue samples in 100% ethanol and froze swabs at −20°C. All handling and collections followed the guidelines outlined in Pessier & Mendelson (2009). Our study was conducted in accordance with the 'Guide for the Care and Use of Laboratory Animals' of the National Institutes of Health, and the protocol was approved by the University of Georgia Institutional Animal Care and Use Committee (AUP A2009 10-175-Y2-A0).

wetland complex was constructed in 2006/2007 to treat storm water discharge from several industrial facilities, including a tritium treatment facility, and it has elevated levels of metals including Cu and Zn (Flynn et al. 2015). From previous research in the H-02 wetlands conducted in 2010/2011, we had tissue samples from 21 amphibians available to include in this study. In the H-02 wetlands, Cu levels averaged 28.51 ± 12.75 (SD) µg l^{-1} in the influent ends and 12.00 ± 4.56 µg l^{-1} in the effluent ends, while Zn levels averaged 29.26 ± 21.58 and 9.16 ± 2.85 µg l^{-1} in the influent and effluent ends, respectively (Flynn et al. 2015). The A-01 wetland complex, constructed in 2000, is a treatment system consisting of a retention basin and 8 cells used to treat industrial runoff, and is elevated in Cu, Zn, and Pb concentrations (Knox et al. 2006). From April 2004 to February 2005, mean (±SD) levels of Cu, Pb and Zn in the influent water were 25.8 ± 11.8, 1.2 ± 1.0, and 18.4 ± 7.5 µg l^{-1}, respectively. Despite these contaminated sites having different 'cocktails' of chemical pollutants, we considered them together as contaminated for the purpose of our study; we note, however, that there is no replication of contamination conditions.

Molecular methods

We extracted DNA from tissue samples using DNeasy Blood and Tissue kit (Qiagen) and followed the standard extraction protocol, with the exception of eluting the DNA in 100 µl of the included elution buffer. All real-time PCR (qPCR) assays were performed on an iCycler IQ PCR detection System (Bio-Rad).

To detect *Bd*, we performed qPCR reactions in 13 µl containing 1× Taqman Universal Master Mix (Life Technologies), 1× of Taqman primer/probe, which yields 0.25 µM of probe and 0.9 µM of each primer and 3.0 µl of DNA template. The primers and probe were as described by Boyle et al. (2004). We reduced the reaction volumes of Boyle et al. (2004) by 50% based on the success of the low-volume qPCR reported by Kerby et al. (2013). We ran each sample

in triplicate. To quantify zoospore loads, we established standard curves for each plate by including 2 replicates each of 100, 10, 1, and 0.1 genome equivalents of *Bd* (isolate JEL404, Maine, USA). The amplification conditions were as follows: 2 min at 50°C, 10 min at 95°C, 50 cycles of 15 s at 95°C, and 1 min at 60°C. To quantify zoospore loads, we considered the 0.1 genome equivalent standard to represent the threshold of detection. Thus, for a sample to be considered positive it had to amplify with a cycle threshold (Ct) lower than the Ct for the 0.1 standard in at least 2 of 3 replicates. When samples were screened from both swabs and tail clips, the results were always consistent. As a result of variation in internal transcribed spacer (ITS) copy number, qPCR estimates of zoospore load may be incorrect when ITS copy number is not known in the *Bd* standards or sampled strains of *Bd* (Longo et al. 2013). Our standards and local strains are uncharacterized, and thus our numbers may be incorrect and should be considered as relative loads.

To detect frog virus 3 (FV3)-like ranaviruses, we performed qPCR reactions in 20.0 µl containing 1× IQ Syber Green supermix (Bio-Rad), 0.4 µM of MCP forward and reverse primer (Forson & Storfer 2006) and 2 µl of DNA template. To establish a standard curve for absolute quantification, we used a synthetic 250-bp oligo that represents a fragment of the MCP gene and includes the priming sites. We serially diluted the oligo from 3.82×10^4 to 3.82 copies µl^{-1} and included 2 replicates of each dilution in every run. We also included negative controls in every plate. The amplification conditions were as follows: 2 min at 95°C, 40 cycles of 20 s at 95°C, 20 s at 59°C and 1 min at 95°C, 1 min at 55°C, 80 cycles of 10 s at 5°C. All samples were run in triplicate. To quantify viral loads, we considered the 3.82 standard to represent the threshold of detection. Thus, for a sample to be considered positive it had to amplify with a lower Ct than that standard in at least 2 of 3 replicates.

Statistical analyses

We conducted our sampling efforts across wetlands (n = 11), species (n = 21), wetland type (metal-contaminated vs. reference), and life stage (aquatic/larval or post-metamorphic/terrestrial). The opportunistic nature of our sampling for larvae and adults present at different times throughout the year, combined with the absence of some species and/or life stages from some wetlands, resulted in an unbalanced design. Therefore, we used logistic regression

models (PROC LOGISTIC; SAS 2011) to analyze *Bd* and ranavirus presence/absence in relation to single factors and species or species groups. Specifically, we used reduced models to test the effect of wetland type on disease prevalence in (1) adult southern toads *Anaxyrus terrestris* and southern leopard frogs *Lithobates sphenocephalus* and (2) larval *L. sphenocephalus*, as these were the only species with sufficient adult and/or larval captures at both wetland types. We also tested whether pathogen patterns differed among the 3 species of adult ambystomatid salamanders from the same 3 reference wetlands (Flamingo Bay, Ellenton Bay, and Rainbow Bay), as well as among adult toads from 4 reference wetlands (Craig's Pond, Flamingo Bay, Ellenton Bay, and Rainbow Bay) that represent 3 families: *A. terrestris* (Bufonidae), *Gastrophryne carolinensis* (Microhylidae), and *Scaphiopus holbrookii* (Scaphiopodidae). These models were of the form DISEASE (0 for negative test, 1 for positive test) = TREATMENT, where wetland type, stage, or species were individually substituted for TREATMENT. When pairwise differences were significant, we summarized those using the logistic regression odds ratio estimates to compare prevalence between groups.

We also used a logistic regression model to test for an effect of wetland type on co-infection rates for individuals that tested positive for *Bd*, ranavirus, or both. To test for an association between testing positive for ranavirus and the likelihood of also being *Bd* positive, we used the Pearson chi-squared test in a contingency table analysis (PROC FREQ; SAS 2011). We calculated 95% Clopper-Pearson binomial confidence intervals for percent prevalence of both *Bd* and ranavirus in each species, wetland, and wetland type. For animals that tested positive for either disease, we then tested whether zoospore numbers (*Bd*) and viral loads (ranavirus) differed between wetland types (reference vs. contaminated) and locations (= wetland) using a general linear model with location nested within wetland type as a random effect (PROC GLM; SAS 2011), which was used as the error term to test for a wetland type effect. Zoospore and viral loads were log$_{10}$ transformed to meet normality assumptions for the model residuals, which we tested with the Kolmogorov-Smirnov test (p > 0.05). In general, for each analysis, we first tested all species pooled data, and then analyzed a subset of the data for species that had sufficient sample size at the same life stage across wetland types; typically, these analyses were for either *A. terrestris* or *L. sphenocephalus*. For adult *A. terrestris*, which had the most widely distributed captures across all wetlands, we used a 6 yr

record of the hydroperiod of the wetlands to test for any correlation between pathogen loads and hydroperiod using Spearman's rank correlations.

RESULTS

Overall patterns of pathogen prevalence

In total, we sampled 1004 individuals representing 11 wetlands (Table 1) and 21 species (Table 2); 190 samples were from aquatic larvae and 814 from post-metamorphic animals. We found positive *Bd* samples in 9 species and 97 of 1004 (9.7%) individuals. Within a species, prevalence ranged from 0 to 48% for *Bd*. *Pseudacris ornata* had the highest *Bd* prevalence (adults, 46.4%), followed by *Lithobates catesbeianus* (primarily larvae, 27.4%) and *L. sphenocephalus* (primarily adults, 24.4%). These 3 species, along with *Anaxyrus terrestris*, represent 88% of all positive samples. Three salamander species tested *Bd*-positive, including *Ambystoma tigrinum*, *A. opacum*, and *A. talpoideum*, although all at low levels (Table 2); the ambystomatids did not differ from each other in *Bd* occurrence ($\chi^2 = 1.2$, df = 2, p = 0.5491). Animals from 7 wetlands were *Bd*-positive, and *Bd* prevalence of infected individuals within a wetland ranged from 0 to 27.3% (mean 7.9 ± 2.9%). Five wetlands accounted for 91% of all *Bd*-positive samples in *Ambystoma* spp. Comparison of adults from the 3 toad species/families showed a significant species effect on *Bd* occurrence ($\chi^2 = 7.0$, df = 2, p = 0.0301), with *Anaxyrus terrestris* having 95 and 380% greater likelihood of *Bd* infection than *Scaphiopus holbrookii* and *Gastrophryne carolinensis*, respectively. No individuals had overt clinical signs of chytridiomycosis.

Of the 988 tissue samples analyzed for ranavirus infection, 37.4% tested ranavirus-positive, including individuals of 15 species. Within a species, prevalence ranged from 0 to 100% for ranavirus, and when only considering species with more than 2 individuals sampled, *P. ornata* again had the highest prevalence of ranavirus infections (adults, 100%), followed by *Ambystoma tigrinum* (adults, 85.7%), *A. talpoideum* (adults, 52.5%), and *A. opacum* (adults, 45.9%) (Table 2). The ambystomatids differed from each other in ranavirus occurrence ($\chi^2 = 14.4$, df = 2, p = 0.0002), with *A. tigrinum* having 83 to 85% higher likelihood of ranavirus infection than *A. opacum* and *A. talpoideum*. For ranavirus, *Anaxyrus terrestris* accounted for the highest proportion (adults, 21%) of all positive samples attributed to one species; ambystomatids accounted for 30%, and *L. sphenocephalus* for 15% (larvae and adults). Comparison of adults from the 3 toad species/families showed a significant species effect on ranavirus occurrence ($\chi^2 = 22.4$, df = 2, p < 0.0001), with *S. holbrookii* and *A. terrestris* having 3.1 and 4.9 times greater likelihood of ranavirus infection than *G. carolinensis*, respectively. Toads from 10 wetlands were positive for ranavirus and

Table 1. Wetlands included in the analyses, including number of sampling events in 2012 and 2013, total number analyzed (N), number that tested positive for *Bd* (N_{Bd}), number that tested positive for ranavirus (N_{RV}), number that were co-infected (N_{Co}), and the geographic coordinates for each wetland. CI%: lower and upper Clopper-Pearson binomial confidence limits for percent prevalence. UTM_E, UTM_N: geographic coordinates for wetlands, in Universal Transverse Mercator (zone 175)

Wetland	2012	2013	N	N_{Bd}	CI%	N_{RV}	CI%	N_{Co}	UTM_E	UTM_N
Reference sites										
Castor Bay	8	0	83	0	0	10	5.0–19.1	0	438966.0	3677435.0
Craig's Pond	0	5	74	14	10.0–27.8	74	100	14	455352.7	3683153.0
Ellenton Bay	9	8	146	12	3.8–12.6	88	52.3–68.2	8	430374.8	3675926.0
Fire Pond	3	0	29	0	0	1	0–10.0	0	437515.3	3685909.0
Flamingo Bay	26	6	194	9	1.7–7.6	68	28.3–41.8	2	436822.2	3688669.0
Risher Pond	2	0	63	11	8.1–26.8	17	16.0–37.9	6	432022.0	3673653.0
Rainbow Bay	11	2	137	0	0	31	15.6–29.6	0	441229.8	3680374.6
Twin Bay	1	0	13	0	0	0	0	0	442661.1	3666341.0
Total			739	46	4.5–7.9	289	35.6–42.6	30		
Contaminated sites										
A-01 wetlands	5	2	87	9	3.9–16.7	24	18.2–36.9	3	432651.5	3689320.2
H-02 wetlands	12	5	154	42	20.2–34.3	55	28.2–43.2	15	439769.2	3683491.2
Tim's Branch	2	0	24 (8)[a]	0	0	1	0–35.4	0	432991.7	3688264.1
Total			265 (249)[a]	51	14.5–23.9	80	26.3–37.9	18		
Grand total			**1004 (988)[a]**	**97**		**369**		**48**		

[a]Number in parentheses represents the number sampled for ranavirus caused by a lack of DNA after testing for *Bd*

Table 2. Samples included in the analyses, with the number of wetlands each species was sampled from (N_w), the total number analyzed (N), the number that tested positive for *Bd* (N_{Bd}), the number that tested positive for ranavirus (N_{RV}), and the number that were co-infected (N_{Co}). CI%: lower and upper Clopper-Pearson binomial confidence limits for percent prevalence

Species	N_w	N	N_{Bd}	CI%	N_{RV}	CI%	N_{Co}
Salamanders							
Ambystoma talpoideum	5	80	4	0.2–9.7	42	41.6–63.4	1
A. opacum	3	61	1	0–4.8	28	33.4–58.4	0
A. tigrinum	3	49	1	0–6.0	42	75.9–95.5	0
Notophthalmus viridescens	1	1	0	0	1	100	0
Plethodon chlorobryonis	2	3	0	0	2	13.3–100	0
Pseudotriton ruber	1	1	0	0	0	0	0
Eurycea quadridigitata	2	2	0	0	0	0	0
Total	5	197	6	3.0	115		1
Anurans							
Scaphiopus holbrookii	6	95	0	0	32	24.2–43.2	0
Anaxyrus terrestris	9	218	13	2.8–9.1	77	28.9–41.6	11
Gastrophryne carolinensis	6	99	2	0–4.8	14	7.3–21.0	1
Acris gryllus	1	1	0	0	0	0	0
Pseudacris crucifer	5	39	0	0	4	0–19.7	0
P. ornata	4	28	13	27.9–64.9	28	100	13
Hyla cinerea	2	23	0	0	8	15.3–54.3	0
H. gratiosa	2	2	0	0	0	0	0
H. femoralis	1	1	0	0	0	0	0
H. squirella	1	1	0	0	0	0	0
H. chrysoscelis	1	2	0	0	2	100	0
Lithobates catesbeianus	5	73	20	17.2–37.6	21	18.4–39.1	7
L. clamitans	4	57 (41)[a]	3	0–11.1	14	19.6–48.6	1
L. sphenocephalus	10	168	41	17.4–30.2	54	25.1–39.2	14
Total	11	807 (791)[a]	92		254		47
Grand total	**12**	**1004 (988)[a]**	**98**		**369**		**48**

[a]Number in parentheses represents the number sampled for ranavirus caused by a lack of DNA after testing for *Bd*

prevalence within a wetland ranged from 0 to 100% (mean 29.8 ± 8.8%). As with *Bd*, 5 wetlands accounted for a vast majority of all ranavirus-positive samples. Interestingly, 4 wetlands (H-02, Craig's Pond, Ellenton Bay, and Flamingo Bay) accounted for the majority of positive samples for both *Bd* and ranavirus (Table 1). We observed no mortalities or overt symptoms of ranaviral disease in any individuals.

Co-infection

For the 988 individuals of all species that were tested for both pathogens (Table 2), 321 (32.5%) tested positive solely for ranavirus, 49 (5.0%) only for *Bd*, and 48 (4.9%) were co-infected with both pathogens. Individuals that were ranavirus-positive had a 5% higher *Bd*-infection frequency ($\chi^2 = 6.2$, df = 1, p = 0.0127) than individuals that were ranavirus-negative. *P. ornata* had very high co-infection rates (adults, 46.4%), driven by the 100% prevalence of ranavirus in this species. We analyzed co-infection associations separately for adults of *A. terrestris*, *L. catesbeianus*, and *L. sphenocephalus*, the only 3 spe-

cies in our sample that occurred at 3 or more wetlands and which had >2 co-infected individuals at the same life stage. For adult *A. terrestris*, we observed a 13% higher *Bd* infection rate in individuals that were ranavirus-positive ($\chi^2 = 11.1$, df = 1, p = 0.0009), but no difference in adult *L. sphenocephalus* ($\chi^2 = 0.6$, df = 1, p = 0.4506) or *L. catesbeianus* ($\chi^2 = 0.01$, df = 1, p = 0.8960), or in larval *L. catesbeianus* ($\chi^2 = 1.4$, df = 1, p = 0.2286).

Life stage

Pooled across all individuals, wetlands, and species, *Bd* presence/absence did not differ between larval and post-metamorphic life stages ($\chi^2 = 2.7$, df = 2, p = 0.0994). For the 2 species for which we had sufficient larval and adult captures at multiple locations (*L. sphenocephalus* and *L. catesbeianus*), the odds of *Bd* infection were 45 times greater in adult *L. sphenocephalus* than in larvae ($\chi^2 = 13.7$, df = 1, p = 0.0002), and 4.6 times greater in adult *L. catesbeianus* ($\chi^2 = 6.7$, df = 1, p = 0.0098). Life stage was also strongly associated with ranavirus occurrence

($\chi^2 = 20.8$, df = 1, p < 0.0001), with the odds of infection in post-metamorphic animals 2.5 times more likely than in larvae; this pattern held when limited to analysis of *L. sphenocephalus* ($\chi^2 = 6.2$, df = 1, p = 0.0127), with the odds of ranavirus in adults 2.5 times greater than in larvae (Fig. 2), but did not hold for *L. catesbeianus* ($\chi^2 = 0.3$, df = 1, p = 0.5872).

Contaminated and reference wetlands

We sampled and *Bd*-screened 739 amphibians from historically reference wetlands (e.g. Hopkins et al. 1997, Metts et al. 2012) and 265 from documented contaminated wetlands (Lance et al. 2013). Lower proportions of individuals at reference wetlands were *Bd*-positive than at contaminated wetlands (6.2 vs. 19.3%, respectively; $\chi^2 = 33.5$, df = 1, p < 0.0001). The odds ratio of an individual testing *Bd*-positive in reference versus contaminated wetlands was 0.285 (95% CI: 0.186 to 0.436), indicating that the odds of *Bd* infection on average were 71% greater in contaminated wetlands.

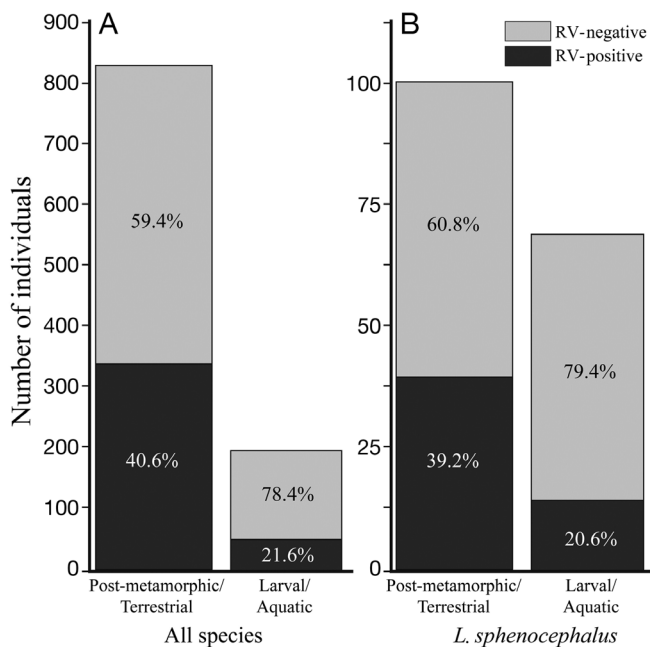Only 2 species, *A. terrestris* and *L. sphenocepha-*

Fig. 2. Differences in ranavirus (RV) prevalence between amphibian life stages (aquatic larval vs. terrestrial juvenile or adult) for 21 amphibian species sampled on the Savannah River Site (SRS) during 2012 and 2013. Data are pooled across species, years, seasons, wetlands, and wetland types for (A) all species, and (B) presented separately for *Lithobates sphenocephalus*, the single species with sufficient aquatic and terrestrial captures across multiple wetlands (3 larval wetlands, 8 juvenile/adult)

lus, had sufficient captures of adults at both wetland types to enable a comparison of *Bd* prevalence at the same life stage. Both species showed a higher *Bd* prevalence in adults at contaminated sites (*A. terrestris*: $\chi^2 = 5.0$, df = 1, p = 0.0249; *L. sphenocephalus*: $\chi^2 = 9.9$, df = 1, p = 0.0016), with odds of infection 75 to 77% greater in contaminated sites. Only *A. terrestris* occurred at the same stage (adult) in all 4 permanent ponds—2 reference (Fire and Risher) and 2 contaminated (A-01 and H-02)—and the odds of *Bd* infection in the 2 contaminated wetlands was 94% higher (nearing statistical significance; $\chi^2 = 3.4$, df = 1, p = 0.0674) than in the 2 permanent reference ponds. Only 1 species, *L. sphenocephalus*, had sufficient captures of larvae in both wetland types, and *Bd* prevalence did not differ between wetland types ($\chi^2 = 0.6$, df = 1, p = 0.4357).

We analyzed 988 tissue samples for ranavirus; 739 amphibians from reference wetlands and 249 from contaminated wetlands. Slightly lower proportions of individuals at contaminated wetlands were ranavirus-positive than at reference wetlands (32.1 vs. 39.1%, respectively; $\chi^2 = 3.9$, df = 1, p = 0.0494). Again, comparing only adult *A. terrestris* and *L. sphenocephalus*, ranavirus prevalence did not differ between wetland types for either species (*A. terrestris*: $\chi^2 = 2.9$, df = 1, p = 0.0872; *L. sphenocephalus*: $\chi^2 = 0.8$, df = 1, p = 0.3663). Ranavirus prevalence also did not differ between wetland types for larval *L. sphenocephalus* ($\chi^2 = 2.4$, df = 1, p = 0.1222). The overall odds ratio of testing ranavirus-positive in reference versus contaminated wetlands was 1.357 (95% CI: 1.001 to 1.839); averaged across species and life stage, the odds of ranavirus infection were 36% higher in reference wetlands, due largely to the 100% prevalence of ranavirus in *P. ornata* at Craig's Pond and the occurrence of ambystomatids solely in reference ponds. When we limited the analysis to adult *A. terrestris* in the 4 permanent ponds, the odds of ranavirus infection in contaminated ponds was 70% higher than in reference ($\chi^2 = 4.3$, df = 1, p = 0.0374).

We compared co-infection rates between reference and contaminated wetlands in adult *A. terrestris* and *L. sphenocephalus*, and odds of co-infection were 23% higher in contaminated wetlands for *A. terrestris* adults ($\chi^2 = 4.4$, df = 1, p = 0.0361) but no different for *L. sphenocephalus*.

Zoospore and viral loads

For animals that tested *Bd*-positive, higher zoospore numbers occurred in some wetlands than in

others. That is, there was a significant wetland effect ($F_{4,92}$ = 5.27, p = 0.0007), but no effect of wetland status ($F_{1,4}$ = 0.07, p = 0.8054). A reference wetland (Craig's Pond) and a contaminated wetland (H-02) had the highest mean *Bd* zoospore counts, with 434 ± 191 and 114 ± 58 zoospores per positive individual, respectively. For ranavirus-positive animals, the viral load also differed among wetlands ($F_{8,357}$ = 12.33, p < 0.0001) but not between wetland types ($F_{1,8}$ = 0.06, p = 0.8109), with the highest ranavirus viral counts in a reference wetland (Ellenton Bay; 109 538 ± 14 581 ind.$^{-1}$) and second highest in the H-02 wetlands (76 624 ± 27 745). Zoospore and viral loads varied widely across species (Fig. 3), with the highest zoospore loads in terrestrial *P. ornata*, followed by *Ambystoma talpoideum*, *Anaxyrus terrestris*, and *L. sphenocephalus*; the highest viral loads occurred in terrestrial *L. clamitans*, followed by *A. talpoideum*, *L. sphenocephalus*, and *A. opacum*. Adult *Anaxyrus terrestris* were captured in 9 of the 11 wetlands across the full hydroperiod spectrum, but we observed no correlation between zoospore and viral counts and average wetland hydroperiod in adults of this species (Spearman's rank correlations; r < 0.02, p > 0.87). We were only able to compare zoospore and viral loads between life stages for 2 ranid species: *L. sphenocephalus* and *L. catesbeianus*. Zoospore loads did not differ between stages for either species (p > 0.3975), although viral load was marginally elevated in adult *L. sphenocephalus* compared to larvae (p = 0.0601; Fig. 3).

DISCUSSION

Overall patterns of pathogen prevalence

Across the SRS, *Bd* has been present at low frequency since at least the late 1970s (Daszak et al. 2005). Specifically, *Bd* was documented in 3 (2 *Lithobates catesbeianus* and 1 *L. sphenocephalus*) of 137 specimens collected on the SRS between 1940 and 2001 (Daszak et al. 2005), and in 64% of *L. catesbeianus* tadpoles sampled from the A-01 created wetlands in 2006 (Peterson et al. 2007). These previous studies were limited in both wetland and species scope, and no studies have examined the incidence and/or prevalence of ranavirus on the SRS. Our study examined 11 wetlands and 21 species, and combining all samples, yielded an average prevalence of 9.7 and 37.4% for *Bd* and ranavirus, respectively. In previous studies in the southeastern USA, the overall prevalence averaged 17.8% for *Bd* (Rothermel et

al. 2008) and 60% for ranavirus (Hoverman et al. 2012a), hence we found lower infection prevalence for both pathogens on the SRS.

Our study supports some, but not all, of the previous findings from studies in the southeastern USA on within- and among-species patterns of disease prevalence. Overall, the anurans in our study had a higher prevalence of *Bd* than the caudates, and appear to be important carriers (Rothermel et al. 2008). However, we found *Bd*-positive *Ambystoma tigrinum*, *A. talpoideum*, and *A. opacum*, which had not been previously reported for the region (Rothermel et al. 2008). To our knowledge, this is also the first study to document infection in wild populations of *Anaxyrus terrestris*, with adults having 95 and 380% greater likelihood of *Bd* infection than *Scaphiopus holbrookii* and *Gastrophryne carolinensis*, respectively. In laboratory exposures, *Anaxyrus terrestris* incurred some of the highest infection rates when compared to other anuran species, and it may also suffer significant *Bd*-related mortality (Searle et al. 2011). Unexpectedly, *Pseudacris ornata* had the highest prevalence for both *Bd* and ranavirus. To our knowledge, *P. ornata* has not been tested before for either pathogen. However, other *Pseudacris* species have tested *Bd*-positive (Ouellet et al. 2005, Rothermel et al. 2008) and ranavirus-positive (Hoverman et al. 2012a), but they had much lower prevalence than we found for *P. ornata*. None of the *P. crucifer* in our study tested positive for *Bd*, highlighting the potential high susceptibility of *P. ornata* and the need to further examine the pathogen dynamics within this species. *L. sphenocephalus*, *P. ornata*, and *A. terrestris* appear to be important carriers of both *Bd* and ranavirus on the SRS. Among the pond-breeding salamanders, *Ambystoma tigrinum* had much higher odds of ranavirus infection than *A. opacum* and *A. talpoideum*.

The opportunistic nature of our sampling limits the degree to which we can examine the unique contribution of each separate factor (life stage, species, wetland site, wetland type) on pathogen prevalence. Nonetheless, we observed patterns that warrant additional, more structured sampling. For example, life stage affected pathogen prevalence estimates; in general, post-metamorphic animals had increased pathogen prevalence, particularly for ranavirus, compared to aquatic larvae. Typically, studies of *Bd* and ranavirus only examine one life stage, making comparisons across life stages in the same species and locations difficult. However, in experimental studies the prevalence of *Bd* is lowered after metamorphosis, suggesting that individuals may clear the
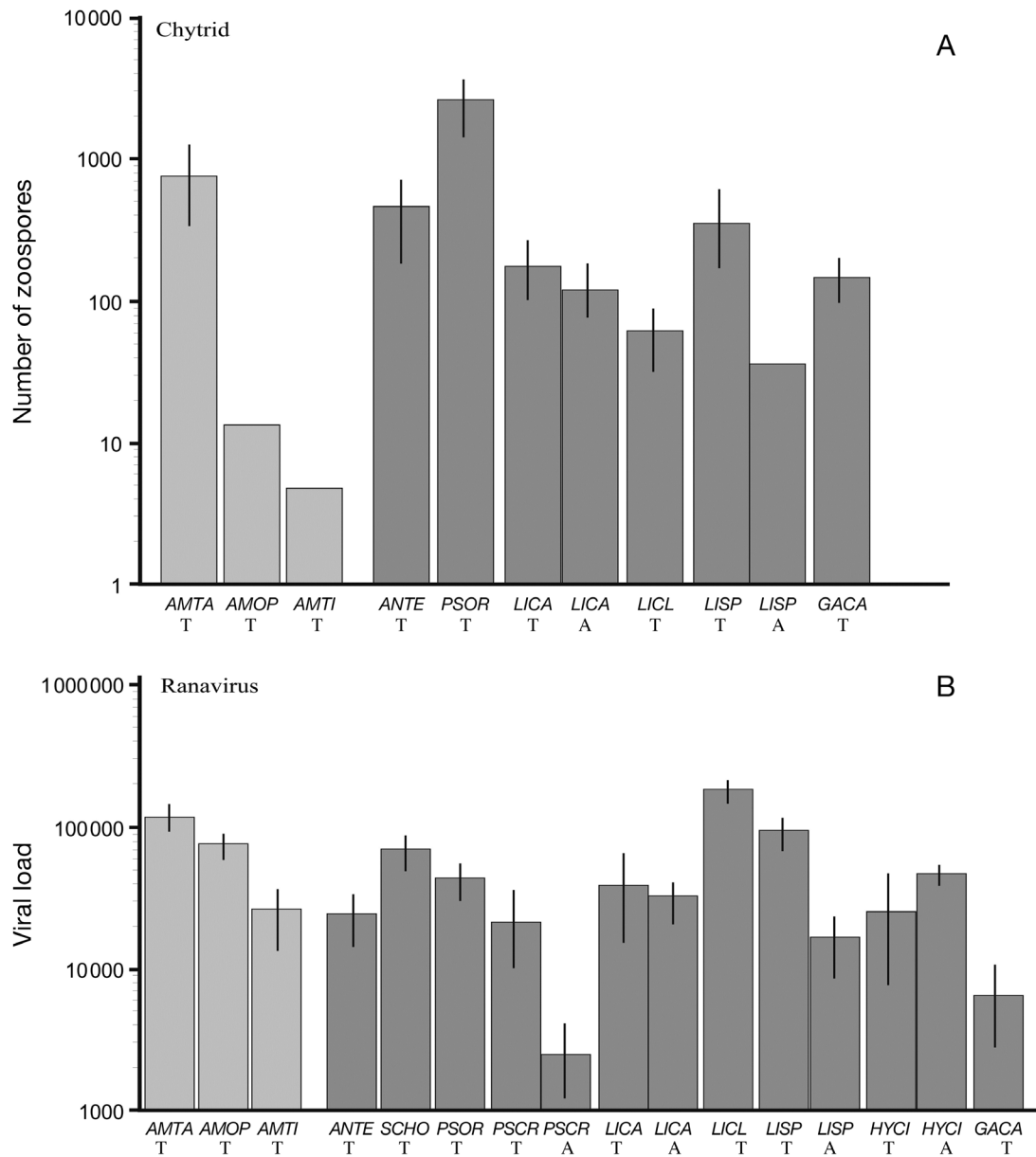
Fig. 3. Mean (±1 SE) (A) chytrid zoospore and (B) ranavirus viral load estimates for species on the Savannah River Site with >3 captures that tested positive in at least 1 capture. No error bars are shown when only 1 sample was positive. Estimates are pooled across wetlands and wetland type. Dark gray bars: anurans; light gray: salamanders. AMTA: *Ambystoma talpoideum*; AMOP: *Ambystoma opacum*; AMTI: *Ambystoma tigrinum*; ANTE: *Anaxyrus terrestris*; GACA: *Gastrophryne carolinensis*; HYCI: *Hyla cinerea*; LICA: *Lithobates catesbeianus*; LICL: *L. clamitans*; LISP: *L. sphenocephalus*; PSCR: *P. crucifer*; PSOR: *P. ornata*; SCHO: *S. holbrookii*. 'T': post-metamorphic juvenile or adult in the terrestrial stage; 'A': larvae in the aquatic stage

infection (Searle et al. 2013). In our case, because we did not directly sample the mouthparts of tadpoles, our estimates of prevalence are underestimates. Still, the prevalence of *Bd* in terrestrial *L. sphenocephalus* and *L. catesbeianus* is high enough to suggest that they are not clearing the infection at metamorphosis, but rather maintaining it without overt symptoms. This has important implications for the maintenance

of *Bd* in populations and for examining tolerance. Studies that examine *Bd* prevalence in both life stages may be better suited to explain seasonal patterns (Duncan Pullen et al. 2010) or the effects of *Bd* at the population level (Lips 1999).

Ranavirus has been shown to infect larval amphibians at greater rates than adults (Gantress et al. 2003), although our study shows the opposite trend.

Adult *L. sphenocephalus* — the species for which we had the greatest numbers and widespread distribution of both adults and larvae — were 2.5 times more likely to be infected with ranavirus than larvae. Only *Hyla cinerea* had slightly higher counts in larvae than in adults. Another ranavirus, *Ambystoma tigrinum* virus (ATV) is found in both life stages. With ATV it appears that because of the complex life cycle of the salamanders studied, adults that survived infection as larvae can act as intraspecific reservoirs that may return the virus to the aquatic system during breeding events, where it is amplified in larval populations (Brunner et al. 2004, 2007, Collins et al. 2004). With these experimental studies of ATV, the prevalence in recent metamorphs can be quite high but survival of infected individuals is low, resulting in low prevalence in adults. In our study, the prevalence of ranavirus in *L. sphenocephalus* was higher in terrestrial individuals, and a majority of those were breeding age adults. Individuals were sampled leaving the wetlands and thus we cannot determine if they entered the wetland already infected or were infected during a breeding bout. In addition, we have few adults and larvae from the same wetland with which to make direct comparisons of prevalence across stages. Future studies should address the role of adults in the transmission dynamics of FV3 to determine if it follows similar patterns as ATV. We also found high ranavirus load in post-metamorphic *L. clamitans*, unlike Forzán & Wood (2013), who found low detection of ranavirus after larval mortality. Their study suggested that *L. clamitans* could be poor reservoirs in Canada, but our study shows that both *L. sphenocephalus* and *L. clamitans* could be important intra- and inter-specific reservoirs for ranavirus persistence in populations in the southeastern USA.

Contaminated and reference wetlands

It has been hypothesized that amphibian disease susceptibility may increase due to exposure to environmental pollution and other anthropogenic, abiotic stressors (Daszak et al. 1999). Some wetlands on the SRS have a well-documented history of contamination and the SRS also encompasses numerous contaminated natural wetlands (Punshon et al. 2003, Lance et al. 2013). As our sampling scheme included contaminated and uncontaminated (reference) wetlands on the SRS, we investigated differences in disease prevalence between these 2 wetland types to further inform this area of research. A majority of

studies on the interaction of contaminants and disease have understandably focused on controlled laboratory experiments (e.g. Forson & Storfer 2006, Kerby & Storfer 2009, Buck et al. 2012), leading to a lack of information on whether amphibian pathogens are more prevalent in contaminated habitats. Our results differed for *Bd* and ranavirus when comparing contaminated and reference wetlands: *Bd* prevalence was greater in contaminated wetlands, whereas the overall prevalence of ranavirus was slightly higher in reference wetlands due to the occurrence of ambystomatid salamanders solely in the reference sites. Overall, ranavirus was found in all contaminated wetlands and all but one reference wetland, but the proportion of ranavirus-positive animals was quite high in 2 reference wetlands: Craig's Pond (100%) and Ellenton Bay (60%). The incidence of *Bd* was less consistent, with only half of the reference and all but one of the contaminated wetlands containing *Bd*-positive individuals. Individuals infected with *Bd* were more likely to be found at contaminated wetlands, but that pattern was driven by the very high proportion of *Bd*-positive samples coming from the H-02 wetlands. However, the pattern held when only examining the same life stage (adult) in *L. sphenocephalus* and *Anaxyrus terrestris*. *Bd* was not found in Tim's Branch, a natural wetland contaminated with Ni and U. The other contaminated wetlands (A-01 and H-02) are constructed treatment wetlands with high levels of Cu and Zn. It is unclear whether the increased incidence of *Bd* was related to the presence of metals in the treatment wetlands, or perhaps another factor of these constructed systems such as permanent continuous flooding. We compared pathogen prevalence in adults of a single species, *A. terrestris*, that occurred in the 2 contaminated ponds as well as our only 2 permanent reference ponds. We found a higher prevalence of ranavirus and marginally higher rates of *Bd* at the contaminated sites, suggesting a potential contaminant connection when controlling for hydroperiod. Given that immunosuppression can result from exposure to heavy metals (Koller 1980) such as Cu (Mitra et al. 2012), a higher incidence and prevalence of both pathogens in our contaminated wetlands may be expected.

Alternatively, the difference in pathogen prevalence may be connected to differences between constructed and natural wetlands other than, or in addition to, the presence of metals. Two of our 3 contaminated wetlands (all but Tim's Branch) are man-made and have a permanent hydroperiod, less natural vegetative cover, and steep banks. Artificial

wetlands are likely to have an altered amphibian species composition (Venesky et al. 2011), overall density (Rachowicz & Briggs 2007), and diversity (Searle et al. 2011) — all of which can affect pathogen dynamics. Constructed wetlands can differ from natural wetlands in species diversity (Denton & Richter 2013), and the ones we sampled are atypical wetlands for the SRS in that salamanders have not colonized them, despite being common in reference wetlands. Thus, the absence of salamanders from contaminated wetlands on the SRS could be affecting our finding of elevated ranavirus prevalence in some reference sites. Our observation of ranavirus infections in all 3 contaminated wetlands suggests that other species are also carriers for this pathogen on the SRS. The permanent hydroperiod in these constructed wetlands allows for overwintering of *L. catesbeianus* and *L. clamitans* tadpoles. These species may be important ranavirus reservoirs that maintain a transmission cycle throughout the year (Gray et al. 2009) like salamander species in reference wetlands. But again, pathogen prevalence in adult toads from the 2 permanent reference ponds, which also have overwintering *L. catesbeianus* and *L. clamitans* tadpoles, was lower than in the contaminated ponds. If our results of increased pathogen prevalence in contaminated wetlands are a consequence of constructed, permanent treatment wetlands, this has important implications for amphibian populations, as man-made wetlands are becoming an increasing proportion of the wetlands landscape. In fact, from 2004 to 2009, while the area of natural freshwater ponds continued to decline in the USA, urban and industrial pond areas grew by 18.0 and 9.9%, respectively (Dahl 2009). Additional studies are needed to disentangle the interacting effects of constructed, permanent, and/or contaminated wetlands, as well as amphibian species composition, on *Bd* and ranavirus occurrence.

When comparing pathogen loads between contaminated and reference wetlands, we found significant differences among wetlands, but no effect of wetland type. Cu is widely used as a fungicide (Newbold 1975), and in a previous study, exposure to Cu ameliorated some of the negative effects *Bd* exposure had on time to metamorphosis (Parris & Baud 2004). Thus, metals could potentially hinder the growth of *Bd*; however, our Cu-contaminated H-02 wetland had one of the highest zoospore loads. Interestingly, the wetland with the highest prevalence of ranavirus (Craig's Pond, 100%) also had the highest overall *Bd* zoospore load.

Co-infection

Few studies have reported co-infection rates of *Bd* and ranavirus in the field, despite the overlap in range of the 2 pathogens (Miller et al. 2008, Schock et al. 2010) and the potential for co-infection to affect the severity of one or both pathogens. Fox et al. (2006) and Hoverman et al. (2012b) reported co-occurrence of both pathogens within the same wetland but not within the same host. Three of the studies examining both pathogens found co-infection rates between 5 and 6% (Souza et al. 2012, Whitfield et al. 2013, Rothermel et al. 2016),which is nearly identical to our rate of 4.8%. We found only 1 salamander sample (*Ambystoma talpoideum*) to exhibit co-infection; all other co-infections occurred in our anuran samples. The predominance of co-infections in anurans may have resulted from greater numbers of anurans being sampled, and higher rates of *Bd* infection, in comparison to caudates in our study. The greater co-infection in anurans could also be a result of wetland type, as anurans were the only species that occupied the constructed and contaminated wetlands in our study. We found an overall rate of co-infection in contaminated wetlands of 15.9 compared to 9.8% in reference wetlands. In adult *Anaxyrus terrestris*, the co-infection rate was 23% higher in contaminated wetlands, suggesting a possible interplay between wetland characteristics and infection susceptibility. Whitfield et al. (2013) found no spatial patterns in *Bd* and ranavirus infection between 3 habitat types across all 20 species they sampled, yet they did not look at wetland characteristics, leaving many questions still unanswered. We also found that individuals of at least 1 species (*Anaxyrus terrestris*) testing positive for ranavirus were more likely to also test positive for *Bd.* Given the importance of these pathogens to amphibian populations it seems critical for future studies to examine them simultaneously to better understand their ecology, particularly given that interactive effects of parasite co-infection have been shown to change pathogen dynamics and host fitness in other host/parasite interactions (Budischak et al. 2012, James et al. 2015).

CONCLUSIONS

Our data highlight the differences in host species susceptibility to *Bd* and ranavirus as well as the possible interplay of wetland characteristics. Although

more detailed surveys would be needed to assess disease effects, a better understanding of the distribution and prevalence of these pathogens is a critical step toward understanding their impacts on amphibian populations. To identify what may be triggering die-offs in particular situations it would be beneficial to have large-scale monitoring efforts to collect data on background distribution, prevalence, and habitat characteristics before a die-off occurs. Although outbreaks can easily go unnoticed, no known die-offs have occurred from either *Bd* or ranavirus on the SRS. However, based on our data, it is evident that the prevalence of *Bd* has increased since the 1970s (Daszak et al. 2005), and the prevalence of ranavirus is similar to other studies in the southeastern USA (Hoverman et al. 2012a). Determining what leads to disease outbreaks will require understanding more about 'cold spots' (James et al. 2015). The SRS represents a large National Environmental Research Park with high amphibian biodiversity (Gibbons & Semlitsch 1991), a large diversity of wetlands with varying amphibian communities (Snodgrass et al. 2000), long-term (Daszak et al. 2005) and historic (Gibbons et al. 2006) population data on amphibian communities, and historic genetic samples (Nunziata et al. 2015). Given those aspects of the SRS and the apparently widespread distribution of both *Bd* and ranavirus, we suggest the wetlands of the SRS make an ideal area to examine 'cold spots' (James et al. 2015). Future studies need to characterize the interacting effects of wetland characteristics on *Bd* and ranavirus, use an experimental approach to examine the impacts of contaminants on susceptibility, and implement community-wide studies to elucidate the disease dynamics of these 2 prominent amphibian pathogens.

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