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Multi-year dynamics of ranavirus, chytridiomycosis, and co-infections in a temperate host assemblage of amphibians

Jennifer C. Olori[1,*], Rachel Netzband[1,2], Nathan McKean[1], Jason Lowery[1], Kirsten Parsons[3], Sofia T. Windstam[1,4]

[1]Department of Biological Sciences, State University of New York (SUNY) at Oswego, Oswego, NY 13126, USA
[2]Department of Biological Sciences, University at Albany, SUNY Albany, Albany, NY 12222, USA
[3]Department of Statistics, Oregon State University, Corvallis, OR 97306, USA

[4]*Present address:* Institution of Biosystems and Technology, Swedish University of Agricultural Sciences, Alnarp, 23053 Sweden

ABSTRACT: Chytridiomycosis and ranavirosis are 2 emerging infectious diseases that have caused significant global amphibian decline. Although both have received much scrutiny, little is known about interactions between the 2 causative agents *Batrachochytrium dendrobatidis* (*Bd*) and ranavirus (Rv) at the individual host and population levels. We present the first longitudinal assessment of *Bd*, Rv, and co-infections of a temperate amphibian assemblage in North America. From 2012 to 2016, we assessed the temporal oscillations of *Bd,* Rv and co-infection dynamics in a sample of 729 animals representing 13 species. *Bd*, Rv, and co-infected amphibians were detected during all 5 yr. *Bd*, Rv, and co-infection prevalence all varied annually, with the lowest instances of each at 2.1% (2013), 7.9% (2016), and 0.6% (2016), respectively. The highest *Bd*, Rv, and co-infection prevalence were recorded in 2012 (26.8%), 2016 (38.3%), and 2015 (10.3%), respectively. There was no association between *Bd* or Rv infection prevalence and co-infection, either when assessing the entire amphibian assemblage as a whole (odds ratio 1.32, 95% CI: 0.83–2.1, $p = 0.29$) or within species for amphibians that were more numerically represented ($n > 40$, $p > 0.05$). This suggests neither *Bd* nor Rv facilitate host co-infections within the sampled host assemblage. Instead, the basis for co-infections is the spatiotemporal distribution of both pathogens. Despite lack of interplay between *Bd* and Rv in this population, our study highlights the importance of considering numerous pathogens that may be present within amphibian habitats in order to properly anticipate interactions that may have direct bearing on disease outcomes.

KEY WORDS: *Batrachochytrium dendrobatidis* · Ranavirus · Co-infections · Host assemblage · Longitudinal

INTRODUCTION

Emerging infectious diseases (EIDs) have been implicated in population declines and extirpations of amphibians, and chief among these EIDs are chytridiomycosis and ranavirosis (Miller et al. 2011, Fisher et al. 2012, Olson et al. 2013). Chytridiomycosis is caused by *Batrachochytrium dendrobatidis* (*Bd*), a zoospore-producing fungus known to infect over 500 species of amphibians (Olson et al. 2013). Ranavirosis can be caused by numerous strains of ranavirus (Rv) including frog virus 3 (FV3), *Ambystoma tigrinum* virus (ATV), and common midwife toad virus (CMTV), all of which are members of the *Iridoviridae*

*Corresponding author: jennifer.olori@oswego.edu

family (Brunner et al. 2015). The host range of Rv is broad and the virus is known to be infectious to at least 70 amphibian species from 14 clades (Miller et al. 2011). *Bd* has caused mass declines and species extirpations in Australia, Central and South America, and the western part of the USA (Berger et al. 1998, Skerratt et al. 2007, Briggs et al. 2010, Vredenburg et al. 2010), while Rv has precipitated mortality events on 5 continents (Miller et al. 2011). Considering how widely distributed both pathogens are and that they co-occur quite frequently (e.g. Hoverman et al. 2012, Olson et al. 2013, James et al. 2015, Rosa et al. 2017, Smith et al. 2017, Stutz et al. 2018), it is reasonable to predict that co-infections by both *Bd* and Rv are commonplace. Recently, findings emerged indicating that amphibian co-infection by both pathogens is not a rarity, although most data come from tropical regions and animals in captivity (e.g. Fox et al. 2006, Miller et al. 2008, Whitfield et al. 2013, Soto-Azat et al. 2016, Warne et al. 2016).

Principally, parasites or pathogens that co-infect hosts may interact with one another through either direct means, for example by competing for resources, or via indirect means, which usually is mediated by the immune system (Graham 2008, Budischak et al. 2015). Regardless of whether interactions are direct or indirect, they may lead to an antagonistic interaction in which infectivity of one or more of the interacting parasites is reduced (Pedersen & Fenton 2007). Alternatively, the infectious agents can facilitate each other, which leads to exacerbated morbidity and/or transmissibility (Pedersen & Fenton 2007, Graham 2008), or co-infecting pathogens may have a neutral interaction. The very limited data that exist suggest that, at least for some Costa Rican, Peruvian, and Portuguese amphibian populations, infection by one of the pathogens (e.g. either *Bd* or Rv) does not increase the likelihood of co-infection by both (Whitfield et al. 2013, Warne et al. 2016, Rosa et al. 2017). *Craugastor fitzingeri* has been the exception, exhibiting a facilitative interaction between *Bd* and Rv (Whitfield et al. 2013). The varying propensity of different amphibian species for co-infection suggests that community composition and/or species richness of amphibians may be important to consider when evaluating co-infection dynamics.

Assessing the risk and relative significance of *Bd* and Rv co-infections for amphibians requires first establishing how commonplace co-infections are, how they are distributed spatiotemporally, and whether they have a differential impact on the host amphibian populations. Co-infections have been reported in both captive (Miller et al. 2008) and wild populations (Souza et al. 2012, Whitfield et al. 2013, Warne et al. 2016). *Bd* and Rv co-infections or co-occurrences are documented mostly in South America (Fox et al. 2006, Soto-Azat et al. 2016, Warne et al. 2016, Whitfield et al. 2013), including Argentina, Chile, Costa Rica, and Peru, but also in Portugal and the USA (Miller et al. 2008, Hoverman et al. 2012, Souza et al. 2012, Rosa et al. 2017, Smith et al. 2017). In California there were 2 yr of sporadic and smaller ranavirosis outbreaks in tadpoles of mountain yellow-legged frog *Rana mucosa* populations that previously were decimated by chytridiomycosis (Smith et al. 2017), whereas a small captive anuran population included individuals infected by *Bd*, Rv, and also bacterial *Aeromonas* sp. (Miller et al. 2008). Finally, co-infection also was reported in eastern hellbenders *Cryptobranchus alleganiensis alleganiensis* in eastern Tennessee (Souza et al. 2012). Due to more imminent needs, most of those studies focused on specific amphibian populations and thus were limited in scope (Fox et al. 2006, Miller et al. 2008, Souza et al. 2012, Soto-Azat et al. 2016, Warne et al. 2016); understanding how entire amphibian assemblages are impacted by co-infections or pathogen co-occurrences, especially over multiple years, are more rare (Hoverman et al. 2012, Whitfield et al. 2013, Rosa et al. 2017). Ultimately, aggregation patterns of parasites and pathogens can also be used to predict the mechanistic underpinnings of resulting infection and disease patterns (Wilber et al. 2017).

Our study addressed the following objectives:(1) to evaluate temporal oscillations of *Bd* and Rv infection prevalence in an amphibian assemblage in a temperate climate, and (2) to investigate whether infection by one of the pathogens pre-disposes amphibians to co-infections due to facilitative dynamics. Based on the limited data available from other co-infection studies, we predicted that neither *Bd* nor Rv infections facilitate co-infections in our native (Central New York State, NYS) amphibian assemblage as a whole (Whitfield et al. 2013, Warne et al. 2016), but that facilitation may happen for one or 2 individual species (Whitfield et al. 2013).

MATERIALS AND METHODS

Field locations and sampling procedure

Amphibians were collected from 4 different field sites (Fig. 1); Independence Park (IP), Minetto (MI), Rice Creek Field Station (RCFS), and Snake Swamp (SS). All 4 sites are located in Oswego County, NYS,

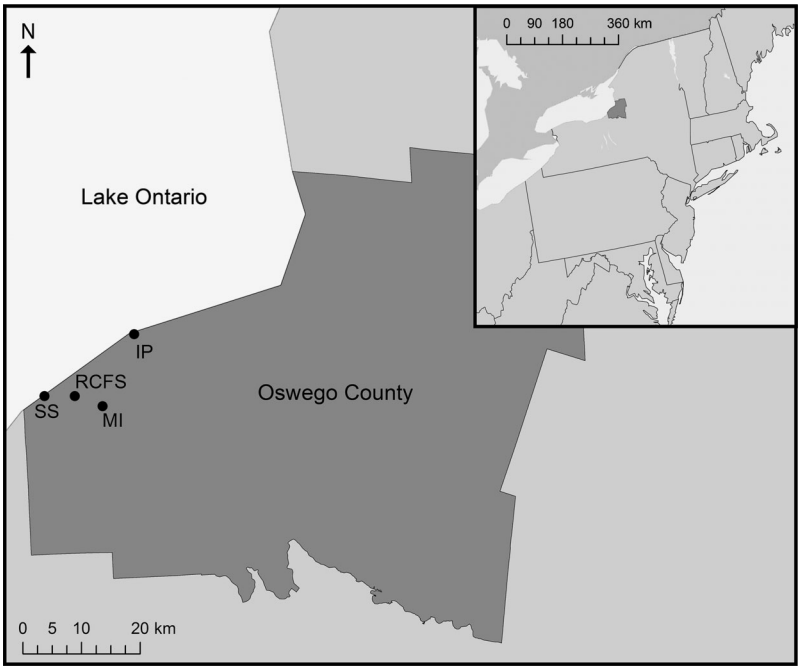

Fig. 1. Field sampling locations in Oswego County, NY, relative to the southeastern shore of Lake Ontario. Inset depicts Northeastern USA; the position of Oswego County within New York State is shown in dark gray. IP = Independence Park; MI = Gray Rd., Minetto; RCFS = Rice Creek Field Station; SS = Snake Swamp. Base map modified from Northeast US Boundaries (CT ECO 2017)

within 6.5 km of the Lake Ontario shoreline, and represent mainly secondary growth temperate deciduous forest with small wetlands and vernal pools. All 4 sites are zoned as residential areas and the MI and RCFS sites also border on land that is part of agricultural district 11 in Oswego County. The distance between the 4 field sites is as follows; RC–IP: 6.6 mi (10.6 km), RC–SS: 2 mi (3.2 km), IP–SS: 8 mi (13.02 km), RC–MI: 2 mi (3.2 km), SS–MI: 4 mi (6.4 km), and IP–MI: 6mi (9.6 km).

Sampling in 2012 began as a small pilot study including only RCFS and SS. In 2013, the study was expanded to IP in order to include amphibian species commonly encountered in upstate NY (e.g. *Lithobates sylvatica, Plethodon cinereus*) but rarely were present at RCFS and SS. MI was added in 2016 when we discovered that it is a prime road crossing for early season explosive breeders like *Ambystoma maculatum*, a species that was undersampled in prior years of our study.

Field sampling was carried out during months when amphibian activity was detected and thus occurred between April and October during all years. Additional sampling was done in March in 2016 due to earlier emergence of amphibians. Sampling in 2012, 2015, and 2016 was extended into November due to delays of amphibians entering hibernation. Sampling frequency was once to twice weekly in April and May, weekly in June, and once every 2 to 4 wk for the rest of the season. The total number of sampling occasions where animals were captured was 11 (2012), 28 (2013), 32 (2014), 33 (2015), and 28 (2016), with most sessions lasting for 3–4 h. Sampling procedures and handling of animals were in accordance with approved NYS DEC scientific license 1739 and SUNY Oswego IACUC permit 2014.06.2. Precautions were taken to prevent cross contamination by utilizing fresh nitrile gloves for each animal handled and decontaminating boots and other equipment with 10% bleach according to Northeastern Partners in Amphibian and Reptile Conservation guidelines (http://northeastparc.org/disinfection-protocol/).

Amphibians were opportunistically collected by either hand or net and the majority of sampling efforts were concentrated around dusk, yielding primarily adult individuals, though larvae and juveniles were captured when possible. When sampling vernal pools or creeks, the entire perimeter of the body of water was walked at least once, both on banks and in the water when possible. Captured animals were placed in individual, unused plastic zip-top bags to prevent contamination of tools. Snout to vent length was measured at least twice with a digital caliper (Bioquip) to generate 2 measurements that differed by no more than 5%, and weight was recorded using spring scales (Pesola). Any abnormalities, injuries, ectoparasites, or gross lesions were noted, and all individuals were photographed to record individual differences in dorsal and ventral markings and any external signs of disease. In order to test for *Bd*, amphibians were swabbed with rayon-tipped sterile swabs (Medical Wire) on hands, abdomen, thighs and feet, and swab tips were broken off into sterile screwcap vials containing 1 ml of 70% ethanol. Tissue was collected from each animal for detection of Rv, but also for standard marking and recapture. For frogs and larger salamanders, such as *Ambystoma* spp., the distal phalanx of a digit was removed using sterile scissors, whereas for smaller salamanders,

such as *P. cinereus* and *E. bislineata*, no more than 5 mm of the tip of the tail was taken. Tissue samples were stored in sterile screwcap vials containing 70% ethanol. Incisions were treated with triple antibiotic ointment (active ingredients: 400 U × g^{-1} Bacitracin zinc, 0.35% Neomycin sulfate, 5000 U × g^{-1} Polymyxin B sulfate) before releasing animals back to their site of capture. Vials containing swabs and tissues were kept at ambient temperature until returning to the lab, where they were transferred to and kept at −20°C until analyzed.

Current air temperature at the site of capture was recorded along with water temperature if the animal was caught in water. Total monthly precipitation and average air temperature for Oswego County were obtained from the National Centers for Environmental Information, NOAA, station Oswego East NY US USC00306314.

Molecular PCR-based detection of *Bd* and Rv

Swabs were aseptically transferred to sterile screwcap tubes containing 30–40 mg of zirconium-silica beads (particle size 0.5 mm) and 50 µl of Prepman Ultra (Applied Biosciences) was added to each tube. Contents were mechanically disrupted by agitating for 50 s using a Biospec Mini Beadbeater (Biospec), then chilled on ice and spun at 13 000 rpm (16.2 × *g*) for 1 min. Bead beating, chilling, and centrifuging was repeated, then tubes were incubated at 100°C for 10 min, placed on ice for 2 min and then spun at 13 000 rpm for 3 min. Twenty µl of supernatant was transferred to clean tubes.

PCR reactions for *Bd* detection were carried out in either 10 or 20 µl volumes and containing 1X PCR buffer, 1 U of Taq polymerase, 0.25 mM dNTP's, 3 mM $MgCl_2$, 0.9 µM forward primer ITS1-3 Chytr (5′-CCT TGA TAT AAT ACA GTG TGC CAT ATG TC-3′), 0.9 µM reverse primer 5.8S Chytr (5′-AGC CAA GAG ATC CGT TGT CAA A-3′), molecular grade water and 2 or 4 µl template diluted to 1/10 (Boyle et al. 2004, Garland et al. 2011). Molecular grade water was used in place of template for negative control. The positive control was DNA extracted from *Bd* cultures. Initially positive control DNA was acquired courtesy of Dr Kelly R. Zamudio at Cornell University; later, DNA was extracted from *Bd* JEL404 cultures acquired from Dr Joyce Longcore at Maine University, using the MoBio microbial DNA extraction kit (MoBio) according to manufacturer's instructions. A 1–10% solution of pure pathogen DNA was used as template for the positive control. PCR was carried out using the following temperature protocol: initial denaturation at 95°C for 4 min followed by 50 cycles of denaturing at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s (Garland et al. 2011).

To investigate Rv presence, DNA from tissue samples was extracted by either a manual salt-based extraction (Sambrook & Russell 2001) followed by a chloroform-phenol-isoamyl alcohol (25:24:1, v/v/v) purification and ethanol precipitation, or by using commercial kits (Qiagen DNeasy DNA extraction or Promega Wizard Genomic Extraction) according to manufacturer's instructions. Overall genomic DNA yield from the 2 kits used is similar, ranging from 10–25 µg from 1.2 cm tissue vouchers and 10–30 µg for 0.5–1 cm vouchers for the Qiagen and Promega kits, respectively. Specific protocols for extracting DNA from small tissue vouchers from animals, usually mice, were followed for all extraction procedures used. Extracted DNA was quantified and concentrations were adjusted to 20–100 ng × $µl^{-1}$. PCR reactions contained 1X PCR buffer, 1 U of Taq polymerase, 0.2 mM dNTP's, 1.5 mM $MgCl_2$, 0.2 µM MCP-5 (5′-GTC TCT GGA GAA GAA GAA-3′) and MCP-4 (5′-ACT TGG CCA CTT ATG AC-3′) primers, 2–20 ng × $µl^{-1}$ DNA template, and molecular grade water to make the final volume 10 µl (Mao et al. 1997, Greer et al. 2005, Harp & Petranka 2006, Brunner et al. 2007, Greer & Collins 2007). Molecular grade water replaced template in the negative control. The positive control was DNA extracted from viral titers courtesy of Dr Jesse Brunner at Washington State University. Thermocycling was conducted using the following settings: 94°C for 5 min, 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, cycled 35 times, followed by an extension of 72°C for 2 min (Greer & Collins 2007).

PCR fragments from both *Bd* and Rv analysis were resolved using 1.5–2% agarose gels in 1X TAE buffer for endpoint detection (i.e. neither pathogen was quantified for infection load). DNA ladders (100 bp) were employed to estimate fragment sizes. Criteria for *Bd* versus Rv positive assignment was the presence of a 150 (*Bd*) and 500 (Rv) bp band following 50 and 35 PCR cycles in 2 independent PCR rounds, respectively (Mao et al. 1997, Garland et al. 2011).

Statistical analysis

Statistical analysis was performed using the Epi Info™ v7.2 software package from the Centers for Disease Control and Minitab v17 (Minitab) and

analysis was conducted on pooled data sets. Infection prevalence was calculated along with 95% confidence intervals on binomial data. Chi-square tests were performed to determine association between infection prevalence (*Bd*, Rv, and co-infection) and year, sampling location, or month but also to determine if individual years, location and months deviated from the overall prevalence encountered. Chi-square tests were also performed to determine if there was a difference in *Bd*, Rv, and co-infection prevalence among the more numerous species (*E. bislineata, L. catesbeiana, L. clamitans, L. sylvatica, P. cinereus, Pseudacris crucifer*). In cases in which a category had a count of fewer than 5 animals, Fisher's exact test was employed instead of chi-square. Odds ratios were calculated to assess whether infection by one pathogen (*Bd* or Rv) was associated with an increased risk of being infected by the second pathogen. Finally, simple and multiple linear regression was employed, using '*Bd* prevalence' and/or 'Rv prevalence' as predictors, and 'co-infection prevalence' as response variable. Regression analyses were carried out using annual, overall prevalence data. Tests yielding $p \leq 0.05$ were considered statistically significant.

RESULTS

A total of 729 individuals were sampled from 13 amphibian species. The number of animals recaptured later in the season and sampled again was negligible (0.6%; individuals were not removed from statistical tests). Similarly, dead or moribund animals were encountered rarely, accounting for only 1.2% of all individuals sampled; of those, 22.2% were *Bd* positive and 11.1% were Rv positive. Despite the consistent presence of both pathogens across the study period, external symptoms of disease (e.g. lesions, subcutaneous hemorrhaging, lack of righting reflex, emaciation, lethargy), were observed in just 4.1% of all captured animals, 20% of which were *Bd* positive, and 23.3% of which were Rv positive. No mass mortality events were observed at our sites during our study period.

There was significant annual variation in *Bd*, Rv, and co-infection prevalence (Fig. 2). *Bd* prevalence peaked in 2012 and 2015, with 26.8 and 25.1% of animals testing positive for the pathogen, respectively (Fig. 2A). In the intervening years, *Bd* infection prevalence was much lower (2.1–6.2%). Although year was significantly associated with Rv prevalence, the pattern over time was quite distinct from that of

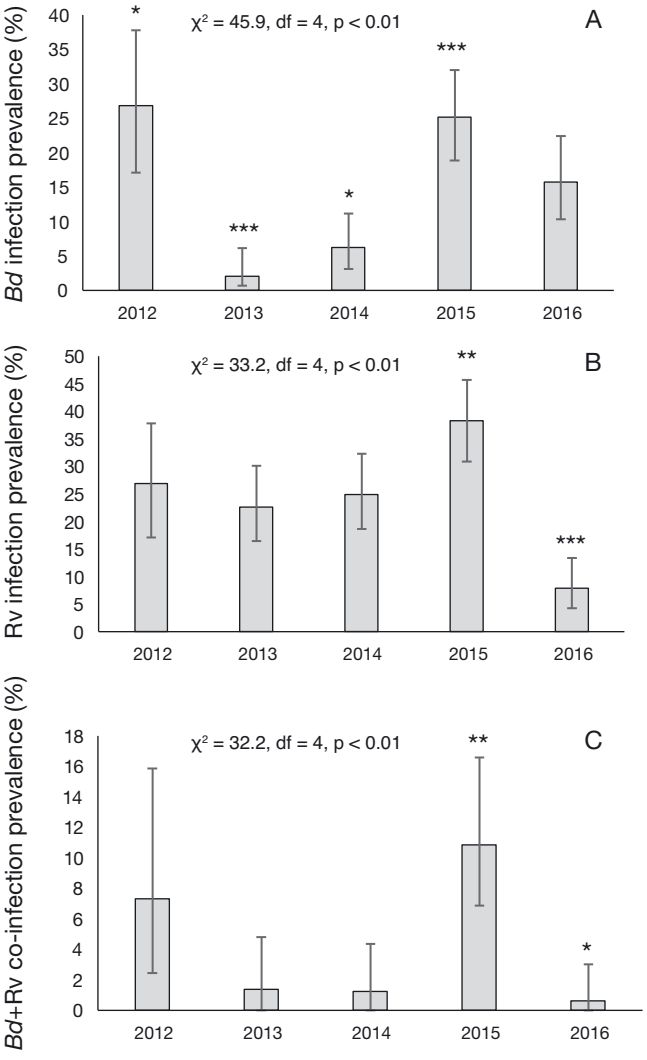

Fig. 2. Annual infection prevalence of (A) *Bd*, (B) Rv, and (C) animals co-infected by both pathogens. Error bars denote the 95% CI around the mean. Number of animals sampled annually ranged from 82 (2012) to 175 (2015), and a total of 729 specimens were analyzed for each pathogen. Asterisks indicate that observed prevalence was significantly different from the expected prevalence: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Bd (Fig. 2B). Between 2012 and 2014, Rv infection remained relatively stable at 23.3 to 25.6%. In 2015 Rv infection prevalence peaked at 38.3% and then fell to its lowest the following year, when only 7.9% of animals carried the pathogen. Co-infections by both pathogens also was significantly associated with sampling year (Fig. 2C) and ranged between 0.6% (2016) and 10.3% (2015). Those last 2 years also were significantly different from the overall infection prevalence. Infection by either pathogen did not yield an increased risk of co-infection, as evidenced by the non-significant odds ratio with a 95% confi-

dence interval spanning 1 (Fig. 3). When segregating the more numerously sampled amphibians (n > 40) and calculating their odds ratios and associated 95% confidence intervals within each individual species, none of the odds ratio confidence intervals indicated that being infected with either *Bd* or Rv yielded a higher risk for co-infection (data not shown). Neither *Bd* (r^2 = 67.13, p = 0.09) nor Rv (r^2 = 66.05, p = 0.10) prevalence by itself was correlated significantly with co-infection, but a regression model using both pathogen prevalences was significantly correlated with co-infection (r^2 = 0.974, p = 0.026). Within this multiple regression model both predictors were found to be significant (*Bd* p = 0.040, Rv p = 0.039).

Sampling month was a significant predictor for both *Bd* and Rv infection prevalence (Fig. 3). *Bd*

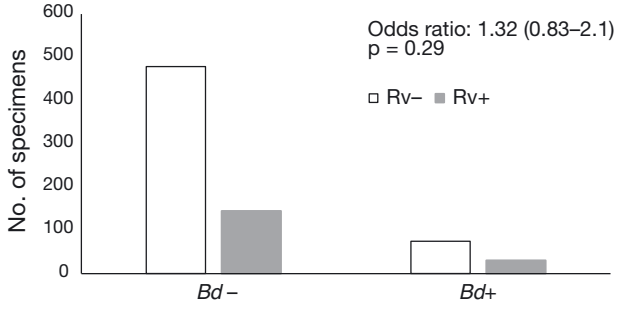

Fig. 3. Overall co-infection patterns in 12 species of sampled amphibians with calculated odds ratio, with 95% CI in parentheses. This represents the entire pooled data set of 729 sampled animals. The p-value denotes the result from a corrected 2-tailed chi-square analysis. + pathogen present, – pathogen absent. *Bd* status is represented on the *x*-axis and Rv status is represented by bars

showed an approximately bimodal pattern, with infections peaking in May at 24% and then again in September and November, although neither of the latter months had a significantly higher infection prevalence (Fig. 3A). July, August, and October had the lowest percentages of *Bd*-positive animals. Rv prevalence was quite stable across the sampled months (Fig. 3B), and most months did not deviate from the overall infection prevalence, except for March which was lower at 7.1%, and peaks in July and November when Rv prevalence was 35.8 and 65%, respectively. The association between month and co-infection prevalence could not be carried out due to the low number of co-infected animals for some months. However, none of the months sampled had a statistically significantly different co-infection prevalence from overall values, and co-infection prevalence ranged between 0 to 7.1% (Fig. 3C).

Bd was detected on 9 of the 13 amphibian species sampled and the prevalence rate ranged from 2.9% of *Lithobates pipiens* to 22.2% of *Notophthalmus viridescens*, although it is noteworthy that only 9 *N. viridescens* were sampled (Table 1). All species for which at least 35 animals had been sampled included individuals positive for *Bd*, with the exception of *Plethodon cinereus,* for which none of the 44 sampled animals carried *Bd*. Among the 6 species (*E. bislineata*, *L. catesbeiana, L. clamitans, L. sylvativa, P. cinereus, Pseudacris crucifer*) with a minimum sample number of 40, species itself was not significantly associated with *Bd* prevalence (χ^2 = 8.9, df = 5, p = 0.114). Only *Plethodon cinereus* had a significantly different and lower (0%) observed *Bd* prevalence

Table 1. Prevalence of *Bd*, Rv, and co-infection by species sampled between 2012 and 2016. Prevalence is expressed in percent followed by the 95% confidence interval in parentheses

Species	Prevalence (%)			Total sampled	Year(s) sampled
	Bd	Rv	*Bd* + Rv		
Ambystoma laterale	0 (0–100)	0 (0–100)	0 (0–100)	2	2015
A. maculatum	5.3 (0–26.3)	5.3 (0–26.3)	0 (0–15.8)	19	2014–2016
Ambystoma sp.	0 (0–60)	0 (0–60)	0 (0–80)	5	2016
Anaxyrus americanus	0 (0–100)	0 (0–100)	0 (0–100)	1	2013
Eurycea bislineata	17.8 (8.9–31.1)	33.3 (20–48.9)	6.7 (2.2–17.8)	45	2012–2016
Hemidactylum scutatum	0 (0–100)	0 (0–100)	0 (0–100)	1	2014
Hyla versicolor	9.1 (0–45.5)	0 (0–100)	0 (0–27.3)	11	2013–2016
Lithobates catesbeiana	16.7 (7.4–29.6)	33.3 (0–27.3)	5.6 (1.9–14.8)	54	2012–2016
L. clamitans	15.5 (11.7–19.8)	36.4 (20.4–48.1)	5.5 (3.5–8.5)	343	2012–2016
L. pipiens	2.9 (0–14.3)	8.6 (31.2–41.7)	0 (0–11.4)	35	2012–2016
L. sylvatica	10.6 (4.3–23.4)	4.3 (0–14.9)	0 (0–8.5)	47	2013–2016
Lithobates sp.	0 (0–100)	100 (n/a)	0 (0–100)	1	2014
Notophthalmus viridescens	22.2 (0–55.6)	0 (0–33.3)	0 (0–33.3)	9	2013, 2014, 2016
Plethodon cinereus	0 (0–9.1)	0 (0-9.1)	0 (0–9.1)	44	2012–2016
Pseudacris crucifer	19.4 (12.2–28.6)	4.1 (1.0–10.2)	0 (0–4.1)	98	2012–2016

compared to the overall value ($p < 0.01$). Similar to *Bd*, co-infection prevalence is not associated with amphibian species (Table 1, $\chi^2 = 10.8$, df = 5, $p = 0.06$).

Rv, on the other hand, was significantly associated with amphibian species ($\chi^2 = 54.6$, df = 5, $p < 0.001$) when assessing the 6 most commonly sampled species (Table 1). Four species had significantly different observed Rv prevalences; *L. clamitans* ($p < 0.05$), *L. sylvatica* ($p < 0.01$), *P. cinereus* ($p < 0.01$) and *Pseudacris crucifer* ($p < 0.001$), in which 36.4, 4.3, 0, and 4.1% of animals carried Rv, respectively. Of those 4 species, *L. sylvativa*, *Plethodon cinereus*, and *Pseudacris crucifer* had Rv prevalence rates significantly lower than the rest of the assemblage

Sampling site did not significantly influence overall detection rate of *Bd*, Rv, or co-infections (data not shown). However, *Bd* was not detected on any of the animals sampled at SS, and although *Bd* prevalence at SS statistically was not different from that of IP and MI, it was significantly lower than at RCFS ($p < 0.05$).

Air temperature, precipitation, and snowfall were collected from a local weather station and showed monthly average temperatures generally peaking in July (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/d130p187_supp.pdf). Air and water temperature collected at field sites at sampling show that animals that test positive for *Bd* were collected at significantly lower air and water temperatures than those that test negative (Fig. S2). No such trend was discerned for Rv.

DISCUSSION

To our knowledge, our report represents the first longitudinal assessment of *Bd* and Rv co-infections of an amphibian assemblage in North America. We found low levels of co-infection, ranging from 0.6% in 2016 and 10.3% in 2015 (Fig. 1) that also fluctuated significantly from year to year. This is similar to results from Souza et al. (2012), which showed significant annual variation in co-infection prevalence among *Cryptobranchus alleganiensis alleganiensis* sampled in Tennessee over a 2 yr period.

Co-infections were detected in 3 out of the 13 species that we sampled; *Erycea bislineata*, *Lithobates catesbeiana*, and *L. clamitans* (Table 1). This is the first time that co-infections are reported for these 3 amphibian species. Among them, co-infection prevalence was fairly similar, ranging from 5.5% of *L. clamitans* to 6.7% of *E. bislineata* (Table 1). Co-infection rates reported in the literature vary widely depending on amphibian species assessed and location sampled, but for wild amphibian populations it was reported to range from 1.7% for *Xenopus laevis* in Chile to 50% for *Rhinella manu* in Peru, although the latter included only 4 animals (Soto-Azat et al. 2016, Warne et al. 2016). Following *R. manu*, the second highest co-infection prevalence in wild amphibian populations was 29.6% of *Craugastor fitzingeri* in Costa Rica (Whitfield et al. 2013). In the USA, co-infection prevalence among wild amphibians was reported to be 2.9% for *Rana muscosa* and *R. sierrae* in California and 5.2% for *C. alleganiensis alleganiensis* in Tennessee, which is very similar to rates we observed (Souza et al. 2012, Smith et al. 2017).

We detected *Bd* and Rv on *L. pipiens*, *L. sylvatica* and *Pseudacris crucifer*, yet co-infection was not detected for these species (Table 1). Both *L. sylvatica* and *P. crucifer* showed significantly lower Rv prevalence than other species, which may explain the lack of co-infections. The low Rv prevalence in *L. sylvatica* was surprising, given that their tadpoles are especially susceptible to Rv (Haislip et al. 2011), but it is possible that our bias toward sampling adults meant that we primarily encountered the more resistant individuals that survived to metamorphose. Additionally, across the 5 yr we sampled, *Plethodon cinereus* never tested positive for *Bd* nor Rv, consistent with previously reported data (Ouellet et al. 2005, Gratwicke et al. 2011, Miller et al. 2011, Windstam & Olori 2014). With the exception of a study in which 14% of *P. cinereus* tested positive for *Bd* (Richards-Hrdlicka et al. 2013), larger surveys, both historical and field based, have found *Bd* prevalence to range between 0 to 0.3% (Gratwicke et al. 2011, Muletz et al. 2014). The mechanistic explanation for *Bd* resistance of *P. cinereus* may result from its association with skin microbiota, including antibiotic-producing bacteria such as *Pseudomonas* sp. and *Janthinobacterium lividum* (Harris et al. 2009a,b, Rollins-Smith 2009, Becker & Harris 2010, Wiggins et al. 2011, Loudon et al. 2014;).

Our initial prediction was that neither *Bd* nor Rv infections would facilitate co-infections for the sampled amphibian assemblage as a whole. Considering that the 95% confidence interval for the odds ratio was non-significant ($p = 0.29$) and included 1 (0.83–2.1), we conclude that amphibian infection by *Bd* or Rv is not associated with co-infection and there is no evidence for one of the infectious agents pre-disposing animals to co-infections (Fig. 3). This is corroborated by several other studies (Whitfield et al. 2013, Warne et al. 2016, Rosa et al. 2017, Smith et al. 2017;) that noted a similar lack of association between

either Rv or *Bd* infections and co-infection. Furthermore, in a prior study, co-infected animals also were not more likely to carry heavier infection loads of *Bd* or Rv than amphibians only infected by one of the infectious agents (Warne et al. 2016). A notable exception was *C. fitzingeri*, sampled in Costa Rica, which showed that infection by one of the pathogens facilitated co-infection (Whitfield et al. 2013). When we analyzed species that were co-infected and had an n > 40, we did not find a within species pathogen facilitation effect for *E. bislineata*, *L. catesbeiana*, or *L. clamitans* (data not shown, p > 0.05).

Within our sampled amphibian assemblage, it is more likely that co-infection prevalence is directly a function of overall *Bd* and Rv prevalence in the environment at any given time. This is supported by a multiple regression analysis in which annual co-infection prevalence was predicted to a very high degree (R^2 = 97.4, p = 0.026) by our annual prevalence of both *Bd* and Rv, when both predictors were significant (*Bd* p = 0.040, Rv p = 0.039). Furthermore, prior work suggested that different species also should display similar patterns in their co-infection rates if indeed the basis for co-infection is simply overall spatiotemporal abundance of infectious agents (Whitfield et al. 2013). That also is congruent with our findings, because species was not a predictor significantly associated with co-infection prevalence in our study (Table 1, χ^2 = 8.9, df = 5, p = 0.114), nor were any of the within species odds ratios predictive of infection by one pathogen facilitating co-infection for any individual species (data not shown, p > 0.05). Finally, if *Bd* and Rv co-infections are not facilitative, but rather determined by spatiotemporal distribution of both infectious agents, then amphibians held in captivity or in aquaculture should be more likely to become co-infected than wild populations due to being kept at higher densities and in close quarters (Warne et al. 2016). Our work supports that prediction because our low co-infection rates align with those reported in previous investigations of wild populations (1.7–50%; Souza et al. 2012, Whitfield et al. 2013, Soto-Azat et al. 2016, Warne et al. 2016, Smith et al. 2017) and are much lower than those reported from studies of captive amphibians (48.8–100%; Miller et al. 2008, Warne et al. 2016). However, one caveat to using co-infection correlates is that competition between parasites is sometimes masked and not deduced from correlates of co-infection dynamics alone (Johnson & Buller 2011).

The temporal segregation of peak *Bd* and Rv prevalence throughout a season is most likely the best explanation for the low co-infection rates we observed between 2012 and 2016 (Fig. 4). For example, Rv prevalence peaks in July and November, whereas *Bd* prevalence during the same months is either significantly lower than during the rest of the year (July), or not significantly different than expected (Fig. 4). We suggest that co-infections are not species- nor site-driven because neither parameter is associated with

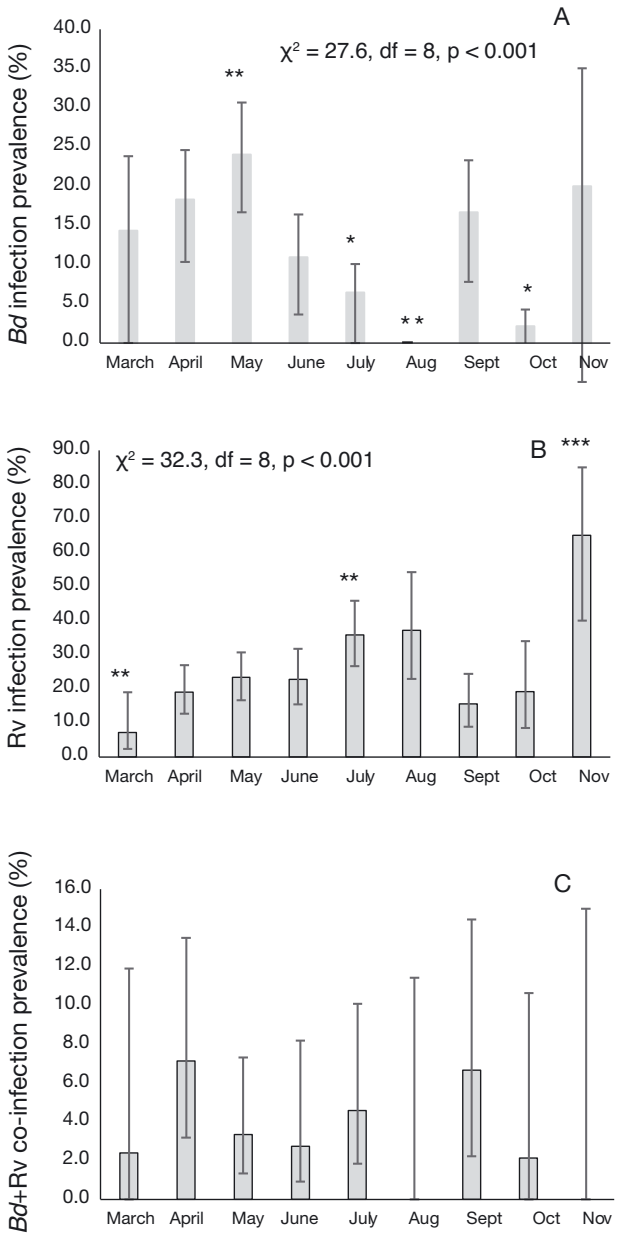

Fig. 4. Overall seasonal infection prevalence of (A) *Bd*, (B) Rv, and (C) co-infection by both *Bd*+Rv between 2012 to 2016. Error bars denote the 95% CI around the mean. Number of animals sampled per month ranged from 20 (November) to 150 (May), with a total n = 729. Asterisks indicate that observed prevalence was significantly different from the expected overall prevalence: *p < 0.05, **p < 0.01, ***p < 0.001

co-infection prevalence. The lack of species association with *Bd* prevalence could imply that all the amphibians in the assessed assemblage are similarly susceptible to *Bd* infection, with the notable exception of *P. cinereus*, and the observed temporal pattern of *Bd* prevalence would instead be a function of environmental factors that impact both *Bd* and host physiology (Woodhams et al. 2003, Piotrowski et al. 2004, Raffel et al. 2006). For example, the inverse relationships between the 2 pathogens in late summer may be driven by temperatures that are too high for *Bd* growth (Piotrowski et al. 2004), rather than competition between the pathogens. Indeed, weather data during the study period indicates the highest air temperature when *Bd* prevalence is low (Fig. S1). Furthermore, air and water temperature recorded at field sites at the time of sampling show as association between *Bd* positive animals and lower air and water temperature (Fig. S2). Considering that Rv infection is associated with species, this bias might explain temporal prevalence patterns of Rv that are linked to peak activities of different species within the assemblage, such as the long, late summer breeding season of *L. clamitans*, our most abundantly sampled species. Because our sampling was opportunistic we tended to sample animals during their peak activity.

Despite an articulated need, until recently there has been a lack of long-term field studies on *Bd* and Rv dynamics. Recent publications are starting to shed light on oscillations of Rv (Sutton et al. 2015) in plethodontid populations, *Bd* in anurans (Seimon et al. 2017), and co-occurrences of both pathogens in amphibian assemblages (Rosa et al. 2017). Our longitudinal study revealed broadly different long-term patterns of *Bd* and Rv infections within the same amphibian populations, something that cannot readily be gleaned from one or even 2 yr investigations. Specifically, *Bd* showed larger annual fluctuations in prevalence, and reflecting what is known about *Bd* physiology, appears to be more sensitive to environmental parameters than Rv. However, despite the consistent presence of both pathogens in our area across all study years, co-infections remain relatively rare, and there is a lack of evidence for either facilitative or antagonistic interactions. Instead, co-infections are driven primarily by local spatio-temporal patterns of *Bd* and Rv prevalence. As more information emerges about co-occurrences and co-infections of amphibians, we are starting to understand that amphibians often exist in environments where they may be challenged by more than one pathogen or parasite at a time (Miller et al. 2008, Hoverman et al. 2012, Souza et al. 2012, Whitfield et al. 2013, Warne et al. 2016). This underscores the importance of mapping the nature of pathogen co-occurrences and co-infections, and also conducting field- and laboratory-based assays to investigate the interaction among amphibian parasites. Superimposed on this are the interactions among cutaneous microbes, amphibians, and pathogens, which also are consequential to amphibian health (Loudon et al. 2014, Rynkiewicz et al. 2015, Burkart et al. 2017), as well as the influence of a changing climate on both host and pathogen physiology (James et al. 2015), which may shift current spatio-temporal patterns of infection. We may very well be moving away from a paradigm of studying one pathosystem at a time to integrating a more network-based approach that flexibly considers host populations, complements of microbial communities with which hosts associate, and the pathogen population to inform our conclusions about disease outcomes.

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