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Factors influencing detection and co-detection of *Ranavirus* and *Batrachochytrium dendrobatidis* in Midwestern North American anuran populations

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ABSTRACT: Amphibian populations are in decline worldwide as they face a barrage of challenges, including infectious diseases caused by ranaviruses and the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*). Here we describe seasonal dynamics of *Bd* and ranavirus detection in free-ranging post-metamorphic wood frogs *Lithobates sylvaticus*, boreal chorus frogs *Pseudacris maculata/triseriata*, and gray treefrogs *Hyla versicolor/chrysoscelis*, sampled over a 3 season gradient in Minnesota (USA) wetlands. We detected *Bd* in 36% (n = 259) of individuals sampled in 3 wetlands in 2014, and 33% (n = 255) of individuals sampled in 8 wetlands in 2015. We also detected ranavirus in 60% and 18% of individuals sampled in 2014 and 2015, respectively. Ranavirus and *Bd* were detected concurrently in 26% and 2% of animals sampled in 2014 and 2015, respectively. We report clinical signs and associated infection status of sampled frogs; of the clinical signs observed, skin discoloration was significantly associated with ranavirus infection. Using generalized estimating equations, we found that species, season, wetland, and a species × season interaction term were significant predictors of *Bd* detection, whereas test year approached significance as a predictor of ranavirus detection. The odds of detecting both pathogens concurrently was significantly influenced by species, season, a species × season interaction term, year, and environmental ammonia. We propose an amphibian health monitoring scheme that couples population size surveys with seasonal molecular surveys of pathogen presence. This information is crucial to monitoring the health of remaining strongholds of healthy amphibian populations, as they face an uncertain future of further anthropogenic change.

KEY WORDS: Amphibian decline · Amphibian disease · *Ranavirus* · Amphibian chytrid fungus · Gray treefrog · Wood frog · Boreal chorus frog

INTRODUCTION

In response to the current crisis of worldwide amphibian decline, in which over a third of amphibian species face population decline or extinction (Stuart et al. 2004), scientists have been racing to identify the key drivers of this trend and understand their dynamics. Pathogens such as *Batrachochytrium dendrobatidis* (*Bd*) and viruses in the genus *Ranavirus* contribute heavily to this decline, along with other factors such as climate change, habitat loss, pollution, species introduction, and exploitation (Beebee & Griffiths 2005, Rohr & Raffel 2010, Chan et al. 2014, Polo-Cavia et al. 2016, Whitfield et al. 2016).

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However, these phenomena are not mutually exclusive, and there is likely synergism among these factors. For instance, seasonal temperature fluctuations have been shown to decrease immune function in amphibians (Raffel et al. 2006), which suggests that increased temperature variability attributable to climate change could affect disease prevalence in the future; however, temperature variability could also potentially influence pathogen viability. Environmental pollution interacts with pathogens in complex ways. Negative synergistic effects have been documented between ranavirus and the insecticides carbaryl and thiamethoxam (De Jesús Andino et al. 2017, Pochini & Hoverman 2017), while studies have yielded mixed results on the interactions of atrazine and *Bd* (Hanlon & Parris 2012, Paetow et al. 2012). In addition, introduced species have the potential to act as carriers of both *Bd* and ranavirus, highlighting the importance of animal movement and trade (Daszak et al. 1999, Sharifian-Fard et al. 2011). Clearly, many factors contributing to amphibian decline are dynamic, and several likely work synergistically. Thus, baseline data describing pathogen dynamics in stable amphibian populations are necessary for detecting shifts in environmental factors and pathogen presence that could threaten these strongholds in the future.

Both *Bd* and ranavirus have been detected on all continents except Antarctica (Duffus et al. 2015, USGS National Wildlife Health Center 2016). *Bd* is an aquatic chytrid fungus that parasitizes amphibian skin cells, leading to disruption of osmotic regulation, and in severe cases, to death (Voyles et al. 2011). *Bd*-related mass mortalities, some contributing to species-level declines, are frequently reported from tropical regions in Central America, South America, and Australia (Skerratt et al. 2007), as well as the western regions of the USA (Muths et al. 2003, Vredenburg et al. 2010); however, relatively few studies have investigated the ecology of *Bd* and ranavirus in amphibian populations in the temperate Midwestern region of North America, which has distinct patterns of temperature and rainfall.

Ranaviruses belong to the family *Iridoviridae*, and also persist in aquatic ecosystems. Unlike *Bd*, ranaviruses can infect multiple ectothermic taxa, including fish, reptiles, and amphibians (Brenes et al. 2014). Ranaviral infection causes cutaneous and visceral hemorrhage, and necrosis, and often results in death (Gray et al. 2009, Miller et al. 2015). Ranaviruses have been associated with amphibian mass mortalities, although more studies are needed to clarify the role of these pathogens in population-level decline (Price et al. 2014). Mortalities in free-ranging populations can be difficult to detect, especially at the beginning of an epidemic (Todd-Thompson 2010), and relatively high prevalences have been noted in populations in which no clinical signs were observed (Duffus et al. 2008, Greer et al. 2009). Therefore, studies of apparently healthy populations, carried out over a seasonal gradient, are needed to clarify the dynamics of ranavirus ecology (Teacher et al. 2010, Brunner et al. 2015, Gray et al. 2015).

Analysis of museum specimens revealed that *Bd* has been present in the Midwestern USA since at least 1888 (Talley et al. 2015), while the first described ranavirus came from specimens collected in Wisconsin and Minnesota in the 1960s (Granoff et al. 1966). Sporadic mass mortalities of amphibians have been recorded in Minnesota and surrounding states since 1996, several of which are likely attributable to *Bd* and ranavirus infection (Green et al. 2002). Given the recognized synergistic effects of amphibian population threats (e.g. environmental contamination, climate change) and disease, it is important to understand the ecological dynamics of these pathogens in otherwise stable populations, particularly in the North American Midwest, where relatively little is known regarding the dynamics of these pathogens. The aim of this study was to document the seasonal variation in *Bd* and ranavirus prevalence in 3 regionally common species of anurans, and to provide baseline data on the dynamics of *Bd* and ranavirus ecology in anuran populations in temperate North American wetlands.

MATERIALS AND METHODS

Study site and field sampling

This study was conducted from May to September 2014 and 2015 across wetlands in Dakota County, Minnesota (Fig. 1). In 2014, we collected disease and water quality data from 3 wetlands within undeveloped areas of the Minnesota Zoo grounds (44.768° N, 93.199° W). Wetlands with distinct vegetation communities were selected based on the habitat delineations assessed by Applied Ecological Services, Inc. as part of a habitat management plan for the site; habitat types included vernal pool, mixed emergent marsh, mixed emergent marsh (seasonally flooded), palustrine open water, and tamarack swamp (Chapman et al. 2012). To expand the study to include a greater spectrum of wetland areas and land uses, we included 5 additional sampling locations in the

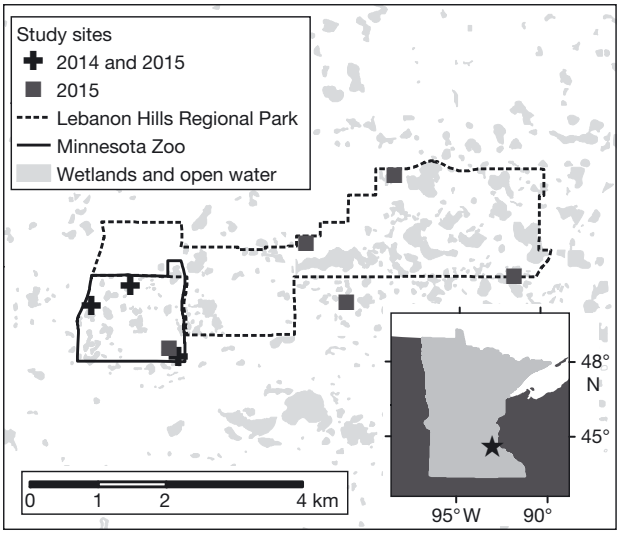

Fig. 1. Anuran sampling locations in Dakota County, MN (USA), in 2014 and 2015

neighboring Lebanon Hills Regional Park and a public golf course in the 2015 field season, for a total of 8 wetlands in 2015.

To understand the influence of seasonality on pathogen presence, we sampled frogs from each wetland 3 times yr^{-1}. In 2014, the spring sampling session occurred from 29 May to 11 June, the summer session from 23–25 July, and the fall session from 17–20 September. In 2015, the spring session took place from 19 May to 4 June, the summer session from 6–23 July, and the fall session from 14–29 September.

During each sampling session, we captured post-metamorphic juvenile and adult wood frogs *Lithobates sylvaticus*, boreal chorus frogs *Pseudacris maculata/triseriata* (species distinctions unresolved), and gray treefrogs *Hyla versicolor/chrysoscelis* (species morphologically indistinguishable); these species were selected because they were the most frequently observed species among the study locations. Sampling was conducted from late afternoon into evening, with the goal of sampling 20 or more individuals of each species per site. Each frog was captured by hand and placed in an individual, vented container, along with water from the wetland being sampled. To avoid redundant sampling, frogs were maintained in individual holding containers, and released into the wetland of origin at the end of each sampling session.

Because our study was originally conceived as a model for a non-invasive ranavirus monitoring program, we collected diagnostic samples through skin swabs, rather than toe clips. Sampling included the collection of separate oral and skin swabs, and documentation of any observed clinical signs of disease from each individual. Swabbing methods and materials followed Pessier & Mendelson (2010); briefly, this included swabbing the oral cavity of each frog to sample for ranavirus, and collecting samples for *Bd* by gently rolling a swab along the ventral surfaces of the pelvic patch, toe webbing, and inner thighs. We assessed the health of each frog by noting the presence or absence of clinical signs of disease including bloating, swelling, lacerations, discoloration, and morphologic abnormality (Fig. 2). Dead frogs were fixed in 10% neutral buffered formalin and sent to the Amphibian Disease Laboratory (San Diego, CA) for necropsy and histopathology.

To prevent the spread of *Bd* and ranavirus, each field member wore nitrile gloves during sampling, and changed them between handling individual frogs. Field members also wore rubber boots during sampling; after each session, boots were scrubbed of organic debris and rinsed in a Virkon-S bath (DuPont), followed by fresh water (Pessier & Mendelson 2010). Amphibian holding containers and nitrile gloves were discarded after use.

During each sampling session, we also collected a 1 l sample of surface water from the focal wetland, collected approximately 5 m from the shoreline. Each wetland had dedicated collection bottles to avoid cross contamination between sites. We restricted our analyses of water quality to ammonia, as it is known to be toxic to aquatic organisms. Samples were kept at ambient temperature and were tested for ammonia within 24 h at the Minnesota Zoo's Life Support lab. In 2014, we quantified unionized ammonia using a test kit (Hach 2428700; range of 0–2.4 mg l^{-1}, quantified in steps of 0.2 mg l^{-1}), and in 2015, we used both the test kit and dipstick-style test strips (Hach 2755325; range of 0–6 mg l^{-1}, quantified in steps 0, 0.25, 0.5, 1.0, 3.0, and 6.0 mg l^{-1}).

PCR analyses for *Bd* and ranavirus

Skin swab samples for *Bd* Taqman qPCR were analyzed using the methods, primers, and probe of Boyle et al. (2004), with modifications as previously described (Jones et al. 2012). Samples were analyzed in triplicate and scored as positive, equivocal, or negative (Hyatt et al. 2007). Positive samples tested positive in 2 or more wells; equivocal samples tested positive in 1/3 wells; and negative samples were negative in 3/3 wells. Oral swab samples for ranavirus were also analyzed through Taqman qPCR, using the primers and probe of Pallister et al. (2007) with modifications as previously described (Cheng et al. 2014).

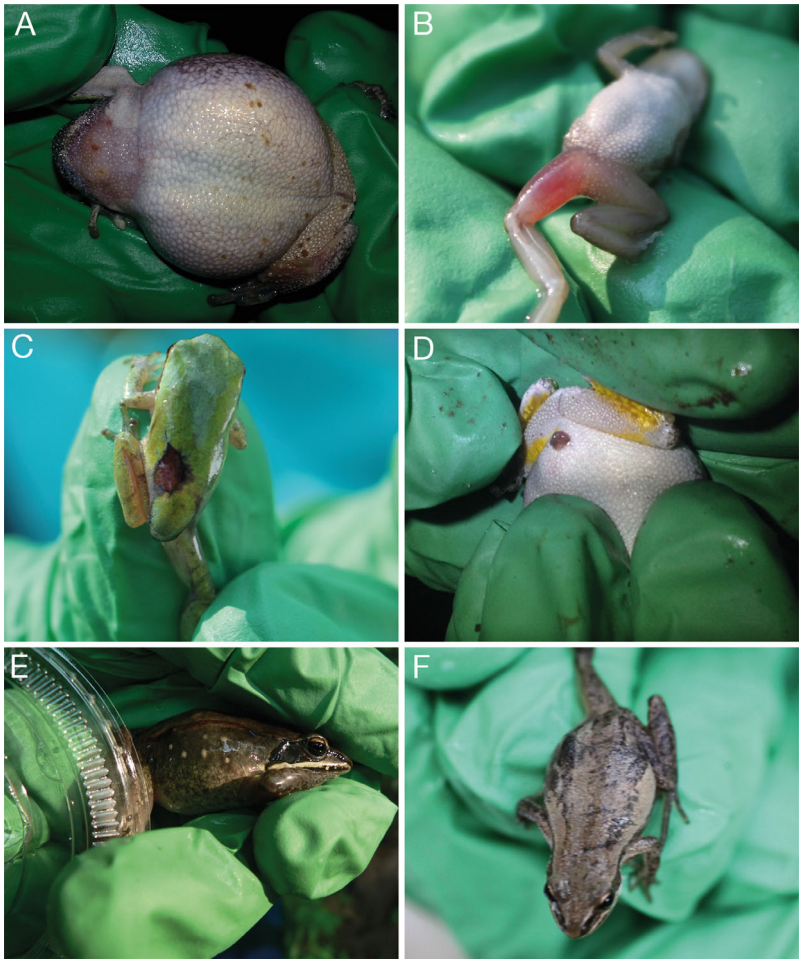

Fig. 2. Examples of clinical signs of disease, including bloating, swelling, discoloration, lacerations, and morphological abnormalities: (A) Bloating: gray treefrog *Hyla versicolor/chrysoscelis*. (B) Swelling: hindlimb edema in a boreal chorus frog *Pseudacris maculata/triseriata*. (C) Discoloration: dermal ulcer in a gray treefrog. (D) Morphological abnormalities: ventral hernia in a gray treefrog. (E) Morphological abnormalities: raised lateral spots on a wood frog *Lithobates sylvaticus*. (F) Lacerations: dorsal lacerations on a boreal chorus frog

Statistical analyses

To evaluate factors associated with pathogen detection, we built multivariate mixed regression models. Samples that were reported as equivocal by the Amphibian Disease Laboratory were characterized as positive for these analyses. This decision was based on appropriate performance of all qPCR controls for DNA contamination suggesting that the equivocal results were due to a low number of *Bd* zoospores in the original sample (Hyatt et al. 2007). Ammonia data did not meet the normal distribution assumption and were log transformed for analyses. To deal with a large number of 0-level detection data within the ammonia data set that remained skewed toward very low values even after transformation, we analyzed $\log^{10}$ (measurement + F), where F is the 2.5^{th} percentile of the positive measurements; F is needed to avoid taking the logarithm of 0.

Multivariate mixed logistic regression models were created as generalized estimating equations (GEEs) through PROC GENMOD in SAS software, Version 9.4 of the SAS System for Windows (© 2002–2012, SAS Institute). Three sets of models were created with *Bd* detection, ranavirus detection, and co-detection as dependent variables. In all models, we included a nested season and wetland variable as a random effect to account for repeated sampling in wetlands within seasons, resulting in a 20-level random effect variable. The independent correlation matrix was specified as the working covariance structure. Species was considered a confounding variable due to an *a priori* understanding of its association with pathogen susceptibility and season (e.g. distribution in habitat, and thus researchers' ability to locate, can vary based on the seasonal behavior of different species) (Rosenblum et al. 2010). Thus, species was included as a fixed effect in all multivariate models. Fixed effects that were varied by model included season, wetland, capture year, ammonia, and an interaction term for species and season. We used a forward stepwise approach to fitting our models; best models were selected using the lowest quasi-likelihood under the independence model criterion (QICu) (Cui 2007).

GEEs were also used to examine associations between clinical signs and pathogen detection. Models were structured as above, but the following clinical sign variables were modeled individually as binomial predictors of *Bd*, ranavirus, or co-detection: bloating, swelling, lacerations, discoloration, and morphologic abnormality. A final clinical sign variable was created to represent the presence of any combination of the former clinical signs and modeled as an ordinal variable, with '0' as absence of clinical signs, '1' as mild to moderate clinical signs, and '2' as moribund or dead. Species was included as a confound-

ing variable based on a significant association with pathogen detection in the previous models and the *a priori* understanding that the detection sensitivity of the specified clinical signs by researchers in the field may vary by species.

RESULTS

In 2014, wetland ammonia values ranged from 0.02–0.94 mg l^{-1}, and in 2015, values ranged from 0–1 mg l^{-1}. During the 2014 and 2015 field seasons, 259 and 255 post-metamorphic frogs were sampled, respectively; data represented both adult and juvenile frogs. Both ranavirus and *Bd* were detected in all species sampled, during all seasons sampled, in both 2014 and 2015 (Table 1). In 2014, 36% (95% CI = 30–42%, n = 259) of samples tested positive for *Bd*, while 33% (95% CI = 28–39%, n = 255) tested positive for *Bd* in 2015. There were 3 models of factors associated with *Bd* detection that fit the data best (ΔQICu < 2) and differed by only a single additional variable (year or ammonia). Among these 3, the best (QICu = 456.66) was the most parsimonious and included species, season, the season × species interaction term, and wetland as predictors (Table 2). This model revealed significantly higher odds of detecting *Bd* in spring and fall than in summer (χ^2 = 55.4, df = 2, p < 0.0001). Gray treefrogs had significantly lower odds of *Bd* detection than boreal chorus frogs or wood frogs (χ^2 = 117.85, df = 2, p < 0.0001), and the interaction between season and species (χ^2 = 12.43, df = 4, p = 0.0145) was significant. Finally, wetland was also a significant predictor of *Bd* detection in frogs (χ^2 = 50.46, df = 6, p < 0.0001).

Ranavirus was detected in 60% (95% CI = 54–66%, n = 259) of samples in 2014, and 18% (95% CI = 13–23%, n = 255) in 2015 (Table 1). Among the ranavirus models, the best model (QICu = 575.63, ΔQICu >2) included species, wetland, year, and ammonia as predictors (Table 3). Although this model demonstrated a significantly better fit to the data, year was the only variable that approached significance as a predictor of detection (χ^2 = 3.61, df = 1, p = 0.0574), with odds of ranavirus detection 7.6 times higher in 2014 than 2015 (95% CI = 0.94–61.9).

Both pathogens were detected in 26% (95% CI = 21–32%, n = 259) of samples in 2014, and 2% (95% CI = 1–5%, n = 255) of samples in 2015. Of the models examining factors in association with detection of

Table 1. Detection prevalence (%) of *Batrachochytrium dendrobatidis* (*Bd*) and ranavirus, and co-detection of both pathogens, by season and species in Dakota County, MN (USA), in 2014 and 2015. Prevalence ranges given in 95% confidence intervals

Variable	Year	n	*Bd*	Ranavirus	Co-detection
Season					
Spring	2014	69	72 (61–82)	90 (81–95)	67 (55–77)
	2015	106	57 (47–66)	4 (1–9)	0 (0–4)
Summer	2014	96	20 (13–29)	66 (56–74)	17 (11–25)
	2015	97	16 (10–25)	3 (1–9)	0 (0–4)
Fall	2014	94	24 (17–34)	32 (23–42)	6 (3–13)
	2015	52	17 (9–30)	73 (60–83)	10 (4–21)
Species					
Boreal chorus frog	2014	67	43 (32–55)	57 (45–68)	19 (12–30)
	2015	86	63 (52–72)	8 (4–16)	3 (1–10)
Gray treefrog	2014	135	16 (11–23)	51 (43–59)	15 (10–22)
	2015	125	15 (10–23)	26 (19–35)	1 (0–4)
Wood frog	2014	57	72 (59–82)	84 (73–91)	61 (48–73)
	2015	44	27 (16–42)	11 (5–24)	2 (0–12)
All samples	2014	259	36 (30–42)	60 (54–66)	26 (21–32)
	2015	255	33 (28–39)	18 (13–23)	2 (1–5)

Table 2. Results of best fitting multivariate generalized estimating equations model of environmental and species factors for association with *Batrachochytrium dendrobatidis* detection (n = 514). OR: odds ratio; measures of association are adjusted for correlation within seasons and wetlands by inclusion of a season–wetland random effect variable. *Significant at alpha = 0.05

Independent variable	OR	95% CI	p	Coefficient	SE
Species					
Gray treefrog	0.09*	0.04–0.19	<0.0001	−1.81	0.53
Boreal chorus frog	1.14	0.43–2.98	0.7970	0.09	0.44
Wood frog	0	0	0	0	0
Boreal chorus frog vs. Gray treefrog[a]	12.756*	7.06–23.05	<0.0001		
Season					
Spring	17.53*	7.78–39.51	<0.0001	2.26	0.31
Fall	2.71*	1.19–6.22	0.0181	2.17	0.83
Summer	0	0	0	0	0
Fall vs. Spring[a]	0.15*	0.08–0.31	<0.0001		
Species × Season			0.0145		
Wetland			<0.0001		

[a]These figures represent the differences of the least squares means between the 2 named variables

Table 3. Results of best fitting multivariate generalized estimating equations model of environmental and species factors for association with ranavirus detection (n = 514). OR: odds ratio; measures of association are adjusted for correlation within seasons and wetlands by inclusion of a season–wetland random effect variable

Independent variable	OR	95% CI	p	Coefficient	SE
Ammonia[a,b]	1.25	0.77–2.05	0.3710	0.22	0.25
Year					
2014	7.62	0.94–61.88	0.0574	2.03	1.07
2015	0	0	0	0	0
Species					
Gray treefrog	0.67	0.30–1.49	0.3234	–0.41	0.41
Boreal chorus frog	0.44	0.12–1.64	0.2233	–0.81	0.67
Wood frog	0	0	0	0	0
Boreal chorus vs. Gray treefrog[b]	0.67	0.31–1.41	0.2906		
Wetland			0.2788		

[a]Two-fold change in effect
[b]These figures represent the differences of the least squares means between the 2 named factors

both pathogens, 2 models performed similarly well (ΔQICu < 2), the difference between the 2 being the inclusion of ammonia. The best model (QICu = 277.5) included species, season, the species × season interaction, year, and ammonia (Table 4). According to this model, gray treefrogs and boreal chorus frogs had significantly lower odds of pathogen co-detection than wood frogs (χ^2 = 19.6, df = 2, p < 0.0001). There were significantly higher odds of co-detection in spring than in summer and fall (χ^2 = 8.7, df = 2, p = 0.0126), and the interaction between species and season was significant (χ^2 = 16.3, df = 4, p = 0.0027). There were significantly higher odds of co-detection in frogs screened in 2014 than 2015 (χ^2 = 6.5, df = 1, p = 0.0108). Increases in ammonia (χ^2 = 7.3, df = 1, p = 0.0068) were associated with significantly increased odds of detecting both ranavirus and *Bd*.

Clinical signs were noted in 82 animals over both study years. Of these, 2 displayed bloating, 7 had swelling, 10 had lacerations, 49 had discoloration of the skin, 14 demonstrated morphological abnormality, and 9 were found dead or moribund. In 2014, 8 post-metamorphic frogs were found dead or moribund at capture; in 2015, we found 1 dead frog. Of the 8 dead and moribund frogs collected in 2014, 6 were histologically examined. Ranavirus was detected in 4 of these individuals through PCR analysis, and 2 of these, both wood frogs, had histopathologic lesions consistent with significant ranaviral disease. These lesions included splenic congestion and hemorrhage with basophilic intracytoplasmic inclusion bodies in reticuloendothelial cells, hemorrhage in the gastrointestinal tract, and varying degrees of multifocal hepatocellular and epidermal necrosis with similar inclusion bodies. *Bd* was detected in 1 dead wood frog through PCR, and histologically, this individual had mild to moderate epidermal hyperplasia and hyperkeratosis with intralesional chytrid fungal thalli consistent with *Bd* (chytridiomycosis). A dead boreal chorus frog was PCR negative for both *Bd* and ranavirus, and death was attributed to acute traumatic injury to a leg. A dead gray treefrog, PCR negative for *Bd* and positive for ranavirus, had atrophy of the gonadal fat bodies and small to moderate numbers of echinostome-type trematode parasites

Table 4. Results of best fitting multivariate generalized estimating equations model of environmental and species factors for association with co-detection of *Batrachochytrium dendrobatidis* and ranavirus (n = 514). OR: Odds ratio; measures of association are adjusted for correlation within seasons and wetlands by inclusion of a season–wetland random effect variable. *Significant at alpha = 0.05

Independent variable	OR	95% CI	p	Coefficient	SE
Ammonia[a]	1.32*	1.08–1.61	0.0068	0.28	0.10
Year					
2014	19.42*	1.99–189.94	0.0108	2.97	1.16
2015	0	0	0	0	0
Species					
Gray treefrog	0.14*	0.06–0.34	<0.0001	–1.87	0.79
Boreal chorus frog	0.31*	0.14–0.71	0.0055	0.26	0.41
Wood frog	0	0	0	0	0
Boreal chorus frog vs. Gray treefrog[b]	2.19*	1.12–4.28	0.0221		
Season					
Spring	5.90*	1.78–19.51	0.0037	2.65	0.34
Fall	1.17	0.36–3.81	0.7907	0.77	1.15
Summer	0	0	0	0	0
Fall vs. Spring[b]	0.20*	0.05–0.83	0.0272		
Species × Season			0.0027		

[a]Two-fold change in effect
[b]These figures represent the differences of the least squares means between the 2 named factors

in the kidney. Ranavirus was detected by PCR on the single individual found dead in 2015, but a sample for *Bd* was not obtained; no histological analysis was performed on this specimen because of its advanced state of autolysis.

To evaluate associations between clinical signs and pathogen detection, 509 frogs with complete data sets were included in the models. As species was significantly associated with the detection of *Bd* and co-detection, it was included as a confounding variable in these analyses, but not for ranavirus-only models, which included clinical signs. Because of the low number of frogs documented with bloating, associations with pathogen detection could not be estimated. No other clinical signs were associated with *Bd* detection, whereas discoloration was significantly associated with ranavirus detection. Frogs with discoloration had 1.5 times higher odds of ranavirus detection than frogs without (95% CI = 1.01–2.18). The presence of any clinical sign was also significantly associated with co-detection of *Bd* and ranavirus (χ^2 = 17.2, df = 2, p = 0.0002), and frogs with mild to moderate clinical signs had 1.9 times higher odds of co-detection than frogs without clinical signs (95% CI = 1.4–2.5).

DISCUSSION

Our results document relatively high detection prevalences of *Bd* compared to other surveys of apparently healthy anuran populations (Kriger & Hero 2007, Whitfield et al. 2012, Love et al. 2016, Warne et al. 2016). Comparison of ranavirus detection prevalence between 2014 and 2015 suggests a possible epidemic during the 2014 field season. Although mass mortalities were not detected, more dead or dying frogs were found in 2014 than in 2015, and the majority were associated with ranavirus either by qPCR detection or by observation of histopathologic lesions typical of ranaviriosis. During the 2014 field season, we also found a relatively high prevalence of co-detection compared to similar studies (Souza et al. 2012, Whitfield et al. 2013, Warne et al. 2016). We were surprised not to see clinical signs associated with *Bd* infection, given its association with osmotic dysregulation; however, skin discoloration may provide a potential syndromic indicator of ranavirus infection. These findings underscore the importance of monitoring both pathogen prevalence and population demographics, as population declines might be difficult to detect in the absence of observed mass mortality events, and such events are rare.

Seasonal and species-based differences in pathogen detection

We observed seasonal fluctuation in *Bd* detection, with the highest odds of detection in the spring, and slightly higher odds of detection in fall compared to summer. Similarly, odds of co-detection were significantly higher in spring compared to summer and fall; this trend was likely driven by the increased odds of *Bd* detection in spring. Increased *Bd* prevalence during cooler seasons has been reported in similar studies (i.e. Kriger & Hero 2007, Kinney et al. 2011, Whitfield et al. 2012, Rowley & Alford 2013); this pattern is likely a synergistic effect of increased *Bd* growth (Piotrowski et al. 2004) and amphibian immunosuppression (Bradley et al. 2002, Raffel et al. 2006), which both occur at cooler temperatures. The significant species × season interaction term in the *Bd* and co-detection models suggest that breeding phenology plays a role in in the transmission of *Bd* and ranavirus. Direct contact among individuals during breeding is likely the most efficient route of intraspecific *Bd* transmission, as individual zoospores travel approximately 2 cm or less in water before encysting (Piotrowski et al. 2004). This hypothesis is supported by a lower likelihood of *Bd* detection in gray treefrogs compared to wood frogs and boreal chorus frogs. In Minnesota, wood frogs and boreal chorus frogs breed early in the spring (March–April), while gray treefrogs breed in late spring and early summer (May–June). Thus, boreal chorus frogs and wood frogs are more likely to harbor *Bd* because they breed earlier in the spring, during optimal temperatures for *Bd* growth, and when amphibian immune systems may not be functioning optimally.

These species-based differences in *Bd* detection are consistent with previous studies showing that wood frogs are susceptible to *Bd* infection (Searle et al. 2011, Gahl et al. 2012), and that Pacific chorus frogs (Reeder et al. 2012) can serve as reservoirs of *Bd*. Differences in dermal microbial communities and immune response among the species sampled (Rollins-Smith et al. 2011) may contribute to the differences in odds of *Bd* detection. This pattern of detection may also be attributable to species-specific differences in habitat use. Gray treefrogs use elevated perches for foraging, calling, and shelter, whereas wood frogs and boreal chorus frogs do not. Dry, elevated perches would be expected to reduce *Bd* zoospore exposure (but see Kolby et al. 2015), as a previous study has shown decreased *Bd* prevalence in boreal toads using terrestrial habitat, compared to toads using aquatic habitat (Hossack et al. 2013).

Perches could also facilitate behavioral fever in gray treefrogs through basking, as has been seen in the congener *Hyla cinerea* and other anuran species (Kluger 1977, Richards-Zawacki 2010). From a conservation standpoint, ensuring adequate dry, warm refuges may be key to managing species impacted by *Bd*.

Effects of wetland pollution

Ammonia is the most toxic form of inorganic nitrogen in aquatic systems (Camargo & Alonso 2006), and our models show increased levels of ammonia to be associated with increased odds of detecting both pathogens concurrently. Ammonia naturally occurs in freshwater wetlands as waste from aquatic animals and decaying organic matter; however, agricultural and residential fertilizer treatments, livestock waste, and wastewater effluents can pollute wetlands through runoff (Marco & Ortiz-Santaliestra 2009). Many of the wetlands in our study system were located adjacent to residential areas, likely exposing them to lawn treatment runoff; the wetlands located on Minnesota Zoo grounds are also likely exposed to livestock waste runoff from animal exhibits. It is reasonable to hypothesize a synergistic relationship between ammonia and pathogens, perhaps through an increased challenge to the amphibian immune system. Previous studies have shown unionized ammonia to have detrimental effects on amphibian development at concentrations as low as 0.6 mg l^{-1} for some species (Jofre & Karasov 1999), and concentrations were much higher in several of our wetlands.

It is also possible that increased ammonia levels could be a secondary effect of increased levels of decaying animal tissue following the putative 2014 ranavirus epidemic. The significant relationship between wetland identity and *Bd* detection further supports the likelihood that factors such as wetland water chemistry might affect the prevalence of amphibian pathogens. Future studies should aim to collect longitudinal water chemistry data to help clarify the impact of water quality dynamics on the physiology and life history of amphibian pathogens.

Co-detection of *Bd* and ranavirus

Thus far, few studies have investigated the prevalence of *Bd* and ranavirus concurrently in wild post-metamorphic anurans (Whitfield et al. 2013, Love et al. 2016, Patla et al. 2016, Warne et al. 2016), and none have yet done so in the Midwestern region of North America. One of the most notable features of our dataset is the remarkable decline in co-detection from 2014 to 2015. The significantly lower odds of co-detection in 2015 is likely driven by the dramatically lower prevalence of ranavirus in the 2015 samples, compared to the 2014 dataset. Indeed, our finding that both *Bd* and ranavirus were detected in 61% of individual wood frogs sampled in 2014 is higher than any yet reported in wild amphibians, to our knowledge (Souza et al. 2012, Whitfield et al. 2013, Love et al. 2016, Warne et al. 2016). Further studies are needed to understand the effects of co-infection within individual frogs, as well as the population-level impacts of concurrent presence of both pathogens. Additional studies are also needed to validate the use of clinical signs as syndromic indicators of co-infection, as the significant association between co-detection and clinical signs detected by our model might be driven primarily by ranavirus.

Detecting potential epidemics

The striking decline in ranavirus detection from the highest prevalence in spring 2014 to low prevalence in spring and summer 2015, followed by a resurgence in fall 2015, suggests that we may have sampled through epidemic and inter-epidemic periods. A recent study has shown that PCR analysis of oral swabs taken from frogs experimentally infected with ranavirus does not detect the virus until advanced stages of disease; thus, PCR of oral swabs underestimates prevalence of ranavirus in asymptomatic animals, and the actual prevalence of ranavirus in our population was likely higher than we observed (Forzán et al. 2017). This, along with the documented ranavirus-associated mortalities, supports the likelihood of a 2014 epidemic.

Although mass mortalities of adults were not noted during the 2014 field season, it is possible that they occurred between sampling sessions, and therefore went unnoticed. Also, it is possible that mass mortalities of tadpoles, which are particularly susceptible to ranavirus outbreaks, may have occurred unnoticed (Miller et al. 2015). Because population declines are extremely difficult to detect in the absence of mortality events, which might go unobserved, frequent surveys incorporating population size estimates of adults and tadpoles are necessary for amphibian conservation. The results of our clinical sign data sug-

gest that discoloration may be a sentinel indicator of an outbreak; however, further validation of this syndromic sign is needed.

Few studies of *Bd* and ranavirus prevalence have paired estimates of population size and pathogen prevalence over time, and this information is also needed to determine endemic levels of these pathogens. Such studies are of utmost importance in the face of climate change and other anthropogenic changes, which have the potential to affect both amphibian behavior and pathogen ecology (Blaustein et al. 2001, 2010, Raffel et al. 2006, Woodhams et al. 2008). Monitoring programs would ideally couple seasonal molecular sampling with population abundance estimates, perhaps using an *N*-mixture model applied to spatially replicated count data (Royle 2004). However, because mass mortalities are rarely seen in the North American Midwest, amphibian pathogen monitoring has been a low priority for many conservation programs. Alternatively, increasingly popular citizen science-style anuran call surveys offer a cost-effective and efficient method of detecting relative shifts in population sizes and species richness, and historical data are also available through these programs (Shirose et al. 1997, Nelson & Graves 2004). Coupling citizen science call surveys with seasonal molecular pathogen sampling would present a cost-effective means for monitoring amphibian health, especially in areas where mass mortalities have not been reported.

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