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NOTE

Amphibian pathogens at northern latitudes: presence of chytrid fungus and ranavirus in northeastern Canada

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ABSTRACT: Infections by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) and members of the genus *Ranavirus* (Rv) are increasingly reported as significant determinants of amphibian population die-offs. The complexity associated with their transmission and spatial distribution leads to an increase in demand for comprehensive reporting systems and global mapping of their distribution. Here, we document the distribution of these 2 pathogens in a remote northern temperate lowland where environmental sensitivity is high, providing important insight into the pathogens' natural history and infection patterns. Wood frog *Lithobates sylvaticus* tissues were collected from the James Bay area in northeastern Canada and were screened for the presence of *Bd* and Rv using conventional and real-time PCR. Both pathogens were present in the study area, which is the northernmost record in eastern North America. Interestingly, different patterns of distribution were observed between the eastern and western coasts of James Bay, suggesting differences in the spatial and transmission dynamics for each pathogen. Anthropogenic introduction may still influence the distribution patterns observed, even at these latitudes. The presence of infections in this remote area also raises further questions on the risk these pathogens pose to northern amphibian communities. We encourage further research in remote locations for a better understanding of these pathogens, their transmission dynamics, and especially their respective impacts on amphibian populations worldwide.

KEY WORDS: Global amphibian decline · Emergent infectious diseases · Transmission dynamics · Hudson Bay lowlands · James Bay · Wood frog

INTRODUCTION

As part of the overall biodiversity crisis, many amphibian populations are in decline worldwide (Blaustein et al. 1994). The severity and large geographic scale of this decline in conjunction with the ecological importance of amphibians has been coined as one of the greatest conservation issues of the 21st century (Alford & Richards 1999, Daszak et al. 1999, Wake & Vredenburg 2008). Among the factors responsible for amphibian declines, emerging infectious diseases (EIDs), such as chytridiomycosis caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*), and ranaviral infections caused by members of the *Ranavirus* genus (Rv, family *Iridoviridae*) are increasingly reported as significant determinants of amphibian population die-offs (Gray et al. 2009, Kilpatrick et al. 2010, Miller et al. 2011, Lesbarrères et al. 2012).

Possible vectors have been investigated for both diseases, including contaminated water and amphibian trade (Brunner et al. 2004, 2007, Johnson &

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Speare 2005, Harp & Petranka 2006, Greer et al. 2008, Picco & Collins 2008, Nazir et al. 2012), and although transmission of the chytrid fungus and the ranavirus can be influenced by anthropogenic disruptions (St-Amour et al. 2008), such as habitat destruction and fragmentation (Phillips 1990), their presence in seemingly undisturbed areas led to alternate hypotheses about their transmission routes. For instance, migrating birds or other animals can act as vectors (Johnson & Speare 2005, Garmyn et al. 2012), and climate change has been shown to alter host and pathogen distributions (Parra-Olea et al. 2005, Pounds et al. 2006) as well as susceptibility and virulence (Rojas et al. 2005, Altizer et al. 2013). Comprehensive reporting systems of prevalence and global mapping of the distribution of *Bd* and ranaviral infections are thus key to identify catalysts of disease transmission (Olson et al. 2013).

The detection of infection by *Bd* and Rv in remote locations is cause for concern, as it suggests that the pathogens' transmission dynamics may be more complex than what we initially predicted (Gahl & Calhoun 2008). High latitudes in the northern hemisphere present such venues, where epidemiological studies are rare and even basic ecological knowledge is often lacking because of logistic constraints. *Bd* and Rv are present at high northern latitudes (Reeves 2008, Ariel et al. 2009, Reshetnikov et al. 2014), indicating the risk that *Bd* and Rv pose to these fragile amphibian communities. Species are often at the margin of their distribution range at high latitudes (Ariel et al. 2009, Patrelle et al. 2012), and peripheral populations often experience increased isolation, segmented distributions, and climatic conditions close to their physiological limit (Brooks 2000). These conditions likely make northern populations more susceptible to disease, and understanding pathogen–host dynamics at high latitudes becomes critical in light of the general biodiversity crisis.

In this study, we opportunistically conducted *Bd* and Rv screenings in the James Bay area, spanning across the Hudson Bay Lowlands, covering over 600 000 km^2 across the provinces of Ontario and Québec and the Nunavut territory in northeastern Canada. No publications have yet described the infection status of amphibian populations in this northern temperate lowland (but see Global *Bd*-Mapping Group [2013] for other reports of *Bd* infection statuses in southeastern Canada). We aimed at extending our knowledge of the pathogens' distribution in remote environments at high latitudes, thus increasing the predictive power of future models pertaining to the ecology, distribution, and evolution of EIDs (Ron 2005). To this end, we screened tissue samples from wood frogs *Lithobates sylvaticus*, a common species throughout the study area. The wood frog is susceptible to both the chytrid fungus and ranaviruses (Schock et al. 2010) and is likely one of the most susceptible species to ranaviral infection as compared to 18 other North American amphibian species (Hoverman et al. 2011). The wood frog is the most widespread anuran in Canada (MacCulloch 2002), and infection in this species could put other species with more restricted distributions at risk (Schock et al. 2010), in turn potentially influencing community stability (see Olson et al. 2013). Documenting the presence of these 2 EIDs across high-latitude landscapes thus becomes crucial to evaluate the threat on northern amphibian communities.

MATERIALS AND METHODS

Study area and sampling

Samples were collected between May 26 and August 14 in 2009 and between June 10 and August 15 in 2010. Each sampled site was only visited once during this time period because of logistical constraints. We covered 3 areas around James Bay in Canada (labelled hereafter Ontario, Québec, and Nunavut; see Table 1), from Peawanuck in Ontario to Radisson in Québec, sampling wood frog populations between these localities around the southern part of the bay (Fig. 1). Sampling sites were mostly accessible by road, with the exception of Akimiski Island in Nunavut (see Fig. 1; large island in James Bay by the coast of Attawapiskat, Ontario), where we travelled by helicopter and bush plane. Wood frogs were found opportunistically, walking through apparent suitable habitats (woodlots close to wetlands, humid grassy trails) and around the perimeter of possible breeding sites.

Collection and DNA isolation

A total of 480 wood frogs were sampled from wild populations in 17 localities around James Bay. Toe clips were collected from adults and juveniles (Laurentian University Animal Care protocol #2009-03-04), while the whole body of tadpoles was collected. Individuals were caught either using a small dip net or by hand. To prevent cross-contamination between individual samples, a new pair of nitrile gloves was used to capture each individual, and toe-clipping

Table 1. Sampled localities (see also Fig. 1). n = number of tested individuals, Prevalence (%) = prevalence of infection for *Batrachochytrium dendrobatidis* (*Bd*) and ranavirus (Rv) (no. of infected ind./no. tested × 100%), CI 95% = 95% confidence interval, Co-infection (%) = co-infection rate

Locality	Date visited	Coordinates Latitude (°N)	Coordinates Longitude (°W)	n	Life stage Adult	Life stage Juvenile	Life stage Tadpole	Prevalence (%) *Bd* (CI 95%)	Prevalence (%) Rv (CI 95%)	Co-infection (%)
Ontario										
Winisk River	Jul 2009	54.7358	85.2580	12	6	1	5	33.3 (14–61)	8.3 (1–35)	0
West Attawapiskat River	Jun 2010	52.8358	86.4948	7	5	1	1	0 (0–35)	0 (0–35)	0
Victor Mine	Aug 2010	52.8628	83.9312	3	2	1	0	0 (0–56)	0 (0–56)	0
Attawapiskat	Jun 2010	52.9301	82.4471	29	9	7	13	17.2 (8–34)	0 (0–12)	0
Fort Albany	Jun 2010	52.2160	81.6854	30	5	9	16	10 (3–26)	0 (0–11)	0
Kenogami River	Jul 2009	50.3268	84.4478	19	19	0	0	36.8 (19–59)	0 (0–17)	0
Moosonee	Jun 2010	51.2692	80.6491	44	12	3	29	13.6 (6–27)	6.8 (2–18)	2.3
Missisicabi River	Jul 2009	51.2121	79.6586	6	6	0	0	0 (0–39)	0 (0–39)	0
Nunavut										
Akimiski Island West Point	Jun 2009	53.0245	81.9933	17	2	0	15	0 (0–18)	0 (0–18)	0
Akimiski Island Research Area	Jun 2009	53.1026	81.0294	172	68	25	79	8.1 (5–13)	8.1 (5–13)	0.6
Akimiski Island East	Jun 2009	52.9436	80.8152	13	5	0	8	7.7 (1–33)	0 (0–23)	0
Québec										
Matagami	Jul 2009	49.8871	76.9922	5	1	4	0	60 (23–88)	40 (12–77)	20
Waskaganish	Jul 2009	51.4681	78.7793	14	6	4	4	35.7 (16–61)	7.1 (1–32)	0
Eastmain	Aug 2009	52.2137	78.5065	32	8	20	4	25 (13–42)	12.5 (5–28)	6.3
Wemindji	Aug 2009	52.9818	78.6714	35	25	9	1	51.4 (36–67)	5.7 (2–19)	2.9
Chisasibi	Aug 2009	53.7139	78.8214	24	0	21	3	8.3 (2–26)	8.3 (2–26)	0
Radisson	Aug 2009	53.6840	77.7307	20	3	16	1	40 (22–61)	20 (8–42)	10
Total				482				17.4	6.8	1.7

equipment was disinfected with 95% ethanol between each use, which is a limitation of the study. To confidently avoid potential cross-contamination between samples, bleach rather than ethanol should be used, but because of the remoteness of the area and the associated logistics, we used ethanol in the wetlands in fear of harming wildlife with any spill and only used bleach at base camp, where it could be disposed of safely. All gear, nets, and boots were disinfected in a 15% bleach solution between sites (Phillott et al. 2010). Tissue samples were preserved in 95% ethanol and then stored at –20°C until laboratory analysis. DNA was subsequently extracted using Qiagen's DNeasy blood and tissue kit, according to the manufacturer's instructions.

Bd DNA screening

Presence of *Bd* was assessed using quantitative PCR (qPCR) assays in a reaction volume of 10 µl (Kriger et al. 2006, Piovia-Scott et al. 2011), according to the protocol by Boyle et al. (2004). Double-blind screenings were done for each sample, with a third screening to confirm infection in case of discrepancies between the first 2 results (Kriger et al. 2006). Standard curves were not constructed; thus, our results only reflected the presence or absence of *Bd* infection (Voordouw et al. 2010). To optimize sensitivity to fluorescence, white-well qPCR plates were used (Eppendorf twin.tec #0030132726).

Rv DNA screening

To check for Rv infection, a double-blind PCR was performed using a primer pair known to successfully amplify a portion of the major capsid protein within the frog virus 3 genome: MCP-ranavirus-F (5′-GAC TTG GCC ACT TAT GAC-3′) and MCP-ranavirus-R (5′-GTC TCT GGA GAA GAA GAA-3′) (Mao et al. 1997). We followed the PCR conditions listed in Mao et al. (1997), using 1.5 µl of template DNA, cycled 40 times. Rv-positive samples were determined by the

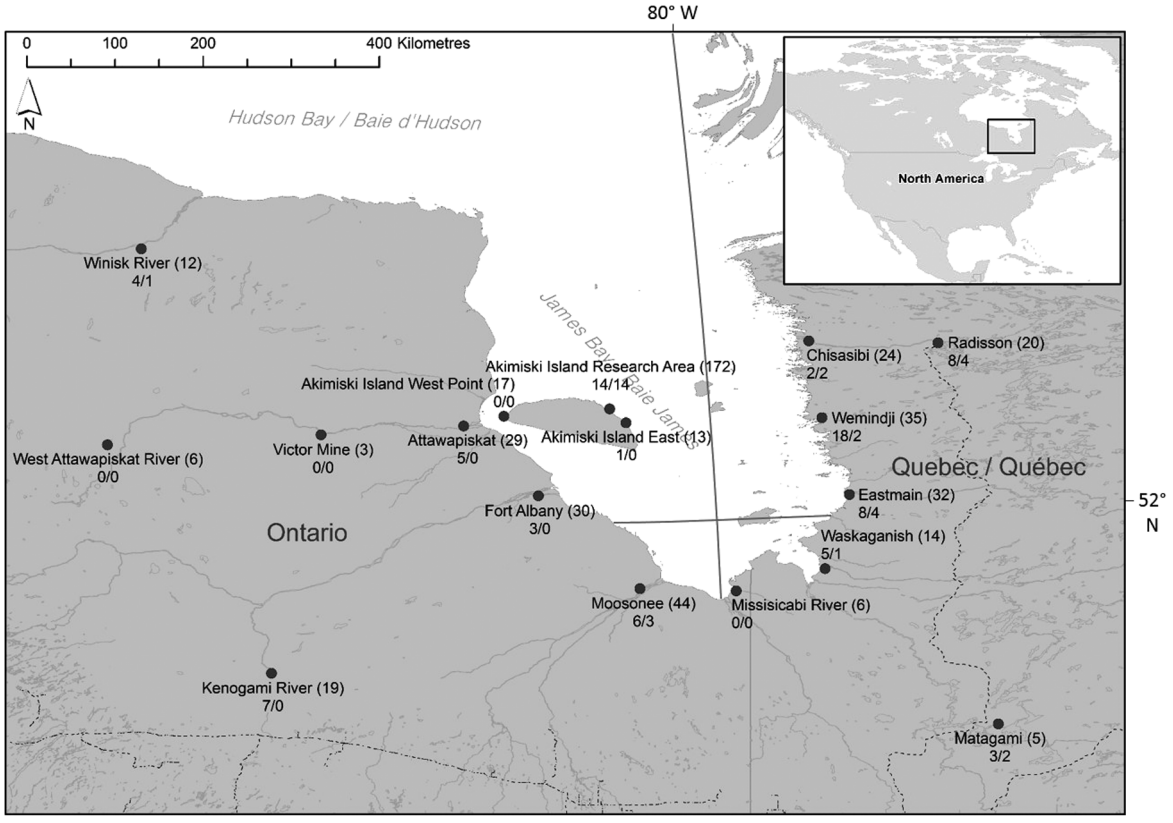

Fig. 1. Study area. The numbers in brackets beside each locality name indicate the number of screened individuals. The numbers under each name indicate the numbers of individuals that screened positive for chytrid fungus and ranaviral infections, respectively. Note that the total number of infected individuals is not always equal to the total number of screened individuals, as some localities had co-infected individuals (i.e. individuals that screened positive for chytrid fungus and ranaviral infections). Highways are represented by thin dotted lines

presence of a 500 bp band, and a sample known to be infected was used as a positive control (St-Amour et al. 2008). Only individuals showing 2 positive amplifications were considered infected.

Statistical comparisons

While prevalence data were not normally distributed, they met the homogeneity of the variance (Levene statistic, p = 0.25 and p = 0.23 for *Bd* and Rv, respectively). A Kruskal-Wallis test was thus used to analyse differences in prevalence among the 3 areas of sampling, and significance was assessed with the exact method. Mann-Whitney post hoc tests without correction were used for specific comparisons between areas. Finally, a Fisher's exact probability test was used to assess the likelihood that individuals would be co-infected by chance alone.

RESULTS

Bd was detected in 17.4% (84 samples) of all individuals tested. Of the 17 sampled localities, 13 tested positive for chytrid fungal infection (5 in Ontario, 6 in Québec, and 2 in Nunavut; Fig. 1), and we detected differences in prevalence among the 3 main areas of sampling (Kruskal-Wallis, $H = 7.12$, df = 2, p = 0.02). Prevalence of *Bd* reached 36.75% among all localities sampled in Québec, with 3 localities showing a prevalence higher than 40% (Table 1). *Bd* was also prevalent in Ontario but with a significantly lower prevalence (13.88%, Mann-Whitney test, p = 0.046 for Québec vs. Ontario). The lowest prevalence of chytrid infection was observed on Akimiski Island (7.43%, Mann-Whitney test, p = 0.02 for Québec vs. Nunavut). No difference was observed between the prevalence of *Bd* in Nunavut and the Ontario side of James Bay. Some of our localities had a small sample size (Table 1), but according to Skerratt et al. (2008),

only 3 of our localities fall short of the numbers required to have a 95% confidence in detecting at least 1 positive frog with our observed prevalence (minimum N = 17): West Attawapiskat River, Victor Mine, and Missisicabi River.

Ranavirus infection was detected in 6.8% (33 samples) of all individuals tested (Table 1). Of the 17 sampled localities, 9 tested positive for Rv (2 in Ontario, 6 in Québec, and 1 in Nunavut; Fig. 1), and we detected differences in prevalence among the 3 main areas of sampling (Kruskal-Wallis, H = 7.68, df = 2, p = 0.022). All sampled localities in Québec had at least 1 ranavirus-infected individual, for an average prevalence of 15.62%, on the eastern side of James Bay. Ranaviruses were also present on Akimiski Island but with significantly lower prevalence (2.51%, Mann-Whitney test, p = 0.05 for Québec vs. Nunavut). The lowest prevalence was detected on the Ontario coast of James Bay, with only 1.89% (Mann-Whitney test, p = 0.01 for Québec vs. Ontario) of screened individuals scoring positive and 2 of 8 localities presenting at most 3 infected individuals. No difference was observed between the prevalence of Rv in Nunavut and on the Ontario side of James Bay.

Overall, 109 of 482 (22.6%) individuals tested positive for one of the diseases, and 8 individuals (1.7%) tested positive for co-infection (Table 1). This number does not deviate significantly from the prevalence expected by chance alone (1.2%, p = 0.34; 2-tailed Fisher's exact probability test), given the overall prevalences for *Bd* and Rv in our study. Of these 8 co-infected individuals, 6 were located in Québec, while Ontario and Nunavut each had 1 individual carrying both pathogens. In 4 of the 17 screened localities, no infection was detected, while 4 localities contained individuals infected by only one of the pathogens, and 9 localities presented both ranaviral and chytrid infections. Notably, no visible signs of ranaviral or chytrid infections were observed on any captured or sighted amphibian.

DISCUSSION

The chytrid fungus was detected throughout the study area, with the exception of 3 localities northwest and 1 locality in the southern part of the bay. These 4 localities are in fact the only ones where neither pathogen was detected. Despite a limited sample size in some localities (West Attawapiskat River, Victor Mine, and Missisicabi River), most presented a large enough sample size to confidently assess prevalence (Skerratt et al. 2008). By contrast, Rv infections were more sporadic and few were detected west of the bay. While every locality on the Québec side tested positive for ranaviral infection, only 3 of the 11 localities in Ontario and Nunavut did. This discrepancy between both coasts may result from different transmission methods between both pathogens in that environment, historical contingencies related to pathogen introduction, or life history phenological differences between coasts for pathogens or hosts. One hypothesis is that the distribution of pathogens may be facilitated by direct anthropogenic introductions through potential hosts (St-Amour et al. 2008). On the western coast of James Bay, localities are very isolated (no road or railway connects them in the aestival season), and flights are costly and limited, thus reducing contact between these localities. By contrast, the eastern coast localities were linked in 2002 by the James Bay Highway, a 620 km paved highway starting in Matagami and ending in Radisson, just south of Hudson Bay in the province of Québec. This highway is very popular for fishing enthusiasts, and multiple camping stops and boat launches decorate the rivers' estuaries. Human involvement in the spread of pathogens has already been documented (Garner et al. 2005, Jancovich et al. 2005, Fisher & Garner 2007, Phillott et al. 2010). In wild tiger salamanders *Ambystoma tigrinum* from the western USA, ranavirus isolates were more closely related to sport fish strains than to amphibian strains, and strains cultured in salamanders in bait shops in Phoenix, Arizona, were also found in the wild (Jancovich et al. 2005). The importation of amphibians from other, possibly infected, sources up the James Bay Highway may act as a transmission vector, creating the dissimilar patterns of infection we observed between the western and eastern coasts of the bay. With the possibility of a new highway connecting communities up the Ontario coast of James Bay, steps to minimize risk associated with importing infected individuals need to be implemented.

Both amphibian pathogens were detected in the James Bay area, including in the territory of Nunavut (Akimiski Island, 53.0008° N, 81.3246° W). While further efforts would be needed to indeed confirm absence of *Bd* and Rv in localities with low sample sizes, this study adds the northernmost record for these pathogens in eastern North America (but see Schock et al. 2010 for results from the Northwest Territories) and extends our knowledge about the risk that *Bd* and Rv infections could pose to northern amphibian communities. Throughout our field work, we also encountered other species of amphibians

such as spring peepers *Pseudacris crucifer*, American toads *Anaxyrus americanus*, boreal chorus frogs *P. maculata* (only encountered in Ontario and on Akimiski Island), mink frogs *Lithobates septentrionalis* (encountered in Québec), 1 northern leopard frog *L. pipiens* (encountered near the Kenogami River locality), and blue-spotted salamanders *Ambystoma laterale* (encountered in Québec). These sightings were opportunistic and in low numbers, the most abundant species encountered by far being the wood frog. All of these species are susceptible to infection by *Bd* or Rv (Green et al. 2002, Greer et al. 2005, Schock et al. 2010, Voordouw et al. 2010, Gahl et al. 2012, Goodman & Ararso 2012), and some could potentially act as vectors to the diseases (Gahl et al. 2012). Our results thus open the door to further research on community-level effects of both pathogens at high latitudes. These locations can in fact provide an ideal platform to study these dynamics, as the relatively low levels of biodiversity can simplify epidemiological models and provide important insight into the pathogens' natural history and transmission dynamics (Schock et al. 2010).

Mapping out the distribution of *Bd* and Rv is the first step to our understanding of the ecology and epidemiology of these amphibian pathogens. However, one caveat of the current distribution assessment is their inherent association with scientists' interests, historical research locations, and practical logistics. With increasing screening outside biodiversity hotspots, we will better identify the potential vectors and transmission corridors of these pathogens and propose and implement conservation strategies to secure regional biodiversity (Olson et al. 2013). Our results suggest that anthropogenic introduction is a possible cause of the distribution patterns observed in the area, and future studies should more consistently address the presence of both pathogens and their distribution if we are to understand their respective impact on population dynamics. With local members of isolated Cree communities around James Bay confirming the decrease in frog calls since their childhoods (F. Wesley pers. comm.), amphibian ecological research in this area may unveil important information about the health and decline of amphibian populations worldwide, especially in remote and pristine areas.

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