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NOTE

Low occurrence of ranavirus in the Prairie Pothole Region of Montana and North Dakota (USA) contrasts with prior surveys

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ABSTRACT: Ranaviruses are emerging pathogens that have caused mortality events in amphibians worldwide. Despite the negative effects of ranaviruses on amphibian populations, monitoring efforts are still lacking in many areas, including in the Prairie Pothole Region (PPR) of North America. Some PPR wetlands in Montana and North Dakota (USA) have been contaminated by energy-related saline wastewaters, and increased salinity has been linked to greater severity of ranavirus infections. In 2017, we tested tissues from larvae collected at 7 wetlands that ranged in salinity from 26 to 4103 mg Cl l^{-1}. In 2019, we used environmental DNA (eDNA) to test for ranaviruses in 30 wetlands that ranged in salinity from 26 to 11 754 mg Cl l^{-1}. A previous study (2013–2014) found that ranavirus-infected amphibians were common across North Dakota, including in some wetlands near our study area. Overall, only 1 larva tested positive for ranavirus infection, and we did not detect ranavirus in any eDNA samples. There are several potential reasons why we found so little evidence of ranaviruses, including low larval sample sizes, mismatch between sampling and disease occurrence, larger pore size of our eDNA filters, temporal variation in outbreaks, low host abundance, or low occurrence or prevalence of ranaviruses in the wetlands we sampled. We suggest future monitoring efforts be conducted to better understand the occurrence and prevalence of ranaviruses within the PPR.

KEY WORDS: Pathogens · Contaminants · Salinity · Amphibians

1. INTRODUCTION

Ranaviruses (family *Iridoviridae*) are emerging pathogens that infect amphibians, reptiles, and fishes and have been associated with amphibian mortality events worldwide (Duffus et al. 2015). Ranaviruses are globally widespread, have been detected in amphibians in more than 25 countries, and have been reported in most states in the USA (Duffus et al. 2015). However, ranaviruses are likely more widespread than reported because monitoring efforts are lacking in some regions, amphibians can be rare or cryptic, signs of infection can be missed, and mortality events and decomposition of amphibians can occur quickly (Brunner et al. 2015, Miller et al. 2015).

One area where ranavirus data are scarce is in North America's Prairie Pothole Region (PPR), which has thousands of potholes (i.e. wetlands) and is

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affected by anthropogenic activities such as farming and energy extraction. The high density of wetlands and connectivity among populations in the PPR could increase transmission and occurrence of ranaviruses (Gray et al. 2004, Greer & Collins 2008, Firkins 2015). Transmission and occurrence of ranaviruses could also be affected by habitat degradation or chemical contamination, which can increase physiological stress, reduce immune responses, and increase susceptibility to diseases (Greer & Collins 2008, Hall et al. 2020). In the eastern USA, salinity stress from road salt contamination can increase the severity of ranavirus epidemics (Hall et al. 2020). In the PPR, energy extraction over the past 50 yr and accidental spills of high-salinity wastewaters (hereafter, wastewaters) have contaminated surface waters there (Gleason & Tangen 2014), suggesting the potential for the environment to increase disease risk.

Surveys for ranaviruses are lacking in the PPR, including in Montana and North Dakota (USA). A few studies have detected ranaviruses in the PPR of South Dakota (USA) and Saskatchewan and Alberta (Canada) (Bollinger et al. 1999, Vilaça et al. 2019, Davis et al. 2020). Only one study in Montana has reported verified ranavirus infections, and it was from a montane environment >500 km from the PPR (Patla et al. 2016). In North Dakota, a comprehensive state-wide survey during 2013–2014 revealed that ranaviruses infected multiple amphibian species and ranaviruses were prevalent throughout the state (35% of amphibians tested and 56% of sites with at least one infected amphibian; Firkins 2015). However, we are unaware of studies of variation in ranavirus occurrence across a large salinity gradient where amphibians occur (e.g. <26–4103 mg Cl l^{-1}), which is common in the PPR (Gleason & Tangen 2014, Hossack et al. 2018). With the goal of gaining insight into the potential influence of salinity on ranavirus dynamics, we sampled larvae of 3 amphibian species from wetlands in 2017 and sampled water from 30 wetlands across a salinity range of <26–11 754 mg Cl l^{-1} in 2019 to test for environmental DNA (eDNA) evidence of ranavirus.

2. MATERIALS AND METHODS

2.1. Study species and sample collection

Three habitat-generalist amphibian species occur throughout the study area (Fig. 1): boreal chorus frogs *Pseudacris maculata*, northern leopard frogs *Lithobates pipiens*, and barred tiger salamanders (also known as western tiger salamanders) *Ambystoma mavortium*. Abundance of each species in wetlands is affected by contamination from wastewaters (Hossack et al. 2018). Although wood frogs (*Lithobates sylvatica*) can occur at the edge of the study area (e.g. in Lostwood National Wildlife Refuge), they are rare and were not sampled.

In 2017, we used dipnets in mid-June to early July to collect 1–9 larvae of each species that occurred in each of 7 wetlands (3–18 larvae per wetland collectively; Fig. 1, Table 1). We sampled larvae across a gradient of wastewater contamination. Larvae were collected for a separate study wherein interrenal glands were dissected (Tornabene et al. 2021), and we opportunistically sampled these larvae for ranavirus infection. We tested salinity of wetlands in 2017 and 2019 using Hach QuanTab chloride titration strips with a lower limit of detection of 26 mg Cl l^{-1} (±10% accuracy).

In late June 2019, we expanded our efforts and collected water samples from 30 wetlands to assay for ranavirus DNA (Fig. 1, Table 1). eDNA methods provide a noninvasive method of assaying for ranavirus, and eDNA concentrations of ranavirus have been found to be highly correlated with ranavirus infection severity of individuals (Hall et al. 2016). We collected 3 replicate filter samples from a pooled water sample at each wetland and negative field controls (Goldberg et al. 2018). Prior to use, we sterilized 1 l polypropylene bottles with ≥10% bleach and triple rinsed with tap water. To collect a pooled water sample, we triple rinsed bottles with site water and then collected equal amounts of water at each of ≥4 points in each wetland. Water samples were placed in coolers with ice, filtered within 24 h using single-use filter funnels (47 mm, 5 µm mixed cellulose ester filters; Millipore SMWP04700), then preserved in 1 ml of 99% ethanol. We used 5 µm filters because we assayed for multiple pathogens (B. R. Hossack and C. S. Goldberg unpubl. data) and to maximize volume of water filtered at our study sites with high turbidity and organic matter (Barnes et al. 2021). Per-filter volumes ranged from 300 to 1000 ml (mean ± SD = 741 ± 123 ml; Table 1).

2.2. Ranavirus DNA extraction and quantification

For larval tissue samples collected in 2017, we dissected kidney, liver, and spleen from each larva using flame-sterilized tools and pooled tissues. We assayed for ranavirus infection in pooled tissues following the methods of Hall et al. (2020). Briefly, we

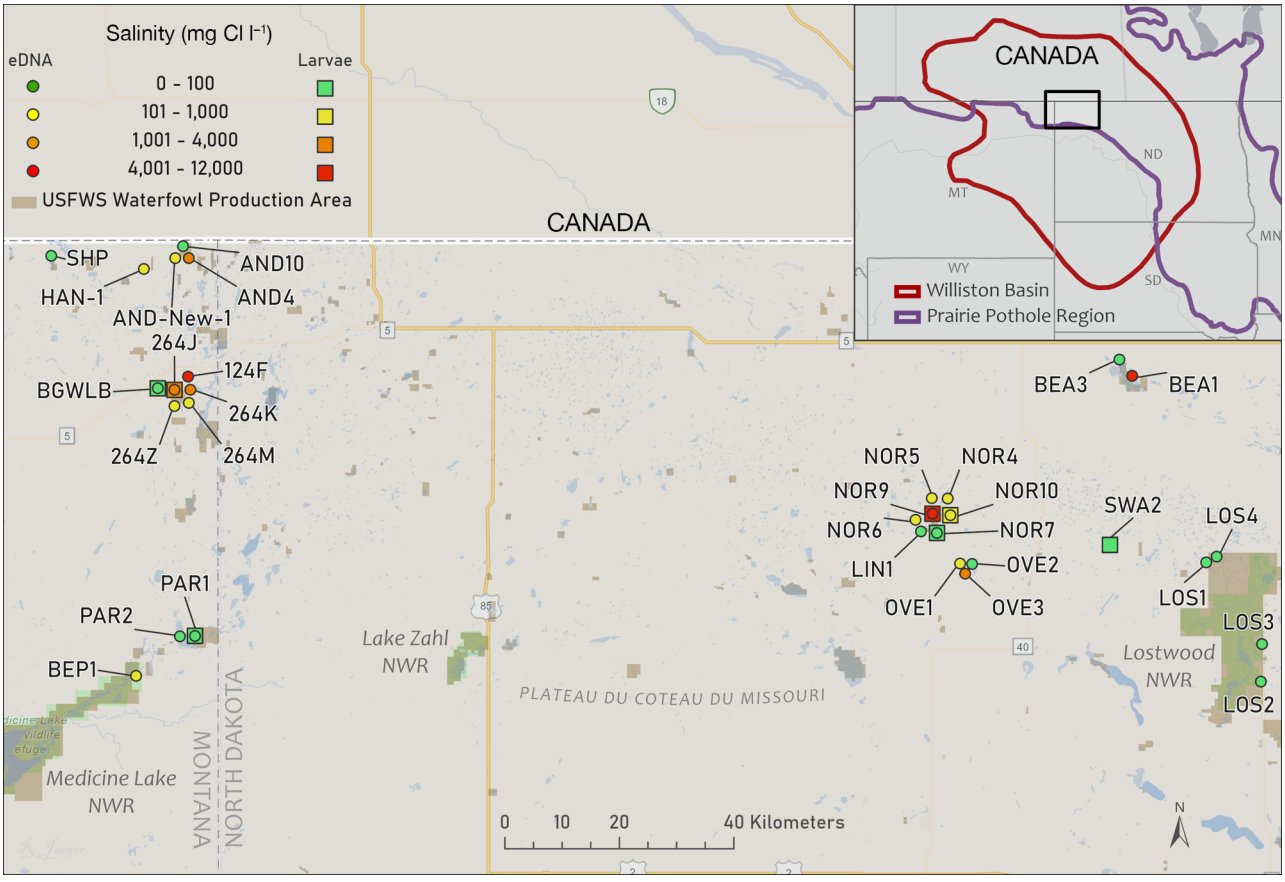

Fig. 1. Study area in Prairie Pothole Region of Montana and North Dakota, USA, where we collected larval tissue samples (squares) and eDNA (circles). No eDNA samples were positive for ranavirus, and only 1 larval barred tiger salamander was positive for ranavirus at site NOR9. See Table 1 for site attributes. USFWS: US Fish and Wildlife Service. Baselayer sources: Esri, HERE, Garmin, SafeGraph, FAO, METI/NASA, USGS, EPA, NPS, NOAA, and State of North Dakota

used Qiagen DNA Blood and Tissue kits following manufacturer protocols to extract DNA. To quantify and detect ranavirus DNA, we used a TaqMan real-time quantitative PCR assay specific to ranavirus capsid protein gene (Picco et al. 2007). We used synthetic gBlock standards (5×10^2 to 5×10^6 copies; IDT) to quantify against unknown samples (Stilwell et al. 2018), and negative extraction controls were included with each set of extractions. eDNA samples were analyzed using the same methods, except with adding a Qiashredder (Qiagen) step prior to extraction (Goldberg et al. 2011), substitution of Environmental Master Mix in the assay to overcome inhibition common in eDNA samples, and multiplexing with the assay of Boyle et al. (2004). Inhibition was detected by including an internal positive control in each well (Qiagen or Thermofisher). Samples where Cq was increased by >3 were considered inhibited. We cleaned any samples testing as inhibited using a OneStep PCR Inhibitor Removal Kit (Zymo); these samples were then re-analyzed and confirmed to be uninhibited using this criterion. Extraction and PCR setup were conducted in a dedicated, restricted-access lab. For both tissue and eDNA samples, we defined positive samples as those with exponential amplification in 2 or 3 wells and negative samples as those without amplification in any wells. One eDNA sample tested positive in one well on the initial run and none on a follow-up run; we considered this sample as negative.

3. RESULTS AND DISCUSSION

We found scarce evidence of ranavirus in our samples in both 2017 and 2019. Of the tissues from 69 larvae from 7 wetlands, only 1 tiger salamander larva was positive for ranavirus infection (at site NOR9; Table 1). From 30 wetlands spanning nearly 130 km in eastern Montana and western North Dakota, no

Table 1. Study sites in the Prairie Pothole Region of Montana (MT) and North Dakota (ND), USA, sampled for amphibian larvae in 2017 and eDNA in 2019. WPA: Waterfowl Production Area; NWR: National Wildlife Refuge; BCF: boreal chorus frog; NLF: northern leopard frog; BTS: barred tiger salamander; NC: those species were not captured in that site during sampling. The asterisk (*) denotes the site where 1 larval barred tiger salamander was positive for ranavirus (NOR9). For 2017 and 2019, a dash (–) denotes that no larval samples were taken from that site in 2017 or no eDNA samples were taken from that site in 2019. The lower detection limit for salinity was 26 mg Cl l^{-1}. Volume filtered (ml) refers to water volume filtered for eDNA in 2019; a dash (–) denotes that eDNA was not collected at that site

Site	State	Location	Salinity (mg Cl l^{-1})	2017			2019	Volume filtered (ml)	
				BCF	NLF	BTS	eDNA	Mean (SD)	Sum
264J	MT	Rabenberg WPA	2495	NC	NC	3	Y	785 (79)	2355
BGWLB	MT	Private	<26	NC	NC	3	Y	800 (200)	2400
NOR10	ND	Norman WPA	727	9	5	NC	Y	750 (50)	2250
NOR7	ND	Norman WPA	44	NC	1	8	Y	773 (46)	2320
NOR9*	ND	Norman WPA	4103	NC	4	9	Y	770 (61)	2310
PAR1	MT	Pary WPA	30	NC	9	NC	Y	785 (26)	2355
SWA2	ND	Swanson WPA	<26	9	9	NC	–	–	–
124F	MT	Rabenberg WPA	11754	–	–	–	Y	483 (126)	1450
264K	MT	Rabenberg WPA	1002	–	–	–	Y	798 (100)	2395
264M	MT	Rabenberg WPA	445	–	–	–	Y	775 (139)	2325
264Z	MT	Rabenberg WPA	552	–	–	–	Y	792 (14)	2375
AND-10	MT	Anderson WPA	<26	–	–	–	Y	733 (379)	2200
AND-4	MT	Anderson WPA	3371	–	–	–	Y	748 (50)	2245
AND-New-1	MT	Anderson WPA	179	–	–	–	Y	760 (53)	2280
BEA1	ND	Beaver WPA	4089	–	–	–	Y	703 (6)	2110
BEA3	ND	Beaver WPA	<26	–	–	–	Y	717 (29)	2150
BEP1	MT	Berger Pond WPA	164	–	–	–	Y	813 (23)	2440
HAN-1	MT	Hansen WPA	476	–	–	–	Y	539 (248)	2155
LIN1	ND	Lindell WPA	<26	–	–	–	Y	763 (55)	2290
LOS1	ND	Lostwood NWR	<26	–	–	–	Y	800 (0)	2400
LOS2	ND	Lostwood NWR	<26	–	–	–	Y	793 (12)	2380
LOS3	ND	Lostwood NWR	<26	–	–	–	Y	803 (6)	2410
LOS4	ND	Lostwood NWR	<26	–	–	–	Y	767 (58)	2300
NOR4	ND	Norman WPA	151	–	–	–	Y	780 (159)	2340
NOR5	ND	Norman WPA	164	–	–	–	Y	750 (50)	2250
NOR6	ND	Norman WPA	377	–	–	–	Y	753 (81)	2260
OVE1	ND	Private	137	–	–	–	Y	617 (29)	1850
OVE2	ND	Private	<26	–	–	–	Y	600 (0)	1800
OVE3	ND	Private	1521	–	–	–	Y	783 (29)	2350
PAR2	MT	Pary WPA	71	–	–	–	Y	783 (76)	2350
SHP	MT	Shoveler Puddle WPA	<26	–	–	–	Y	767 (58)	2300

water samples had detectable ranavirus eDNA. Our findings contrast previous sampling in the same area that found moderately high prevalence of ranaviruses in 2013 and 2014 (35% of amphibians and 56% of sites; Firkins 2015). There are several possibilities for these different results, including our low sample sizes, mismatch in study designs, temporal variation in outbreaks, low occurrence or prevalence of ranavirus and amphibian abundance in the wetlands we sampled, and our eDNA sampling methods.

The very low number of larvae we collected could have affected our ability to detect ranavirus, particularly if occurrence or prevalence were also low in our study area (Gray et al. 2015). For instance, if population sizes and prevalence are generally low within a wetland (e.g. 100 and 2%, respectively) we would have needed to sample 75 larvae to detect ranavirus; however, even with higher prevalence (e.g. 20%) we would have needed to sample 8 larvae and we generally sampled fewer than that per species (Gray et al. 2015). Future research in the study area should include collecting much higher sample sizes in case prevalence is low.

Presence of ranavirus is often detected via mortality events, which generally occur in mid- to late summer and are often attributed to immunosuppression during metamorphosis (Green et al. 2002, Greer et al. 2005). The previous survey near our study area (Firkins 2015) had a longer sampling period (i.e. April through September), and most ranavirus-positive amphibians were adults and juveniles (post-metamorphosis). Over 5 yr, we did not observe mortality

events at wetlands we sampled for ranavirus and monitored amphibian populations (2015–2019; Hossack et al. 2018), whereas mortality events are evident in the Greater Yellowstone Ecosystem annually, even in early spring (Patla et al. 2016). In contrast, our sampling concluded by early July in both years, and we only collected larvae. Larvae are often more susceptible to death from ranavirus than adults and juveniles, which could have also reduced our ability to find infected individuals if they quickly perish and decompose (Duffus et al. 2015). We may have also missed ranavirus outbreaks because their occurrence can be sporadic among years (Brunner et al. 2015).

Although ranavirus has been detected near our study system, it could have low occurrence or prevalence within the wetlands we sampled due to regular drying of temporary wetlands or low host abundances. Some wetlands in our study system are temporary and dry regularly, which could decrease persistence of ranaviruses in wetlands among years (Brunner et al. 2007, 2015). Lower amphibian abundance, possibly as a result of wastewater contamination, could limit the number of hosts and reservoirs and thereby decrease transmission (Greer et al. 2008, Hossack et al. 2018). However, we did not detect ranavirus eDNA in water samples from 4 uncontaminated wetlands at Lostwood National Wildlife Refuge where amphibians are more abundant and a previous study found a ranavirus-positive northern leopard frog (Firkins 2015). Other potential hosts are also limited in our wetlands because we sampled from fishless wetlands and rarely saw reptiles.

Larger pore size of our filters could have limited our detection of ranavirus in eDNA samples. We used larger pore size relative to other ranavirus eDNA studies (e.g. 5 compared to 0.22 µm; Hall et al. 2016) because of tradeoffs between volume of water processed and capture efficiency (Barnes et al. 2021) and because we sampled for multiple pathogens (B. R. Hossack and C. S. Goldberg unpubl. data). Previous studies have suggested that using larger pore sizes can increase volume filtered, and capture most eDNA, when detection is the primary objective (Barnes et al. 2021). However, because of the larger pore size used, our data likely reflect detection of infected tissues compared to smaller, free-floating ranavirus particles (i.e. detection of outbreaks compared to detection of ranavirus in the system). Nevertheless, other studies have detected ranavirus using similar filter pore size and water volumes (B. R. Hossack and C. S. Goldberg unpubl. data). Future studies are warranted to better understand capture efficiency of ranavirus eDNA using filters with different pore sizes.

Amphibian populations are affected by multiple interacting stressors in the PPR (Firkins 2015, Gustafson & Newman 2016, Hossack et al. 2018). Unfortunately, our results do not provide insight into factors linked with ranavirus prevalence or dynamics. The disparity between our results and a previous study in the same area emphasizes the need for continued monitoring efforts to better understand the occurrence and prevalence of ranaviruses and other pathogens, and factors influencing their occurrence and prevalence, in the PPR.

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