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Detection of spatiotemporal variation in ranavirus distribution using eDNA

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Abstract

Amphibian population declines have been associated with emerging diseases including ranaviruses, which can cause mass die-offs across entire amphibian communities. Understanding and mitigating disease spread requires knowledge of spatial and temporal patterns of pathogen distribution, but also how environmental factors influence pathogen occurrence. We applied environmental DNA (eDNA) detection tools to survey spatial and temporal distributions of ranaviruses by sampling 103 waterbodies in southeastern Ontario, Canada and assessed the role of abiotic factors as predictors of pathogen occurrence. Ten waterbodies sampled during June–August (>30 km between sites) revealed that ranavirus was marginally more prevalent (p = .055) during the latter part of the summer. Ninety-three sites sampled at a finer scale (<10 km between sites) exhibited seasonal variability in ranavirus detection (site prevalence: 56% May; 66% July). Occupancy modeling revealed that wetland size and elevation influenced ranavirus occurrence while sampling date and water temperature influenced probability of detection. These findings indicate that biotic factors, such as host density and alternative hosts, should be investigated further as likely determinants of ranavirus prevalence across the landscape. Further, these results highlight the sensitivity of eDNA for detecting widespread presence of ranavirus and that abiotic factors may have a limited role in determining its prevalence and infectivity.

KEYWORDS

amphibian, environmental DNA, pathogen surveillance, *Ranavirus*, wildlife disease

1 | INTRODUCTION

Anthropogenic influences can have many negative impacts on biodiversity (Daszak, Cunningham, & Hyatt, 2001; Keesing et al., 2010), including changing disease dynamics that may facilitate the spread of pathogens into formerly naïve populations (Echaubard et al., 2014). Primary examples are the mass mortalities and amphibian population declines that have been associated with several emerging pathogens (Scheele et al., 2019). While enhanced understanding of pathogen persistence and spread through disease surveillance are key to mitigating impacts from emerging disease (Belant & Deese, 2010), to date many potentially important pathogens have been difficult and/or too costly to survey in natural environments. Therefore, the development of fast and reliable detection methods is key for a

rapid detection and potential prevention of epidemics in vulnerable populations.

Detection assays using environmental DNA (eDNA) allow for aquatic pathogen detection without the need for invasive sampling from host species (Chestnut et al., 2014). Aquatic eDNA captures DNA and cells in water to test for the presence of specific organisms via species-specific PCR primers (Lodge et al., 2012). Generally, pathogen detection using eDNA has the potential to be more effective and representative than traditional methods and can be a valuable in detecting alternative hosts, shedding of infective stages, and disease range shifts (Bass et al., 2015; Sengupta et al., 2019). eDNA can also provide an opportunity for more extensive sampling, often with enhanced sensitivity for detecting pathogens (Boothroyd, Mandrak, Fox, & Wilson, 2016; Jane et al., 2015). Further, pathogen detection via traditional host sampling techniques may inaccurately portray prevalence rates because practical considerations often limit sample size of captured specimens (Cooch, Conn, Ellner, Dobson, & Pollock, 2012), particularly in the case of diseases with low prevalence or biased detection based on visibly infected dead specimens (Cameron & Baldock, 1998; Gray, Brunner, Earl, & Ariel, 2015; Hall, Crespi, Goldberg, & Brunner, 2016; Spens et al., 2017). There are limitations to pathogen detection from eDNA, however, such as the fact that sampling the environment, instead of host tissue, does not quantify prevalence or intensity of infection, nor does eDNA detection of pathogens directly imply that potential hosts are indeed infected by the detected pathogens. It should also be acknowledged that eDNA methods cannot distinguish between DNA from an infectious pathogen (e.g., virion) and a fragment of naked DNA. eDNA sampling also requires careful scrutiny to ensure adequate detection probabilities and proper method validation in an attempt to quantify false positive and negative detections. Overall, however, eDNA is a powerful way to detect minute pathogen DNA quantities in the environment, including when hosts are not present, and allows for relative risk assessments for potential hosts in the waterbody. Therefore, provided that eDNA surveillance tools receive prior validation and sensitivity testing before deployment in new environments, they should provide a robust means for pathogen surveillance in a variety of systems.

Many pathogens are harmful to amphibians, including *Saprolegnia ferax*, *Ribeiroia ondatrae*, and *Batrachochytrium dendrobatidus* (*Bd*) (Daszak et al., 2003; Johnson et al., 2002; Kiesecker, Blaustein, & Belden, 2001; Romansic, Diez, Higashi, Johnson, & Blaustein, 2009). Among these pathogens, ranaviruses are notable given their known impacts on amphibian populations, affecting both larval and adult life stages (D'Aoust-Messier, Echaubard, Billy, & Lesbarrères, 2015; Duffus et al., 2015). *Ranavirus* (family *Iridoviridae*) is a genus of double stranded DNA viruses that circulate in the host's bloodstream and cause systemic infections (Gantress, Maniero, Cohen, & Robert, 2003). Ranaviruses are also known to be especially lethal to amphibians during metamorphosis, when tadpole mortality can reach 90% (Green, Converse, & Schrader, 2002; Greer, Berrill, & Wilson, 2005). Ranaviruses are presumed to be widespread across North America (The Global Ranavirus Reporting System, https://mantle.io/grrs/) and have the highest lethality to amphibians; however, the precise distribution of this virus across the continent is not well known because of patchy surveillance efforts and undetected die-offs (Duffus et al., 2015). Further, the ephemeral nature of ranavirus die-offs likely leads to underreporting, as carcasses are scavenged or quickly decomposed (Brunner, Storfer, Gray, & Hoverman, 2015; Harp & Petranka, 2006). Ranavirus disease dynamics are not well understood, but these viruses are thought to remain at low levels in the water, sediment, or within hosts that did not clear infection, until conditions for propagation are again presented (i.e., high host density or introduction of naïve larvae) (Brunner et al., 2015; Hall, Goldberg, Brunner, & Crespi, 2018; Roy & Kirchner, 2000). Given the nature of ranavirus, surveillance using eDNA may provide a more accurate portrayal of pathogen abundance without capturing infected specimens or carcasses (Hall et al., 2016, 2018).

eDNA-based studies for ranavirus detection have previously been published (Hall et al., 2016, 2018; Kolby et al., 2015; Miaud, Arnal, Poulain, Valentini, & Dejean, 2019). Using an eDNA approach, Kolby et al. (2015) found that ranaviruses were introduced to Madagascar from two commercial amphibian export facilities. Likewise, Hall et al. (2016) found that ranavirus titers (i.e., viruses concentrations in a sample) were detectable with eDNA samples ~15 days before and ~30 days after initial die-off events, suggesting ranavirus is detectable in water even if mortality is not observed. Further, work by Hall et al., (2018) in 8 ponds in Connecticut, United States, suggest that mortality from ranavirus was not necessarily driven only by pathogen presence in the waterbody, but also potentially by water temperature or developmental stage of tadpoles. In France, using eDNA samples from water, Miaud et al. (2019) monitored one pond in the Alps over the summer and reported ranavirus detections at the end of summer months (August and September), even outside die-off events. While eDNA appears to be viable for ranavirus monitoring, systematic, spatial and temporal surveys of ranavirus in the environment are lacking. For example, viral titer levels may be highest during the early summer, when there are many susceptible tadpoles hatching in the waterbody (Bayley et al., 2013; Greer et al., 2005), or in late summer, when amphibians are in later stages of metamorphosis when prospective hosts are highly susceptible to pathogens (Green et al., 2002; Hall et al., 2018). Accordingly, eDNA methods hold promise for pathogen surveillance in amphibian habitats and may be successfully applied across broader spatiotemporal scales than are normally adopted using standard monitoring approaches.

In this study, we investigated the use of eDNA as a surveillance tool for ranaviruses across southern Ontario, Canada. The goals were to: (a) provide insight into the temporal and spatial extent of ranavirus in south central Ontario waterbodies and (b) assess abiotic factors that may influence ranavirus detection. By sampling an extensive array of waterbodies, our overarching goal was to further the understanding of ranavirus presence in southern Ontario.

2 | MATERIALS AND METHODS

2.1 | Field collections—coarse geographic sampling

In a first phase of the study, we sampled 10 waterbodies during June, July, and August of 2016 and 2017 in south central Ontario, Canada, that were known to be occupied by various amphibian species (Figure 1). Sites were selected based on previous tissue sampling for *Ranavirus* and Bd in 2012, where ranavirus had been previously detected (Echaubard, *unpublished results*). At each study site, three water samples (250 ml) were collected for subsequent, in-laboratory eDNA filtering from 10 cm below surface level to avoid surface contaminants. Each collection bottle was dried with paper towel before being placed in a cooler or stored at 4°C until filtering. At each site, type of waterbody, weather, air temperature, and time collected were recorded. All equipment was decontaminated between sites (e.g., boots, hip waders, bottles, and caps) using 10% bleach and left to sit for 15 min before rinsing with deionized water (as per Hall et al., 2016). eDNA samples were filtered within 24 hr of collection through 0.2 µm cellulose nitrate filters (Whatman, CAT# 10401312; GE Healthcare Life Sciences) using a powered vacuum pump (EMD Millipore Corporation) and magnetic funnels (Pall Corporation). Post filtration, filters were placed in 1.5 ml microcentrifuge tubes and stored at −20°C. Prefilter blanks were processed with deionized water through each funnel and cup prior to filtering samples, followed by postfilter blanks after samples were run, to ensure materials were properly decontaminated.

2.2 | Field collections—fine-scale geographic sampling

In a second phase of the study starting in 2017, 93 sites with unknown ranavirus presence were sampled near Peterborough, Ontario, Canada (44°18'0.43"N, 78°18'58.43"W), with water samples collected during two sampling rounds between late May and July (Figure 1). During the July sampling period, 7 of the 93 sites had dried up or were otherwise inaccessible. Only samples included in both sampling periods are reported (n = 86). Given further improvements in eDNA sampling methods (Spens et al., 2017), we changed our filters from "open" (i.e., cellulose nitrate filters) to "closed" filters to reduce handling and possible contamination. Each site was sampled from five different points around the waterbody and filtered through Sterivex 0.22-µm (Millipore) capsule filters, following a sampling protocol described previously (Chestnut et al., 2014). Therefore, five samples were obtained per site for each month, totaling 10 capsules per site. Samples were drawn into 60 ml syringes that had been rinsed three times in situ water, then pushed through a Sterivex capsule until clogged with natural debris. Total volumes per capsule ranged from 60 ml to 500 ml, averaging around 250 ml. Capsules were then flushed with 50 ml of 0.01 mol/L Phosphate-buffered saline (PBS) followed by an additional expiration of air to drain all liquid. The outflow end of the capsule was closed with hematocrit sealant clay, and 0.9 ml of lysis buffer solution was injected for preservation, before being capped. Capsules were then sealed and labeled in individual plastic bags and kept in a cooler until returned to the laboratory where they were refrigerated at 4°C until extraction. Abiotic environmental variables were also measured during each sampling occasion. Estimated wetland body size was approximated from satellite imagery. Water temperature was measured using an ExStik II Dissolved Oxygen Meter (Ex Instruments), conductivity was measured using an ECOTestr conductivity meter (Oakton Instruments), and pH was measured using an aquarium pH testing kit (API Aquarium Pharmaceuticals).

2.3 | eDNA sample extraction

eDNA filters for the coarse geographic sampling (n = 180) had 280 µl of 1X lysis buffer (4 mol/L urea, 0.2 mol/L NaCl, 0.5% n-lauroyl sarcosine, 10 mmol/L 1,2-cyclohexanediaminetetraacetic acid, and 0.1 mol/L Tris-HCl, pH 8.0) and 20 mg/ml proteinase K (Qiagen Inc.) added and incubated at 56°C. Samples were vortexed every 30 min for the initial three hours of incubation then left overnight at 56°C (Goldberg, Pilliod, Arkle, & Waits, 2011). Samples were spun down, and the filter papers were removed using forceps sanitized in a concentrated (50%) solution of Extran 300 (an effective detergent in removing organic materials such as blood and proteins; VWR International) and rinsed with deionized water. A total of 230 µl of lysate was transferred to a clean deep well plate and extracted by magnetic beads using a MagneSil® Blood Genomic Max Yield System (Promega Corporation) on a Janus 96-well automated liquid handler (Perkin Elmer). One filter negative control and one filter positive control were included with each set of extractions. Filter positives were 250 ml of deionized water spiked with 2×10^6 plaque-forming units (pfu/ml) of frog virus 3 (FV3) filtered through a 0.2 µm cellulose nitrate filter.

Capsule filters used for the fine-scale sampling (n = 860) were extracted following the $SX_{CAPSULE}$ protocol from Spens et al., (2017). All capsule filters were extracted within thirteen weeks of sampling (Chestnut et al., 2014). Each set of extractions were completed along with a negative to ensure no contamination of samples. All equipment (caps and syringes) were decontaminated between samples and days by soaking in 10% bleach for 15 min and rinsed with distilled water at least three times.

2.4 | qPCR assay

qPCRs were performed using primers (Picco, Brunner, & Collins, 2007) targeting a 70 bp segment of the major capsid protein gene (MCP).To assess the influence of different amplification kits on PCR inhibition that commonly present false negative results for environmental samples, we compared the TaqMan Universal Master Mix and TaqMan Environmental Master Mix 2.0 (Applied Biosystems) on a subset of 33 samples from the coarse scale sampling to determine

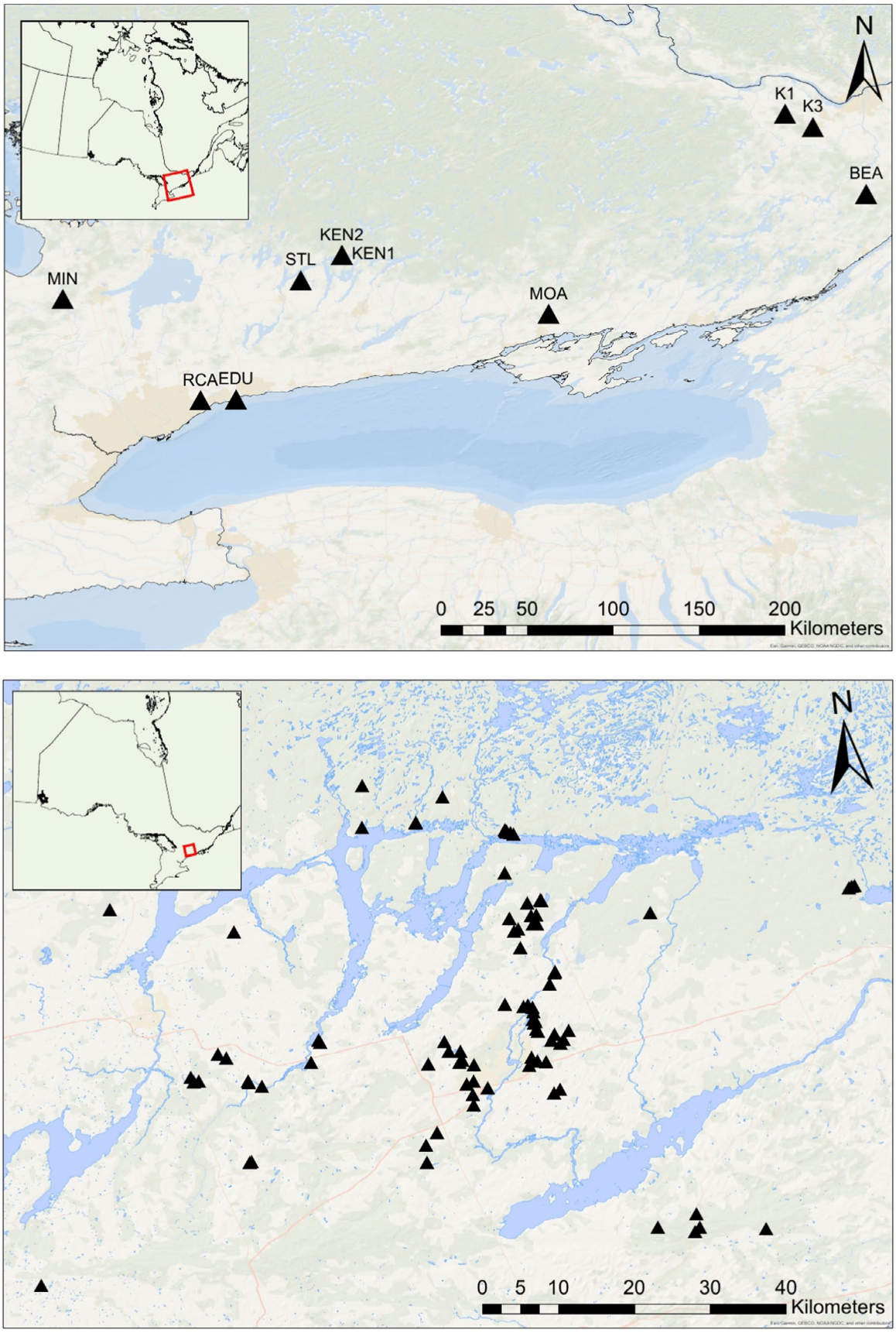

FIGURE 1 Spatial distribution of sampling sites. Above: Map of south central Ontario, Canada identifying coarse geographic sampling sites for eDNA collection. eDNA samples were taken three times in 2016 and 2017 summer seasons (June, July, and August); Below: Map of 2017 fine-scale eDNA sample sites surrounding Peterborough, Ontario, Canada

which yielded the best DNA amplification. The detailed methodology and results are shown in Appendix S1.

Standard curves for the qPCR assay were generated using 10-fold dilutions between 10^7 and 10^2 pfu/ml of FV3-like cultured in *epithelioma papulosum cyprini* (EPC) cells (Fijan et al., 1983). Standards and eDNA samples were run in triplicate in reaction volumes of 20 µl. Each reaction was run with 5 µl of unknown concentrations of template DNA, 1X TaqMan Environmental PCR Master Mix 2.0, 0.3 µmol/L of forward (5′-ACACCACCGCCCAAAAGTAC-3′) and reverse (5′-CCGTTCATGATGCGGATAATG-3′) primers (Picco et al., 2007), and 0.15 µmol/L fluorescent probe (5′-FAM-CCTCATCGTTCTGGCCATCAACCAC-MGB-3′). The primers are specific to *Ranavirus* and do not amplify the related genera L*ymphocystivirus a*nd M*egalocytivirus* (Hall et al., 2016), although this assay was not tested for all *Ranavirus* lineages/species. Reactions were run with an initial holding stage of 50°C for 2 min, then 95°C for 10 min, followed by a cycling stage of 95°C for 15 s and 58°C for 1 min for 50 cycles. All runs were conducted using the Applied Biosystems 7,900 detection system, and data analysis was conducted using the Applied Biosystems StepOnePlus™ system following the protocols for standard curve experiment. To test for inhibition in our samples, we spiked the samples with a known concentration of the MCP synthetic DNA, similar to Biggs et al. (2015) and Miaud et al. (2019), and used a probe specific to the synthetic DNA (Wilson, Wozney, & Smith, 2016). Samples were run as described above and were considered as inhibited if the final concentration was less than the expected initial concentration.

2.5 | Statistical analysis—Coarse geographic sampling

For cellulose nitrate filters, we used identical thresholds as Hall et al., (2016) since the same methodology was employed. We tested whether there was a temporal pattern of ranavirus presence in eDNA samples over summer months by analyzing samples filtered across 2016 and 2017 using a logistic regression with the *glmer* function in R version 3.4.2 (R Core Team 2017), following a binomial distribution with logit-link function and pond included as a random effect.

2.6 | Statistical analysis—Fine-scale sampling

To minimize the chance of misassigning pathogen occurrence due to false positive and false negative detections given that capsule filters were used for ranavirus detection for the first time in this study, we established qPCR thresholds based on a limit of detection (LOD). The LOD was determined using the Ct values of the qPCR standard dilution series of all sample runs and plotting them as per Hunter et al. (2017). The purpose of calculating LOD was to determine the lowest amount of ranavirus DNA that is both detectable and distinguishable from the concentration plateau as per Hunter et al. (2017).

Based on the threshold established by the LOD, capsule samples were considered positive if two or more qPCR replicates were ≥40 pfu/ml (see Results). A site was considered positive if one of the five capsules was positive.

To evaluate whether abiotic factors predicted pathogen presence at a site, we constructed single-season occupancy models using the *unmarked* package in R (Fiske & Chandler, 2011) . These models simultaneously estimate the site-level probability of ranavirus occurrence (Ψ) while accounting for factors that influence the probability of detection for each observation (p; Chestnut et al., 2014) . In our models, we treated each capsule as an observation, resulting in five observations per site per sampling period, for a total of ten observations per site. Our models contained all possible combinations of eight site-level covariates thought to influence ranavirus presence and five covariates thought to influence probability of detection. We investigated surrounding land use, elevation, distance to the nearest road, waterbody type (i.e., pond, wetland, retention pond, ephemeral pool, and forest pond), estimated waterbody size, average summer water temperatures of each site, average summer pH of each site, and average summer conductivity of each site as potential covariates that could influence ranavirus occupancy of a site. Land use, elevation, and distance to the nearest road were estimated using ArcGIS v10.4.1 using the tools Near (for distance nearest road) and Extraction (for elevation and land use). Maps describing these three features for Ontario were obtained from the ESRI online database. Sampling month, volume filtered through each capsule, water temperature, conductivity, and pH recorded at the time of sampling were each included as potential covariates influencing the probability of detection. All continuous covariates were standardized (z-transformed, with zero mean and standard deviation of one) prior to analysis. The resulting models were ranked using Akaike's information criterion (AIC), with the model with the lowest AIC and those within two AIC units of the top model being considered as indistinguishable (Burnham & Anderson, 2004). Adequacy of model fit was assessed using a parametric bootstrap and Chi-squared (χ^2) statistic (MacKenzie & Bailey, 2004).

To evaluate how abiotic factors influenced the abundance of eDNA detected (i.e., copy number), we constructed alternative negative binomial hurdle models in R using the *glmmTMB* package (Brooks et al., 2017) . We again treated each capsule as an observation and included the same factors that were used in the occupancy modeling as potential covariates determining either the copy number or the zero counts. All continuous covariates were standardized (z-transformed, with zero mean and standard deviation of one) prior to analysis. Resulting models were again compared with AIC, and models within two AIC units were considered as equally supported.

To infer the spatial autocorrelation of our data, we calculated the Global Moran's I statistic (I) in ArcGIS v10.4.1. If $I > 0$ and Z-value > 1.96, or $I < 0$ and Z-value <−1.96, with a p-value < .05, it indicated that the distribution of positive sites was clustered in the whole area; otherwise, the distribution of the positive cases was random.

3 | RESULTS

3.1 | qPCR Master Mix comparison and inhibition test

Environmental DNA (eDNA) samples that had been spiked with 10^5 copies of synthetic virus had an average recovery of 19%, with 18 samples showing total inhibition when using the Universal Master Mix (Appendix S1). However, the levels of inhibition significantly decreased when using the Environmental Master Mix, resulting in a consistent >90% recovery rate (Appendix S1). Due to the significant increase in DNA recovery, all eDNA samples were subsequently processed with the Environmental Master Mix.

Using a known concentration of spiked DNA, we did not observe signs of PCR inhibition based on the values from the amplification of synthetic DNA in all samples. This suggests that the few negative samples in our study were true negatives and not a product of inhibition.

3.2 | Ranavirus detection—Coarse geographic sampling

Standard curves of cultured FV3 ranged from 10^2 to 10^7 pfu/ml and had slopes averaging at −3.5 with R^2 values around .99. In 2016, 4/10 sites were positive in June, 5/10 were positive in July, and 7/10 were positive in August. Only one site had observed signs of infection (lethargic tadpoles observed swimming near the water's surface). This site also had high pfu values of ranavirus (average 9.09×10^4 pfu/ml) (Table S1).

In 2017, all 10 sites were positive for ranavirus at least once throughout the three sampling periods (Table S1), and 9/10 sites sampled across both years were positive across both years, and only one site was negative during 2016, but positive during 2017 (STL). In 2017, 6/10 sites were positive.

3.3 | Temporal eDNA analysis—Coarse geographic sampling

eDNA samples taken via cellulose nitrate filters across three months in 2016 and 2017 were analyzed to determine variation in the likelihood of presence, depending on the time of the season. Ranavirus detection over time was marginally not significant (Figure S1; $p = .055$) showing that the pathogen was more likely to be detected in August than June.

3.4 | Ranavirus detection—Fine-scale sampling

A statistical model for LOD was determined for a 10-fold serial dilution points from 10^7 to 10^2 pfu/mL where LOD was estimated to be 30.1 pfu/ml (21–41 95% CI). We chose to use a conservative LOD estimate, and LOD was considered as the upper interval of the CI (40 pfu/ml). Out of our 86 sites, we observed contamination in seven sites sampled in May, as positive amplifications from the synthetic DNA specific probe were detected during qPCR. Therefore, we excluded seven sites from our analysis and report results for 79 sites. In May, 44 of the 79 sampled sites (56%) yielded positive ranavirus detection, whereas in July, 52 sites were positive (65%). A total of 30 sites (36%) were positive across both sampling periods, 14 (19%) were positive only in May, 22 (28%) only in July, and 13 (16%) sites were negative during both months (Figure 2). Positive samples ranged from 40 pfu/ml to 229,600 pfu/ml, with the highest site being opportunistically observed during a mass die-off event. We tested for spatial autocorrelation in our samples (number of positive capsules per site per month, average pfu/ml per site per month, presence/absence per site per month, and combined values per site for both months) and detected no significant spatial correlation (all $p > .05$).

Occupancy analyses were conducted on 78 of the 79 sites due to missing abiotic data for one site. Occupancy modeling corroborated the observed temporal trend in ranavirus detection, with the top four, equally supported models containing the effect of sampling date on the probability of detection (Table 1, Table S2). Qualitatively (based on the marginally lower AIC for the top model), we inferred that probability of detection estimates for May were lower than July (mean ± *SE*; 0.30 ± 0.03 vs. 0.42 ± 0.03, respectively). Among the supported models, we noted that the probability of ranavirus occupancy appeared to be positively related to wetland size (Figure 3) and site elevation (logit estimate ± *SE*: −0.009 ± 0.33), and that probability of detection was related to water temperature (logit estimate ± *SE*: 0.011 ± 0.11), but these coefficient estimates have a large amount of error, so their potential effects should be interpreted with caution.

The most supported model for the abundance of ranavirus included the influence of sampling month (Table S3). Furthermore, there was a greater abundance of eDNA detected in July than in May (Figure 4).

4 | DISCUSSION

In this study, we report the widespread presence of ranavirus eDNA in waterbodies in southeastern Ontario. We used extensive sampling during the summer season to define the presence of ranavirus in a large sample of waterbodies divided in two geographical scales. Our results show that ranavirus is present throughout southern Ontario during summer months and is more likely to be detected during late summer. We were unable to distinguish between ranavirus occupancy models, leaving only qualitative support for the role of time of sampling and water temperature on probability of detection, and the role of waterbody size and elevation on the probability of site occupancy.

To ensure we were using an optimal methodology to detect ranavirus, we improved a qPCR protocol for sampling ranavirus from waterbodies using eDNA. We found that the Environmental Master Mix worked to eliminate almost 100% of inhibition (90% recovery rate vs. 19% with the Universal Master Mix) from the water samples tested. Had we not used Environmental Master Mix, most of

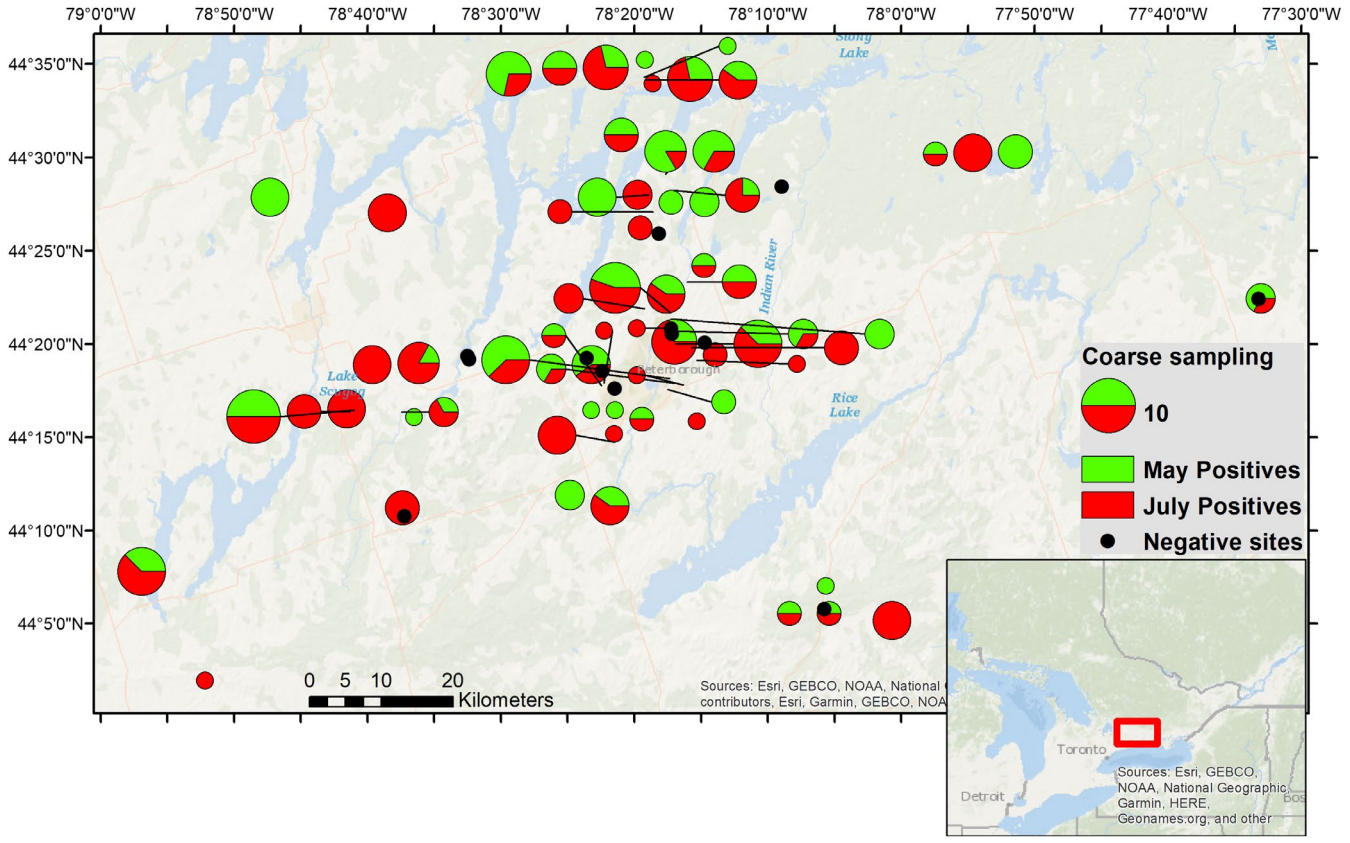

FIGURE 2 Spatial distribution of positive sites for ranavirus. The size of the circles represents the number of positive capsules in each waterbody (minimum = 1, maximum = 10), while the color represents number of the positive capsules in each month (May: green, July: red). Negative sites for both months are shown as a black circle

our samples would have shown inhibition, and this would have undermined detectability of the virus. Further, we established a limit of detection (LOD) determine a threshold of detection to strengthen the validity designating samples as positives. It is important to note that our results were based on our ability to detect the virus using filtered water samples, and that a site considered negative may still have viral particles within hosts and in the surrounding soils and sediment (Brunner & Yarber, 2018).

TABLE 1 Model selection results for alternative occupancy models of ranavirus in 78 sites across Ontario based on the influence of eight site-level covariates, including waterbody size (size) and elevation (elevation), and five observation-level covariates, including sampling date (date; May or June) and water temperature at the time of sample collection (temp)

Model	*K*	AIC	ΔAIC	*w*	X^2 *p*-value
Ψ(.)p(date)	3	918.24	0	0.38	.72
Ψ(size)p(date)	4	919.36	1.12	0.21	.27
Ψ(.) p(date + temp)	4	920.23	1.99	0.14	.55
Ψ(elevation) p(date)	4	920.24	2.00	0.14	.46

Note: The *p*-value of Chi-squared tests for model adequacy are also included; *p*-values > .05 suggest adequate model fit.

Ranaviruses such as frog virus 3 are presumed as being widespread across North America, yet the exact temporal and spatial extent of the virus is unknown because of limited surveillance (Duffus et al., 2015). At a coarse geographic scale (>30 km between sites, 62,200 km^2), ranavirus was detected at nearly all sites across years. At a finer geographic

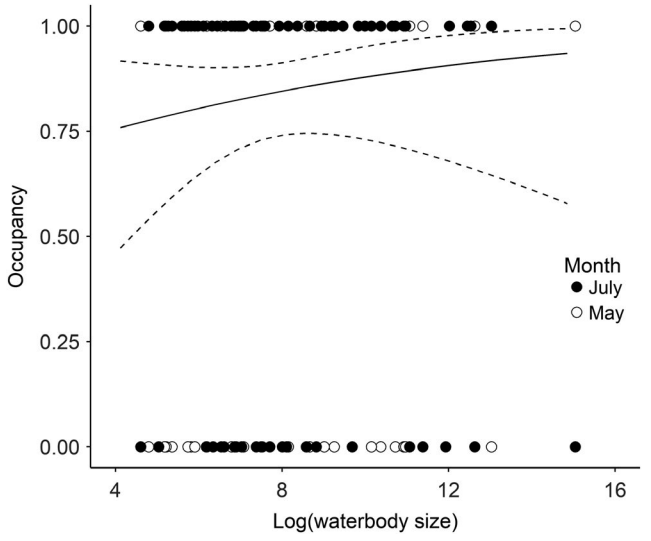

FIGURE 3 Site occupancy of ranavirus at 78 Ontario sites based on waterbody size and sampling date

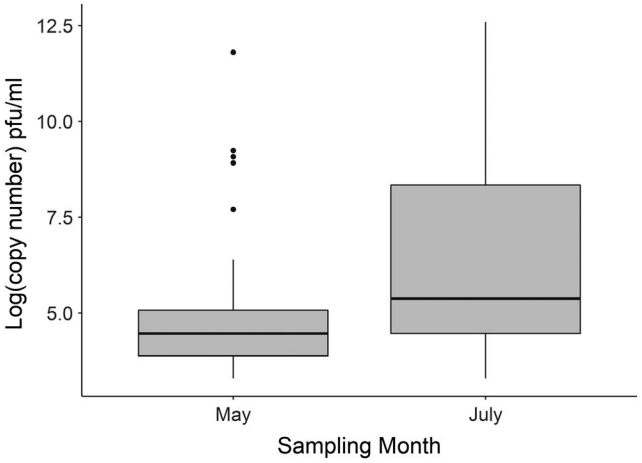

FIGURE 4 Box plot showing the amount of eDNA (log pfu/ml) detected at 78 Ontario sites in July and May 2017

scale (<10 km between sites, 7,150 km^2), we found that ranavirus was detected in 56% sites in May, and 65% sites in July, with 37% of sites being positive during both sampling rounds. Considering the capsule sampling alone, 84% of sites were positive at some point throughout the two sampling periods. While the extent of ranavirus presence found in the sampled regions does not necessarily extrapolate directly to other regions, eDNA-based surveillance methods in this study demonstrate their utility in enhancing our understanding of ranavirus abundance with a higher degree of certainty.

Our occupancy models found no abiotic factors strongly associated with increased ranavirus detection, although it is notable that sampling date was present in all supported models. Temperature may be tied to the temporal trend in increased ranavirus presence as the summer progressed, which is consistent with results from the recent study by Hall et al., (2018). It is understood that host–pathogen dynamics in aquatic systems can be influenced by temperature, as viruses replicate faster at their ideal temperature (Ariel et al., 2009; Bayley et al., 2013). In our fine-scale samples, average water temperature taken during May and July was 14.9°C and 20.5°C, respectively, and the latter month also was associated with higher ranavirus detection and higher pfu values per site. By comparison, Ariel et al. (2009) found that FV3 had an optimal temperature of 24°C when propagated in vitro. Bayley et al. (2013) found that FV3-infected tadpoles had a mortality rate of 96% at 20°C, compared with only 32% mortality at 15°C. However, Echaubard et al. (2014) found a different trend, where *Rana pipiens* tadpoles infected with an FV3-like ranavirus had a higher likelihood of death at 14°C (67% mortality) compared with 22°C (51%). These studies suggest that ranavirus replication is likely highest around 20°C; however, mortality may also be host- and strain-dependent, as the virus used in Echaubard et al. (2014) was an FV3-like isolate, not wild type FV3 (Brunner et al., 2015; Morrison et al., 2014). As such, biotic factors may be stronger predictors for ranavirus presence and intensity, such as taxonomic richness, species presence, and predator presence (Tornabene et al., 2017). A more likely explanation for this trend may be the presence of more metamorphic amphibians within the waterbodies (Brunner et al., 2015). In wood frog tadpoles, for example, the odds of mortality when exposed to ranavirus increased with each Gosner development stage (Warne, Crespi, & Brunner, 2011). Epidemics are often observed in amphibians that are going through metamorphosis, as it is an energy-taxing process that causes immunosuppression (Carey, Cohen, & Rollins-Smith, 1999; Greer et al., 2005; Rollins-Smith, 1998). An alternative explanation may involve alternative ectothermic hosts or reservoirs. Both fish and turtles are known to host ranavirus, including FV3 (Duffus et al., 2015; Lesbarrères et al., 2012) . In 2018, the first two cases of ranavirus (FV3) infecting turtles in Ontario were reported (McKenzie et al., 2019) . As turtles can move across the landscape and were often seen in our sampling sites, an infected turtle can potentially transmit ranavirus to various waterbodies.

Previous studies have associated landscape features and abiotic factors with ranavirus presence. High elevation and high catchment position were shown to be important factors of ranavirus presence, although spatial autocorrelation was not found to predict ranavirus presence in Acadia National Park in Maine, USA (Gahl & Calhoun, 2008) . Our results showed that at a both large and small spatial scales, time of sampling was an important factor in detecting ranavirus, with later summer months having more positive sites and higher pfu values. We found qualitative support that waterbody size and elevation influenced ranavirus occupancy, but, like Gahl & Calhoun (2008), other abiotic factors were found to not influence ranavirus presence. The large number of sample sites allowed us to determine that ranavirus is widespread in southeast Ontario and may represent a conservation concern for amphibian populations.

Our study worked to advance ranavirus eDNA detection protocols as a surveillance tool by decreasing qPCR inhibition and documenting the spatiotemporal patterns of presence over multiple months and seasons and determine factors which predicted ranavirus presence. While the eDNA assay was more likely to detect ranavirus in later summer months, eDNA-based sampling was optimal when used across multiple months, as ranavirus outbreaks and detectability is sporadic across the season. Waterbodies where there are constant, low levels of ranavirus should be further studied to understand what causes the preservation and reemergence each season. Furthermore, estimated plaque-forming unit (pfu) (i.e., active viruses) concentrations do not take into account factors that can affect DNA degradation and shedding rate. Future studies should also evaluate how biotic (e.g., microorganisms) and abiotic (e.g., UV) factors influence ranavirus DNA degradation and, therefore, detection probability. We also urge researchers to use metabarcoding assays for eDNA detection to determine which ranavirus lineages are present in North American wetlands, as different lineages with multiple recombination events were described in Canada with possible increased virulence (Grant et al., 2019; Vilaça, Bienentreu et al., 2019) . As a final note, as amphibian pathogens are a growing concern across Ontario and North American waters, the application of eDNA surveillance should be further considered in order to track how widespread these

diseases impend, as our results suggest that ranavirus appears to be ubiquitously distributed. Conservation and ranavirus disease containment efforts in Ontario should be directed toward disease prevention, control of pathogen pollution by preventing pathogen translocation directed by humans (e.g., contaminated recreation gear, Casais et al., 2019) , and surveillance.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

CJK, CRB, and CCW conceived the original idea; SAG and MC performed sampling; SAG carried out experiments; LB performed statistical analysis; STV and CJK contributed to the interpretation of the results; STV, SAG, and CJK wrote the manuscript with critical feedback from all authors.

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DATA AVAILABILITY STATEMENT

Results are available from Dryad https://doi.org/10.5061/dryad.59zw3r23h (Vilaça, Grant et al., 2019).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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