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Evaluating environmental DNA-based quantification of ranavirus infection in wood frog populations

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Abstract

A variety of challenges arise when monitoring wildlife populations for disease. Sampling tissues can be invasive to hosts, and obtaining sufficient sample sizes can be expensive and time-consuming, particularly for rare species and when pathogen prevalence is low. Environmental DNA (eDNA)-based detection of pathogens is an alternative approach to surveillance for aquatic communities that circumvents many of these issues. Ranaviruses are emerging pathogens of ectothermic vertebrates linked to die-offs of amphibian populations. Detecting ranavirus infections is critical, but nonlethal methods have the above issues and are prone to false negatives. We report on the feasibility and effectiveness of eDNA-based ranavirus detection in the field. We compared ranavirus titres in eDNA samples collected from pond water to titres in wood frog (*Lithobates sylvaticus*; $n = 5$) tadpoles in sites dominated by this one species ($n = 20$ pond visits). We examined whether ranavirus DNA can be detected in eDNA from pond water when infections are present in the pond and if viral titres detected in eDNA samples correlate with the prevalence or intensity of ranavirus infections in tadpoles. With three 250 mL water samples, we were able to detect the virus in all visits with infected larvae (0.92 diagnostic sensitivity). Also, we found a strong relationship between the viral eDNA titres and titres in larval tissues. eDNA titres increased prior to observed die-offs and declined afterwards, and were two orders of magnitude higher in ponds with a die-off. Our results suggest that eDNA is useful for detecting ranavirus infections in wildlife and aquaculture.

Keywords: amphibian, disease surveillance, environmental DNA, ranavirus

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Introduction

Infectious disease is a growing concern to ecologists because of the potential threat disease outbreaks pose to population dynamics and biodiversity. Accurately, characterizing the presence and absence of a pathogen is necessary for understanding host–pathogen ecology and protecting vulnerable populations from disease-related die-offs. Pathogen detection is also critical for identifying environmental conditions conducive to disease outbreaks. Furthermore, detecting infections when they are rare affords wildlife managers the opportunity to intervene and potentially prevent epidemics.

Detecting pathogens in wildlife populations presents a variety of challenges. First, sampling tissues for surveillance can be invasive or lethal for the hosts. Also, obtaining sample sizes large enough to have statistical confidence, particularly when the pathogen prevalence is low, can be difficult. For example, to be 95% confident that the prevalence of infection is <10%, one must find zero infections in ~30 individuals with a perfectly sensitive diagnostic test; this sample size doubles to detect infections at <5% prevalence with the same level of certainty (Cameron & Baldock 1998). Obtaining sufficient sample sizes can be expensive and time-consuming, particularly for elusive and rare species, which are often a priority in conservation biology. Lastly, there may be unknown and unaccounted-for biases in the probability of capturing infected vs. uninfected individuals, which can skew estimates of prevalence and other important metrics of infection or disease burden (Cooch *et al.* 2012).

Environmental DNA (eDNA)-based detection of pathogens is an alternative approach to pathogen surveillance for aquatic species that circumvents many of these issues. Rather than directly isolating and detecting the pathogen of interest in hosts, DNA is extracted from an environmental sample such as pond water and tested with species-specific PCR primers (Ogram *et al.* 1987). Here we define eDNA as trace DNA in an environmental sample (e.g. water or soil) which can be

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contained in shed or degraded host tissue, or pathogens free in the environment (see definition in Bohmann *et al.* 2014). Because eDNA sampling does not involve capturing or handling animals, there is little stress or impact on the hosts. Importantly, collecting water samples is much easier and more cost-effective than sampling large numbers of individual animals and does not become more difficult with rare species. Furthermore, eDNA samples can potentially detect very low levels of pathogen DNA and even multiple pathogens simultaneously. Simple field collection with minimal specialized equipment, fast processing time, high sensitivity and relatively low cost make eDNA an attractive method for disease surveillance in aquatic wildlife populations.

Because of these benefits, eDNA-based methods have been used by the United States Environmental Protection Agency and others for testing wastewater effluent and groundwater for human diseases with high analytic sensitivity and specificity (e.g. Mattison & Bidawid 2009; Staley *et al.* 2012), and for aquacultural pathogens of fish (Minamoto *et al.* 2009, 2011). These assays, however, have been used to simply detect pathogen presence or absence in the environment and have not been compared to rates of infection in hosts. As pathogen distribution is generally aggregated in host populations (Crofton 1971), and pathogen shedding rate can be affected by host physiology or environmental factors, the relationship between pathogen eDNA levels and prevalence of infection is not straightforward and is probably different than the relationship between eDNA levels of vertebrate species and their abundances. In short, if eDNA-based pathogen detection is to be truly useful in pathogen surveillance programmes, we must determine its diagnostic sensitivity (probability of detecting a truly positive or infected population) and diagnostic specificity (probability of correctly identifying uninfected populations as negative) as well as estimate the correlation between pathogen titres in eDNA samples and the prevalence and intensity of the pathogen in hosts living in the environment.

Environmental DNA-based methods of pathogen surveillance would be particularly useful for monitoring pathogens in amphibian populations. Emerging pathogens, such as *Batrachochytrium dendrobatidis* (Bd), *Batrachochytrium salamandrivorans* (Bs) and ranaviruses, are major threats to amphibian populations (Alford & Richards 1999; Stuart *et al.* 2004; Mendelson *et al.* 2006; Rachowicz *et al.* 2006; McCallum 2007; Hayes *et al.* 2010; Blaustein *et al.* 2011; IUCN 2013; Martel *et al.* 2013). Walker *et al.* (2007) detected Bd eDNA in ponds in Europe and were able to detect a minimum of one Bd zoospore in 50 mL of water, a high analytic sensitivity; because they did not sample animals, the diagnostic sensitivity is unknown. Kirshtein *et al.* (2007) evaluated recovery of Bd eDNA in spiked pond water and had an average of 37.4 ± 15.1% recovery rate. Hyman & Collins (2012) used an eDNA-based approach to detect Bd in amphibian breeding ponds and had an estimated detection probability of 0.45 per sample (Schmidt *et al.* 2013). Recently, Chestnut *et al.* (2014) detected Bd in 47% of sites with eDNA samples when occupancy modelling predicted 61% detection probability. These results are promising for monitoring Bd; still there is no assessment of the feasibility of using eDNA to detect ranaviruses, which are difficult to detect using other nonlethal methods.

Ranaviruses are emerging pathogens of amphibians and other ectothermic vertebrates associated with mass mortality events around the world (Gray *et al.* 2009; Miller *et al.* 2011; Chinchar & Waltzek 2014; Duffus *et al.* 2015). Ranavirus infections and die-offs are common in wood frog populations (Duffus *et al.* 2015). Unfortunately, consistently detecting ranavirus infections with nonlethal methods, such as tail or toe clips, and skin swabs, remains a challenge for amphibians (Greer & Collins 2007; St-Amour & Lesbarrères 2007; Gray *et al.* 2012) because they produce false negatives and, at least with swabs, false positives. Accurate diagnosis of ranavirus infections generally requires testing liver or interrenal tissues and thus lethal sampling (Greer & Collins 2007). Because ranavirus is shed into the water from infected individuals (Brunner & Collins 2009; Robert *et al.* 2011) detecting ranavirus eDNA from water samples should be possible.

In this study, we report on the feasibility and effectiveness of eDNA-based ranavirus detection in the field. We compared ranavirus titres in eDNA collected from water to titres in wood frog (*Lithobates sylvaticus*) tadpoles from sites dominated by this one species. Our questions were as follows: (i) can ranavirus DNA be detected in eDNA samples from ponds when infected tadpoles are present, and (ii) do viral titres detected in eDNA samples correlate with the prevalence and intensity of ranavirus infections in tadpoles?

Methods

Ethics statement

This research was approved by the Animal Care and Use Committee (Protocol #04366-002) of Washington State University. Collections were approved by the Connecticut Department of Energy and Environmental Protection (Scientific collections permit #1114003) and Yale Myers Forest (YMF).

Field collections

As part of a project examining landscape epidemiology of wood frog—ranavirus dynamics, we surveyed 20

wood frog ponds in the mixed hardwood forests of YMF in northeastern Connecticut, USA, twice between 16 June and 7 July 2013. The permanent and ephemeral wetlands in YMF have been monitored for amphibian population dynamics and demography for at least a decade (Skelly 2004; Skelly *et al.* 2006). Anecdotal observations of amphibian larvae with swollen hind limbs and bodies and 'red leg' during die-off events in previous years are consistent with ranavirus infection, but also with infection by other parasites such as digenean flatworms (Kiesecker & Skelly 2000). In a retrospective study, ethanol-preserved, moribund wood frog tadpoles and spotted salamander larvae collected from several ponds in this area tested positive for ranavirus with PCR as early as 2002 (J. L. Brunner, unpublished), suggesting that at least some of these die-offs may have been caused by ranavirus infections.

While several amphibian species occur in these ephemeral wetlands such as spotted salamanders (*Ambystoma maculatum*), green frogs (*Lithobates clamitans*) and bullfrogs (*Lithobates catesbeianus*), from spring to early summer wood frog larvae account for the majority of vertebrate biomass (e.g. Skelly 2004). For this reason and because wood frog larvae are highly susceptible to ranavirus infection (Harp & Petranka 2006; Warne *et al.* 2011), we used ranavirus detected in wood frog tadpoles as a surrogate for detection at the site level. However, apparent false positives in eDNA samples could be accounted for by infection in other species. We therefore selected fishless vernal pools in which wood frogs were known to breed at high abundance for our study.

Ponds were mostly homogenous single basin vernal pools; however, some sites had heterogeneity in microhabitat where small connected pools were created by tussocks, logs and bushes of mountain laurel. Sites were considered independent if there were no above-ground hydrological connections seen and were chosen based on high wood frog larvae abundance. Most sites also had spotted salamander larvae at much lower densities (ca. 10× lower), and occasionally, several adult green frogs and bullfrogs were observed. Because mortality events cannot be predicted and laboratory experiments have shown susceptibility to ranavirus in wood frog larvae varies by development stage (Warne *et al.* 2011; but see Haislip *et al.* 2011), we attempted to standardize collection by development stage. We collected samples before wood frogs reached metamorphic climax (when larvae develop front limbs and begin tail reabsorption) and after metamorphic climax but before the new metamorphs dispersed. Timing of these events varied between sites due to differences in pond characteristics which influence development rate. We also attempted to avoid ponds where concurrent herpetological research was occurring; however, we had difficulty finding enough ponds with abundant wood frog larvae that met this criterion, so 9 of the 20 had at least some visits by other researchers. All equipment and waders were decontaminated with 10% bleach between pond visits during this study.

Environmental DNA sampling (water filtering) occurred before entry into each pond. Three water samples were collected at distinct locations (north, east and west) along the shore. At each location, 250 mL of surface pond water (ca. 10 cm deep) was collected using a disposable paper cup and filtered through a sterile 0.2-μm cellulose nitrate filter (Nalgene Analytical Filter funnels, CAT# 145-2020; ThermoScientific, Waltham, MA, USA) using a hand pump and a vacuum flask. The filter was then removed with a clean nitrile glove, folded and stored in 100% ethanol in 2-mL microcentrifuge screwcap tubes.

We approximated larval density per unit effort on the first visit of each pond using dip net surveys standardized by the pond area. Surveys were completed by one person (E.M. Hall) at each site. Ponds <300 m^2 were surveyed for 10 min, ponds between 300 and 1000 m^2 were surveyed for 20 min, 1000 and 1500 m^2 surveyed for 30 min, and ponds >1500 m^2 were surveyed for 40 min. All wood frog and spotted salamander larvae were counted separately and then released, and the presence of amphibian species was also noted as they are possible carriers of ranavirus.

Lastly, in 12 of the 20 ponds monitored, we collected five larval wood frogs during each pond visit immediately after collecting eDNA samples. The tadpoles were selected haphazardly along a transect through the length of the pond. Larvae were immediately euthanized in 10% benzocaine and then stored individually in 10 mL of 100% ethanol until they could be developmentally staged according to Gosner (1960) and liver tissue dissected for DNA extraction.

We checked sites once a week for carcasses to observe ranavirus-associated die-off events, however, due to time constraints could not take eDNA samples at all of those times. We considered the start of a die-off event the first day that ≥5 amphibian carcasses were observed. Carcasses were collected and screened for ranavirus DNA to verify its involvement in observed mortality. Because of the unpredictable nature and rapid onset of die-off events, we could not sample water at the same point during the progression of an epidemic at each site, thus both the number of carcasses and live larvae were counted (using aforementioned survey protocol) to determine the proportion dead at the time of the pond visit which concurred with a die-off event.

Extractions and qPCR

Filters were extracted using the Qiashredder/DNeasy Blood & Tissue kit (Qiagen, Valencia, CA, USA) method

of Goldberg *et al.* (2011) in a laboratory dedicated to low-quantity DNA samples. Researchers are required to shower and change clothes before entering this room after being in a high-quality DNA or post-PCR laboratory, and no amphibian tissues have been handled in this room. One DNA extraction was conducted for each filter. A negative extraction control was included with each set of extractions.

We dissected liver from wood frog larvae in a separate laboratory using flame-sterilized instruments and extracted DNA using manufacturer's protocol of the DNA Blood and Tissue kit (Qiagen). Both eDNA and tissue samples were diluted to 20 ng/μL of DNA (measured with NanoDrop-2000; Thermo-Scientific) to meet the recommended concentration of template DNA in PCR assays, then screened for *ranavirus* DNA in triplicate 20 μL reactions (TaqMan Gene expression master mix; Applied Biosystems, ThermoFisher) on 96-well plates with 5 μL of DNA template (~100 ng) using a TaqMan real-time quantitative PCR assay specific to the major capsid protein (MCP) gene of ranaviruses (Picco *et al.* 2007; Brunner & Collins 2009). A 10-fold serial dilution of DNA extracted from a Frog Virus 3-like ranavirus grown in Epithilium papilloma cyprinia cells from 10^2 to 10^7 plaque-forming units (pfu/mL) was used as a standard against which unknown samples were quantified. Samples with amplification in two or three wells were scored as positive. Those without amplification in any of the wells were scored as negative. Ambiguous samples (1 well testing positive) were rerun and if at least one well showed amplification on the second run, the sample was scored as positive. Triplicates with a coefficient of variation >15% were also rerun. Filter samples that tested negative were rerun undiluted with an exogenous internal positive control (Applied Biosystems, ThermoFisher) to detect PCR inhibition. Viral quantities for positive larval samples are reported as the mean of the $\log_{10}$ pfu across all wells of the sample, including zeros, per ng of DNA in the reaction. Viral quantities for positive eDNA samples were back-transformed to the mean $\log_{10}$ pfu per filtered volume.

The primers and probe used in this qPCR assay perfectly match the corresponding sequences of all known members of the genus *Ranavirus* from North America, except for a single base pair mismatch with a Bohle-like ranavirus isolated from a captive collection (Cheng *et al.* 2014) and considerable mismatching with the Santee Cooper ranaviruses, which are a genetically unique lineage found in fish (T. Walztek and K. Subramaniam, personal communication). It does not amplify members of the two other genera of the family Iridoviridae that infect vertebrates (the insect iridoviruses are even more genetically divergent from *Ranavirus*). We could not amplify the MCP gene of the lymphocystis disease virus (genus *Lymphocystivirus*) and Infectious spleen and kidney necrosis virus (genus *Megalocytivirus*). Furthermore, other than known ranaviruses, a PRIMER-BLAST search with the primers of this assay only matched with one organism, a sea anemone (Appendix S1, Supporting information).

Statistical analysis

To test whether the average titre in eDNA samples increased with the average titre in the livers of wood frog larvae, we regressed eDNA titres against those from wood frogs using the *lm* function in R version 3.0.2 (R Core Team 2013). We also included the density (per unit effort) of wood frogs and spotted salamander larvae individually and amphibian species diversity (count of species observed) in these regressions as covariates and compared these models with a log-likelihood test using the *lrtest* function in the package LMTEST (Zeileis & Hothorn 2002).

To test how eDNA titres varied with time relative to when a die-off event occurred (i.e. when carcasses were noted), we compared a linear model with a model including a second-order polynomial using the *lm* function. We found the maximum (peak titre in eDNA samples) of the second-order polynomial using the package POLYNOM (Venables *et al.* 2015). We did the same to look at how larval titres varied with time relative to when a die-off occurred. We also tested whether concurrent research in a pond affected eDNA titres using a student's *t*-test of the average eDNA titre in each pond, or occurence of a die-off with a Barnard's exact test.

Results

Mortality events, which appeared to decimate larval populations, were observed in seven of the 20 ponds from 3 June to 3 July 2013 (Fig. 1). All carcasses collected in these events were positive for ranavirus infection (Table 1), suggesting observed die-offs were due to ranavirus infections. One pond contained no wood frog larvae during sampling, presumably because predatory larval marbled salamanders (*Ambystoma opacum*) were present, thus a ranavirus-associated wood frog die-off was not possible in this pond, although this salamander may also carry ranavirus. The remaining 12 ponds had no observed mortality events.

Seventeen of the 20 ponds had at least one positive eDNA sample from each visit, two were negative in all of the samples from the first visit, but had at least one positive sample during the second visit, and one pond was negative in all of the samples collected. In ponds with observed die-offs, all eDNA samples were positive during both sampling periods, with titres ≥3.05 pfu/mL.

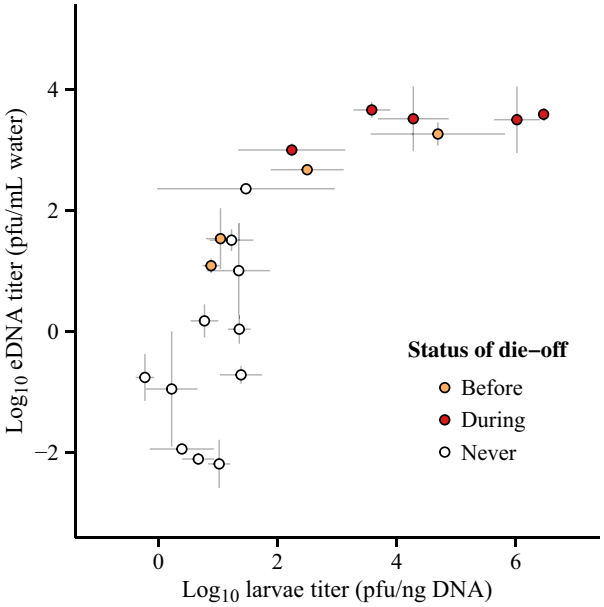

Fig. 1 Log_{10} average ranavirus environmental DNA (eDNA) titre by Log_{10} average larvae titre (ponds may be represented twice, $n = 12$) at each visit where both live animals and eDNA filters were collected. Colours represent the status of the die-off at the visit date, or if there was never a die-off observed at that pond. Error bars represent standard error of the mean. Three filters and five larvae (except in three visits during die-offs in which fewer larvae were collected, see table for collection details) were collected at each pond visit.

Note that this includes samples taken over a month after the observation of a die-off. The average eDNA titre was over two orders of magnitude higher in ponds where a mortality event occurred than ponds without observed mortality events (average = $10^{-0.34}$ pfu/mL vs. $10^{2.46}$ pfu/mL; $\beta_{\text{die-off}} = 1.971 \pm 0.863$, $z = 2.284$, $P = 0.022$). One pond where a die-off was not observed had relatively high titres in eDNA samples and in larvae ($10^{2.36}$ pfu/mL and $10^{1.47}$ pfu/ng, respectively). However, metamorphs emerging from this pond were infected and dying (collected for a different experiment, E. M. Hall, unpublished), and thus, the die-off in this pond may have occurred in the more elusive metamorphic life stage.

We did not observe signs of PCR inhibition in the exogenous internal positive control reaction in the negative eDNA samples, which suggests that these were true negatives. We reran negative DNA samples undiluted in case the dilution to ~100 ng/reaction left the concentration of ranavirus DNA below the detection threshold. Twelve of 33 samples that were negative when run with ~100 ng of DNA in the reaction (the concentration recommended by the manufacturer of the master mix) showed clear amplification when run undiluted (150–730 ng DNA per reaction). In most cases, inconsistencies among eDNA samples (i.e. not all samples negative or positive) were associated with low titres. In three visits, two of the three eDNA filters were positive (at one pond the two positives had titres ~10–27 pfu/mL while the third was negative with no sign of inhibition), and at four pond visits, one of the three samples were positive with a very low titre (<2 pfu/mL) while the other two samples were negative. Note that two eDNA filters clogged before 250 mL could be filtered, ending up with around 200 mL filtered, and three pond visits had less than five larvae collected due to high mortality.

In the 20 visits at 12 ponds from which both eDNA and larvae were collected, there was a strong relationship between viral titres in the eDNA sample and those found in the larvae ($\beta_{\text{titre}} = 0.838 \pm 0.155$ $t_{18} = 5.391$, $P < 0.001$), although titres in eDNA samples appear to saturate with increasing titres in tadpoles (Fig. 1). Adding wood frog or spotted salamander larval density did not improve the fit of the model ($\chi^2 = 0.916$, d.f. = 1, $P = 0.339$; $\chi^2 = 3.394$, d.f. = 1, $P = 0.065$), nor did including observed vertebrate species richness ($\chi^2 = 0.178$, d.f. = 1, $P = 0.673$).

Across the seven ponds in which mortality events were observed, eDNA titres increased as the pond visit date approached the date mortality was observed (Fig. 2); the peak titre predicted by in a second-order polynomial fit to these data was at 3.7 days (day 0 = day when ≥5 carcasses were observed; $\beta_{\text{days}} = 0.033 \pm 0.015$, $\beta^2_{\text{days}} = -0.004 \pm 0.001$, $F_{2,11} = 26.57$, $P < 0.001$). Virus was detectable in these eDNA samples at least 2 weeks before and 5 weeks after the observed onset of the event. Ranavirus titres in wood frog livers also increased as the pond visit date approached the observed mortality event and continued to increase afterwards (Fig. 3; linear regression: $\beta_{\text{days}} = 0.176 \pm 0.051$, $t_{4.26} = 3.507$, $P = 0.022$), and larval titres were higher when animals were collected from ponds during die-offs compared to before the die-off was observed and ponds without die-offs ($\beta_{\text{before}} = -1.9738 \pm 0.6129$, $t_{9.501} = -3.221$, $P = 0.009$; $\beta_{\text{never}} = -3.523 \pm 0.6982$ $t_{12.134} = -5.047$, $P < 0.001$).

In the eight ponds with research activities, four experienced a die-off and four did not, suggesting research activities were not associated with die-offs (Barnard's exact test, $P = 0.307$); nor with higher eDNA titres ($t_{14.593} = -0.352$, $P = 0.73$).

Discussion

We were able to detect ranavirus eDNA in pond water in the field and found that titres in eDNA samples increased with titres in tadpoles collected concurrently. Also, the titres in ponds with die-offs increased leading up to observed die-offs and declined afterwards, all of

Table 1 Summary data from each pond visit

Date	Pond	Log_{10} Avg. eDNA titre	eDNA pos/neg	Log_{10} Avg. larval titre	Larvae assayed	Log_{10} Avg. Carcass titre	Carcasses assayed	Status of die-off	Avg. Larval stage	*Lithobates sylvaticus* Larval Density	*Ambystoma maculatum* larval density	*Lithobates clamitans* presence	*Lithobates catesbeianus* presence
6/18	ACH	1.086	3/0	0.884	5	NA	0	B	37	5.9	0.1	N	Y
7/3	ACH	3.510	3/0	4.278	5	NA	0	D	42	6.2	0		
6/28	AW	–	0/3	NA	0	NA	0	N	35	9.75	1	Y	N
7/10	AW	–	0/3	NA	0	NA	0	N	38				
6/17	BS	3.494	3/0	6.018	5	NA	0	D	35	4.2	3	Y	N
7/3	BS	1.744	3/0	NA	0	6.559	5	D	36				
7/1	BT	−0.758	2/1	−0.227	5	NA	0	N	36	47.1	0.6	Y	N
7/6	BT	1.507	3/0	1.226	5	NA	0	N	41				
6/30	C3	**−0.949**	1/2	0.222	5	NA	0	N	36	6.28	0.43	Y	Y
7/9	C3	−0.624	1/2	NA	0	NA	0	N	42				
6/16	CAV	1.531	3/0	1.041	5	NA	0	B	33	20.1	0.1	N	Y
7/2	CAV	3.039	3/0	NA	0	5.848	4	D	38	4.8	0		
6/16	CPY	0.038	3/0	1.357	5	NA	0	N	36	2.5	0	Y	Y
7/2	CPY	2.355	3/0	1.469	4	6.528	1	N	42				
6/27	CUR	**−2.185**	3/0	1.020	5	NA	0	N	40	6.3	0	Y	Y
7/3	CUR	−0.715	3/0	1.387	5	NA	0	N	42				
7/1	DT	–	0/3	NA	0	NA	0	N	NA	2.1	0	N	N
7/9	DT	**−0.795**	1/2	NA	0	NA	0	N	40				
6/28	E8	−0.600	1/2	NA	0	NA	0	N	37	2.45	0.3	N	N
7/9	E8	**−1.777**	2/1	NA	0	NA	0	N	40				
6/30	KH	**−2.104**	3/0	0.670	5	NA	0	N	34	5.9	4.3	Y	N
7/4	KH	**−1.939**	3/0	0.393	5	NA	0	N	37				
6/18	KL	3.258	3/0	4.691	4	NA	0	B	34	22	0.1	Y	N
7/3	KL	3.583	3/0	6.465	1	4.206	2	D	NA	0.1	0		
6/30	LP	1.378	3/0	NA	0	NA	0	N	40	2.13	0.33	N	N
7/8	LP	1.682	3/0	NA	0	NA	0	N	NA				
6/27	NC	2.669	3/0	2.497	5	NA	0	B	34	14.9	1.2	Y	N
7/6	NC	2.995	3/0	2.238	5	NA	0	D	36	9.3	0.5		
6/20	QU	−1.581	3/0	NA	0	NA	0	NA	NA	0	0.4	Y	N
7/6	QU	**−2.006**	3/0	NA	0	NA	0	NA	NA				
7/8	RE	−1.747	3/0	NA	0	NA	0	N	40	4.6	0.1	Y	Y
6/23	SA	0.175	3/0	0.773	5	NA	0	N	36	4	0.03	Y	Y
7/4	SA	1.004	3/0	1.348	5	NA	0	N	39				
7/2	TS	−0.415	2/1	NA	0	NA	0	N	38	1	0.25	Y	Y
7/10	TS	0.056	3/0	NA	0	NA	0	N	NA				

Table 1 (Continued)

Date	Pond	Log_{10} Avg. eDNA titre	eDNA pos/neg	Log_{10} Avg. larval titre	Larvae assayed	Log_{10} Avg. Carcass titre	Carcasses assayed	Status of die-off	Avg. Larval stage	*Lithobates sylvaticus* Larval Density	*Ambystoma maculatum* larval density	*Lithobates clamitans* presence	*Lithobates catesbeianus* presence
6/26	WF	3.655	3/0	3.580	5	NA	0	D	40	4.4	0	Y	Y
7/4	WF	3.344	3/0	NA	0	5.046	5	D	41	2.3	0		
6/27	WP	0.525	3/0	NA	0	NA	0	A	NA	0.03	0.1	Y	Y
7/7	WP	−0.026	3/0	NA	0	NA	0	A	NA		0.1		

Log_{10} Avg. environmental DNA (eDNA) titre units are pfu/mL water, dashes signify negative samples with no amplification. Log_{10} Avg. larval titre units are pfu/ng DNA. Numbers in bold contain sample(s) which were below our sensitivity threshold. Filter pos./neg. shows the number of positive/negative filters of the three collected. Status of die-off signifies whether the collection occurred before (B), during (D) or after (A) ≥5 carcasses were found in the pond, or if there were never any carcasses found (N).

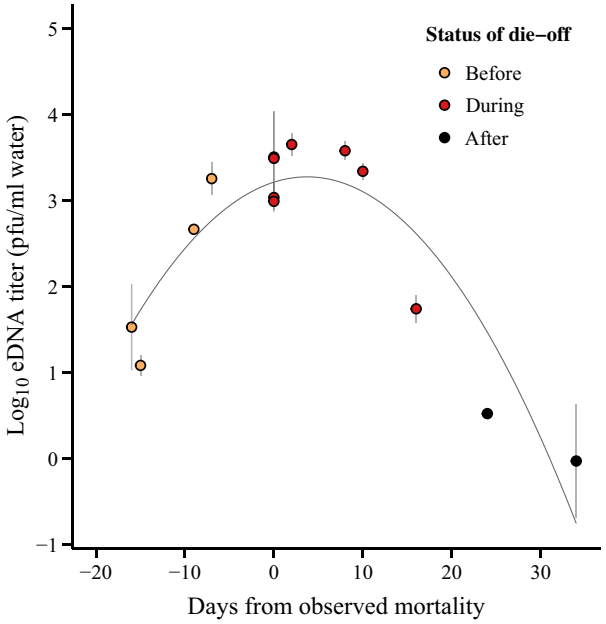

Fig. 2 Log_{10} average ranavirus environmental DNA titre by days from when a die-off event was observed (day = 0, ≥5 carcasses were observed) for each visit of a pond with an observed die-off (ponds are represented twice). Status of die-off is represented by colour, which signifies whether the collection occurred before, during or after carcasses were found in the pond. Three filters were collected at each visit, twice at each pond. Error bars represent standard error of the mean.

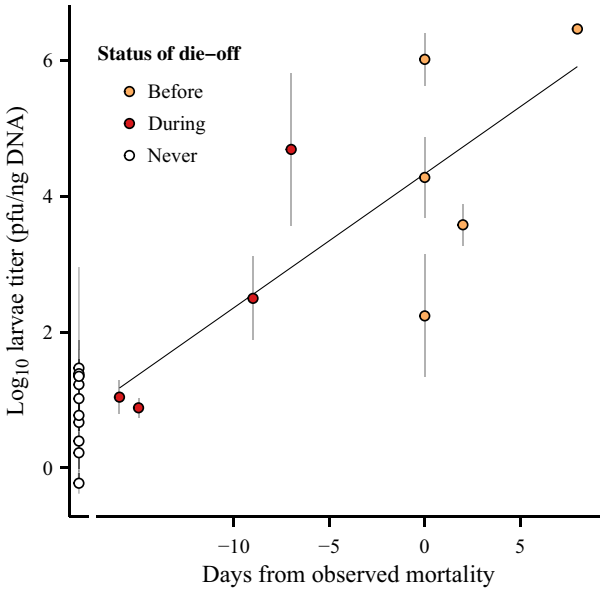

Fig. 3 Log_{10} average ranavirus larvae titre by days from when a die-off event was observed (day = 0, ≥5 carcasses were observed) and in ponds where a die-off event was never observed (represented on the left). Up to five larvae were collected at each visit, twice at each pond (see table for collection details). Status of die-off is represented by colour. Error bars represent standard error of the mean.

which suggests that eDNA detection methods are very useful for understanding the distribution and dynamics of ranaviruses in aquatic amphibians. Notably, we found a large difference in virus titre in eDNA samples between sites with and without observed die-offs, which suggests with further study we could develop a predictive model to determine probability of epidemics based off this measure.

Current methods of disease surveillance require lethal sampling and screening large numbers of animals to have reasonable confidence of detecting ranavirus infection. The eDNA method allows biologists to bypass these issues and determine the infection status of an aquatic system (e.g. pond) without sampling animals. Although not done here, the same eDNA sample can be used to detect and quantify DNA of other pathogens (e.g. Bd, Bs) or of amphibian species of interest (Rees *et al.* 2014). Also, with further qPCR reactions, estimates of the ratio of the viral DNA to host DNA to determine ratios of pathogens to host densities are possible. Not only is eDNA detection effective for detecting ranavirus in ponds, but also this information could potentially be used to develop models to predict the likelihood of mortality events in this system. Altogether, our results support the use of this method for monitoring amphibians for ranaviruses in wild populations, and potentially in other contexts (e.g. aquaculture and the pet trade) as well. On the other hand, if the intent is to calculate the prevalence of ranavirus infection in a target species, eDNA methods are not ideal as wetlands may be occupied by multiple amphibian species and all infected hosts are contributing to eDNA levels. We will first discuss our results about eDNA-based detection in the context of establishing the distribution (presence/absence) of ranavirus among ponds and then discuss how they might be used to understand the dynamics of ranavirus epidemics.

If the goal of surveillance is to document the presence or absence of ranavirus at a site, then our results are very encouraging and suggest surveillance based solely on the presence of die-offs underestimates the occurrence of ranavirus infections within wood frog populations. With three 250 mL water samples, we were able to detect the virus in every pond visit with infected larvae, which equates to a diagnostic sensitivity of 1 relative to the gold standard of tadpole livers, or 0.92 if we consider each eDNA sample separately. We are unable to estimate the diagnostic specificity (true negative rate) of our eDNA samples as none of our animal samples tested negative. Although because these ponds are occupied occasionally by several species that may serve as carriers of ranavirus (e.g. bullfrogs), our estimate may not be inferable to co-occurring species. Therefore, this technique could be used to investigate the presence or absence of ranavirus infection at a site, and then upon finding positive results, further sampling of animal tissue could determine the infected host species.

Beyond documenting presence or absence of a pathogen, current disease surveillance efforts aim to estimate the prevalence of infection in amphibian populations. Ideally, we could relate pathogen eDNA concentrations to pathogen abundance, as observed between concentrations of target amphibian eDNA and their observed densities (Thomsen *et al.* 2012; Pilliod *et al.* 2013). We constructed a model to explore this relationship (see Appendix S2, Supporting information). If we assume that virus is shed into the water by infected tadpoles and decays in water at constant rates, which are fast relative to changes in host density or prevalence, then the equilibrium amount of virus (V^*) in the water is

$$V^* \approx \text{Larval density} \times \text{prevalence} \times \frac{\text{shedding}}{\text{decay}}.$$

This equation suggests that one could correlate viral titres in water with the density of infected larvae (*larval density* × *prevalence*). We did not, however, find such a relationship, perhaps because of the limitations of our data (e.g. rudimentary measures of density and all animals tested positive in a small sample size), or perhaps because one or more of the assumptions of the above solution—constant shedding rates and rapid decay—do not hold.

Indeed, results suggest the shedding rate is not constant. We found a strong relationship between viral titres in eDNA samples and the intensity of infection in tadpoles, as well as a sharp increase in viral titres in water and tadpoles collected nearing observed die-offs. Both of these observations suggest that shedding rates are not constant, but instead increase throughout individual infections and as an epidemic progresses. Brunner *et al.* (2007) found that larval tiger salamander (*Ambystoma mavortium*) became more infectious to naïve conspecifics with time since exposure, which is consistent with increasing virus production and shedding rate. If shedding rates increase throughout an epidemic, our equation becomes more complex (see Appendix S2, Supporting information). To account for variable shedding rates, we would need to integrate over the time since initial infection for all individuals in the model and need to understand how host characteristics affect shedding rate (e.g. body size, development stage, immune response).

An increase in shedding rate over the course of an epidemic helps explain the increasing, but saturating relationship we observed between titres in eDNA and tadpole samples. As the density of infected individuals increases early in an epidemic and infections of individu-

als increase in severity, we would expect to detect more viral DNA in the water if shedding rates also increase. However, mortality during the later stage of an epidemic may reduce the density of infected larvae, so while the individual larvae may have more intense infections and shed more viruses into the water on average, there are fewer of them doing so. To the extent that these two effects balance each other, the amount of virus in the water may be fairly constant at the epidemic peak. Alternatively, there may be a threshold when the water becomes saturated with ranavirus DNA, as Pilliod *et al.* (2013) and Thomsen *et al.* (2012) saw with vertebrate DNA, which should be investigated further.

Whether ranavirus eDNA degrades at a constant or rapid rate is also not entirely clear. If ranavirus DNA decays in the environment as rapidly as that of other viruses (e.g. Poliovirus in groundwater decays at −0.79 pfu/day; Yates *et al.* 1985) and vertebrate hosts (e.g. bullfrog DNA was detected for 25 days after source was removed; Dejean *et al.* 2011), then ranavirus eDNA detected in these vernal ponds would be from current or recent infections. Johnson & Brunner (2014) observed a rapid decline in the viability and detectability of ranavirus in inoculated pond water. This suggests that the ranavirus DNA in our eDNA samples had been recently shed from tadpoles. However, Johnson & Brunner (2014) and another study on the persistence of ranavirus in the environment (Nazir *et al.* 2012) used virus grown in culture. Hypothetically, virus particles shed in amphibian cells, mucous or other fomites may be protected from microorganisms, exogenous nucleases, UV-radiation and other factors that inactivate virus and degrade DNA (Wommack & Colwell 2000). Taken together, an important focus of research should be to further characterize the persistence of ranavirus particles and DNA in natural environments to better understand what eDNA titres represent.

In summary, while eDNA titres did not correlate to the prevalence of infection in a pond in our samples and may not have a simple relationship with prevalence in general, this method can be used to determine whether ranavirus is present at a site of interest. eDNA titres may even indicate whether an epidemic is about to happen (low titres in eDNA samples representing low densities of infected animals and/or low rates of shedding) or is already underway (high titres). We were able to detect ranavirus DNA in eDNA samples at least 2 weeks before and several weeks after observed die-offs even when carcasses were no longer found by dip netting. In the case of surveillance programmes designed to detect ranavirus in a pond, this suggests a potential to determine whether an unoccupied pond has suffered a recent disease-associated die-off. Ranavirus-related mortality events are generally very quick in amphibian populations inhabiting vernal pools, from days to a couple weeks (e.g. Brunner *et al.* 2011; Wheelwright *et al.* 2014). This means that frequent visits are necessary to have any confidence of observing a die-off or even ranavirus infection with moderate sample sizes. In contrast, our results suggest that surveillance programmes using eDNA sampling may have a fairly wide window of time in which to detect ranavirus infections in a pond.

Note that because we did not collect field negative control samples (ranavirus-free, DNA-free water filtered in the field) during this experiment, we cannot rule out contamination of samples in the field. However, we have reason to think that this did not occur. First, field negatives collected in the following field season by the same researcher using with the exact same protocol and collector had no PCR amplification. Second, the negative eDNA samples were collected immediately after samples from positive wetlands, suggesting that the protocol was robust to contamination in the field. If the low titres detected in some wetlands were the result of contamination, our estimate of the sensitivity of this method is inflated, although the overall patterns hold (i.e. Figs 1–3). Use of field negative controls, regardless of sampling technique, will help ensure that samples are free of contamination and the data can be used with confidence.

While our data suggest that eDNA methods are a viable and attractive alternative to traditional methods for detecting and quantifying ranavirus in aquatic amphibian populations, several issues will need to be addressed before this method is widely used for routine surveillance. First, although the eDNA samples had a high diagnostic sensitivity to detect ranavirus infections in sites with high titres in animals and where mortality was observed, in one pond visit ranavirus was detected at very low levels in only one of three of the eDNA samples while titres in larvae were low ($10^{0.2}$ pfu/mL). There is potentially a threshold density of infected larvae below which detection is less likely, such as early in epidemics, and sampling design may have to be adjusted to compensate for this. Future experiments should determine the relationship between sampling design and this threshold, both in terms of virus concentrations in water and densities of infected larvae. This latter threshold could also vary between host species because they may shed virus at different rates. Determining whether these thresholds are influenced by characteristics of the environment (e.g. water chemistry) or host population (e.g. stage, density) should be addressed in future research.

Second, determining the proportion of viral DNA in water that is detected by eDNA-based methods is critical to interpreting results. The efficiency of the method will affect the detection threshold. Johnson & Brunner (2014) estimated this efficiency at ~40%, which is encouraging, but they used a different filter and extraction protocol.

Based on the observation that initially negative eDNA samples tested positive when used undiluted in the qPCR reaction, one could increase the analytic and diagnostic sensitivity of these methods using undiluted extracted DNA and then diluting or otherwise removing inhibitors from the sample only if the exogenous internal positive control shows signs of PCR inhibition (a problem we did not have).

Finally, a note of caution: the high analytic sensitivity of qPCR, which enables detection of extremely rare targets, also makes it prone to false positives due to even trace amount of target DNA. Extreme caution must therefore be taken to prevent contamination (Darling & Mahon 2011). Field negatives should be included in the sampling protocol and subsequent processing. These are filters treated identically as the other eDNA samples, only filtering clean water (e.g. store-bought spring water or molecular-grade water from the laboratory) instead of pond water. This will help identify when contamination has occurred. Furthermore, as ranaviruses are multiple-host pathogens, co-inhabiting fish and/or reptile species can pose a challenge if using eDNA sampling for surveillance of infection in amphibian populations.

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References

Alford RA, Richards SJ (1999) Global amphibian declines: a problem in applied ecology. *Annual Review of Ecology and Systematics*, **30**, 133–165.

Blaustein AR, Han BA, Relyea RA *et al.* (2011) The complexity of amphibian population declines: understanding the role of cofactors in driving amphibian losses. *Annals of the New York Academy of Sciences*, **1223**, 108–119.

Bohmann K, Evans A, Gilbert MTP *et al.* (2014) Environmental DNA for wildlife biology and biodiversity monitoring. *Trends in Ecology & Evolution*, **29**, 358–367.

Brunner JL, Collins JP (2009) Testing assumptions of the trade-off theory of the evolution of parasite virulence. *Evolutionary Ecology Research*, **11**, 1169–1188.

Brunner JL, Schock DM, Collins JP (2007) Transmission dynamics of the amphibian ranavirus Ambystoma tigrinum virus. *Diseases of Aquatic Organisms*, **77**, 87–95.

Brunner JL, Barnett KE, Gosier CJ *et al.* (2011) Ranavirus infection in die-offs of vernal pool amphibians in New York, USA. *Herpetological Review*, **42**, 76–79.

Cameron AR, Baldock FC (1998) A new probability formula for surveys to substantiate freedom from disease. *Preventive Veterinary Medicine*, **34**, 1–17.

Cheng K, Jones ME, Jancovich JK *et al.* (2014) Isolation of a Bohle-like iridovirus from boreal toads housed within a cosmopolitan aquarium collection. *Diseases of Aquatic Organisms*, **111**, 139–152.

Chestnut T, Anderson C, Popa R *et al.* (2014) Heterogeneous occupancy and density estimates of the pathogenic fungus *Batrachochytrium dendrobatidis* in waters of North America. *PLoS One*, **9**, e106790.

Chinchar VG, Waltzek TB (2014) Ranaviruses: not just for frogs. *PLoS Pathogens*, **10**, e1003850.

Cooch EG, Conn PB, Ellner SP, Dobson AP, Pollock KH (2012) Disease dynamics in wild populations: modeling and estimation: a review. *Journal of Ornithology*, **152**, 485–509.

Crofton H (1971) A model of host–parasite relationships. *Parasitology*, **63**, 343–364.

Darling JA, Mahon AR (2011) From molecules to management: adopting DNA-based methods for monitoring biological invasions in aquatic environments. *Environmental Research*, **111**, 978–988.

Dejean T, Valentini A, Duparc A *et al.* (2011) Persistence of environmental DNA in freshwater ecosystems. *PLoS One*, **6**, e23398.

Duffus AL, Waltzek TB, Stöhr AC *et al.* (2015) Distribution and host range of ranaviruses. In: *Ranaviruses* (eds Gray MJ, Chinchar VG), pp. 9–57. Springer International Publishing.

Goldberg CS, Pilliod DS, Arkle RS, Waits LP (2011) Molecular detection of vertebrates in stream water: a demonstration using rocky mountain tailed frogs and idaho giant salamanders. *PLoS One*, **6**, e22746.

Gosner KL (1960) A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica*, **16**, 183–190.

Gray MJ, Miller DL, Hoverman JT (2009) Ecology and pathology of amphibian ranaviruses. *Diseases of Aquatic Organisms*, **87**, 243–266.

Gray MJ, Miller DL, Hoverman JT (2012) Reliability of non-lethal surveillance methods for detecting ranavirus infection. *Diseases of Aquatic Organisms*, **99**, 1–6.

Greer AL, Collins JP (2007) Sensitivity of a diagnostic test for amphibian ranavirus varies with sampling protocol. *Journal of Wildlife Diseases*, **43**, 525–532.

Haislip NA, Gray MJ, Hoverman JT, Miller DL (2011) Development and disease: how susceptibility to an emerging pathogen changes through anuran development. *PLoS One*, **6**, e22307.

Harp EM, Petranka JW (2006) Ranavirus in wood frogs (*Rana sylvatica*): potential sources of transmission within and between ponds. *Journal of Wildlife Diseases*, **42**, 307–318.

Hayes TB, Falso P, Gallipeau S, Stice M (2010) The cause of global amphibian declines: a developmental endocrinologist's perspective. *Journal of Experimental Biology*, **213**, 921–933.

Hyman OJ, Collins JP (2012) Evaluation of a filtration-based method for detecting batrachochytrium dendrobatidis in natural bodies of water. *Diseases of Aquatic Organisms*, **97**, 185–195.

IUCN (2013) *IUCN Red List*. Available from http://www.iucnredlist.org/initiatives/amphibians.

Johnson A, Brunner J (2014) Persistence of an amphibian ranavirus in aquatic communities. *Diseases of Aquatic Organisms*, **111**, 129–138.

Kiesecker JM, Skelly DK (2000) Choice of oviposition site by gray treefrogs: the role of potential parasitic infection. *Ecology*, **81**, 2939–2943.

Kirshtein JD, Anderson CW, Wood JS, Longcore JE, Voytek MA (2007) Quantitative PCR detection of *Batrachochytrium dendrobatidis* DNA from sediments and water. *Diseases of Aquatic Organisms*, **77**, 11–15.

Martel A, Spitzen-van der Sluijs A, Blooi M *et al.* (2013) *Batrachochytrium salamandrivorans* sp. nov. causes lethal chytridiomycosis in amphibians. *Proceedings of the National Academy of Sciences*, **110**, 15325–15329.

Mattison K, Bidawid S (2009) Analytical methods for food and environmental viruses. *Food and Environmental Virology*, **1**, 107–122.

McCallum ML (2007) Amphibian decline or extinction? Current declines dwarf background extinction rate. *Journal of Herpetology*, **41**, 483–491.

Mendelson JR, Lips KR, Gagliardo RW *et al.* (2006) Biodiversity—confronting amphibian declines and extinctions. *Science*, **313**, 48.

Miller D, Gray M, Storfer A (2011) Ecopathology of ranaviruses infecting amphibians. *Viruses (Basel)*, **3**, 2351–2373.

Minamoto T, Honjo MN, Uchii K *et al.* (2009) Detection of cyprinid herpesvirus 3 DNA in river water during and after an outbreak. *Veterinary Microbiology*, **135**, 261–266.

Minamoto T, Honjo MN, Yamanaka H *et al.* (2011) Detection of cyprinid herpesvirus-3 DNA in lake plankton. *Research in Veterinary Science*, **90**, 530–532.

Nazir J, Spengler M, Marschang RE (2012) Environmental persistence of amphibian and reptilian ranaviruses. *Diseases of Aquatic Organisms*, **98**, 177–184.

Ogram A, Sayler GS, Barkay T (1987) The extraction and purification of microbial DNA from sediments. *Journal of Microbiological Methods*, **7**, 57–66.

Picco AM, Brunner JL, Collins JP (2007) Susceptibility of the endangered California tiger salamander, *Ambystoma californiense*, to ranavirus infection. *Journal of Wildlife Diseases*, **43**, 286–290.

Pilliod DS, Goldberg CS, Arkle RS, Waits LP (2013) Estimating occupancy and abundance of stream amphibians using environmental DNA from filtered water samples. *Canadian Journal of Fisheries and Aquatic Sciences*, **70**, 1123–1130.

R Core Team (2013) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. Available from http://www.R-project.org/.

Rachowicz LJ, Knapp RA, Morgan JAT *et al.* (2006) Emerging infectious disease as a proximate cause of amphibian mass mortality. *Ecology*, **87**, 1671–1683.

Rees HC, Maddison BC, Middleditch DJ, Patmore JR, Gough KC (2014) REVIEW: the detection of aquatic animal species using environmental DNA–a review of eDNA as a survey tool in ecology. *Journal of Applied Ecology*, **51**, 1450–1459.

Robert J, George E, De Jesus Andino F, Chen G (2011) Waterborne infectivity of the Ranavirus frog virus 3 in *Xenopus laevis*. *Virology*, **417**, 410–417.

Schmidt BR, Kéry M, Ursenbacher S *et al.* (2013) Site occupancy models in the analysis of environmental DNA presence/absence surveys: a case study of an emerging amphibian pathogen. *Methods in Ecology and Evolution*, **4**, 646–653.

Skelly DK (2004) Microgeographic countergradient variation in the wood frog, *Rana sylvatica*. *Evolution*, **58**, 160–165.

Skelly DK, Bolden SR, Holland MP *et al.* (2006) Urbanization and disease in amphibians. In: *Disease Ecology: Community Structure and Pathogen Dynamics* (eds Collinge SK, Ray C), pp. 153–167. Oxford University Press, Cary, NC, USA.

Staley C, Gordon KV, Schoen ME, Harwood VJ (2012) Performance of two quantitative PCR methods for microbial source tracking of human sewage and implications for microbial risk assessment in recreational waters. *Applied and Environment Microbiology*, **78**, 7317–7326.

St-Amour V, Lesbarrères D (2007) Genetic evidence of Ranavirus in toe clips: an alternative to lethal sampling methods. *Conservation Genetics*, **8**, 1247–1250.

Stuart SN, Chanson JS, Cox NA *et al.* (2004) Status and trends of amphibian declines and extinctions worldwide. *Science*, **306**, 1783–1786.

Thomsen PF, Kielgast J, Iversen LL *et al.* (2012) Monitoring endangered freshwater biodiversity using environmental DNA. *Molecular Ecology*, **21**, 2565–2573.

Venables BH, Hornik K, Maechler M (2015) polynom: A Collection of Functions to Implement a Class for Univariate Polynomial Manipulations. *R package version 1.3-8*. Available from http://CRAN.R-project.org/package=polynom.

Walker SF, Baldi Salas M, Jenkins D *et al.* (2007) Environmental detection of *Batrachochytrium dendrobatidis* in a temperate climate. *Diseases of Aquatic Organisms*, **77**, 105–112.

Warne RW, Crespi EJ, Brunner JL (2011) Escape from the pond: stress and developmental responses to ranavirus infection in wood frog tadpoles. *Functional Ecology*, **25**, 139–146.

Wheelwright NT, Gray MJ, Hill RD, Miller DL (2014) Sudden mass die-off of a large population of wood frog (*Lithobates sylvaticus*) tadpoles in Maine, USA, likely due to ranavirus. *Herpetological Review*, **45**, 240–242.

Wommack KE, Colwell RR (2000) Virioplankton: viruses in aquatic ecosystems. *Microbiology and Molecular Biology Reviews*, **64**, 69–114.

Yates MV, Gerba CP, Kelley LM (1985) Virus persistence in groundwater. *Applied and Environment Microbiology*, **49**, 778–781.

Zeileis A, Hothorn T (2002) Diagnostic checking in regression relationships. *R News*, **2**, 7–10.

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Data accessibility

The data underlying the figures are in Dyrad. DOI: 10.5061/dryad.k797r.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Supplemental qPCR method details.

Appendix S2. Model parameter estimates.

Appendix S3. Model of eDNA concentration.