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Persistence of an amphibian ranavirus in aquatic communities

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ABSTRACT: Host–parasite dynamics can be strongly influenced by interactions with other members of the biotic community, particularly when the parasite spends some fraction of its life in the environment unprotected by its host. Ranaviruses — often lethal viruses of cold-blooded vertebrate hosts transmitted by direct contact, and via water and fomites — offer an interesting system for understanding these community influences. Previous laboratory studies have shown that ranaviruses can persist for anywhere from days to years, depending on the conditions, with much longer times under sterile conditions. To address the role of the biotic community and particulate matter on ranavirus persistence, we experimentally inoculated filter-sterilized, UV-treated, and unmanipulated pond water with a frog virus 3 (FV3)-like ranavirus and took samples over 78 d, quantifying viral titers with real-time quantitative PCR and plaque assays. Viral counts dropped quickly in all treatments, by an order of magnitude in under a day in unmanipulated pond water and in 8 d in filter-sterilized pond water. In a second experiment, we measured viral titers over 24 h in virus-spiked spring water with *Daphnia pulex*. Presence of *D. pulex* reduced the concentration of infectious ranavirus, but not viral DNA, by an order of magnitude in 24 h. *D. pulex* themselves did not accumulate the virus. We conclude that both microbial and zooplanktonic communities can play an important role in ranavirus epidemiology, rapidly inactivating ranavirus in the water and thereby minimizing environmental transmission. We suspect that interactions with the biotic community will be important for most pathogens with environmental resting or transmission stages.

KEY WORDS: Ranavirus · Persistence · Indirect transmission · Microbial community · Zooplankton · Environmental DNA

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INTRODUCTION

Although host–parasite interactions are often considered in isolation, they are in fact influenced by larger communities of interacting species. These community interactions can influence the densities, distributions, and behaviors of both hosts and parasites, all of which can alter transmission dynamics (Ostfeld & Holt 2004, Thieltges et al. 2008, Johnson & Thieltges 2010). Environmentally transmitted parasites, such as those borne through water, are by definition free in the environment, unprotected by their host, for at least part of their life cycles. While they are often resistant to harsh abiotic conditions (e.g. heat, UV radiation), the propagules of these parasites may be subject to ecological interactions that can both reduce or promote transmission (Rosenheim 1998, Cottingham et al. 2003, Matz & Kjelleberg 2005, Meixell et al. 2013).

Water-borne pathogens, for example, may be actively consumed as a food source or incidentally inactivated or degraded by microbes, scavengers, or other community members (Gonzalez & Suttle 1993, Keiser & Utzinger 2005). Specifically, zooplankton such as *Daphnia* spp. have been shown to filter avian influenza viruses (Abbas et al. 2012) and the infectious zoospores of the fungal pathogen *Batrachochytrium dendrobatidis* (Buck et al. 2011, Woodhams et al. 2011, Schmeller et al. 2014) from the water, presumably reducing their potential for transmission.

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Additionally, filter-feeding *Daphnia carinata* have been shown to inactivate the bacterial pathogen *Campylobacter jejuni* (Schallenberg et al. 2005). In contrast, Minamoto et al. (2011) found that the filter-feeding activity of zooplankton, such as rotifers, simply concentrated the cyprinid herpesvirus-3 in their bodies. Depending on feeding preferences of the host, this might promote or reduce transmission of this virus via trophic interactions (i.e. hosts feeding on the filter-feeders). Pathogens may simply adhere to other aquatic organisms, with similar consequences (Tarsi et al. 2000). Finally, indirect interactions with other community members may also influence persistence of pathogens in aquatic environments. For example, toxins released by several types of bacteria can be used to kill other neighboring bacteria as a means of competition for space and nutrients (Chao & Levin 1981) and might have non-specific effects on pathogens in the environment.

Despite the demonstrated importance of biotic interactions to pathogen persistence, particularly those in aquatic communities, most studies have estimated pathogen persistence times under sterile or near-sterile conditions (Johnson & Speare 2003, Brown et al. 2009, Lebarbenchon et al. 2011, Nazir et al. 2012). We suggest that only under natural, decidedly non-sterile conditions with all of the interacting community members, can we accurately estimate persistence times and thus the potential for pathogens to cause recurrent epidemics (e.g. from year to year), to be transported between sites in contaminated water (e.g. Johnson & Speare 2003), or more generally, to be transmitted through the water (Breban 2013). We set out to quantify the effects of 2 ubiquitous aquatic community members — microbes and zooplankton — on the persistence of an amphibian ranavirus.

Viruses in the genus *Ranavirus* (family *Iridoviridae*) infect amphibians, fish, and reptiles and are associated with large-scale mortality events in the wild and aquaculture (Chinchar 2002, Miller et al. 2011, Chinchar & Waltzek 2014). These double-stranded DNA viruses are transmitted by direct contact, as well as indirectly from water, sediment, and fomites (Harp & Petranka 2006, Brunner et al. 2007). The ranavirus–amphibian system is an interesting one to use because the majority of ranavirus-induced mortality is seen in larval amphibian stages, which are confined to ponds (Green et al. 2002, Gray et al. 2009a). It is not well understood, however, how important indirect transmission through water is to overall transmission, partially because it is unclear how long ranaviruses remain viable in the water. Estimates of persistence time vary among published experiments, which often use different experimental methods. Some research teams allowed the virus to directly interact with biotic components of pond water, whereas others did not (Jancovich et al. 1997, Reinauer et al. 2005, Brunner et al. 2007, Nazir et al. 2012).

We conducted 2 laboratory experiments to estimate the rate of loss and persistence time of a frog virus 3 (FV3)-like ranavirus (AEC37; Brunner et al. 2011) with and without normal microbial communities and with and without the zooplankter *Daphnia pulex*. We expected that the microbial community would inactivate the virus much more rapidly than under sterile conditions. Zooplankton, however, might either inactivate ranavirus particles in water by filter-feeding or concentrate the virus and thus increase ranavirus transmission to the amphibians that prey upon them (Dodson & Dodson 1971, Hamilton et al. 2012).

MATERIALS AND METHODS

Expt 1: Persistence in pond water

Water was collected from each of 5 ponds with a history of amphibian breeding near Moscow, Idaho, USA on 16–17 September 2011. These included a pond at an arboretum, a pond in a wheat field, a cattle pond, a pond in a park historically used for farming, and an upper elevation pond in a forested area. These ponds were selected to represent the diversity of habitats in which amphibians are found in the area. At each pond, six 1 l plastic Nalgene (Nalge Nunc International) screw-cap containers were filled with water from the surface, so as not to disturb sediments on the bottom of ponds, returned to the lab, and transferred to 1 l glass screw-cap bottles. Water from each pond was divided into each of 3 treatments: unmanipulated, filtered, or UV-treated. Pond water in the filtered treatment was passed through sterile 0.22 µm Millipore Express Plus vacuum filters (EMD Millipore) in order to remove both microorganisms and particulate matter, essentially sterilizing the water samples. UV-treated water was circulated through individual 9 W Turbo Twist-3X UV clarifiers (Coralife) using an Eheim Universal pump (~300 l h^{-1}) and plastic aquarium tubing for 90 min in order to kill any living organisms, but allowing all particulate matter to remain. Pond water in the unmanipulated treatment was not processed in any way and contained all biotic and abiotic components collected from each pond. After exposing the water from each pond to the appropriate treatment, 800 ml of each experimental unit (5 ponds × 3 treatments ×

2 replicates = 30 units) was spiked with 0.32 ml of the AEC37 ranavirus, resulting in a final concentration of 10^5 plaque-forming units (pfu) per ml. The virus was grown in flathead minnow (FHM) cells with 10% fetal bovine serum (FBS) in Hank's Minimum Essential Media (HMEM; Eagle) in a 1.5% CO_2 environment at ~22°C and titrated with a standard plaque assay. After the water was spiked, the bottles were inverted 20 times to ensure homogeneous solutions and stored at room temperature (range ≈ 22–24°C) with a 12 h light:12 h dark cycle. Samples were taken immediately following inoculation and mixing of the water in each jar (Time 0, although up to 30 min passed before samples were taken), and then again 1, 2, 4, 6, 8, 10, 14, 29, 43, 57, 71, and 78 d post-inoculation. At each of these time points, jars were inverted 20 times, then a 10 ml and a 1 ml sample were collected and frozen at −80°C for real-time quantitative PCR (qPCR) and cell culture analysis, respectively.

Expt 2: Persistence in water with *Daphnia pulex*

Lab-reared *Daphnia pulex* of mixed ages (Aquatic Research Organisms) were kept in aerated spring water (Crystal Geyser Natural Alpine Spring Water). *D. pulex* were fed *Pseudokirchneriella subcapitata* algae and a yeast, cereal leaf, trout chow mix (Aquatic Research Organisms).

Populations at one of 5 densities (0, 1, 2, 5, or 10) of *D. pulex* per 9.6 ml of spring water were created in 50 ml sterile plastic screw-cap conical tubes. As daphniids have been shown to exhibit a type III functional response (Sarnelle & Wilson 2008), 2 food-density treatments were used in this experiment: medium density, corresponding to about 90 µg C l^{-1}, and high density, corresponding to about 1000 µg C l^{-1}, which amounted to 1.17×10^4 and 1.30×10^5 *P. subcapitata* cells ml^{-1} respectively, based on the conversion factor of 1 mg C $l^{-1} \cong 1.30 \times 10^8$ cells l^{-1} (Evjemo & Olsen 1999). Immediately before adding the appropriate number of *D. pulex*, 0.4 ml of the same virus stock as in the first experiment was added into each tube for a final volume of 10 ml and final concentration of 10^6 pfu ml^{-1}. The food–virus combination was mixed thoroughly, then *D. pulex* were added using standard disposable transfer pipettes. The tubes were kept on a 12 h light: 12 h dark cycle at room temperature (mean ≈ 22.3°C, range ≈ 22–24°C).

At 0, 5, and 24 h after inoculation, 3 tubes were destructively sampled from each treatment (2 food levels × 5 *D. pulex* densities × 3 replicates = 30 samples per time point). Tubes were inverted into a funnel containing grade No. 1 Whatman filter paper (GE Healthcare Bio-Sciences) over a 10 ml sterile plastic screw-cap conical centrifuge tube in order to strain *D. pulex* from the water. A 1 ml water aliquot was set aside from each replicate to be inoculated onto cell culture, and the remaining water was set aside for DNA extraction and real-time qPCR. *D. pulex* were rinsed on the filter paper with distilled water, then transferred into sterile 2 ml screw-cap microcentrifuge tubes containing 200 µl HMEM for subsequent inoculation onto cell culture or real-time qPCR. All water and *D. pulex* samples were frozen at −80°C.

Cell culture assays of viral titers

Samples were assayed for live, replicating ranavirus by mixing the water with cell culture media and then inoculating this mixture onto FHM cells in a standard plaque assays design. Briefly, 1 ml water samples were each pushed through sterile Millex-GV PVDF 0.45 µm filters (EDM Millipore), mixed 1:1 with 4% FBS in 2× HMEM and 0.1% Pen-Strep-Neomycin, and then serially diluted in 10-fold increments in 2% FBS in HMEM. Monolayers at about 90% confluency were inoculated with 100 µl of each dilution into 2 wells of a 6-well plate (Corning Cell-BIND; Corning). Sterile Cell Culture-grade water (Mediatech) mixed 1:1 with media served as a negative control in 2 wells of each assay. Samples were considered negative if no cytopathic effects were observed after 2 wk of incubation. Otherwise, the monolayers were stained with 200 µl of a 1% crystal violet in 10% buffered formalin and plaques counted using a stereo microscope. The average number of plaques in the pairs of wells with the highest number of clearly identifiable plaques was used to estimate the viral titer at that dilution and then back-transformed to estimate the titer (log_{10}[pfu] ml^{-1}) in the undiluted sample.

A subset of 12 *D. pulex* samples per food-level × density combination were also assayed for live virus. They were ground in 200 µl 2× HMEM using clean plastic 1.5 ml pellet pestles. Sample volume was brought to 2 ml by adding 1.8 ml HMEM, then pushed through sterile Millex-GV PVDF 0.45 µm filters using 3 ml sterile Luer-Lok plastic syringes. Samples were then split into two 1 ml samples, for qPCR analysis and cell culture. The subsequent cell culture protocol followed that of the water samples.

Extraction of viral DNA from water and *Daphnia pulex*

DNA was extracted from water samples following the methods of Kirshtein et al. (2007) and the Puregene Kit A protocol for tissue (Gentra Systems), as modified by Hyman & Collins (2012). Briefly, 10 ml water samples were thawed in the refrigerator (~4°C) until completely liquid, typically overnight. The water was then pushed through Millex-GV PVDF 0.22 µm filters (Millipore) using new, sterile 12 ml Luer-Lok plastic syringes, followed by 10 ml of Modified Dulbeco's Phosphate Buffered Saline without magnesium or calcium (HyClone Thermo Fisher Scientific), followed by air to completely push out the fluids from the filters. The filters were then extracted immediately or sealed in individual Ziploc bags at −20°C until later extraction. To remove DNA from the filters, they were sealed with Parafilm, filled with 1.5 µl proteinase K and 100 µl cell lysis buffer, inverted 20 times to force the liquid into the filter membrane, filled to capacity (~250 µl) with cell lysis buffer, and inverted an additional 30 times to ensure mixing. The filter membranes were digested for 1 h at 55°C. After incubation, the lysis solution was drained into 1.5 ml microcentrifuge tubes and the Puregene DNA extraction protocol was followed as per the manufacturer. Extracted samples were stored at 20°C until qPCR analysis. A pilot experiment demonstrated that this method recovered an average of 30% of the virus spiked into sterile water and immediately frozen and then extracted.

D. pulex samples were allowed to thaw overnight, transferred with forceps to clean tubes containing 180 µl ATL Buffer and 20 µl proteinase K (Qiagen DNeasy Blood and Tissue Kit), and then ground using a clean plastic 1.5 ml pellet pestle (Gerresheimer). Samples were then digested and DNA was extracted with the Qiagen DNeasy Blood and Tissue Kit following the manufacturer's instructions.

Quantitative real-time PCR

The amounts of viral DNA in water samples and *D. pulex* were quantified using qPCR methods outlined by Brunner & Collins (2009). Each sample (5 µl of DNA template at ~20 ng µl^{-1} as measured by a NanoDrop 2000 spectrophotometer) was run in triplicate 25 µl reactions on a StepOnePlus Real-Time PCR thermocycler (Applied Biosystems) and compared against a standard curve of DNA extracted from the same virus titrated with a cell culture-based plaque assay. Samples were scored negative if there was no amplification in any of the wells. If 1 of 3 wells was positive, the sample was re-run in triplicate. Samples that were inconsistent or had a coefficient of variance above 15% among wells were sonicated on full power at a 50% duty cycle for 60 s using a Branson Digital Sonifier (Hielscher) to ensure that the DNA solution was homogeneous, and then were re-run. Viral DNA concentrations are presented as the average $\log_{10}$(pfu) ml^{-1} of sample per *D. pulex* across all wells with the sample, except those runs that required sonication, which were reported as the average of just the sonicated wells. A TaqMan Exogenous Internal Positive Control (IPC) kit (Applied Biosystems) was used to check for qPCR inhibition in a subset of samples. Briefly, 2.5 µl 10× Exo IPC Mix and 0.5 µl 50× Exo IPC DNA were added per reaction to every third well of the 96-well plate. PCR inhibitors would reduce the amplification of the IPC assay and so provided a check that negative samples were true negatives.

Analysis

Changes in viral titers ($\log_{10}$-transformed viral titers per milliliter of water or per *D. pulex*) through time were analyzed with linear regression in R (R Development Core Team 2013), except for the viral titers measured by qPCR in the first experiment, which were analyzed with piecewise linear regression using the mle2 function in the bbmle package (Bolker & R Development Core Team 2013). We compared different versions of these models with shared or separate terms (e.g. slopes, breakpoints) for various treatments using Akaike's information criterion, corrected for small sample sizes (AICc), and AICc weights (Burnham & Anderson 2002). When p-values were used, we used a cutoff of 0.05 for significance.

RESULTS

Expt 1: Persistence in pond water

Ranavirus titers, as detected by qPCR, initially declined quickly in all treatments, decreasing in concentration by an order of magnitude across ponds in less than a day in unmanipulated pond water (Table 1), but then declined much more slowly and remained detectable for at least 78 d (Fig. 1). Thus, a piecewise regression model was fit, which allowed the titer to decline at 2 different rates on each side of a

Table 1. Parameters of the piecewise linear regression fit to $\log_{10}$ ranavirus DNA titers through time in 3 pond water treatments. T90 values are estimates of the time required for viral titers to decline by an order of magnitude. Initial and late rates of decline are the slopes on the $\log_{10}$ scale before and after the breakpoint. Breakpoint indicates the estimated day at which the rate of viral decline changed for each treatment (see Fig. 1). Numbers in parentheses are 95% confidence intervals

Treatment	T90 (d)	Rate of decline ($\log_{10}$[pfu] d^{-1}) Initial	Late	Breakpoint day
Filtered	8.018	−0.125 (−0.187 to −0.071)	−0.015 (−0.025 to 0.000)	12.486 (6.965 to 54.265)
UV-treated	1.582	−0.632 (−1.102 to −0.448)	−0.023 (−0.030 to −0.018)	4.685 (2.895 to 6.314)
Unmanipulated	0.839	−1.192 (−1.554 to −0.830)	−0.012 (−0.018 to −0.006)	2.827 (2.323 to 3.732)

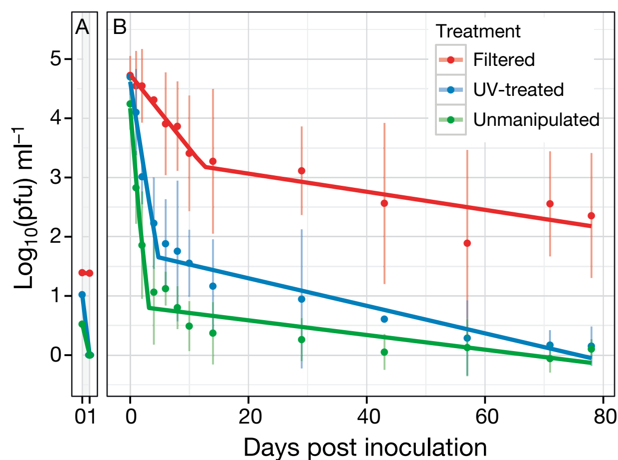

Fig. 1. Average ranavirus titers in virus-spiked water from 5 ponds over 78 d. Water samples from ponds were filtered (0.2 µm), UV-treated, or left unmanipulated, then spiked with a frog virus 3 (FV3)-like ranavirus (initial concentration = 10^5 pfu ml^{-1}). Points represent mean viral titers across all 5 ponds and the vertical lines are 95% confidence intervals. (A) Titers of infectious ranavirus were estimated with cell culture plaque-assays for Days 0 and 1 of the experiment. Solid lines represent the linear model for each treatment. For clarity, confidence intervals are not shown. (B) Ranavirus DNA was quantified by real-time qPCR. Bent lines are best-fit linear piecewise regressions for each treatment

breakpoint that was estimated from the data. There was very little support for a model with a single, common breakpoint across treatments (ΔAICc = 8.0, weight = 0.018), but rather the point at which the rate of decline changed was allowed to vary, from 2.827 d in unmanipulated water to 12.486 d in filtered water (Table 1; AICc weight = 0.982). (Note: there was much more support for a model in which each parameter varied between ponds [ΔAICc = 19.4, AICc weight ≈ 1; Fig. A1 in the Appendix]. However, while the specific rates vary substantially among ponds, the results are qualitatively similar. So for clarity, we present just the model with common parameters across ponds.) The initial rate of decline was significantly faster in the unmanipulated pond water and the UV-treated water than in the filtered water (Table 1). After the breakpoint, the rates of decline were very low — the rate of decline in filtered pond water was not significantly different from zero — and not significantly different among the 3 treatments. The amount of viral DNA detected at the end of the experiment (Day 78) was significantly higher in the filtered pond water than in the other 2 treatments (Fig. 1). This difference was not due to low PCR efficiency in the unfiltered treatments, as there was no indication of PCR inhibition in the TaqMan Exogenous IPC reaction added to the samples of unfiltered water from each pond.

It is important to note that the UV treatment was not completely effective at killing living organisms in the pond water before the virus was added. In several bottles with UV-treated water, as well as in water in the unmanipulated treatment, algae were observed growing late in the experiment.

Titers of live, infectious virus were 3–4 orders of magnitude lower than those indicated by qPCR (Fig. 1). This was at least partially due to the filter sterilization process, which removed about an order of magnitude of virus spiked into sterile water in pilot experiments using the same methods (authors' unpubl. data). Freezing and thawing of the virus-spiked samples was kept to a minimum, but may have also inactivated the virus to some degree. Overall, however, trends in viral titers through time were similar to the initial decline observed in the qPCR data (Fig. 1). The highest average initial (Day 0) titer was observed in the filtered treatment followed by the UV treatment; unmanipulated pond water had the lowest average initial titer. Over the first 24 h, the filtered treatment showed little to no decline in infectious virus, but in the other 2 treatments, the virus had declined to undetectable levels.

Expt 2: Persistence in water with *Daphnia pulex*

There was no detectable difference in ranavirus DNA titers (by qPCR) or in titers of infectious virions

(by plaque assays) between the medium and high food treatments, in either water samples or experimental *Daphnia pulex* (all $p \geq 0.541$). Thus, the 2 food treatments were combined for the following analyses.

Ranavirus DNA titers from the spring water samples did not change significantly over the 24 h period (Fig. 2; $\beta_{Time} = 0.005 \pm 0.003$, estimate ± 1 standard error; $t = 1.601$, $p = 0.113$). There was a detectible difference in the initial amount of viral DNA among the *D. pulex* density treatments ($\beta_{Daphnia} = -0.018 \pm 0.008$, $t = -2.194$, $p = 0.031$), and a significant interaction between *D. pulex* density and time, with viral titers declining more quickly with increasing *D. pulex* densities ($\beta_{Time \times Daphnia} = -0.029 \pm 0.014$, $t = -2.103$, $p = 0.038$). This result was largely driven by the highest density treatment (10 *D. pulex* per 10 ml). When this group was removed from the analysis, the interaction was no longer significant ($\beta_{Time \times Daphnia} = -0.015 \pm 0.015$, $t = -0.993$, $p = 0.324$), and viral titers did not decline through time ($\beta_{Time} < 0.001 \pm 0.004$, $t = 0.169$, $p = 0.866$).

The fraction of infectious ranavirus titers from water samples were between 3 and 4 orders of magnitude lower than DNA titers from qPCR (Fig. 2). The concentration of detectible infectious virions decreased significantly in all density treatments over the 24 h period ($\beta_{Time} = -0.879 \pm 0.196$, $t = -4.493$, $p < 0.001$). There was a significant main effect of *D. pulex* on the initial ranavirus titers ($\beta_{Daphnia} = -0.499 \pm 0.240$, $t = -2.082$, $p = 0.044$), but the interaction between *Daphnia* and time, which would indicate an increasing rate of decline with increasing *D. pulex* density, was not significant ($\beta_{Time \times Daphnia} = -0.066 \pm 0.039$, $t = -1.710$, $p = 0.096$). This is probably because of the large amount of variability in the titers of the control samples. When regressions were fit to each treatment level separately, the slope of the control treatment without *D. pulex* was not significant (-0.445 $\log_{10}$[pfu equivalents] $d^{-1} \pm 0.429$, $t = -1.039$, $p = 0.339$), whereas those for the treatments with *D. pulex* were -1.193 $\log_{10}$(pfu equivalents) d^{-1} or steeper (all $p \leq 0.002$). Second, when all *D. pulex* density treatments were grouped and compared to the control treatment (without *D. pulex*), the interaction was significant ($\beta_{Time \times Daphnia} = -0.890 \pm 0.362$, $t = -2.464$, $p = 0.018$), indicating that concentrations of infectious virions were reduced more quickly in samples containing *D. pulex* than those without.

The amount of ranavirus DNA in each *D. pulex* was about 2–4 orders of magnitude lower than what was detected in each milliliter of water samples (Fig. 2). If we assume that there are about 50 *D. pulex* ml^{-1}, then these are at least roughly equivalent. Viral DNA titers decreased significantly in all density treatments over the 24 h period ($\beta_{Time} = -1.736 \pm 0.408$, $t = -4.256$, $p < 0.001$). However, there was no detectible difference in titer among *D. pulex* density treatments ($\beta_{Daphnia} = -0.089 \pm 0.054$, $t = -1.628$, $p = 0.118$).

As in the previous experiment, the titer of infectious ranavirus particles in the *D. pulex* samples were 2–4 orders of magnitude lower than the DNA titers

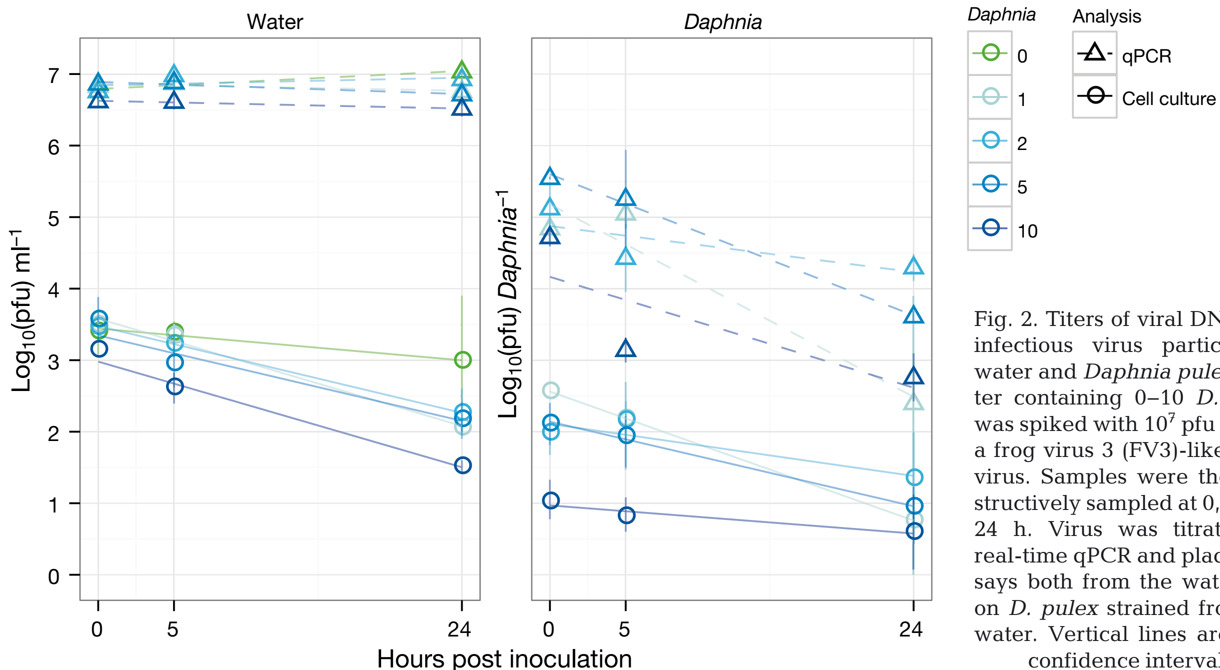

Fig. 2. Titers of viral DNA and infectious virus particles in water and *Daphnia pulex*. Water containing 0–10 *D. pulex* was spiked with 10^7 pfu ml^{-1} of a frog virus 3 (FV3)-like ranavirus. Samples were then destructively sampled at 0, 5, and 24 h. Virus was titrated by real-time qPCR and plaque assays both from the water and on *D. pulex* strained from the water. Vertical lines are 95% confidence intervals

determined by qPCR, and about 1–2 orders of magnitude lower than infectious viral titers detected in water samples (Fig. 2). The highest infectious viral titers were found in samples taken immediately after *D. pulex* were added to the virus-spiked water and decreased significantly over the 24 h sampling period ($\beta_{Time} = -1.466 \pm 0.329$, $t = -4.453$, $p < 0.001$). There was also a significant effect of *D. pulex* density ($\beta_{Daphnia} = -0.157 \pm 0.035$, $t = -4.479$, $p < 0.001$), but not of the interaction between density and time ($\beta_{Time \times Daphnia} = 0.109 \pm 0.060$, $t = 1.829$, $p = 0.076$).

DISCUSSION

Our experimental results demonstrate that interactions within aquatic communities can sharply reduce the persistence of pathogens in the environment. Specifically, we showed that titers of an amphibian ranavirus decline by roughly an order of magnitude per day in the presence of 2 common members of aquatic communities — microbes and zooplankton. At these rates, there is little time for virus particles to encounter and infect new hosts before being inactivated. We thus suspect that environmental transmission of ranaviruses may be largely curtailed in natural environments by microbes, zooplankton, and other aquatic organisms.

The 2 fairly distinct phases of declines in viral DNA (Fig. 1) suggest that there are at least 2 processes at work. The initial, rapid decline in viral DNA (and in titers of infectious virus particles) in the unmanipulated pond water treatment and, to some extent, the UV-treated water was likely due to direct interactions with microbes (e.g. consumption), but also, potentially, by some dissolved factor such as extracellular enzymes released by bacteria (Noble & Fuhrman 1997, Nasser et al. 2002). Noble & Fuhrman (1997), for example, found that extracellular microbial enzymes were likely responsible for about one-fifth of the maximal decay of viruses spiked into natural seawater, and Nasser et al. (2002) found that microbial extracellular enzymes could be highly effective at inactivating viruses. The presence of extracellular enzymes or other solutes might help explain why we observed a relatively rapid decline in the filter-sterilized water samples: these factors would likely have evaded filtration and thus been at work in both unmanipulated and filter-sterilized pond water.

Whatever specific factors might have been responsible for the reduction in virus concentration, they acted very quickly. The initial, day zero samples from unmanipulated water had half an order of magnitude less virus than those from filtered water, with samples from the UV-treated water in between. This suggests that the microbial community, their extracellular enzymes, or whatever factors were at work were able to strongly reduce viral titers in the short time between inoculation and when the initial sample was frozen at −80°C (approximately 30 min). It is also possible that virions adsorbed to particulate matter in the UV and unmanipulated treatments without necessarily being inactivated and were thus filtered out before titration by plaque assay, resulting in underestimates of infectious viral titer (Carlson et al. 1968). Adsorption onto increasingly saturated particles might also help explain the 2-phase decline in viral DNA concentrations. However, this effect alone would not explain why initial viral titers were lower in the unmanipulated pond water than the UV-treated pond water, both of which had the same particulate matter. Moreover, we would have expected the DNA from virions adsorbed to particulate matter to be included in the extracted DNA (although live virus attached to particles would have been removed by the filtration step when cells were inoculated). In any case, it is clear that ranavirus titers declined rapidly in regular pond water and remained orders of magnitude lower than samples from water in which all particulate matter and living organisms were removed.

We are cautious about how to interpret the long detection time of low levels of viral DNA (Fig. 1). First, unfortunately, we do not have data on the activity of ranavirus past 24 h; the viral titers were lower initially than we expected and quickly became undetectable. So we simply do not know whether a very small fraction of the initial ranavirus might have persisted for longer than a few days. Second, it is unclear whether the water samples remained at all representative of the ponds from which they came after many weeks in closed bottles in the laboratory. For instance, we do not know if the microbial community remained active throughout the experiment or conditions (e.g. dissolved O_2 concentrations or pH) changed. Future studies would benefit from better characterizing the water and microbial community in water. Our strongest result is thus that ranavirus and ranavirus DNA are rapidly degraded in pond water.

It is important to note that we cannot completely distinguish between the effects of the microbial community in pond water and the suspended solids in the water. Although the initial rate of viral decline in UV-treated water appeared to be intermediate to filtered and untreated water, it was not significantly different

from the unmanipulated pond water (Table 1). There are 2 possible explanations. First, the virus may have degraded much faster in the UV-treated pond water compared to filter-sterilized water because virions adhered to (and were potentially inactivated by) suspended particulate matter. Alternatively, there may have been enough of the microbial community left in these UV-treated water samples to have caused the virus's decline; the UV treatment did not completely eliminate all biotic components of pond water, as algae were observed growing in UV-treated pond water samples late in the experiment. Further study will be required to separate the relative importance of biotic and abiotic components of pond water. It is clear that the zooplankter *Daphnia pulex* can significantly reduce the persistence time of ranaviruses in water by about an order of magnitude in 24 h. Consistent with recent studies using the zoospores of the fungal pathogen *Batrachochytrium dendrobatidis* (Buck et al. 2011, Searle et al. 2011, Hamilton et al. 2012) and avian influenza viruses (Abbas et al. 2012, Meixell et al. 2013), *Daphnia* spp. can remove pathogens from the water column very quickly. This is presumably incidental, at least for viruses, which are much smaller than the size range of food typically preferred by *Daphnia* spp. (about 0.2–4.7 µm compared to the ≤150 nm diameter of ranaviruses; Burns 1968, Geller & Müller 1981). Indeed, there was no change in concentrations of viral DNA over the 24 h period, whereas titers of infectious virus were reduced in all treatments containing *D. pulex* (Fig. 2). This suggests that *D. pulex* were inactivating virions without completely digesting them. Surprisingly, there was no effect of increasing densities of *D. pulex* on the rate of ranaviral decay in our experiment (although even 1 *D. pulex* per 10 ml tube [= 100 *D. pulex* l^{-1}] is on the high end of natural densities; Kwik & Carter 1975, DeMott 1983), nor of food densities (in contrast to Searle et al. 2013) as would be expected if rates of filtration and inactivation were a simple product of the feeding rates of *D. pulex*. We do not know how to account for this finding.

Lastly, we had hypothesized, like others (e.g. Abbas et al. 2012, Meixell et al. 2013), that *Daphnia* spp. might accumulate virions from the water and thus act as a source of infection to species that prey upon *Daphnia* spp. Instead we found that virus titers in the *D. pulex* themselves declined over time, a result supported by Meixell et al. (2013). It seems unlikely, then, that these zooplankters are an important source of infections to their consumers.

Our estimates of the persistence time of this ranavirus contrast with those of previous studies, which have generally concluded that ranaviruses are environmentally stable, persisting for long periods while frozen (Langdon 1989) or in water (Reinauer et al. 2005, Nazir et al. 2012). Those studies, however, were conducted under sterile or nearly sterile conditions. Nazir et al. (2012), for example, estimated that the concentration of 3 ranaviruses in pond water would decline by an order of magnitude in 22–34 d, but enclosed the virus in a protective filter membrane that prevented microbes or other organisms access to the virions. Nazir et al.'s (2012) estimates in which direct biotic interactions were excluded would thus represent a best-case, upper bound on persistence times, similar to our filtered water treatment. It is also important to note that while the initial rate of decline in replication-competent virus mirrors that of viral DNA concentrations, we were not able to measure live virus titers beyond the first day. Thus most of our data come from measures of viral DNA; the relationship between the two is largely unknown.

There are 2 additional factors that might play an important role in the persistence of ranaviruses and similar pathogens that were not included in our experimental designs. The first is temperature. Ranaviruses, like many other pathogens, degrade faster at higher temperatures (Reinauer et al. 2005, Nazir et al. 2012). Even in filter-sterilized water, we observed an order of magnitude drop in viral titers within 8 d, in contrast to Nazir et al.'s (2012) estimate of ≥22 d for a similar decline, but our samples were held in a room in which the temperature varied between approximately 22 and 24°C, while their study was conducted at a constant 20°C. Since amphibian breeding ponds are often cooler than room temperature, our experiment may underestimate ranaviral persistence in nature. Our laboratory experiment was likely a poor facsimile of many other features of ponds in nature (e.g. sunlight, pond substrates, wind).

Second, like most others (Langdon 1989, Reinauer et al. 2005, Nazir et al. 2012), our study used virus grown in cell culture. It is likely that much of the virus shed by infected animals is embedded in sloughed skin and mucus and protected, at least initially, from bacteria and other microbes. These fomites likely remain infectious longer than free virions. Jancovich et al. (1997), for example, observed that the *Ambystoma tigrinum* virus shed into aged tap water by infected animals lost its ability to infect naïve individuals by about 2 wk, which is considerably longer than our data would suggest. Whether naturally shed virions in fomites persist longer is thus an open question, but if they are, then our estimates of persistence times may be closer to the lower bound.

We conclude that zooplankton, and likely the microbial community in ponds, play an important role in inactivating ranavirus in pond water. In contrast to the conventional wisdom that ranaviruses are environmentally resistant (Gray et al. 2009b, Whittington et al. 2010), they appear to be rapidly degraded by organisms found in the aquatic environment. The short persistence time of ranaviruses suggests that transmission from the environment, while possible, could be less important or common than previously thought. We encourage more research on the effects of the biological community on the persistence and transmission of pathogens in other systems and conditions.

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Appendix. Ranavirus titer data separated by pond

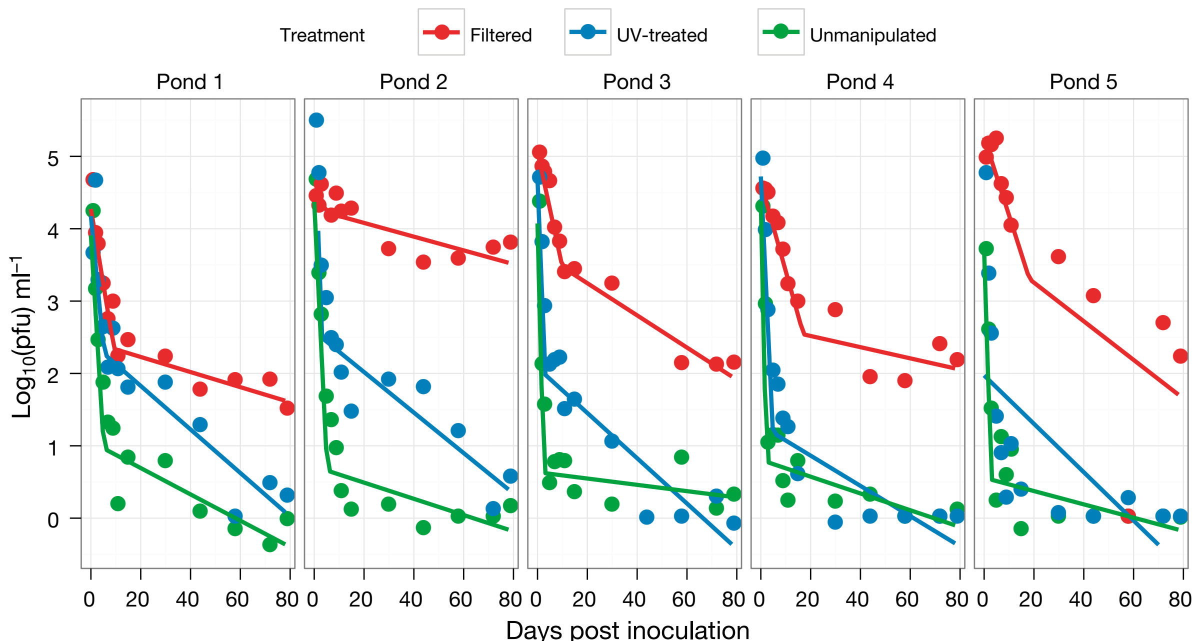

Fig. A1. Ranavirus titers in water from each of 5 ponds (panels) that had been filtered (0.2 µm), UV-treated, or left unmanipulated, then spiked with a frog virus 3 (FV3)-like ranavirus (initial concentration = 10^5 pfu ml^{-1}). Viral titers measured by quantitative real-time PCR. Bent lines are best-fit linear piecewise regressions for each treatment within each pond

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