

# Evaluating the Importance of Environmental Persistence for *Ranavirus* Transmission and Epidemiology

Jesse L. Brunner<sup>1</sup>, Christian M. Yarber

School of Biological Sciences, Washington State University, Pullman, WA, United States

<sup>1</sup>Corresponding author: e-mail address: jesse.brunner@wsu.edu

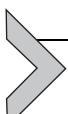
## Contents

1. Introduction	2
2. Persistence on a Continuum: A Framework	3
3. Environmental Influences on Persistence Times	5
4. Persistence in Tissues and Carcasses	9
5. An Epidemic Model to Evaluate the Importance of Persistence Times	10
6. Conclusions	15
Acknowledgments	17
References	17
Further Reading	20

## Abstract

Viruses persist outside their hosts in a variety of forms, from naked virions to virus protected in sloughed tissues or carcasses, and for a range of times, all of which affect the likelihood and importance of transmission from the environment. This review synthesizes the literature on environmental persistence of viruses in the genus *Ranavirus* (family *Iridoviridae*), which are large double-stranded DNA viruses of ectothermic, often aquatic or semiaquatic vertebrates. Ranaviruses have been associated with mass mortality events in natural and captive settings around the world, and with population and community-wide declines in Europe. Early work suggested ranaviruses are environmentally robust and transmission from the environment should be common. More recent work has shown a large effect of temperature and microbial action on persistence times, although other aspects of the environment (e.g., water chemistry) and aquatic communities (e.g., zooplankton) may also be important. Ranaviruses may persist in the carcasses of animals that have died of infection, and so decomposing organisms and invertebrate scavengers may reduce these persistence times. The question is, do persistence times vary enough to promote or preclude substantial transmission from the environment. We built an epidemiological model with transmission from contacts,

free virus in water, and carcasses, to explore the conditions in which environmental persistence could be important for ranavirus epidemiology. Based on prior work, we expected a substantial amount of transmission from the water and that longer persistence times would make this route of transmission dominant. However, neither water-borne nor transmission from carcasses played an important role in the simulated epidemics except under fairly restrictive conditions, such as when there were high rates of virus shedding or high rates of scavenging on highly infectious carcasses. While many aspects of environmental persistence of ranaviruses are being resolved by experiments, key parameters such as viral shedding rates are virtually unknown and will need to be empirically constrained if we are to determine whether environmental persistence and transmission from the environment are essential or insignificant features of *Ranavirus* epidemiology. We conclude by emphasizing the need to place environmental persistence research in an epidemiological framework.



## 1. INTRODUCTION

Viruses of aquatic vertebrates are known primarily from their impacts on host populations. The earliest known animal virus, lymphocystis disease virus, for instance, was initially studied for the wart-like disease it causes in fish (Weissenberg, 1965). The epizootic hematopoietic necrosis virus (EHNV), one of the first viruses of aquatic vertebrates listed by the World Organization for Animal Health (OIE), was of particular concern because of its capacity to cause massive fish die-offs (Langdon and Humphrey, 1987; Langdon et al., 1986). Both of these viruses are members of the *Alphairidovirinae* subfamily of the *Iridoviridae* family—large, icosahedral viruses with double-stranded DNA genomes—which includes three genera, *Lymphocystivirus* and *Megalocytivirus*, which infect fish, and *Ranavirus*, which infects fish, amphibians, and reptiles (Chinchar et al., 2017). Ranaviruses in particular have received a great deal of attention for causing die-offs of tens to tens of thousands of fish and aquatic or semiaquatic amphibians and turtles around the world (Duffus et al., 2015). The recent emergence of a ranavirus in Spain has led to declines in amphibian communities (Price et al., 2014). Ranaviruses also continue to threaten aquaculture and fish hatcheries (e.g., Huang et al., 2011; Kayansamruaj et al., 2017; Waltzek et al., 2014).

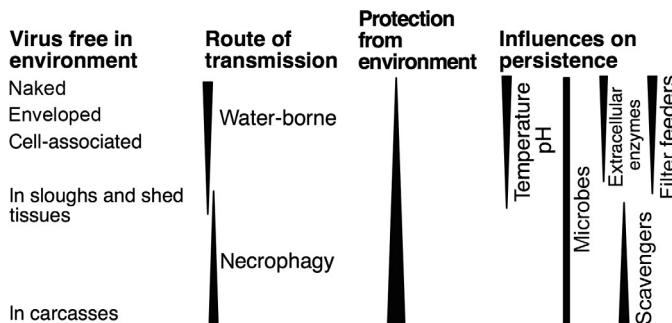
Beyond an impressively broad host range, experiments have shown that ranaviruses can be transmitted through several routes, including via direct contact, consumption of infectious tissues (cannibalism and necrophagy), and adsorption of virions from the water (reviewed in Brunner et al., 2015). The host's gastrointestinal tract is probably the most common site

of entry (Robert et al., 2011). Dose–response studies in fish, salamanders, and frogs (e.g., Brunner et al., 2005; Forzan et al., 2015; Plumb and Zilberg, 1999b) are reasonably fit by a simple model assuming independent action of each virion (J.L. Brunner, unpublished analysis), which suggests a simple per virion hazard of infection (Ben-Ami et al., 2010; Regoes et al., 2002; van der Werf et al., 2011). Different routes of transmission are therefore more or less effective because they deliver more or fewer virions. What is interesting about this perspective is that environmental factors can influence transmission, perhaps strongly, by promoting or limiting the accumulation of infectious viral particles in the environment. If this is the case, it might help explain the sporadic occurrence of *Ranavirus* epidemics and die-offs in endemic regions (e.g., O'Connor et al., 2016; Whittington et al., 2010, #42952). It might also afford some control over the epidemiology of these emerging viruses; by preventing ranaviruses from persisting in the environment, one could perhaps prevent epidemics from occurring.



## 2. PERSISTENCE ON A CONTINUUM: A FRAMEWORK

We find it useful to think of environmental persistence of viruses along a continuum. Most viruses persist outside a living host for a time, but that time may be measured in hours (e.g., Ebola virus in sterilized waste water; Bibby et al., 2015) or days (Human Adenovirus 2 in groundwater; Ogorzaly et al., 2010) or months (avian influenza in distilled water; Brown et al., 2007). Longer persistence times facilitate transmission from the environment (Kotwal and Cannon, 2014), whereas shorter times restrict it, often to a small enough role that it can be effectively ignored. And so, we posit, for viruses such as ranaviruses that infect aquatic animals that the relative importance of environmental transmission is in large part a function of environmental persistence times. Persistence times, in turn, are a function of the environment in which the virus finds itself and the adaptations it may have to persist in that environment. The form of free virus also spans a continuum from naked virus particles to large quantities of virus within newly deceased hosts (Fig. 1). Naked particles are clearly more vulnerable to the chemical and physical environment, extracellular enzymes secreted by microbes, and filter feeding organisms (e.g., zooplankton) while viruses contained in carcasses are somewhat protected from these elements but may be more vulnerable to scavenging or simply being lost from the water column. Propagules embedded in or on sloughed tissues (e.g., skin, stomach lining) or feces from an infected host likely experience intermediate protection.



**Fig. 1** Viruses such as *Ranavirus* spp. can be free in the environment in a variety of forms, from naked virions to virions held within carcasses of infected hosts. The form has consequences for the route by which this free virus can infect susceptible hosts as well as how it persists in the environment. Virions in carcasses, for instance, are largely protected from the physical environment (e.g., water pH, extracellular enzymes), but may be consumed by invertebrate scavengers or necrophagous hosts.

We can also map onto this continuum the route by which propagules are transmitted from the environment to naïve hosts. Because they are small and easily dispersed by Brownian motion or currents, viruses shed directly into water, or associated with small bits of sloughed tissues and fomites (e.g., virus adhered to floating particulate matter), contribute to water-borne transmission. Greater rates of shedding and longer environmental persistence times allow large amounts of virus to accumulate in the water and thus likely promote water-borne transmission. As the sloughs get larger, up to and including infectious carcasses, we can no longer assume that these sources of infection are well mixed in the water column, but rather that susceptible hosts encounter virus in these forms by consumption. Many animals, even putative herbivores like anuran tadpoles, engage in a degree of scavenging, which might lead to consuming infectious materials in the environment as they feed (Altig et al., 2007; Getz, 2011; Wilson and Wolkovich, 2011). At the extreme, some animals, including many fish and amphibians, are necrophagous, seeking out and consuming conspecific carcasses, which can be a very efficient means of transferring large doses of virus (e.g., Diamant, 1997). High viral titers in carcasses, longer persistence times of the carcasses, and high rates of scavenging or necrophagy, and especially sharing of carcasses (Rudolf and Antonovics, 2007), all promote transmission by necrophagy.

While these general patterns seem intuitive, the actual importance of environmental persistence for virus transmission in nonhuman systems is rarely considered quantitatively, especially across the spectrum of forms

of free virus and routes of transmission. Moreover, outside of sporadic studies in groundwater or wastewater (e.g., Bibby et al., 2015; Brown et al., 2007; Ogorzaly et al., 2010), most research on persistence of animal viruses focuses on persistence in hospital settings (e.g., Cook et al., 2015) or on hands or food surfaces (e.g., Kotwal and Cannon, 2014) or other human dominated settings. As such, there is a need to better understand the persistence of nonhuman animal viruses in nature. Here, we explore the factors that affect the persistence of ranaviruses and try to relate persistence times directly to ranavirus transmission. Note that we largely ellipse over environmental persistence between epidemics, although though this is an important issue deserving future attention.



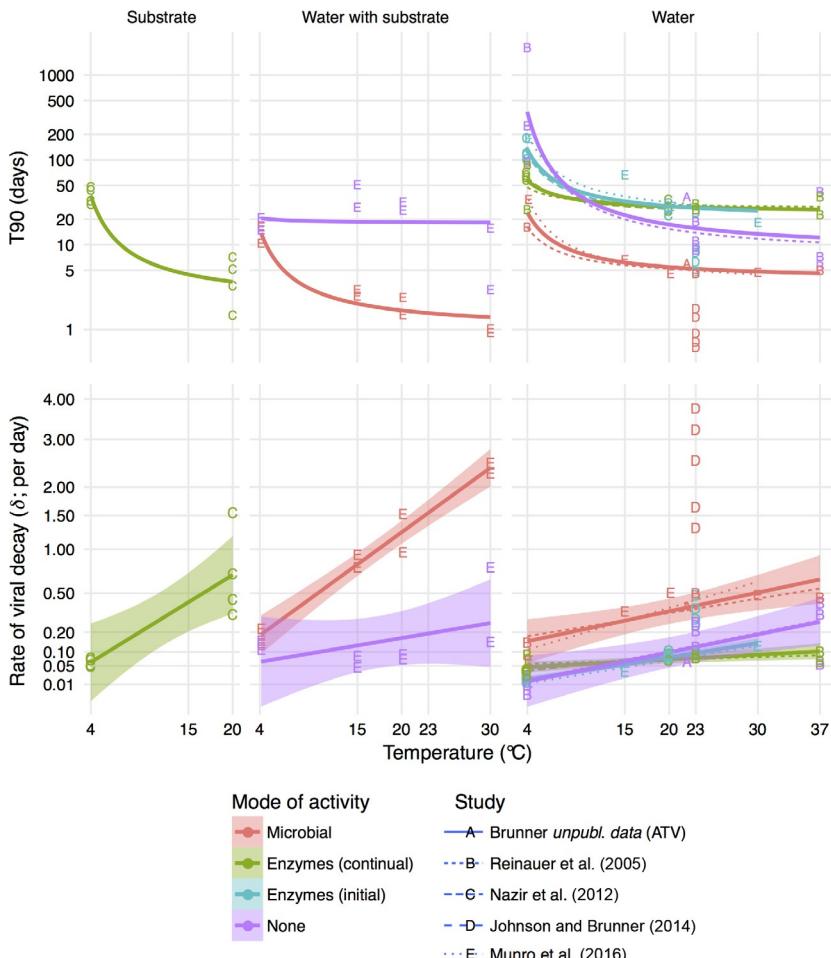
### 3. ENVIRONMENTAL INFLUENCES ON PERSISTENCE TIMES

Until recently, most of what we knew about ranaviral persistence came from anecdotes or small studies with ranavirus grown in culture held under various laboratory conditions (e.g., Jancovich et al., 1997; Langdon, 1989; Speare and Smith, 1992). The Bohle Iridovirus, for instance, can survive 6 h at 25°C and 37°C in culture medium, but is rapidly inactivated at 56°C and titers are dramatically reduced at pH 2, 3, 10, and 11 (Speare and Smith, 1992). EHNV maintained its infectiousness for at least 97 days in sterile water at 15°C and at least 300 days when held at 4°C in tissue culture (Langdon, 1989). While these results suggest that ranaviruses are inherently robust, it is difficult to extrapolate these results from essentially aseptic conditions to natural environments where microbes and other organisms actively or inadvertently inactivate virus particles (Ward et al., 1986).

A series of studies beginning with Reinauer et al. (2005) and Nazir et al. (2012) brought much needed ecological realism to our understanding of persistence of ranaviruses in the environment. These first two studies used “germ carriers,” comprised of virus in tissue culture adsorbed to a filter and sealed within a polycarbonate membrane “sandwich” with a 10-nm pore size, much smaller than most bacteria (Stevik et al., 2004; Young, 2006). This apparatus allows extracellular enzymes to pass through and act on the virus, but prevents contamination of the culture by other microbes. These germ carriers were then placed in soil or in drinking water or pond water that had been filter sterilized or left unmanipulated. If we assume that drinking water lacks appreciable microbes and that filter-sterilizing leaves the extracellular enzymes initially present, these studies collectively

provide estimates of ranavirus persistence under three conditions: (1) without any microbial action; (2) with the extracellular enzymes initially present, but no further action; and (3) with extracellular enzymes continually produced by microbes. However, to understand the role of direct microbial action (e.g., adsorption or endocytosis) on *Ranavirus* persistence, we must look to studies that spiked ranavirus in culture directly into water and then attempted to reisolate and titer the virus (Brunner *unpublished data*, referenced in Brunner et al., 2007; Reinauer, 2004; Johnson and Brunner, 2014; Munro et al., 2016). [Note that Johnson and Brunner's (2014) results are primarily based on quantities of viral DNA rather than live virus titers as viral titers rapidly fell below the limits of detection.] Importantly, there is little if any evidence that ranavirus isolates, even those from amphibians and reptiles, vary in their ability to persist in the environment (Nazir et al., 2012). It is possible that certain strains have been subject to selection for greater environmental resistance, but measurable differences have not been found. We therefore include studies with both amphibian and reptile ranavirus isolates in our synthesis. (Unfortunately, persistence of fish ranaviruses has not been studied quantitatively.) These studies have found a great deal of variation in persistence times, measured as the time it takes for viral titers to be reduced by 90% (T90), but several patterns emerge when the evidence is considered collectively (Fig. 2).

First, soil and pond substrate may reduce persistence. Ranaviruses appear to degrade more rapidly in soil or pond substrate than in water (Fig. 2), especially at warmer temperatures (discussed later). While ranavirus may persist in capsules in the soil or in sterilized pond substrate, as in the experiment by Brunner et al. (2007), retaining its ability to infect 86% of naïve salamander larvae placed in it, when *Ranavirus* is dried in the substrate and then rehydrated, it loses its activity entirely (0% infection; Brunner et al., 2007). This stands in sharp contrast to the long persistence times found when virus was dried on plastic or metal (Langdon, 1989; Nazir et al., 2012) and suggests that soil or sediments may act on the virus directly. Indeed, Munro et al. (2016) found that adding sediments, even autoclave sterilized sediment, to water increased rates of degradation (Fig. 2), or at least reduced viral recovery—perhaps irreversibly binding to it or somehow chemically inactivating it. Viruses can rapidly adsorb to soil particles, at a rate dependent on soil properties, pH, water content, etc. (Sobsey et al., 1980). In any case, it appears that ranavirus is unlikely to persist long in the soil or sediment under warm conditions or when dried but may be able to persist throughout a winter if kept moist and cool. Whether persistence in or on soil plays much if any role in ranavirus transmission within epidemics remains unknown.



**Fig. 2** Estimates of the time it takes for viral titers to be reduced by 90% (T90; top) or, conversely, the rate of viral decay [ $\delta = T90^{-1} / \log_{10} e$ ] as a function of temperature in soil, water with substrate, or water. We classify treatments by the mode of biotic action they allow, from no action (e.g., autoclaved pond water or tap water) to extracellular enzymes initially in the water (when water is filter-sterilized) or continually excreted (when water is left unmanipulated) to allowing direct interaction with microbes. Lines are hyperbolas ( $y = b_0 + b_1 / {}^{\circ}\text{C}$ ) fit to the T90 times and linear fit to the decay rates by study (thin dashed or dotted lines) or to all studies together (thick solid lines), excluding Johnson and Brunner (2014), which focused on viral DNA rather than infectious virus.

Second, other microbes, presumably bacteria and fungi, appear to play a critical role in the inactivation of ranavirus, at least in water (Fig. 2). In particular, when additional microbes are allowed to directly interact with ranavirus, persistence times are four to five times shorter than when the only

mode of action is extracellular enzymes (Fig. 2). Surprisingly, while bacterial proteolytic enzymes may be the primary means of viral inactivation in water (Ward et al., 1986), there were only minor differences between treatments that exposed ranavirus to the action of extracellular enzymes and those that used tap water (Reinauer, 2004) or autoclaved water (Brunner, unpublished data; Fig. 2). Johnson and Brunner (2014) demonstrated a similar role for zooplankton. Concentrations of viral DNA and titers of active ranavirus declined rapidly in spring when *Daphnia pulex* were present ( $T90 < 1$  day) relative to virtually no degradation in spring water alone. Moreover, viral titers on the *Daphnia* themselves declined during the experiment, suggesting these filter-feeding cladocerans do not simply concentrate active virus, which might infect their predators, but rather inactivate the ranavirus they filter. Collectively, these results suggest that aquatic microbes, which interact directly with virions, and the zooplankton that filter them, can play an important role in the inactivation of ranaviruses in freshwater. The degree to which aquatic community composition and abundance influence ranavirus persistence remains an open question.

Lastly, similar to other animal viruses in water (e.g., avian influenza virus; Brown et al., 2007, 2009), there is a clear pattern of increasing rates of degradation of *Ranavirus*, and thus shorter persistence times, with increasing temperatures across substrates and experimental manipulations (Fig. 2).  $T90$  times in soil were on the order of several days under summer conditions ( $\geq 20^{\circ}\text{C}$ ) relative to  $T90$  times  $> 30$  days in the cool spring (Nazir et al., 2012; Reinauer et al., 2005). Persistence times in water vary by an order of magnitude across an ecologically relevant range, from several weeks or more at  $4^{\circ}$ , which is a common temperature in small bodies of water in the winter or early spring, to less than a week at temperatures  $\geq 20^{\circ}\text{C}$  (Fig. 2), which are commonly achieved in the summer. Larger water bodies can also have dramatic temperature clines from deep to shallow waters and so we would expect persistence times to vary a great deal within a single habitat.

The effects of other aspects of the physical aquatic environments (e.g., pH, turbidity) on ranavirus persistence have not been well explored, but are likely to be important (Brown et al., 2009). Indeed, viral persistence times varied dramatically among ponds in Johnson and Brunner's (2014) study, even in filtered water under common garden conditions. A key question is whether and how much this variation in viral persistence times in the environment matter to ranavirus transmission and dynamics. We return to this question later, but first we consider rates of degradation in host tissues.



#### 4. PERSISTENCE IN TISSUES AND CARCASSES

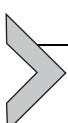
One critique of the studies presented earlier is that they all used virus grown in cell culture, which includes nonenveloped, enveloped, and cell-associated ranavirus particles, but not virus shed from host animals or shed tissues (e.g., sloughed skin or intestinal lining). Virus particles inside of host tissues or sloughs are likely protected to a degree from the action of microbes, plankton, and water chemistry and so the estimates of persistence times in prior experiments (Fig. 2) may underestimate the persistence of naturally shed ranavirus. The production of sloughs can be considerable during late stages of ranaviral disease, and their consumption, intentional or not, can be a very effective route of transmission (Brunner et al., 2007; Jancovich et al., 1997). Extending this reasoning to carcasses of hosts killed by ranavirus infections, we see that the route of transmission and the mode of virus inactivation are likely to change when virus occurs in host tissues rather than free floating in the water.

Infectious carcasses can contain very high viral titers (e.g., Brunner and Collins, 2009), which makes them very capable of infecting scavenging or necrophagous hosts, such as conspecifics or other ectothermic vertebrates in the habitat (Ariel and Owens, 1997; Brunner et al., 2007; Harp and Petranka, 2006; Pearman et al., 2004). Scavenging or inadvertent necrophagy is a common feeding pattern, even among putative herbivores like tadpoles (Altig et al., 2007; Getz, 2011; Wilson and Wolkovich, 2011) and so this route of transmission may be more important than typically considered, at least for pathogens like ranaviruses that remain infectious in dead hosts.

Carcasses are removed from ecosystems by the detrital or decomposer community, through the combined actions of obligate and facultative vertebrate and invertebrate scavengers and microbes (Parmenter and Lamarra, 1991; Regester and Whiles, 2006; Stevenson and Childers, 2004). Rates at which hosts decompose vary substantially depending on the species considered and the environment in which rates are tested. Decomposition rates of freshwater fish biomass vary from T90 times of less than 2 days (mosquito fish in a Florida marsh; Stevenson and Childers, 2004) to over 2 months (bluegill in a Wisconsin lake; Kitchell et al., 1975; reviewed in Regester and Whiles, 2006). Decomposition rates of amphibians are on the shorter end of this range. Regester and Whiles (2006), for instance, estimated that spotted salamander (*Ambystoma maculatum*) larvae have a T90 time of 3 days. Their and

other studies enclosed the carcasses in water in wire mesh of varying sizes, which likely excluded the action of larger scavengers and so may underestimate rates of decay (Regester and Whiles, 2006). Le Sage et al. (in review), for instance, found that a single diving beetle (Dytiscidae) larva or dragonfly (Aeschnidae) naiad in aquaria consumed 84% or 62%, respectively, of the mass of a larval *Ambystoma macractylum* carcass within 2 days, and larval Notonectidae and Gammaridae also consumed some carcass mass. The entire community of scavenging invertebrate animals in addition to microbes plays an important role in removing carcasses (Le Sage et al., in review; Parmenter and Lamarra, 1991; Regester and Whiles, 2006; Stevenson and Childers, 2004). Whether carcass removal plays an important role in ranavirus transmission, however, has been unexplored until very recently.

To test whether scavengers might play an important role in *Ranavirus* transmission, Le Sage et al. (in review) conducted an experiment in the laboratory in which larval salamanders, *A. macractylum*, either had access to the carcass of a conspecific that had died of ranavirus infection or was prevented from contacting (and consuming) the carcass by a mesh barrier. As expected, most (68%) of larvae that could contact and feed on the carcass for 24 h became infected while only 16% of larvae were infected when this contact was prevented. When the carcass was first scavenged by a dytiscid (diving beetle) larva for 24 h before the salamander larvae were introduced, only 23% of salamander larvae became infected. In other words, the scavenging beetle effectively reduced transmission to levels equivalent to those observed when transmission could only occur indirectly, by virions shed into the water. Moreover, scavenging did not appear to spread infectious particles into the water as had been initially hypothesized (a scavenged carcass behind a screen infected 20% of the larvae). Le Sage et al. (in review) also estimated the relative densities of salamander larvae and dytiscid larvae, and the functional response of dytiscid larvae to carcasses, and found that over most of the larval period, the scavengers had the capacity to consume carcasses from the entire salamander population, were all individuals to die at once. While this is only one study, it does suggest that invertebrate scavengers have the potential to minimize or even prevent transmission from carcasses and so dramatically reduce ranavirus transmission within amphibian breeding ponds.



---

## 5. AN EPIDEMIC MODEL TO EVALUATE THE IMPORTANCE OF PERSISTENCE TIMES

While there is still uncertainty about the physical, chemical, and biotic factors that control ranaviruses persistence in various environments, it is clear

that persistence times vary considerably, often by orders of magnitude. Perhaps, the most pressing question is thus whether this variation in persistence times is important for ranavirus transmission and epidemiology. In other words, do long persistence times increase ranavirus transmission through water or from carcasses, relative to short persistence times? We next develop an epidemiological model to help address this question.

First, let us assume for simplicity a single large, well-mixed population of a single host species in a pond or lake with no birth and no natural deaths (i.e., deaths due to factors besides disease). With these assumptions, susceptible hosts are lost only to infections, and so we focus our attention on the change in density of infected hosts:

$$\frac{dI}{dt} = Sb \left( \kappa\eta \frac{I}{N} + \mu\lambda_h C + \omega V \right) - \alpha I.$$

The variables  $S$ ,  $I$ , and  $V$  are the densities of susceptible and infected hosts, and ranavirus in the water, respectively. Susceptible hosts become infected by exposure to virions via (in order of the terms in parentheses) (i) direct casual contact with infected hosts; (ii) consumption of infectious carcasses (at density  $C$  in the pond); or (iii) absorption or ingestion of virus in the water. Susceptible hosts ( $S$ ) have a per virion hazard of infection,  $b$ , and so this formulation makes clear that these three routes of transmission differ only in the concentration of virions they provide to susceptible hosts per unit time. Direct casual contact with an infected individual delivers  $\kappa$  virions per contact, and we assume this route of transmission is frequency dependent, i.e., contacts occur at a constant rate,  $\eta$ , only  $I/N$  of which are with infected hosts (Brunner et al., 2017). Infectious carcasses house  $\mu$  virions, which is presumably much higher than  $\kappa$ , and carcasses are consumed at some rate,  $\lambda_h$ . Exposure to virus particles in the water occurs as hosts move through  $\omega$  volume of water, which has virus concentration,  $V$ . Lastly, infectious hosts ( $I$ ) die from infection at rate  $\alpha$  and enter the “carcass” class,  $C$ .

The density of infectious carcasses can be modeled as,

$$\frac{dC}{dt} = \alpha I - [\lambda_h(S + I) + \lambda_d D]C,$$

which includes the gain from disease-induced mortality ( $\alpha I$ ) and the losses through consumption by hosts (the first term in the brackets) and by microbial decomposition and scavenging by the decomposer community (the second term in the brackets), which we assume exists at a constant density,  $D$ . While scavenging rates by hosts ( $\lambda_h$ ) and by invertebrates ( $\lambda_d$ ) are likely saturating functions of carcass density (a type II functional

response; Le Sage et al., in review), for our current purposes we assume that these are simply constant, linear functions. In any case, this equation emphasizes a sort of competition for carcasses between necrophagous hosts (both infected and uninfected) and nonhost decomposers or scavengers. If carcasses decompose or are scavenged inefficiently, this allows carcasses to be eaten by and infect naïve hosts.

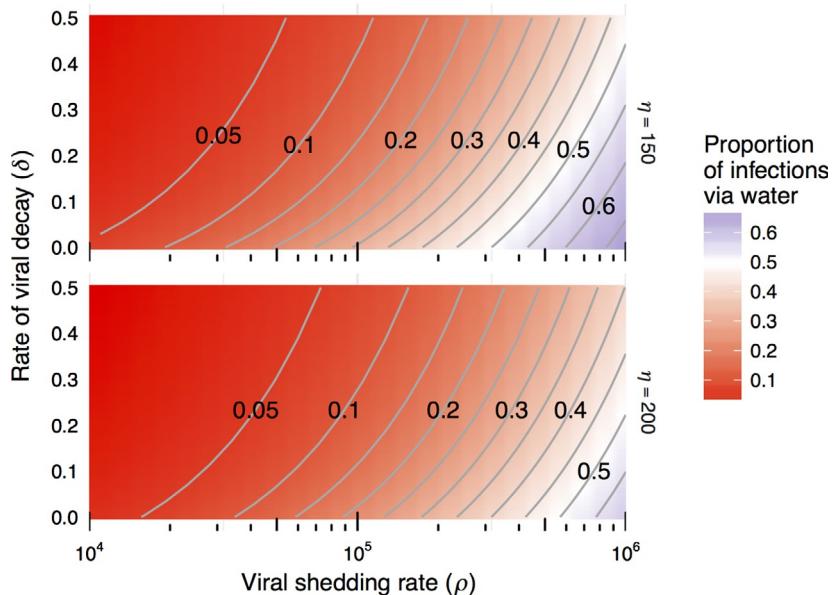
Lastly, we model the change in the density of ranavirus in the water as

$$\frac{dV}{dt} = \rho(I + C) - \delta V,$$

where both infected hosts and carcasses shed ranavirus at some rate,  $\rho$ , which we assume is the same for simplicity—there are few data on shedding rates of live hosts, let alone carcasses, though they appear similarly infectious (Brunner et al., 2007). These virions in the water are then lost at a constant rate,  $\delta$  (Fig. 2). Again, if the virus is degraded slowly relative to the rate of shedding, virus will accumulate in the water and lead to more infections.

The dynamics of this model (with no recovery of susceptible hosts or introduction of new susceptibles, and no resistant hosts), are such that if the infection spreads, it infects the entire population. Therefore, while we numerically solved the equations of this model using the simecol package in R (Petzoldt and Rinke, 2007) to determine the density of hosts in each class through time, we present these data as the *proportion* of the population infected at the end of the epidemic by each route of transmission (code available at [https://github.com/JesseBrunner/RV\\_Persistence](https://github.com/JesseBrunner/RV_Persistence)). It is important to note that this is a simple model that assumes that all individuals within a class are identical (e.g., no stage-specific susceptibility or tolerance; Warne et al., 2011); that infections become immediately transmissible (no latent period or changes in shedding through time; Brunner et al., 2007); that infected hosts do not recover to an immune or still susceptible class (Brunner et al., 2004); and that the exposure dose does not affect the timing of mortality (Brunner et al., 2005). All these assumptions are empirically suspect and relaxing them may change some of the resulting dynamics. However, even this relatively simple model can shed light on the importance of environmental persistence for ranavirus transmission.

The principle result of this modeling exercise is that the two routes of environmental transmission (water-borne and carcass consumption) of ranaviruses become important only under a narrow set of conditions. Water-borne transmission does not play a substantial role (i.e., comprise  $>0.2$  of overall transmission) until shedding rates are high ( $>10^5$  virions per day) and rates of viral decay are low (Fig. 3). There is currently a great

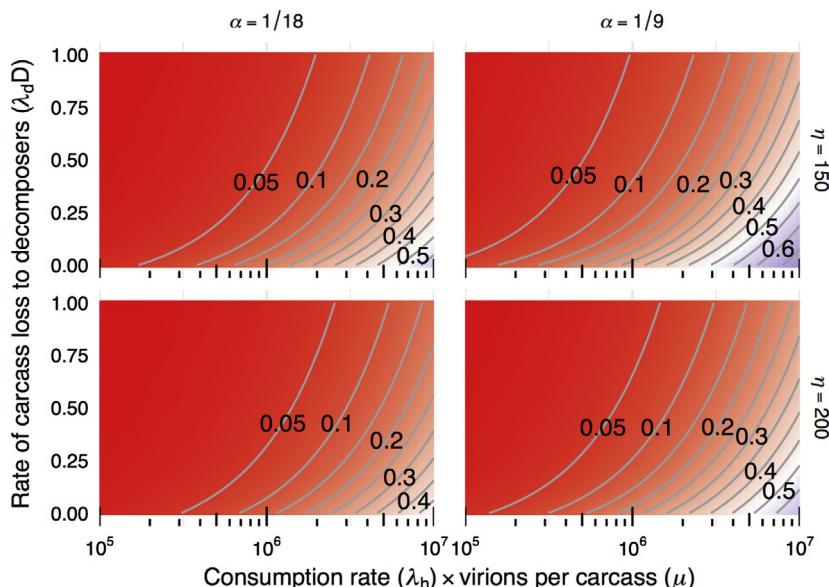


**Fig. 3** The proportion of infections caused by water-borne transmission as a function of viral shedding rate ( $\rho$ ; virions per day per infected host or carcass) and the rate of viral decay ( $\delta$ ; per day, as in Fig. 2). The *top panel* is for  $\eta=150$  contacts per host per day, which produces slow epidemics by direct contact, and the *bottom* is for  $\eta=200$ , which produces epidemics that peak at about 50 days. The baseline conditions for the other parameters are  $b=2.5 \times 10^{-5}$ , which corresponds to an  $ID_{50}$  of  $\sim 10^{4.44}$  pfu in a 1-day exposure;  $\kappa=50$  virions are transferred per casual contact; infected hosts die at a rate of  $\alpha=1/18$  per day; carcasses are removed at a rate of  $\lambda_d D=0.7$  per day by invertebrate scavengers and microbes, and a rate of  $\lambda_h=0.25$  per day per host, but hosts do not become infected as carcasses had  $\mu=0$  virions. Hosts sweep through  $\omega=1L$  day ( $=10^4$  cm long path  $\times 0.1$  cm $^2$  cross section); this parameter was not manipulated as it enters the model as a product with  $\rho$ .

deal of uncertainty in shedding rates and there may well be order-of-magnitude variation among individuals of a species (Brunner et al., in revision). Thus, understanding whether water-borne transmission is important or inconsequential requires additional investigation focused on understanding shedding rates, but it may be highly context dependent. In any case, it appears that at a given shedding rate ( $\rho$ ), the rate of viral decay ( $\delta$ ) can strongly effect the relative proportion of transmission from the water (e.g., from  $>0.3$  with  $\delta \approx 0$  to  $<0.1$  when  $\delta \approx 0.5$  with  $\rho=10^5$  and  $\eta=150$ ; Fig. 3). To achieve such a range of outcomes in nature, however, would require large changes in  $\delta$ , on the order of seasonal changes in temperature in addition to changes in microbial activity, pH, or similar environmental impacts (e.g., lake turnover). More moderate differences

in viral decay should lead to relatively smaller impacts on the rate of water-borne transmission.

This simple model also suggests that transmission via necrophagy by hosts is largely unimportant over a wide range of parameters, and hence rates of invertebrate scavenging and decomposition of carcasses, which limit opportunities for host necrophagy, also may play only minor roles in transmission dynamics. Indeed, only with high virulence ( $\alpha=1/9$ ), large amounts of virus transferred by host necrophagy ( $\lambda_h\mu>5\times 10^6$ ), and low removal by other microbes or invertebrate scavengers ( $\lambda_dD<0.5$ ) does transmission by host necrophagy become important (Fig. 4). This is largely because transmission by necrophagy occurs after hosts have been infected by direct transmission and then die. Since the simulated epidemics usually proceed quickly relative to the average time to death (dynamics not shown), there is not sufficient time or susceptible hosts ( $S$ ) remaining for necrophagous feedbacks to contribute much additional transmission. All that said,



**Fig. 4** The proportion of infections caused by necrophagy as a function of the product of the rate of carcass consumption by hosts ( $\lambda_h$ ; per host per day) and virions per carcass ( $\mu$ ), and the rate of loss to decomposition and invertebrate scavenging ( $\lambda_dD$ ; per day). The top row is  $\eta = 150$  and the bottom is  $\eta = 200$  contacts per host per day, and the left column for the baseline virulence of  $\alpha = 1/18$  per day and the right column for the much faster  $\alpha = 1/9$  per day. The other parameters are as in the caption of Fig. 3, except that  $\rho = 0$ , so that there is no water-borne transmission. The color scale is the same as Fig. 3.

until more empirical data are collected, there remains great uncertainty in these parameters and these restrictive conditions may well be met in some settings or species. Viral titers in carcasses, for instance, are often  $>10^2$  virions and sometimes  $>10^7$  in just a small sample used for DNA extractions (Brunner and Collins, 2009; Hoverman et al., 2010), suggesting that the whole carcass may have very high titers. At any given (high) level of virus transfer, the rate of decomposition and scavenging by invertebrates can strongly influence transmission rates (e.g., from nearly 0.6 to with  $\lambda_d D \approx 0$  to 0.2 when  $\lambda_d D \approx 1$  with  $\lambda_h \mu = 5 \times 10^6$  and  $\eta = 150$ ; Fig. 4). These are very large changes in rates of decomposition and scavenging, but this may well occur seasonally or between ponds, especially if they have very different aquatic invertebrate scavenger communities. It is also worth noting that because of the handling time associated with scavenging a carcass and resultant saturating functional response (Le Sage et al., in review), as well as the fact that scavengers may become sated during die-off events, which can occur very suddenly (Hall et al., 2016; Wheelwright et al., 2014), scavenging may become inefficient in epidemics. Thus, this rate,  $\lambda_d D$ , may be rather low and ineffectual when there is a great deal of host mortality. This suggests that transmission by necrophagy is more important during die-off events, when carcass removal by scavengers may be saturated, than in the earlier phases of ranavirus epidemics. However, while transmission by necrophagy may play a minor role during ranavirus epidemics of a single species, this may be an important route of transmission between host taxa (Belzer and Seibert, 2011). Future work should specifically consider the routes of spillover between species that may not come into contact with each other (hence,  $\eta_{AB} \approx 0$  between species A and B). It may be that environmental persistence and scavenging become especially important in determining whether other host species become involved in *Ranavirus* epidemics.



## 6. CONCLUSIONS

Ranaviruses have long had a reputation for environmental resistance (Langdon, 1989; Whittington et al., 2010). They survive desiccation, freezing, and, under the right conditions, high temperatures in the laboratory. This view has led to the expectation that ranaviruses could generally persist in the environment when hosts were not present and perhaps be translocated with fomites, and that transmission from the environment is important (reviewed in Gray et al., 2009). Simply recognizing that viruses occur free in the environment in a range of forms from naked virions to virus protected

from the physical environment within shed tissues or carcasses suggests that the reality is more complex (Fig. 1). Indeed, a growing number of studies under increasingly realistic conditions have demonstrated that ranavirus persistence times vary by orders of magnitude with environmental conditions (e.g., temperature) and with the activity of the aquatic microbial and planktonic community. Persistence times on the order of a few days would seem to preclude survival in the environment between epidemics, which occur at most annually in highly seasonal environments (Brunner et al., 2015), and likely support only small amounts of environmental transmission. Persistence over several weeks or even months, on the other hand, makes the environment an important source of inoculum for transmission within an epidemic as well as for sparking new epidemics in subsequent seasons or years. As such, we should expect the relative importance of environmental persistence to vary among and even within sites and across seasons. But just how important is environmental persistence for transmission?

Incorporating free virus in the water and in infectious carcasses—two ends of the continuum of persistence—into a simple epidemiological model provided initial assessments of the conditions under which environmental persistence could affect ranavirus transmission and epidemiology. The model indicates that water-borne transmission only becomes a dominant route of infection with high rates of viral shedding and long persistence times. The conditions in which transmission by host necrophagy of infectious carcasses becomes important are even more restrictive, requiring very high rates of necrophagy by susceptible hosts consuming carcasses with very high titers, and low rates of carcass removal by decomposing microbes and invertebrate scavengers. These conditions may well be met, but, we cannot say with certainty. Ironically, this exercise in understanding the importance of environmental persistence in ranavirus epidemiology has shown that we do not know enough about the key quantities related to virus transmission, including the rates of ranavirus shedding, titers in whole carcasses, or rates of host necrophagy. Future studies that better quantify these parameters will help us understand the role that environmental persistence plays in ranavirus epidemiology.

Lastly, it is worth recognizing that even if environmental persistence proves to be unimportant to ranavirus transmission in most situations, it is likely that ranaviruses can persist over the winter, between epidemics, in the environment, at least in some settings, which is critical for the longer-term and larger scale epidemiology of ranaviruses. Persistence times

of free virions in water extend to weeks and months at 4°C, and may be much longer if the virus is protected in host tissues (e.g., frozen in carcasses; Langdon, 1989; Plumb and Zilberg, 1999a) or otherwise protected from microbial action. Ranaviruses may also persist for long periods in soil if kept cool and moist (Brunner et al., 2007; Nazir et al., 2012; Reinauer et al., 2005), although whether and how ranavirus in soil can infect susceptible hosts is still unclear. Indeed, as the previous modeling exercise makes clear, simply demonstrating that long persistence times in the environment are possible is a necessary first step, but is not sufficient to demonstrate that this is an important mechanism of long-term survival for the virus. We hope this review encourages a greater focus on considering the influence of environmental persistence within an epidemiological framework. It is critical to determine not only what is possible, but what is important.

## ACKNOWLEDGMENTS

Many thanks to Rachel Marschang for providing the original data from Reinauer (2004) and Reinauer et al. (2005) and helpful discussions over the years on ranaviral persistence, to Matt Gray for reminding J.L.B. that the relative importance of different routes of transmission is far from settled, and to Carolyn Malmstrom for the invitation to think more deeply about environmental persistence as well as many helpful suggestions for the manuscript.

## REFERENCES

- Altig, R., Whiles, M.R., Taylor, C.L., 2007. What do tadpoles really eat? Assessing the trophic status of an understudied and imperiled group of consumers in freshwater habitats. *Freshw. Biol.* 52, 386–395.
- Ariel, E., Owens, L., 1997. Epizootic mortalities in tilapia *Oreochromis mossambicus*. *Dis. Aquat. Organ.* 29, 1–6.
- Belzer, W., Seibert, S., 2011. A natural history of *Ranavirus* in an eastern box turtle population. *Turtle Tortoise Newsl.* 15, 18–25.
- Ben-Ami, F., Ebert, D., Regoes, R.R., 2010. Pathogen dose infectivity curves as a method to analyze the distribution of host susceptibility: a quantitative assessment of maternal effects after food stress and pathogen exposure. *Am. Nat.* 175, 106–115.
- Bibby, K., et al., 2015. Persistence of Ebola virus in sterilized wastewater. *Environ. Sci. Technol. Lett.* 2, 245–249.
- Brown, J.D., et al., 2007. Persistence of H5 and H7 avian influenza viruses in water. *Avian Dis.* 51, 285–289.
- Brown, J.D., et al., 2009. Avian influenza virus in water: infectivity is dependent on pH, salinity and temperature. *Vet. Microbiol.* 136, 20–26.
- Brunner, J.L., Collins, J.P., 2009. Testing assumptions of the trade-off theory of the evolution of parasite virulence. *Evol. Ecol. Res.* 11, 1169–1188.
- Brunner, J.L., et al., 2004. The role of an intraspecific reservoir in the persistence of a lethal ranavirus. *Ecology* 85, 560–566.
- Brunner, J.L., Richards, K., Collins, J.P., 2005. Dose and host characteristics influence virulence of ranavirus infections. *Oecologia* 144, 399–406.

- Brunner, J.L., Schock, D.M., Collins, J.P., 2007. Transmission dynamics of the amphibian ranavirus *Ambystoma tigrinum* virus. *Dis. Aquat. Organ.* 77, 87–95.
- Brunner, J.L., et al., 2015. Ranavirus ecology and evolution: from epidemiology to extinction. In: Gray, M.J., Chinchar, V.G. (Eds.), *Ranaviruses: Lethal Pathogens of Ectothermic Vertebrates*. Springer International Publishing, Cham, Switzerland, pp. 71–104.
- Brunner, J.L., et al., 2017. Heterogeneities in the infection process drive ranavirus transmission. *Ecology* 98, 576–582.
- Brunner, J.L. et al. (in revision) Performance of nonlethal methods of detecting *Ranavirus* infections in captivity and trade. *Dis. Aquat. Organ.*
- Chinchar, V.G., Waltzek, T.B., Subramaniam, K., 2017. Ranaviruses and other members of the family Iridoviridae: their place in the virosphere. *Virology* 511, 259–271.
- Cook, B.W., et al., 2015. Evaluating environmental persistence and disinfection of the Ebola virus Makona variant. *Virus* 7, 1975–1986.
- Diamant, A., 1997. Fish-to-fish transmission of a marine myxosporean. *Dis. Aquat. Organ.* 30, 99–105.
- Duffus, A.L.J., et al., 2015. Distribution and host range of ranaviruses. In: Gray, M.J., Chinchar, V.G. (Eds.), *Ranaviruses: Lethal Pathogens of Ectothermic Vertebrates*. Springer International Publishing, Cham, Switzerland, pp. 9–57.
- Forzan, M.J., et al., 2015. Clinical signs, pathology and dose-dependent survival of adult wood frogs, *Rana sylvatica*, inoculated orally with frog virus 3 (*Ranavirus* sp., Iridoviridae). *J. Gen. Virol.* 96, 1138–1149.
- Getz, W.M., 2011. Biomass transformation webs provide a unified approach to consumer-resource modelling. *Ecol. Lett.* 14, 113–124.
- Gray, M.J., Miller, D.L., Hoverman, J.T., 2009. Ecology and pathology of amphibian ranaviruses. *Dis. Aquat. Organ.* 87, 243–266.
- Hall, E.M., et al., 2016. Evaluating environmental DNA-based quantification of ranavirus infection in wood frog populations. *Mol. Ecol. Resour.* 16, 423–433.
- Harp, E.M., Petrranka, J.W., 2006. Ranavirus in wood frogs (*Rana sylvatica*): potential sources of transmission within and between ponds. *J. Wildl. Dis.* 42, 307–318.
- Hoverman, J.T., Gray, M.J., Miller, D.L., 2010. Anuran susceptibilities to ranaviruses: role of species identity, exposure route, and a novel virus isolate. *Dis. Aquat. Organ.* 89, 97–107.
- Huang, X.H., et al., 2011. Singapore grouper iridovirus, a large DNA virus, induces non-apoptotic cell death by a cell type dependent fashion and evokes ERK signaling. *Apoptosis* 16, 831–845.
- Jancovich, J.K., et al., 1997. Isolation of a lethal virus from the endangered tiger salamander *Ambystoma tigrinum stebbinsi*. *Dis. Aquat. Organ.* 31, 161–167.
- Johnson, A.F., Brunner, J.L., 2014. Persistence of an amphibian ranavirus in aquatic communities. *Dis. Aquat. Organ.* 111, 129–138.
- Kayansruaj, P., et al., 2017. Outbreaks of ulcerative disease associated with ranavirus infection in barcoo grunter, *Scortum barcoo* (McCulloch & Waite). *J. Fish Dis.* 40, 1341–1350.
- Kitchell, J.F., Koonce, J.F., Tennis, P.S., 1975. Phosphorus flux through fishes. *Verh. Int. Ver. Theor. Angew. Limnol.* 19, 2478–2484.
- Kotwal, G., Cannon, J.L., 2014. Environmental persistence and transfer of enteric viruses. *Curr. Opin. Virol.* 4, 37–43.
- Langdon, J.S., 1989. Experimental transmission and pathogenicity of epizootic haematopoietic necrosis virus (EHNV) in redfin perch, *Perca fluviatilis* L., and 11 other teleosts. *J. Fish Dis.* 12, 295–310.
- Langdon, J.S., Humphrey, J.D., 1987. Epizootic haematopoietic necrosis, a new viral disease in redfin perch, *Perca fluviatilis* L., in Australia. *J. Fish Dis.* 10, 289–297.

- Langdon, J.S., et al., 1986. First virus isolation from Australian fish: an iridovirus-like pathogen from redfin perch, *Perca fluviatilis* L. J. Fish Dis. 9, 263–268.
- Le Sage, M.J., Towey, B.D., Brunner, J.L., in review Are scavengers good for your health? An experimental test of the effect of invertebrate scavengers on ranavirus transmission. Funct. Ecol.
- Munro, J., et al., 2016. Survival of frog virus 3 in freshwater and sediment from an English lake. J. Wildl. Dis. 52, 138–142.
- Nazir, J., Spengler, M., Marschang, R.E., 2012. Environmental persistence of amphibian and reptilian ranaviruses. Dis. Aquat. Organ. 98, 177–184.
- O'Connor, K.M., Rittenhouse, T.A.G., Brunner, J.L., 2016. Ranavirus is common in wood frog (*Lithobates sylvaticus*) tadpoles throughout Connecticut. Herpetol. Rev. 47, 394–397.
- Ogorzaly, L., et al., 2010. Occurrence, survival, and persistence of human adenoviruses and F-specific RNA phages in raw groundwater. Appl. Environ. Microbiol. 76, 8019–8025.
- Parmenter, R.R., Lamarra, V.A., 1991. Nutrient cycling in a freshwater marsh: the decomposition of fish and waterfowl carrion. Limnol. Oceanogr. 36, 976–987.
- Pearman, P.B., et al., 2004. Response of the Italian agile frog (*Rana latastei*) to a Ranavirus, frog virus 3: a model for viral emergence in naive populations. J. Wildl. Dis. 40, 660–669.
- Petzoldt, T., Rinke, K., 2007. Simecol: an object-oriented framework for ecological modeling in R. J. Stat. Softw. 22, 1–31.
- Plumb, J.A., Zilberg, D., 1999a. Survival of largemouth bass iridovirus in frozen fish. J. Aquat. Anim. Health 11, 94–96.
- Plumb, J.A., Zilberg, D., 1999b. The lethal dose of largemouth bass virus in juvenile largemouth bass and the comparative susceptibility of striped bass. J. Aquat. Anim. Health 11, 246–252.
- Price, S., et al., 2014. Collapse of amphibian communities due to an introduced *Ranavirus*. Curr. Biol. 24, 2586–2591.
- Regester, K.J., Whiles, M.R., 2006. Decomposition rates of salamander (*Ambystoma maculatum*) life stages and associated energy and nutrient fluxes in ponds and adjacent forest in southern Illinois. Copeia 2006, 640–649.
- Regoes, R.R., Ebert, D., Bonhoeffer, S., 2002. Dose-dependent infection rates of parasites produce the allee effect in epidemiology. Proc. R. Soc. Lond. Ser. B Biol. Sci. 269, 271–279.
- Reinauer, S., 2004. Tenazitaet Dreier Schildkroetenviren Unter Naturnahen Bedingungen (German) Tenacities of three Turtle Viruses under Close to Nature Conditions (German). Diploma thesis thesis, University of Hohenheim, Stuttgart, Germany.
- Reinauer, S., Bohm, R., Marschang, R.E., 2005. Inactivation of tortoise viruses in the environment. J. Herpetol. Med. Surg. 15, 10–15.
- Robert, J., et al., 2011. Waterborne infectivity of the Ranavirus frog virus 3 in *Xenopus laevis*. Virology 417, 410–417.
- Rudolf, V.H., Antonovics, J., 2007. Disease transmission by cannibalism: rare event or common occurrence. Proc. Biol. Sci. 274, 1205–1210.
- Sobsey, M.D., et al., 1980. Interactions and survival of enteric viruses in soil materials. Appl. Environ. Microbiol. 40, 92–101.
- Speare, R., Smith, J.R., 1992. An iridovirus-like agent isolated from the ornate burrowing frog *Limnodynastes ornatus* in northern Australia. Dis. Aquat. Organ. 14, 51–57.
- Stevenson, C., Childers, D.L., 2004. Hydroperiod and seasonal effects on fish decomposition in an oligotrophic Everglades marsh. Wetlands 24, 529–537.
- Stevik, T.K., et al., 2004. Retention and removal of pathogenic bacteria in wastewater percolating through porous media: a review. Water Res. 38, 1355–1367.
- van der Werf, W., et al., 2011. Heterogeneous host susceptibility enhances prevalence of mixed-genotype micro-parasite infections. PLoS Comput. Biol. 7, e1002097.

- Waltzek, T.B., et al., 2014. New disease records for hatchery-reared sturgeon. I. Expansion of frog virus 3 host range into *Scaphirhynchus albus*. Dis. Aquat. Organ. 111, 219–227.
- Ward, R.L., Knowlton, D.R., Winston, P.E., 1986. Mechanism of inactivation of enteric viruses in fresh water. Appl. Environ. Microbiol. 52, 450–459.
- Warne, R.W., Crespi, E.J., Brunner, J.L., 2011. Escape from the pond: stress and developmental responses to ranavirus infection in wood frog tadpoles. Funct. Ecol. 25, 139–146.
- Weissenberg, R., 1965. Fifty years of research on the lymphocystis virus disease of fishes (1914–1964). Ann. N. Y. Acad. Sci. 126, 362–374.
- Wheelwright, N.T., et al., 2014. Sudden mass die-off of a large population of wood frog (*Lithobates sylvaticus*) tadpoles in Maine, USA, likely due to ranavirus. Herpetol. Rev. 45, 240–242.
- Whittington, R.J., Becker, J.A., Dennis, M.M., 2010. Iridovirus infections in finfish—critical review with emphasis on ranaviruses. J. Fish Dis. 33, 95–122.
- Wilson, E.E., Wolkovich, E.M., 2011. Scavenging: how carnivores and carrion structure communities. Trends Ecol. Evol. 26, 129–135.
- Young, K.D., 2006. The selective value of bacterial shape. Microbiol. Mol. Biol. Rev. 70, 660–703.

## FURTHER READING

- Kimble, S.J., et al., 2015. Mosquitoes as a potential vector of ranavirus transmission in terrestrial turtles. Ecohealth 12, 334–338.