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RESPONSE OF THE ITALIAN AGILE FROG (*RANA LATASTEI*) TO A *RANAVIRUS*, FROG VIRUS 3: A MODEL FOR VIRAL EMERGENCE IN NAÏVE POPULATIONS

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ABSTRACT: *Ranavirus* (family *Iridoviridae*) is a genus of pathogens of poikilotherms, and some ranaviruses may play a role in widespread mortality of amphibians. Ecology of viral transmission in amphibians is poorly known but can be addressed through experimentation in the laboratory. In this study, we use the *Ranavirus* frog virus 3 (FV3) as an experimental model for pathogen emergence in naïve populations of tadpoles. We simulated emerging disease by exposing tadpoles of the Italian agile frog (*Rana latastei*), to the North American *Ranavirus* FV3. We demonstrated that mortality occurred due to viral exposure, exposure of tadpoles to decreasing concentrations of FV3 in the laboratory produced dose-dependent survival rates, and cannibalism of virus-carrying carcasses increased mortality due to FV3. These experiments suggest the potential for ecological mechanisms to affect the level of exposure of tadpoles to *Ranavirus* and to impact transmission of viral pathogens in aquatic systems.

Key words: Amphibian decline, cannibalism, emerging diseases, frog virus 3, iridovirus, IUCN red list, *Ranavirus, Rana latastei.*

INTRODUCTION

The genus *Ranavirus* (family *Iridoviridae*) constitutes globally distributed pathogens that have been found in association with catastrophic mortality events in salamanders and frogs (Cunningham et al., 1996; Jancovich et al., 1997) and may contribute to declines of amphibian populations (Daszak et al., 1999; Chinchar, 2002). While the mechanisms of *Ranavirus* entry into cells, viral replication, and pathogenesis are increasingly understood (reviewed in Chinchar, 2002), *Ranavirus* host-pathogen ecology has not been well studied. Little is known of the epidemiology of *Ranavirus*, the biotic and abiotic factors that influence transmission and host susceptibility, the means for viral persistence in populations, and how the virus disperses among and spreads within host populations. The importance of understanding host-pathogen ecology of amphibians and *Ranavirus* is highlighted by the potential for some anurans to harbor other pathogens that may affect public health (Kozuch et al., 1978; Kostiukov et al., 1985) and the effect of closely related iridoviruses (e.g., largemouth bass virus, Grizzle and Brunner, 2003) on species of economic importance. Ecologists interested in development of mechanistic ecological models that express interactions between *Ranavirus* and their hosts are challenged to develop experimental systems that allow manipulation of both host and pathogen.

Frog virus 3 (FV3) is already used as a model for the study of immunity to *Ranavirus* (Gantress et al., 2003) and for the study of mechanisms of *Ranavirus* viral replication (Chinchar, 2002). We extend the use of FV3 as a model for host/pathogen ecology and pathogen emergence. Pathogens emerge when their geographic range expands, often due to anthropogenic influence, or when they invade new host species and populations (Daszak et al., 2001). Thus by definition, exposure to emerging pathogens largely involves naïve hosts. We simulate disease emergence by challenging tadpoles of a European frog, the Italian agile frog (*Rana latastei*), with a strain of *Ranavirus* from North America.

While *Ranavirus* has a nearly global distribution, differences exist among the strains found in amphibians; FV3 differs at the DNA sequence level from similar *Ranavirus* strains isolated from Europe (Hyatt et al., 2000) and in the principal lesions observed in free-ranging and farmed populations. In North America FV3 and closely related viruses principally affect tadpoles (Tweedel and Granoff, 1968; Green et al., 2002; but see Wolf et al., 1968), while in Europe and Asia adults are commonly affected (Fijan et al., 1991; Cunningham et al., 1996; Kanchanakhan, 1998).

Our aims were to study experimentally the direct effect of viral dose on mortality, and the additional effect of cannibalism on mortality from FV3. Because FV3 and *R. latastei* do not naturally co-occur, we address whether mortality of tadpoles after exposure is an artifact resulting from virus preparation. We also used standardized bath exposure protocols that may facilitate other studies of host-pathogen relationships of FV3 and related ranaviruses. Morbidity and mortality of amphibians can be induced by FV3 and similar viruses in the laboratory (Clark et al., 1968; Tweedel and Granoff, 1968; Wolf et al., 1968; Cullen et al., 1995). Nonetheless, few experiments have addressed factors that influence exposure, transmission, and mortality. For example, while little is known about transmission of FV3 in nature, cannibalism has been hypothesized to increase transmission rates of diseases in fish and amphibians (Pfennig et al., 1991; Ariel and Owens, 1997) and to promote pathogen transmission in humans (Mead et al., 2003) and other vertebrates (e.g., Qureshi et al., 2000). While cannibalism may be sufficient for viral transmission in some aquatic host-pathogen systems (e.g., Jancovich et al., 1997), an incremental effect of cannibalism (or necrophagy for that matter) on pathogen transmission has rarely been demonstrated experimentally in aquatic animals.

METHODS

Virus preparation

Frog virus 3 (provided courtesy of J. K. Jancovich and E. W. Davidson and derived from the type strain) was cultured following a protocol developed for adenoviruses (Greber et al., 1998; Suomalainen et al., 1999). The protocol was modified by substituting modified Eagle's medium for Dulbecco's modified Eagle's medium, using the epithelioma papillosum carp (EPC) cell line (courtesy of J. K. Jancovich and E. W. Davidson), and incubating the cultures at 18 C and 5% CO_2. We extracted FV3 virus from infected EPC cells that showed cytopathic effect. A stock virus solution was prepared by removing cellular debris through extraction with Tris saturated freon, centrifugation at 1,000 × G for 5 min, then removing the aqueous phase and passing it through a 0.2-μm filter. Plaque assay showed this solution to have 5.5×10^8 plaque forming units (pfu)/ml. We also extracted uninfected cells in an identical manner (i.e., a sham extract).

Animals and husbandry

Six populations of *R. latastei* were selected to span the species' range. Two populations were located near the western range limit (near Turin, Italy), three were in northeastern Italy, and one was in western Slovenia (Garner et al., 2004). The east-west direction is the longest axis of distribution of *R. latastei* and the east-west position of populations is the key variable associated with genetic variation in *R. latastei* (Garner et al., 2004). Twenty egg clutches from each population were collected between 10 and 17 March 2003. Multiple amplexus and paternity are not reported for *R. latastei,* so we assume animals from a clutch are full-sib relatives. All families were equalized as to developmental stage by moving hatching clutches to 4 C, a temperature that slows development but is well within the normal environmental range for this species. This is preferable to equalizing tadpoles by size because of ontogenetic changes in the activity of the tadpole immune system (Gantress et al., 2003). All tadpoles were free swimming and began to exhibit feeding behavior within 24 hr of each other (Gosner stage 25, Gosner, 1960). This is the earliest stage at which *R. latastei* tadpoles begin to feed. Tadpoles were held in tap water aged in an open container for 24 hr to allow removal of toxic volatiles (e.g., chlorine). All experiments used water from a single batch of aged tap water. Tadpoles were held on a 12 hr : 12 hr light–dark cycle.

Experiment 1

When tadpoles reached Gosner stage 25 (Gosner, 1960) we randomly selected four families from each of the six populations. For a population, we pooled approximately 100 seemingly healthy tadpoles from each of the four families and from this pool chose 30 active tadpoles to represent the pooled families. We then randomly assigned 10 individuals to be exposed to FV3 and 10 to be exposed to an equivalent dilution of the sham extract. The remaining 10 tadpoles were sacrificed in 0.2% aqueous MS222 (Fluka, Buchs, Switzerland) preserved in 10% formalin, and their developmental stage (Gosner, 1960) was confirmed by inspection with a dissecting microscope. We replicated this procedure for the remaining five populations.

Seventy milliliters of a solution used to expose the tadpoles were prepared by pipetting stock virus solution into aged tap water that was constantly stirred with a bar to a final virus concentration of 2.25×10^6 pfu/ml. Aliquots of 10 ml were pipetted into 10-cm Petri dishes. The groups of 10 experimental tadpoles from the six populations were transferred at random to these Petri dishes using a piece of fiberglass screen. Three milligrams fish food mixture (a 50:50 mixture of finely ground Tetra Tabi Min and Tetraphyll flakes, Tetra GmbH, Melle, Germany) was suspended in water was added to each Petri dish. An identical procedure was used for the sham exposure treatment except that an identical volume of sham extract was used instead of FV3. All animals fed normally during the exposure period. After 24 hr animals were removed from the solution and blotted with a disposable lab tissue, then transferred and held individually in 10-cm culture dishes, each with 20 ml aged tap water. In this experiment and the ones that follow, animals in Petri dishes were held on a single shelf of a climate-controlled room, at 14 C and 90% relative air humidity, with a 12:12 hr dark:light schedule. Tadpoles received 0.005 g fish food mixture in aqueous suspension when their water was changed every 3 days. The difference in survival between the two treatments on the 30th day following exposure was tested for significance using a Wilcoxon signed-rank test (Siegel and Castellan, 1988).

Experiment 2

Twenty egg masses were collected from a single breeding site in southern Switzerland (population PBPCH, Garner et al., 2003) on 15 March 2003 and were held at ambient outdoor temperature (~12 C) until eclosion. We equalized the developmental stage of four clutches, pooling them as described above. To test for dose-dependent survival after FV3 exposure, we observed tadpoles for the onset of feeding behavior (Gosner stage 25, Gosner, 1960) and then assigned 120 developmentally equalized individuals randomly to 12 groups of 10 tadpoles. Two replicate groups were assigned at random to each of the following six exposure treatments: a virus solution of 4.5×10^6 pfu/ml FV3 in aged tap water, one of four serial dilutions of this solution each an order of magnitude lower in virus concentration, or a control solution of aged water. We then conducted exposure, transfer to individual Petri dishes, and feeding as described above.

Experiment 3

Feeder tadpoles: To examine the effect of cannibalism of infected carcasses on infection rates of susceptible tadpoles, we first created virus-exposed tadpole carcasses by allowing randomly chosen groups of 10 developmentally equalized tadpoles at Stage 25 (Gosner, 1960) to swim in 10 ml of a solution of 4.5×10^6 pfu/ml of FV3. Simultaneously, randomly chosen groups of 10 tadpoles (to become control carcasses) were held under identical conditions in aged tap water. After 24-hr exposure, all tadpoles were blotted of solution with a tissue and transferred to new culture dishes with 20 ml fresh aged water. Virus-exposed tadpoles were frozen at −80 C as soon as possible after death (generally within 6 hr). After 19 days the unexposed tadpoles were flash frozen in liquid nitrogen and stored at −80 C for later use.

Experimental tadpoles: An experimental group of developmentally equalized *R. latastei* tadpoles from a single breeding site in Switzerland (population PBPCH, Garner et al., 2003) was constituted by pooling siblings hatched from four egg clutches collected from nature. All individuals were at Gosner stage 25 (Gosner, 1960). Fifty seemingly healthy tadpoles were selected and housed individually in 0.5-l containers with 400 ml aged tap water, on a 12:12 hr dark:light schedule at 14 C and 90% relative air humidity. Each tadpole was randomly assigned to one of five treatments (Table 1), thereby creating 10 independent replicates of each treatment. The treatments consisted of a control for method and two crossed factors, each factor having two levels. These factors were 1) exposure status of feeder tadpole carcasses (virus exposed or freeze killed) and 2) mode of presentation of the feeder tadpole carcass to the experimental tadpole (i.e., in a cotton sack or free in the water). Thus, tadpoles assigned to the cannibal treatment had free access to carcasses upon which they could feed,

TABLE 1. Treatments of tadpoles in experiment 3. Each treatment was replicated 10 times in a 0.5 l container holding randomly assigned *Rana latastei* tadpoles. See text for additional details.

Treatment code	Treatment	Exposure and food	Location of tadpole carcass	Other comments
A	Bag control	Fish food	No carcass	Empty cotton bag
B	Cannibal control	Frozen tadpole carcass	Free in container	Empty cotton bag
C	Cannibal virus exposed	Frog virus 3 exposed tadpole carcass	Free in container	Empty cotton bag
D	Isolated control	Frozen tadpole carcass and fish food	Carcass in cotton bag	
E	Isolated virus exposed	Frog virus 3 exposed tadpole carcass and fish food	Carcass in cotton bag	

while tadpoles assigned to the isolated treatment had a tadpole carcass suspended in their container in a cotton sack, which prevented them from feeding on the carcass. An empty bag (treatment A, Table 1) controlled for the effect of a submerged cotton bag on tadpole mortality. Comparison of mortality rates of animals that fed on freeze-killed carcasses versus those that ate fish food (treatments B versus D, Table 1) controlled for the effect on mortality of cannibalism per se. Comparison of morality rates of tadpoles exposed to an empty bag versus those exposed to bagged, freeze-killed tadpoles (treatments A versus D, Table 1) controlled for the effect on survival of having a dead tadpole in the container. Comparison of mortality in groups exposed to either virus-exposed tadpoles or freeze-killed tadpoles (all in bags, treatments D versus E, Table 1) controlled for an effect on mortality of exposure to an FV3-exposed feeder tadpole carcass. Comparison of mortality in tadpoles with free access to virus-killed tadpole carcasses versus mortality when exposed to bagged, virus-killed feeder tadpole carcasses (treatments C versus E, Table 1) tested for cannibalism's incremental effect on mortality resulting from viral exposure.

Virus-exposed and control feeder tadpole carcasses were randomly assigned to cannibal and isolated treatments to eliminate the possibility of confounding the two factors. Tadpoles in the isolated and the bag control treatments received 0.005 g of fish food mixture every 2 days. All feeder tadpole carcasses, whether bagged or free in containers, were removed (along with bags) and replaced with new randomly chosen feeder tadpole carcasses every 2 days. Experimental tadpoles were observed daily, and the dates of deaths were recorded, along with whether tadpoles had fed on carcasses or fish food. We observed the external appearance of tadpoles for clinical signs. Dead tadpoles were frozen promptly at −80 C. On day 22 surviving tadpoles were anesthetized in MS222 and frozen.

DNA preparation: Whole genomic DNA was extracted from tadpoles chosen from each treatment in experiment 3 using the QIAamp® DNA mini kit (Qiagen AG, Basel, Switzerland). Viral DNA was detected by polymerase chain reaction (PCR). Polymerase chain reaction mixture, FV3-specific primers, and conditions were as required for amplification of a portion of the FV3 major capsid protein gene (Mao et al., 1996; Gantress et al., 2003). Polymerase chain reaction was conducted using 35 cycles of 45 sec denaturation at 95 C, 45 sec annealing at 52 C, and 45 sec extension at 72 C. Samples were then run on a 1% agarose gel and stained with Sybr Gold (Molecular Probes/Invitrogen, Basel, Switzerland). The size of the amplicon was determined by comparison with the Lambda—EcoRI/HindIII ladder (Qbiogene, Inc., Basel, Switzerland). Frog virus 3–specific primers were as follows: forward primer, 5′-GTCTCTGGAGAAGAAGAA-3′, reverse primer 5′-GACTTGGCCACTTATGAC-3′. Each reaction had a total volume of 30 μl and contained 1 μl of each primer (10 pmol), 2 μl genomic DNA (50–100 ng), 3 μl 1.25 mM of each deoxyribonucleotide triphosphate (Promega, Wallisellen, Switzerland), 3 μl 10×PCR buffer, 2 U Taq DNA polymerase (Quantum-Appligene, Illkirch, France), and 20 μl sterile double distilled water. Amplicons from eight positive animals were sequenced at the core facility for the Institutes of Zoology and Molecular Biology, University of Zurich (Switzerland) using both forward and reverse primers.

Statistical analysis

All randomizations conducted during the experiments were done using SAS Proc Plan

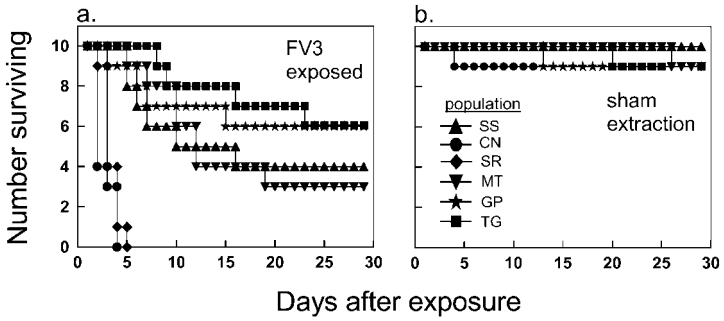

FIGURE 1. Number of tadpoles surviving in 12 groups of 10 from six populations that span the range of *Rana latastei*. Each point refers to the number of surviving tadpoles alive in individual Petri dishes. (a) Tadpoles exposed to frog virus 3 for 24 hr and (b) tadpoles from the same populations when exposed to an equivalent dilution of extract from a sham extraction of uninfected cells. Source populations are shown in a legend as two-letter codes. Populations CN and SR are in northwestern Italy and have low genetic diversity at microsatellite loci. Populations SS, MT, and TG are in northeastern Italy, and GP is a population from Slovenia (see text).

(SAS Institute Inc., 1988). Selection of animals in the studies, when not random, was haphazard, which, as used here, refers to selection without specific use of randomized numbers but without specific knowledge of the condition of the units or animals. We used the Wilcoxon test for differences between survival curves, as implemented in SAS Proc Lifetest, to examine the statistical significance of differences between the survival of tadpoles that consumed carcasses and tadpoles that did not (Allison, 1995). In analyzing data from experiment 3, we tested for differences between survival in treatments C and E first in order to avoid problems associated with making multiple statistical tests using the same data set.

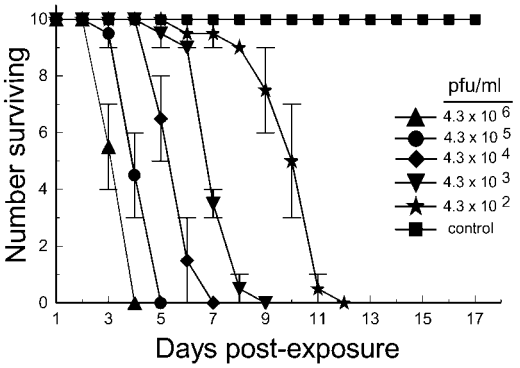

FIGURE 2. Number of surviving tadpoles following 24-hr exposure to five concentrations (pfu = plaque forming units) of frog virus 3 and one control solution of aged tap water. Each observation is the mean of two replicates, with one standard error of the mean shown by bars.

RESULTS

Experiment 1

All experimental animals in the randomly chosen sample of 10 preserved individuals were at Gosner stage 25. Tadpoles from all populations had high mortality rates when exposed to FV3 (Fig. 1a). In contrast, tadpoles exposed to the sham extraction (Fig. 1b) survived significantly better until 30 days after exposure (Wilcoxon signed-rank test, $n=6$, $c=21$, $P=0.016$). Tadpoles from two populations died at a faster rate than did tadpoles from the other four populations (Fig. 1a).

Experiment 2

Exposure to lower concentrations of virus resulted in increasing time to death across the range of doses used in this study (Fig. 2). The narrow standard errors surrounding the mean of the two replicates indicate that the exposure protocol produces highly replicable mortality patterns.

Experiment 3

All tadpoles in cannibal treatments actively fed upon feeder tadpole carcasses. Tadpoles exposed to infected carcasses (treatments C and E) died at a higher rate than tadpoles in any of the control treatments (Fig. 3). Cannibalism of virus-ex-

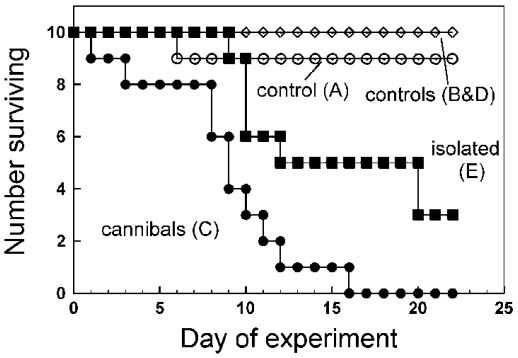

FIGURE 3. Number of surviving tadpoles under five experimental treatments. Each line represents the status of 10 individuals on successive days. Tadpoles in treatments B and C cannibalized carcasses, while tadpoles in treatments D and E were isolated from carcasses by cotton bags. Tadpoles in treatments C and E were exposed to carcasses of tadpoles that died following exposure to frog virus 3 (FV3), while tadpoles in treatments B and D were exposed to carcasses that were killed by flash freezing and had not been exposed to FV3. See text for further description of treatments.

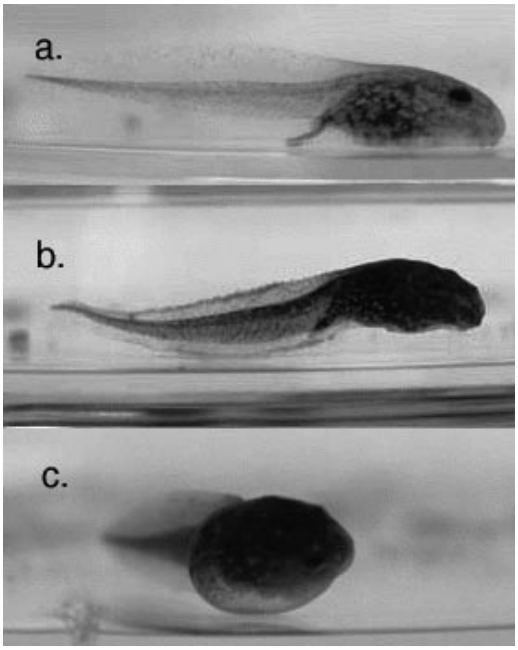

FIGURE 4. External gross lesions in tadpoles exposed to frog virus 3 (FV3). (a) Normal *Rana latastei* tadpole; (b) emaciation representative of that displayed by 12 of 20 tadpoles exposed to FV3; (c) abdominal edema displayed by two of 20 tadpoles exposed to FV3.

posed carcasses increased the death rate of tadpoles compared to tadpoles isolated from infected carcasses by cloth bags (SAS Proc Lifetest, Wilcoxon $\chi^2=6.72$, $P=0.01$). Two clinical signs occurred in exposed tadpoles; they became emaciated (12 tadpoles) or developed edema (two tadpoles, Fig. 4). All affected tadpoles died while only four normal-appearing tadpoles died. Of the 32 tadpoles alive at the end of the experiment, 31 appeared clinically normal and one developed a condition in which the tail was flexed laterally. The association of either of the two clinical conditions (Fig. 4) with mortality was significant (Fisher's exact test, two-tailed test, $P<0.001$).

When PCR primers specific for FV3 were used, DNA amplified from a sample of animals exposed to FV3-exposed feeder tadpole carcasses (Fig. 5). Polymerase chain reaction of a sample of tadpoles from the control treatments did not produce an amplification product when these same primers were used. Polymerase chain reaction product was also obtained from exposed tadpoles that died but for which clinical signs were not observed (Fig. 5). Sequence of the PCR product was compared to sequences in Genbank (http://www.ncbi.nlm.nih.gov/) using BLAST and closely matched existing *Ranavirus* major capsid protein sequences.

DISCUSSION

The results of our studies, including the benign nature of exposure to products of sham extractions (Fig. 1), FV3 dose-dependent mortality of *R. latastei* tadpoles (Fig. 2), transmission of FV3 from exposed to unexposed individuals (Figs. 3, 5), and clinical signs associated with mortality (Fig. 4) indicate that FV3 is a transmissible pathogen in *R. latastei* tadpoles. These results are consistent with other reports of the effect of FV3 exposure on young tadpoles (Tweedel and Granoff, 1968), evidence of intracellular viral replication by FV3 in other anuran species (Clark et al., 1968), and reports of FV3 and closely re-

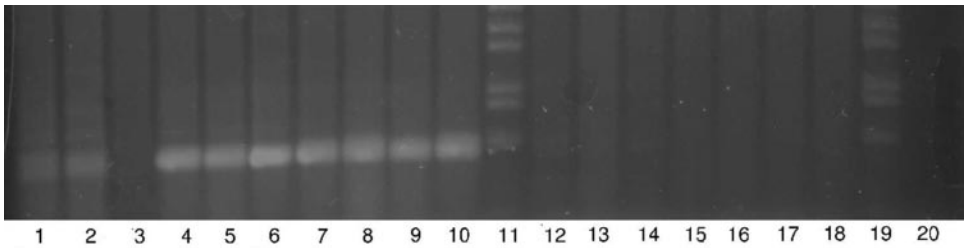

FIGURE 5. Agarose gel showing polymerase chain reaction (PCR) products using frog virus 3–specific primers to amplify from total DNA extractions from individual tadpoles in experiment 3 (see text and Fig. 3 for details). Tadpoles in successive lanes, from left to right, 1) treatment C, died with no visible signs of infection (i.e., a in Fig. 4); 2) treatment C, died with no signs; 3) treatment A, no signs; 4) treatment C, died with signs; 5) treatment C, died with signs; 6) treatment E, died without signs; 7) treatment E, died after developing edema; 8) treatment E, died with signs; 9) treatment E, died without signs; 10) treatment E, died after developing edema; 11) marker; 12) treatment D; 13) treatment B; 14) treatment A survivor; 15) treatment D survivor; 16) treatment B survivor; 17) treatment A survivor; 18) wild caught tadpole from Ticino, Switzerland; 19) marker; 20) negative PCR control.

lated ranaviruses as the source of various lesions of anurans in natural populations and in aquaculture (Wolf et al., 1968; Kanchanakhan, 1998; Zhang et al., 2001). The mechanism(s) underlying pathogenesis in the present study were not determined but may involve cytotoxicity, premature apoptosis, and/or cell lysis due to viral contact or replication (Chinchar, 2002; Chinchar et al., 2003).

Our results (Fig. 1a) also suggest that populations vary in susceptibility to the effects of FV3 exposure. Tadpoles from two Italian populations, CN and SR, survived less well when exposed to virus than tadpoles from the other four populations. Notably, these two populations are known to have reduced levels of population genetic variability in comparison with the other populations (Garner et al., 2003, 2004). While lack of replication within populations prohibits statistical inference regarding this trend, further experiments will be conducted to explore the relationship between population genetic diversity and resistance to effects of novel exposure to FV3.

The second experiment (Fig. 2) demonstrated that variation in levels of FV3 virus exposure may result in different survival rates of tadpoles. This is important because it implies that ecological factors could affect viral acquisition and transmission. While an understanding of the mechanisms of transmission in nature will require additional study, our results suggest that ecological factors that influence concentration of virus by an order of magnitude will affect the time course of tadpole mortality. Controlled exposure protocols will be essential for studying variation in virulence among viral strains, variation in immunocompetence as a function of population genetic composition or additional environmental factors, and variation in susceptibility among species. Understanding these aspects of host-pathogen ecology will facilitate the study of *Ranavirus*-host coevolution and the impact that emergent pathogens may have on amphibian community structure.

Use of FV3-specific primers resulted in detection of a PCR product in exposed animals in experiment 3 but not in unexposed ones, with confirmation of the identity of the amplicons by sequencing. Cannibalism upon tadpole carcasses exposed to FV3 led to higher death rates than exposure to bagged carcasses of tadpoles that died after virus exposure. Exposure to bagged, infected carcasses also increased mortality in exposed tadpoles over background rates. When considered together with the dose-dependency data, the results of these experiments suggest that mortality following FV3 exposure in nature may depend on the degree of contact between in-

fected and uninfected individuals and may be a function of increasing environmental concentrations of FV3. The effect of cannibalism on disease transmission has been hypothesized to influence the distribution of cannibalistic behavior in amphibian populations (Pfennig et al., 1991).

Further, our results suggest that FV3 transmission rates may vary with the complexity of ecologic communities, where scavengers (such as crayfish or other invertebrates) or any number of environmental factors may act to decrease exposure to virus-laden corpses, inactivate virus particles, or concentrate them in a food source for scavenging tadpoles. Virus may be spread in nature by consuming corpses or by contact with virus particles shed upon decomposition. Other ecologic processes may influence transmission rates by affecting the degree to which susceptible individuals aggregate with infected individuals (e.g., Bjornstad et al., 2002). It is not known whether tadpoles prefer uninfected carcasses over infected ones when feeding on conspecifics, as happens in some insects (Boots, 1998).

Challenge of a host with a viral strain that does not naturally occur in the host population accurately simulates some aspects of an anthropogenically mediated emergent pathogen. Our approach here does not, however, simulate aspects of pathogen emergence that result from evolutionary changes in ranaviruses, such as specific mutations or selective pressures that could lead to emergence in additional host species or result in increased virulence. These additional aspects may be addressable in the future as the genetic basis of differences in viral replication among strains, and ultimately differences in pathogenicity, become better understood. Amphibian species may differ in susceptibility to FV3 (Clark et al., 1968), and some evidence suggests that within a single species, susceptibility to FV3 may be related to host size, developmental stage (Tweedel and Granoff, 1968), or genetic composition (Gantress et al., 2003). Susceptibility of amphibians to macroparasites may depend on environmental factors, such as presence of agricultural toxins (Taylor et al., 1999; Kiesecker, 2002; Christin et al., 2003), suggesting that susceptibility to viral infection also may depend on the environment of the host.

Challenge studies that use viral strains that vary in virulence and host species that vary in susceptibility may be useful in identifying both molecular and epidemiologic mechanisms that influence *Ranavirus* host-pathogen ecology. The bath exposure that was used in experiments 1 and 2 was not invasive and did not in itself affect tadpole survival. Low variation in mortality among replicates suggests that bath exposures could be useful for assessing susceptibility to FV3 in quantitative genetic experiments. Finally, exposure protocols that produce low variance among independent replicates are important for identifying the effects of environmental factors that may influence susceptibility to viral infection. Challenge protocols similar to those detailed here may be useful to investigate variation in susceptibility among populations and the genetic basis of that variation.

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