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# Susceptibility of Fish and Turtles to Three Ranaviruses Isolated from Different Ectothermic Vertebrate Classes

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ARTICLE

# Susceptibility of Fish and Turtles to Three Ranaviruses Isolated from Different Ectothermic Vertebrate Classes

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***Abstract***

**Ranaviruses have been associated with mortality of lower vertebrates around the world. Frog virus 3 (FV3)-like ranaviruses have been isolated from different ectothermic vertebrate classes; however, few studies have demonstrated whether this pathogen can be transmitted among classes. Using FV3-like ranaviruses isolated from the American bullfrog *Lithobates catesbeianus*, eastern box turtle *Terrapene carolina carolina*, and Pallid Sturgeon *Scaphirhynchus***

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***albus*, we tested for the occurrence of interclass transmission (i.e., infection) and host susceptibility (i.e., percent mortality) for five juvenile fish and three juvenile turtle species exposed to each of these isolates. Exposure was administered via water bath ($10^3$ PFU/mL) for 3 d and survival was monitored for 28 d. Florida softshell turtles *Apalone ferox* experienced no mortality, but 10% and 20% of individuals became infected by the turtle and fish isolate, respectively. Similarly, 5% of Mississippi map turtles *Graptemys pseudogeographica kohni* were subclinically infected with the turtle isolate at the end of the experiment. Channel Catfish *Ictalurus punctatus* experienced 5% mortality when exposed to the turtle isolate, while Western Mosquitofish *Gambusia affinis* experienced 10% mortality when exposed to the turtle and amphibian isolates and 5% mortality when exposed to the fish isolate. Our results demonstrated that interclass transmission of FV3-like ranaviruses is possible. Although substantial mortality did not occur in our experiments, the occurrence of low mortality and subclinical infections suggest that fish and aquatic turtles may function as reservoirs for FV3-like ranaviruses. Additionally, our study is the first to report transmission of FV3-like ranaviruses between fish and chelonians.**



Transmission of viruses among vertebrate classes (hereafter referred to as interclass transmission) is uncommon. Viral infection is a complex process that involves several steps and exploits a variety of cellular activities (Su et al. 2008; Cronin et al. 2010; Jackson et al. 2010; Paull et al. 2012). The first and perhaps quintessential challenge a virus has to overcome after entering a new host is its replication. Once inside the new cell, a virus has to uncoat, transport its genetic material to the appropriate cellular compartment, gather all the necessary replication machinery, produce copies of its genome and virion components, and package the genome into the capsids (Webby et al. 2004; Acheson 2007). If a virus successfully replicates in the new host cell, there are other obstacles that limit it from infecting its new host. The virus must exit the cell (i.e., exocytosis or lysis of the cell), overcome or avoid the host's immunological response, infect other cells quickly, and be shed from the host so transmission to other hosts can occur (Webby et al. 2004; Bandin and Dopazo 2011; Crispe et al. 2011; Starick et al. 2011).

This complex process of host establishment makes interclass transmission unlikely in most cases. However, several viruses have found ways to overcome these obstacles, and examples of viruses transmitting between species have been recorded (Webby and Kalmakoff 1998; Keesing et al. 2010; Boelle et al. 2011; Swayne 2011). For example, some large double-stranded DNA (dsDNA) viruses in the family *Iridoviridae* are known to infect multiple amphibian species (Hoverman et al. 2011). Iridoviruses enter the cell carrying start-up proteins that are used to initiate genome replication and protein production, thereby facilitating virus replication in the host cell (Chinchar 2002; Chinchar et al. 2011). The highly conserved major capsid protein of the virus and widely distributed cell receptors targeted by the pathogen likely contribute to the wide host range of iridoviruses. Currently, five genera within the family *Iridoviridae* are recognized (King et al. 2012): two genera, *Iridovirus* and *Chloriridovirus*, infect arthropods (Camazine and Liu 1998; Hunter et al. 2001; Marina et al. 2003; Gregory et al. 2006), two genera, *Lymphocystivirus and Megalocytivirus*, infect fish (Sudthongkong et al. 2002; Palmer et al. 2012; Rimmer et al. 2012; Waltzek et al. 2012), and one genus, *Ranavirus*, has been isolated from amphibians, fish, and reptiles (Chinchar et al. 2009; Cinkova et al. 2010; Vesely et al. 2011; Nazir et al. 2012; Robert and Chinchar 2012).

Ranaviruses have been associated with disease and mortality in numerous lower vertebrate species, including amphibians, fishes, and reptiles and are considered a pathogen of ecological and economic importance (Chinchar 2002; Keesing et al. 2010; Robert and Chinchar 2012; Gray and Miller 2013). Currently, the International Committee on Taxonomy of Viruses recognizes six species of ranaviruses (Jancovich et al. 2010; King et al. 2012). Three of the species infect fish exclusively: the epizootic hematopoietic necrosis virus, European catfish virus, and Santee–Cooper ranavirus (Bigarre et al. 2008; Chinchar et al. 2009; Whittington et al. 2010; Bang-Jensen et al. 2011a; Vesely et al. 2011). The other species—Frog Virus 3 (FV3), *Ambystoma tigrinum* virus (ATV), and Bohle iridovirus (BIV)—have been isolated most frequently from amphibian hosts, but might infect and cause disease in other ectothermic vertebrates. For example, ATV is known to cause high mortality in tiger salamanders *Ambystoma tigrinum* (Jancovich et al. 2003; Collins et al. 2004) and has been reported to cause infection in the Largemouth Bass *Micropterus salmoides* (Picco et al. 2010). Also, BIV was originally isolated from an amphibian (Speare and Smith 1992; Cullen et al. 1995; Cullen and Owens 2002; Weir et al. 2012), but can infect fish and turtles (Moody and Owens 1994; La Fauce et al. 2012). Recently, transmission of FV3-like ranaviruses was demonstrated in fish (Bang-Jensen et al. 2009, 2011a; Bayley et al. 2013), chelonians (Allender et al. 2006, 2013; Johnson et al. 2010), and multiple amphibian species (Hoverman et al. 2011).

Despite these findings, the host range of FV3-like ranaviruses remains unclear, especially with North American fish and chelonian species (Gray et al. 2009). Also, the possibility of interclass transmission of FV3-like ranaviruses has not been investigated extensively (Bayley et al. 2013). Our objective was to determine whether three FV3-like ranaviruses isolated from hosts of three different ectothermic classes—amphibians (Amphibia), reptiles (Reptilia), and bony fishes (Osteichthyes)—were able to cause infection and mortality in fish and turtle species known to

coexist with amphibians or that are important to the aquaculture industry in North America. If interclass transmission is possible, fish and turtles may be important reservoirs of FV3-like ranaviruses (Gray et al. 2009), particularly in habitats where amphibians are not present yearlong.

## METHODS

*Ranaviruses and hosts*.—The FV3-like ranaviruses used in our study were isolated from a morbid Pallid Sturgeon *Scaphirhynchus albus* in Missouri (T. B. Waltzek, unpublished data), eastern box turtle *Terrapene carolina carolina* in Kentucky (Ruder et al. 2010), and American bullfrog *Lithobates catesbeianus* in Georgia (Miller et al. 2007). We tested five fish species: Nile Tilapia *Oreochromis niloticus*, Channel Catfish *Ictalurus punctatus*, Western Mosquitofish *Gambusia affinis*, Bluegill *Lepomis macrochirus*, and Fathead Minnow *Pimephales promelas*. All fish species were fingerlings (about 5–10 cm in length) and were obtained from commercial hatcheries (Table 1). Fish were reared from fry in independent, outdoor, concrete troughs with constant water flow and had no contact with other species. Upon purchase and arrival at the University of Tennessee, a random sample of five individuals was humanely euthanized by immersion in a solution of benzocaine hydrochloride (100 mg/L: Iwama and Ackerman 1994) and tested for ranavirus infection using quantitative real-time PCR (qPCR; see methods below); all qPCR results were negative. Prior to the start of the experiments, fishes were acclimated in the laboratory for 1 week in separate 1,200-L tanks with flow-through, dechlorinated water (75.7 L/s) at 26°C with 12 h light : 12 h dark photoperiod. During the acclimation period, fish were fed a commercial high protein fish food (TetraMin, Blacksburg, Virginia) daily ad libitum.

We tested three aquatic turtle species: Florida softshell turtle *Apalone ferox*, eastern river cooter *Pseudemys concinna*, and Mississippi map turtle *Graptemys pseudogeographica kohni.* Turtles were purchased as 15-d-old hatchlings (approximately 5 cm in length) from commercial retailers (Table 1). All species were raised in captivity and in isolation from other species prior to shipment to the University of Tennessee. Turtles were housed under identical conditions as were the fish, except floating platforms were added to the 1,200-L tanks and specialized lamps were provided for thermal and ultraviolet (UV) light exposure (Zoo Med Powersun UV Self-Ballasted Mercury Vapor UVB Lamp, San Luis Obispo, California). A random sample of five individuals per species was collected and euthanized to verify individuals were not infected with ranavirus prior to experimentation; all individuals tested negative by qPCR. Turtles were fed live crickets and bloodworms once daily ad libitum.

*Fish challenges*.—Each experimental trial consisted of four treatments with 20 replicate fish per treatment, totaling 80 experimental units. The treatments were three ranavirus isolates and a negative control. Eighty fish were randomly selected from the 1,200-L tank and placed individually into 4-L (17.7 × 17.7 × 28.5 cm) tubs filled with 2 L of dechlorinated, aged tap water; the tubs were placed on 122 × 244-cm shelving units. Prior to adding the fish, each container was randomly assigned to a viral or control treatment in a randomized block design, in which two shelf heights were the blocking variables. Viral treatments were inoculated with $10^3$ PFU/mL of the appropriate virus isolate, and the controls were inoculated with the same quantity of virus-free media (i.e., MEM Eagle, Sigma-Aldrich, Seelze, Germany). We used $10^3$ PFU/mL because it has been suggested that this concentration is ecologically relevant (Gray et al. 2009). Rojas et al. (2005) reported this titer of ranavirus in water shed by an infected salamander. Dose-dependent studies (e.g., Brunner et al. 2005) show that mortality is low typically when amphibian larvae are exposed to <100 PFU/mL of ranavirus. Less information is available on the dose-dependent relationships of ranavirus and fish hosts, but the viral titer we used is known to cause ranaviral disease in fish (Moody and Owens 1994; Grizzle et al. 2002; Gobbo et al. 2010). Individuals were exposed to the virus in stagnant water for 3 d, which has become standard for ranavirus challenges with amphibians due to no apparent effect of exposure duration on pathogenicity of ranavirus (Hoverman et al. 2010, 2011). Given that fish were negative for ranavirus at the beginning of each experiment, the inoculations likely represented a first-time exposure to the pathogen, which is standard in ranavirus-challenge experiments (Bang-Jensen et al. 2011a; Jaramillo et al. 2012).

TABLE 1. Vendors for specimens during the challenge experiments.

| Species | Vendor |
|---|---|
| Nile Tilapia *Oreochromis niloticus* | Greenwater Fish Farm, Milan, Tennessee |
| Channel Catfish *Ictalurus punctatus* | Greenwater Fish Farm, Milan, Tennessee |
| Western Mosquitofish *Gambusia affinis* | Alabama Aquarium and Pond Services, Birmingham, Alabama |
| Bluegill *Lepomis macrochirus* | Bell Springs Fish Hatchery, Riceville, Tennessee |
| Fathead Minnow *Pimephales promelas* | Bell Springs Fish Hatchery, Riceville, Tennessee |
| Florida softshell turtle *Apalone ferox* | JP Pets, Sanford, Florida |
| Eastern river cooter *Pseudemys concinna* | JP Pets, Sanford, Florida |
| Mississippi map turtle *Graptemys pseudogeographica kohni* | Backwater Reptiles, Sacramento, California |

During experiments, fish were fed high-protein commercial food every day at a ratio of 3% of body mass, which is sufficient for normal growth and development (Budy et al. 2011). The amount of food required was calculated based on the body mass of a separate sample of five nonexperimental fish that were treated in a manner identical to the controls. Fish were monitored twice daily for survival and morbidity. Dead individuals were removed from their containers, necropsied, and any gross signs of ranaviral infection recorded. Fish that exhibited morbidity consistent with ranaviral disease (i.e., petechial hemorrhages, edema, and loss of equilibrium) for >24 h during the experiment were humanely euthanized by immersion in benzocaine hydrochloride solution (100 mg/L). Water was changed (100% of volume) every 3 d to maintain water quality during the experiment (Hoverman et al. 2010), and laboratory temperature was monitored and maintained at 26°C. Duration for all trials was 28 d, which is sufficient duration for morbidity to be observed from ranavirus infection (Bang-Jensen et al. 2009, 2011a; Jaramillo et al. 2012). At the end of each experiment, all surviving individuals were humanely euthanized by immersion in 100 mg/L of benzocaine hydrochloride (Iwama and Ackerman 1994).

*Turtle challenges*.—Turtle experiments followed the same procedures as the fish challenges with three exceptions. First, the turtles were housed in 15.5-L containers ($41.6 \times 28.6 \times 18.7$ cm) containing 2 L of dechlorinated, aged tap water (approximately 3 cm depth). This amount of water was sufficient to allow the turtle to fully immerse its body while maintaining its head above water. Second, during the experiments, turtles were fed two live crickets per day, which was sufficient for normal growth and development (Teece et al. 2001). Lastly, individuals that exhibited gross signs of ranaviral disease (e.g., cutaneous abscessation, oral ulceration or abscessation, respiratory distress, anorexia, and lethargy: Allender et al. 2006; Johnson et al. 2006) and survivors at the end of the experiment were humanely euthanized via intravenous injection of 60–100 mg/kg of sodium pentobarbital. All procedures followed approved University of Tennessee Institutional Animal Care and Use Committee protocol 2052.

*Ranavirus testing*.—Genomic DNA (gDNA) was extracted from a tissue homogenate of the kidney and liver collected during necropsy using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, California).We used a Qubit fluorometer and the Quant-iT dsDNA BR Assay Kit to quantify the concentration of gDNA in each sample (Invitrogen, Carlsbad, California). Quantitative real-time PCR (qPCR) was used to amplify a 70-bp sequence of the ranavirus major capsid protein using primers and protocol identical to Picco et al. (2007). The extracted DNA samples were run in duplicate, and an individual was declared positive if the qPCR cycle threshold (CT) was <30 for both samples. This CT decision rule was determined for our PCR system (ABI 7900 Fast Real-Time PCR System; Life Technologies Corporation, Carlsbad, California) by developing a standard curve with 95% CIs using known quantities of ranavirus. For qPCR-positive individuals, we reported the predicted PFU per 0.25 μg of host gDNA as a relative indicator of viral load. Four controls were included in each qPCR assay: DNA extracted from a ranavirus-positive animal, DNA extracted from a ranavirus-negative animal, DNA extracted from cultured ranavirus, and water.

*Statistical analyses*.—We summarized the results as individuals that died and were infected (case mortality), survived and were infected (subclinical infection), and died but were not infected (natural mortality). For our study, we defined infection as qPCR positive according to our CT decision rule, which is common in transmission studies (e.g., Brunner et al. 2005; Hoverman et al. 2011). Given that quiescent infections are possible with ranaviruses (Robert et al. 2011) and active replication is unnecessary for qPCR to amplify viral DNA (Green et al. 2009), it is possible that our qPCR positive results did not represent active infections. Nonetheless, detection of ranavirus DNA via qPCR is evidence of transmission in our study considering that prescreening resulted in no positive results, and our experiment was designed based on independent water bath challenges. For each species, we tested for the difference in case mortality and infection prevalence (i.e., qPCR positive) among the ranavirus isolates using a *G*-test of maximum likelihood (Sokal and Rohlf 1995). All analyses were performed using SAS 9.3 (SAS 2012) at $\alpha = 0.05$.

## RESULTS

Two fish species experienced case mortality: Channel Catfish and Western Mosquitofish (Figure 1). The catfish experienced 5% mortality when exposed to the fish isolate, while the mosquitofish experienced 10, 10, and 5% mortality when exposed to the turtle, amphibian, and fish isolates, respectively. Average viral load for infected fish tissue (0.25 μg) was 8.9 PFU (Table 2). No statistical differences were detected in case mortality ($G = 5.71$, df = 12, $P = 0.28$) or infection prevalence ($G = 18.94$, df = 12, $P = 0.13$) among the three isolates. Catfish died between 16 and 24 d postexposure, while mosquitofish began to die after 4 d postexposure to the virus (Figure 3).

No deaths were documented in turtles exposed to ranavirus; however, infection occurred in two species (Figure 2). After exposure to the turtle and fish isolates, 10% and 20% of Florida softshell turtles, respectively, were infected. The Mississippi map turtle experienced 5% infection when exposed to the box turtle isolate. Average viral load for infected turtle tissue (0.25 μg) was 228 PFU, and was greatest for Florida softshell turtles exposed to the box turtle isolate (Table 2). No statistical differences were detected in infection prevalence ($G = 7.32$, df = 12, $P = 0.19$) among the three isolates.

## DISCUSSION

Our study documented two new cases of interclass transmission: (1) transmission of a FV3-like ranavirus isolated from a fish to a turtle species, and (2) transmission of a FV3-like ranavirus isolated from a turtle to a fish species. We also documented transmission of a FV3-like ranavirus isolated from an

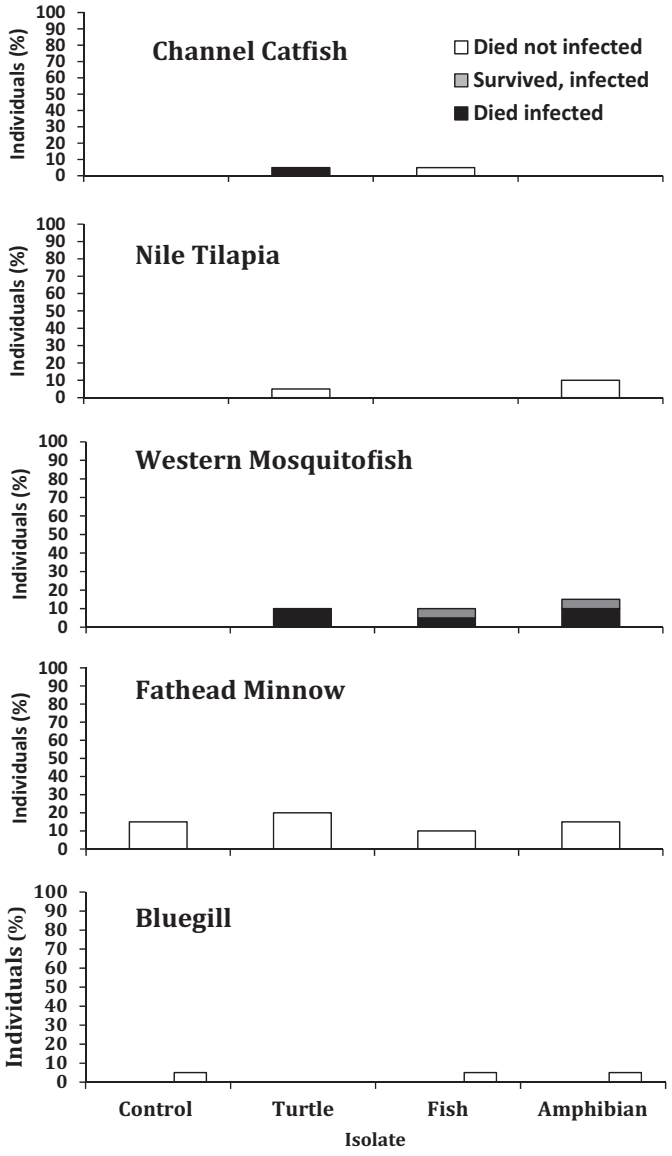


FIGURE 1. Percent mortality and infection of five fish species (Channel Catfish, Nile Tilapia, Western Mosquitofish, Fathead Minnow, and Bluegill) exposed to three ranavirus isolates from different ectothermic vertebrate hosts: turtle, fish, and amphibian. Results are based on exposure of 20 individuals per fish species per ranavirus isolate for 28 d. Infection was determined via qPCR and may not represent occurrence of active virus replication in the host.

TABLE 2. Viral load (PFU) in a homogenate of liver and kidney tissue (0.25 μg) of infected individuals exposed to three FV3-like ranavirus isolates from a morbid turtle (eastern box turtle), a fish (Pallid Sturgeon), and an amphibian (American bullfrog).

| Species | Isolate | PFU |
|---|---|---|
| Western Mosquitofish | Turtle | 11.7 |
| | Fish | 10.2 |
| | Amphibian | 12.1 |
| | | 5.2 |
| Channel Catfish | Turtle | 5.2 |
| Florida softshell turtle | Turtle | 760 |
| | | 606 |
| | Fish | 1.6 |
| | | 1.1 |
| | | 1.4 |
| | | 0.8 |
| Mississippi map turtle | Turtle | 2.6 |

amphibian to a fish species, which has been reported by others (e.g., Bang-Jensen et al. 2009, 2011b; Gobbo et al. 2010; Picco et al. 2010). These results provide additional evidence that FV3-like ranaviruses can be transmitted among ectothermic vertebrate classes.

We documented 5% mortality of Channel Catfish exposed to the turtle isolate, and 5–10% mortality of Western Mosquitofish exposed to fish, turtle, or amphibian isolates. Although this level of mortality is low, these results suggest that ranavirus could negatively impact aquaculture industries (Prasankok et al. 2002; Bang-Jensen et al. 2011b; Vesely et al. 2011). Bang-Jensen et al. (2011b) reported that ranaviruses were a concern to the aquaculture industry in the European Union, and the occurrence of subclinically infected individuals in international fish trade could result in the emergence of ranavirus. Production of Channel Catfish and Western Mosquitofish are major industries in the United States (Mischke et al. 2013; Torrans et al. 2013). Additionally, mosquitofish are commonly released as biological control agents into natural aquatic systems containing native populations of ectothermic vertebrates (Griffin and Knight 2012; Samidurai and Mathew 2013). The fact that mosquitofish can be subclinically infected with FV3-like ranaviruses is a conservation concern.

The species of ranaviruses that are found exclusively in fish hosts (i.e., epizootic hematopoietic necrosis virus, European catfish virus, and Santee–Cooper ranavirus) are known to cause significant morbidity and mortality in several fish species around the world (Bigarre et al. 2008; Picco et al. 2010; Whittington et al. 2010; Bang-Jensen et al. 2011b; Vesely et al. 2011). The ranavirus BIV can cause significant mortality in Barramundi *Lates calcarifer* (Moody and Owens 1994). However, FV3-like and ATV ranaviruses appear to cause subclinical infections and low mortality in fish (Bang-Jensen et al. 2009; 2011a; Gobbo et al. 2010; Picco et al. 2010). The reduced susceptibility of fish to ATV and FV3-like ranaviruses could be a result of host specificity for cell entry and replication, or an inability to bypass the fully functional immune system of fish (Grayfer et al. 2012).

The low susceptibility of the turtles that we tested to ranavirus was unexpected, as cases of ranavirus infection and disease have been reported in at least 11 tortoise and box turtle species (Marschang et al. 1999; De Voe et al. 2004; Benetka et al. 2007; Johnson et al. 2007, 2010; Marschang 2011), red-eared slider turtle *Trachemys scripta elegans* (Johnson et al. 2006, 2010; Allender et al. 2013) and Chinese softshell turtle *Pelodiscus*

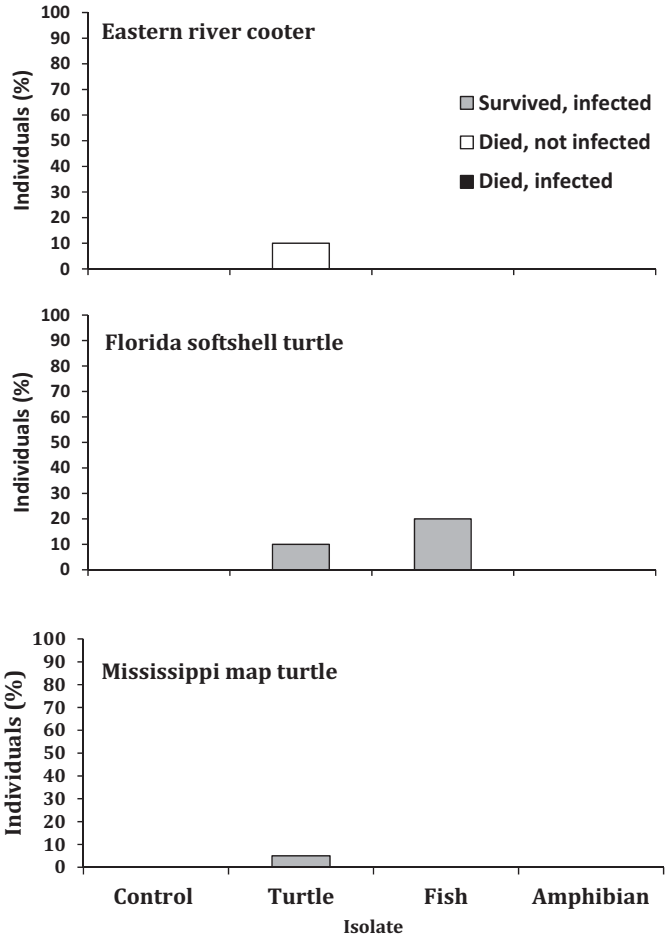


FIGURE 2. Percent mortality and infection of three turtle species (eastern river cooter, Florida softshell turtle, and Mississippi map turtle) exposed to three ranavirus isolates from different ectothermic hosts: turtle, fish, and amphibian. Results are based on exposure of 20 individuals per turtle species per ranavirus isolate for 28 d. Infection was determined via qPCR and may not represent occurrence of active virus replication in the host.

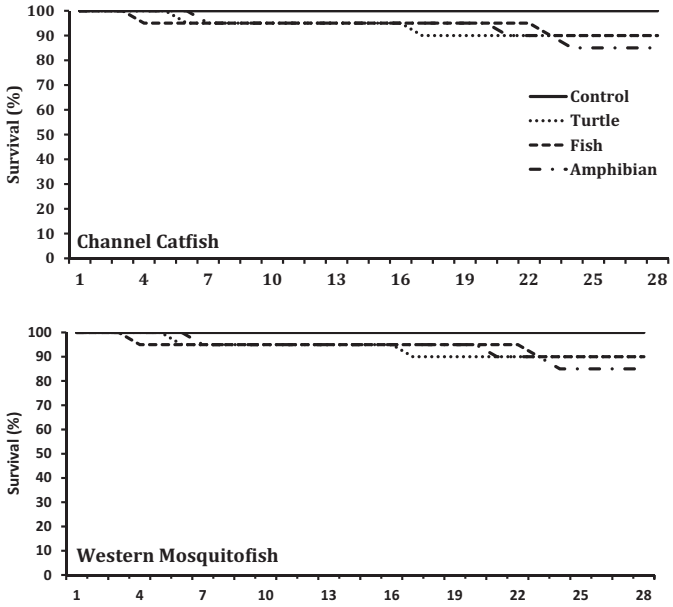


FIGURE 3. Survival curves of fish species (Channel Catfish and Western Mosquitofish) that experienced mortality when exposed to ranavirus isolates from three different ectothermic vertebrate classes (i.e., turtle, fish, and amphibian).

*sinensis* (Chen et al. 1999) in both natural and laboratory environments (Chen et al. 1999; De Voe et al. 2004; Allender et al. 2006; Johnson et al. 2008). However, most of these reports were diagnostic cases on a single individual or challenge experiments via isolate injection, which may be an unrealistic transmission route (Gray et al. 2009). Allender et al. (2013) reported greater susceptibility of adult red-eared slider turtles injected with ranavirus at 21°C compared with 28°C. Given that our experiment was performed at 26°C, the lower infection we observed could have been influenced by temperature. More information is needed on the susceptibility of chelonians to ranavirus, and the role of temperature.

Our susceptibility results likely reflect a best-case scenario inasmuch as our experiments were conducted under controlled conditions with food provided ad libitum. Additionally, factors that contribute to ranavirus emergence such as density-dependent transmission were controlled. In wild or captive populations, multiple infected and morbid individuals can be present, which might increase the likelihood of transmission to other ectothermic vertebrates, particularly those that predate (e.g., fish) or scavenge (e.g., turtles) other hosts.

The majority of individuals in our study tested negative for ranavirus DNA in liver and kidney tissue 28 d following exposure to an isolate. It is possible that individuals became infected and cleared the virus prior to the end of the experiment. For example, Fathead Minnow cells have been used to replicate FV3 in the laboratory for many decades (Green et al. 2009), yet no individuals of this species were positive after 28 d in our study. Short-duration infection could play a role in the epidemiology of ranaviruses, especially where host densities are high. Future transmission studies should consider euthanizing individuals at different postexposure durations to document host susceptibility and improve our understanding of short- versus long-term reservoirs.

Our results indicate that fish and aquatic turtles could function as reservoirs for FV3-like ranaviruses and, through commercial trade, contribute to pathogen pollution (Cunningham et al. 2003). In the United States, 662 million tons of catfish (Hanson 2012) were produced in 2012, and 31.8 million turtles including 17.5 million individual red-eared slider turtles were sold between 2004 and 2005 (Brown et al. 2011; WCT 2013). Our results suggest that fish and turtles infected with ranavirus should be included in the World Organization for Animal Health (OIE) standards for notifiable diseases (Schloegel et al. 2010). Currently, amphibians infected with ranaviruses are the only

taxonomic group listed in the OIE regulations (Schloegel et al. 2010).

Although our results showed that some fishes (Channel Catfish and Western Mosquitofish) and turtles (Florida softshell and Mississippi map turtles) are suitable hosts for FV3-like ranaviruses, additional research is needed on other species in North America. Additionally, experiments are needed to determine whether an infected individual of one vertebrate class can transmit ranavirus through water to a different class. The capacity of fish and turtle species to transmit ranavirus to highly susceptible hosts that inhabit aquatic environments seasonally (e.g., amphibians) will help us understand the reoccurrences of outbreaks in ecosystems with fluctuating species composition (Pearman and Garner 2005; Teacher et al. 2010). This information could be essential for the planning and execution of conservation strategies for areas that exhibit recurrent ranavirus outbreaks, as well as the identification of areas with risk of ranaviral disease.



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