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New disease records for hatchery-reared sturgeon. I. Expansion of frog virus 3 host range into *Scaphirhynchus albus*

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ABSTRACT: In 2009, juvenile pallid sturgeon *Scaphirhynchus albus*, reared at the Blind Pony State Fish Hatchery (Missouri, USA) to replenish dwindling wild stocks, experienced mass mortality. Histological examination revealed extensive necrosis of the haematopoietic tissues, and a virus was isolated from affected organs in cell culture and then observed by electron microscopy. Experimental infection studies revealed that the virus is highly pathogenic to juvenile pallid sturgeon, one of several species of sturgeon currently listed as Endangered. The DNA sequence of the full length major capsid protein gene of the virus was identical to that of the species *Frog virus 3* (FV3), the type species for the genus *Ranavirus*, originally isolated from northern leopard frog *Lithobates pipiens*. Although FV3 infections and epizootics in amphibians and reptiles are well documented, there is only 1 prior report of a natural infection of FV3 in fish. Our results illustrate the broad potential host range for FV3, with the known potential to cause significant mortality in poikilothermic vertebrates across 3 taxonomic classes including bony fishes, anuran and caudate amphibians, and squamate and testudine reptiles.

KEY WORDS: Iridovirus · Ranavirus · Frog virus 3 · Pallid sturgeon · Phylogeny

INTRODUCTION

The family *Iridoviridae* is an assemblage of large dsDNA viruses organized into 5 genera: *Chloriridovirus, Iridovirus*, *Lymphocystivirus*, *Megalocytivirus*, and *Ranavirus.* Although ranaviruses infect fish, amphibians, and reptiles, both lymphocystiviruses and megalocytiviruses are found only in fish while the hosts for members of the genera *Chloriridovirus* and *Iridovirus* are arthropods. Ranaviruses are aquatic animal pathogens of economic and ecological importance causing mass mortality in common and endangered poikilothermic vertebrates, as well as losses to aquaculture industries (Chinchar 2002,

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Gray et al. 2009, Whittington et al. 2010). Globally, epizootics due to ranaviruses have been reported in >70 amphibian (Miller et al. 2011) and 15 reptile (Marschang 2011) species across 5 continents.

Currently, the genus *Ranavirus* contains 3 species naturally infecting and inducing mortality in amphibians (*Ambystoma tigrinum virus*, *Bohle iridovirus*, and *Frog virus 3*; Gray et al. 2009) and 3 species infecting fish (*Epizootic haematopoietic necrosis virus*, *European catfish virus*, and *Santee-Cooper ranavirus*; Whittington et al. 2010). *Frog virus 3* (FV3), the type species for the genus *Ranavirus*, is the sole ranavirus naturally occuring among poikilothermic vertebrates across 3 vertebrate classes: bony fishes, anuran and caudate amphibians, as well as squamate and testudine reptiles (Whittington et al. 2010, Marschang 2011, Gray & Miller 2013). Although natural FV3 epizootics in amphibians and reptiles are well documented, only a single case of natural infection of FV3 in a fish has been reported (Mao et al. 1999). That virus was recovered from a single moribund threespine stickleback *Gasterosteus aculeatus* that was co-infected with myxozoan parasites potentially obscuring the role of the virus in the observed disease (Mao et al. 1999).

Sturgeon belong to an ancient order (Acipenseriformes) of ray-finned fishes that have remained virtually unchanged since the Early Jurassic period 200 million yr ago (Billard & Lecointre 2001). Over the last century, increased demand for caviar and habitat degradation have resulted in the listing of all species as Threatened, with more than half as Critically Endangered (IUCN Red List; Birstein 1993, Billard & Lecointre 2001, Pikitch et al. 2005). Pallid sturgeon *Scaphirhynchus albus* inhabit the Missouri and lower Mississippi River basins in the USA and have been listed by the US Fish and Wildlife Service as endangered since 1990 as a result of habitat modification (e.g. dam construction), lack of natural reproduction, commercial overharvesting, and hybridization with shovelnose sturgeon *S. platorynchus* (SDDGFP 2006). To augment dwindling wild populations, stock enhancement programs were initiated at hatcheries throughout the region. Unfortunately, hatchery rearing of juvenile pallid and shovelnose sturgeon has been hampered by mortality associated with a previously unclassified iridovirus known as the Missouri River sturgeon iridovirus (MRSIV; Kurobe et al. 2010, 2011).

Here we describe the isolation and characterization of a virus clearly different than MRSIV associated with an epizootic and mass mortality in hatchery-reared juvenile pallid sturgeon. Challenge studies confirmed the lethal nature of this virus and reproduced similar gross and microscopic lesions as observed in the hatchery epizootic. The ultrastructural features of the virus and DNA sequence of the major capsid protein gene identify the virus as FV3, and the viral induced pathology and mortality provide the first clear evidence of the pathogenicity of the virus for a fish host.

MATERIALS AND METHODS

Clinical history

The Blind Pony State Fish Hatchery (BPSFH) in Sweet Springs, Missouri, raises approximately 12 000 juvenile pallid sturgeon per year to replenish dwindling stocks within the Missouri River Basin. Adults are spawned at the hatchery and then returned to the wild, and their offspring are reared in flow-through raceways (approximately 2000 fish raceway^{-1}) that receive water from Blind Pony Lake without disinfection or alteration of temperature. From July to October 2009, young of the year pallid sturgeon (approximately 2 mo posthatch in July) experienced significant mortality in 3 separate raceways. The epizootic resulted in approximately 95% cumulative mortality at water temperatures ranging from 16 to 26°C and reached over 500 ind. d^{-1} at the highest water temperatures. Repeated oxytetracycline bath treatments (20 mg l^{-1}) did not reduce mortality. Fish from the BPSFH were submitted to the Bozeman Fish Health Center (BFHC) in Bozeman, Montana, in 2009 for histologic examination, diagnostic virology, and testing for the presence of MRSIV by PCR.

Virology, histopathology, and MRSIV PCR

Fish health screens carried out at the BFHC in September, October, and December 2009 included processing 227 fish as single fish samples or pools of 4, 5, and 6 of whole viscera, kidney/spleen, or kidney/spleen/liver tissues as aseptically removed from moribund (September and October) or overtly healthy (December) juvenile pallid sturgeon. Each sample was diluted 1:25 in Earle's minimum essential medium (Gibco) supplemented with 14 mM HEPES (4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid) buffer and 2% fetal bovine serum (FBS) (MEM-2), homogenized using a Stomacher (Seward) for 30 s, and centrifuged at 2500 × *g* (15 min at 4°C). An equal volume of supernatant was added to a MEM-2 anti-

biotic solution resulting in a final concentration of 500 IU penicillin ml^{-1}, 500 µg streptomycin ml^{-1}, and 12.5 µg fungizone ml^{-1}, 14 mM HEPES, and a pH of 7.2 to 7.6. (Ganzhorn & LaPatra 1994). After 24 h at 4°C, the samples were centrifuged at 2500 × *g* (15 min at 4°C), and 200 µl of the supernatant from each sample were inoculated onto duplicate wells of a 24-well plate with confluent epithelioma papulosum cyprini (EPC), Chinook salmon embryo (CHSE-214), pallid sturgeon gill, pallid sturgeon skin, pallid sturgeon spleen, and white sturgeon skin (WSSK) cell lines (Fijan et al. 1983, Lannan et al. 1984, Hedrick et al. 1991, Kurobe et al. 2011). After virus adsorption, 0.5 ml of MEM-2 was added to each well. All cell lines were incubated at 15°C and checked 2 to 3 times per week for cytopathic effects (CPE) for 14 to 16 d. Negative wells were subjected to a blind passage and monitored for CPE for an additional 14 to 17 d (USFWS & AFS-FHS 2007). Blind passages consisted of transferring all cells and culture medium from a plate well into a tube. Following centrifugation at 2500 × *g* (15 min), 100 µl of the supernatant were used to inoculate each of 2 duplicate wells of a 24-well plate with nearly confluent monolayers of the same cell line used in the initial virus isolation procedure.

The remaining tissues from spleen, kidney, liver, and gills were preserved in Davidson's fixative and processed by standard histological methods for paraffin-embedded tissues (Humason 1979). Tissue sections (3 µm), one stained with Giemsa and the other with haematoxylin and eosin, were examined by light microscopy. The pectoral fins from 30 fish from the September lot and 56 fish from the October lot were tested individually using the MRSIV conventional PCR as previously described (Kurobe et al. 2010).

Electron microscopy

After CPE were observed in cell cultures at the BFHC, the supernatant from presumed viral infected cells was shipped overnight to the Fish Health Laboratory (FHL), University of California, Davis for characterization of the virus. The virus was propagated in EPC cells grown to confluency at 20°C in MEM with 7.5% FBS, 50 IU penicillin ml^{-1}, 50 µg streptomycin ml^{-1}, and 2 mM L-glutamine in 75 cm^2 flasks. Following inoculation with a 1:10 dilution of the virus suspension from the BFHC, MEM-2 was added to the flask. When CPE was complete (2 d), the culture fluid was clarified by centrifugation at 1880 × *g* (20 min at 10°C). Five ml of the clarified supernatant were frozen at −80°C for later virus propagation and extraction of viral DNA (see below); the remainder was centrifuged at 175 000 × *g* (1 h at 10°C) to pellet the virus. The concentrated virus was processed for negative stain electron microscopy by resuspending the pellet in 1% phosphotungstic acid, placing the stained sample on a grid that was then observed with a Philips EM 400 electron microscope.

DNA extraction, PCR amplification, sequencing, BLASTN

Viral DNA was extracted from the clarified supernatant using a QIAamp DNA Mini Kit (Qiagen) following the protocol for free viral DNA from fluids or suspensions. Amplification of the full length major capsid protein (MCP) gene was attempted using ranavirus primers MCP-1 (modified from Hyatt et al. 2000 to exclude the last 3 bases on the 3′ end of the primer) and MCP-6. The 50 µl PCR cocktail contained 1× PCR buffer (Invitrogen), 1.5 mM $MgCl_2$ (Invitrogen), 400 µM deoxynucleoside triphosphate, 0.5 units *Taq* polymerase (Invitrogen), 40 pmol of each primer, and 100 ng of template DNA. The concentration and purity of the template DNA was measured on a NanoDrop 8000 (Thermo Scientific). The mixture was subjected to a precycle at 95°C for 3 min, then 35 cycles of amplification (94°C for 1 min, 50°C for 1 min, 72°C for 2 min), followed by a post-cycle extension at 72°C for 10 min. After electrophoresis, the observed band was gel extracted and purified using a QIAquick gel extraction kit (Qiagen). Purified DNA was sequenced with both the forward and reverse primers on an ABI 3130 DNA sequencer (Applied Biosystems). Following sequence assembly and removal of primer sequences, a general BLASTN search (www.ncbi.nlm.nih.gov/blast/Blast.cgi) of the full length MCP gene sequence was conducted (Altschul et al. 1997).

Challenge study

Young of the year pallid sturgeon were obtained from Gavin's Point National Fish Hatchery (Yankton, South Dakota) and acclimated to 130 l tanks receiving 23°C well water at a flow rate of 0.5 l min^{-1} at the FHL for 1 mo prior to experimentation. Sixty fish (mean ± SD standard length = 21.3 ± 3.0 cm, weight = 39.8 ± 13.9 g) were randomly assigned to 1 of 3 tanks, and after 1 wk were exposed in 30 l of well water to

either 1.3×10^6 $TCID_{50}$ ml^{-1}, 1.3×10^5 $TCID_{50}$ ml^{-1}, or no virus (negative control) for 1 h with aeration, and then water flows to the aquaria were resumed. Fish were monitored for external lesions and behavioral abnormalities for 21 d post virus exposure. Daily mortalities were weighed, standard length measured, evaluated for external parasites, and necropsied to evaluate potential gross and internal signs of infection, as well as selective tissue sampling for virus isolation, qPCR, histology, and bacteriology. Surviving fish at 21 d post virus exposure and 5 control unexposed fish were humanely euthanized (300 mg l^{-1} tricaine methanesulfonate) and processed in an identical fashion to the dead fish described above.

Virus for the challenge study was prepared from a frozen stock inoculated (500 µl $flask^{-1}$) into two 175 cm^2 flasks containing confluent EPC cells with MEM-2 at 20°C. After 2 d, CPE was complete, the supernatants were combined, and clarified by centrifugation at 5000 × *g* (20 min at 10°C). Concentrations of virus in the cell culture media and in infected tissues were estimated by the Reed–Muench method following $TCID_{50}$ endpoint analyses.

The presence and concentrations of virus in dead fish and fish surviving at 21 d post exposure were evaluated by pooling spleen and kidney tissues from each fish. The pooled tissues were processed by standard virological methods (Ganzhorn & LaPatra 1994). Ten-fold dilutions of the tissue extracts were distributed to replicate wells of a 96-well plate (500 µl $well^{-1}$). Immediately afterwards, an additional 500 µl of MEM-2 with suspended EPC cells were added to each well of the plate. The plates were incubated at 20°C and observed daily for CPE for 12 d, at which time blind passages were performed on all samples not showing CPE. After an additional 10 d, all blind passaged samples were scored.

DNA was extracted from the pooled spleen and kidney with a QIAamp DNA Mini Kit (Qiagen) following the protocol for animal tissues. A real-time quantitative PCR targeting the ranavirus major capsid gene was used to verify the presence or absence of ranavirus DNA as previously described by Hoverman et al. (2010). All samples were run in duplicate, and an individual was considered virus positive if the qPCR cycle threshold (C_T) was <30 for both samples on an ABI 7900 Fast Real-Time PCR System (Life Technologies).

For bacteriology, the kidney tissue was streaked onto a blood agar plate (TSA with 5% sheep blood) and incubated at room temperature. Plates were oberved for 1 wk for bacterial growth and if present, colonies were submitted to the University of California, Davis Veterinary Microbiology Laboratory for identification using the API 20E system (bioMerieux).

RESULTS

Examinations of fish at the BFHC

Moribund fish received by the BFHC in September and October 2009 displayed gross external (skin) and internal (swim bladder and liver) haemorrhagic lesions. Fish collected in December 2009 showed no external or internal signs. Microscopic lesions among moribund fish included necrosis of lymphohaematopoietic and endothelial cells with eosinophilic intracytoplasmic inclusion bodies in the spleen, kidney, liver, and pancreas. In particular, marked destruction of the spleen and haemorrhage in the liver were noted (Fig. 1). Nonspecific changes included vacuolation of hepatocytes and attenuation of renal tubular epithelium. All 86 fish tested by conventional PCR at the BFHC were negative for MRSIV. CPE were observed beginning 6 to 8 d post inoculation on all tested cell lines inoculated with tissue extracts (kidney/spleen) collected from fish in September and October 2009. However, only 2 of 30 samples processed on 22 December 2009 displayed CPE. One of these samples exhibited CPE 16 d post inoculation and only on pallid sturgeon gill cells and the second only following blind passage on pallid sturgeon gill cells.

Challenge study

The high and low virus concentration exposures resulted in 90% mortality between Days 8 and 19 (Fig. 2). Virus-challenged fish that succumbed displayed similar gross and microscopic lesions as described above in the natural hatchery outbreak. High virus concentrations were recovered from all fish that died (3.1×10^7 to 6.7×10^8 $TCID_{50}$ g^{-1}) and all were qPCR positive ($C_T \leq 30$), with C_T values that ranged from 14 to 25 (mean ± SD = 18.32 ± 2.21). Virus was recovered from 2 of 4 survivors at 21 d post virus exposure, and these samples were qPCR negative. Bacterial growth was observed within 48 h at room temperature (23°C) in 5 of the fish that died, and all were identified as *Aeromonas hydrophila*. No control fish died during the experiment, and the 5 sampled control fish were qPCR negative for ranavirus. Neither bacterial growth (after 7 d) nor virus (after 22 d, which included a blind passage) were recovered from the 5 control fish.

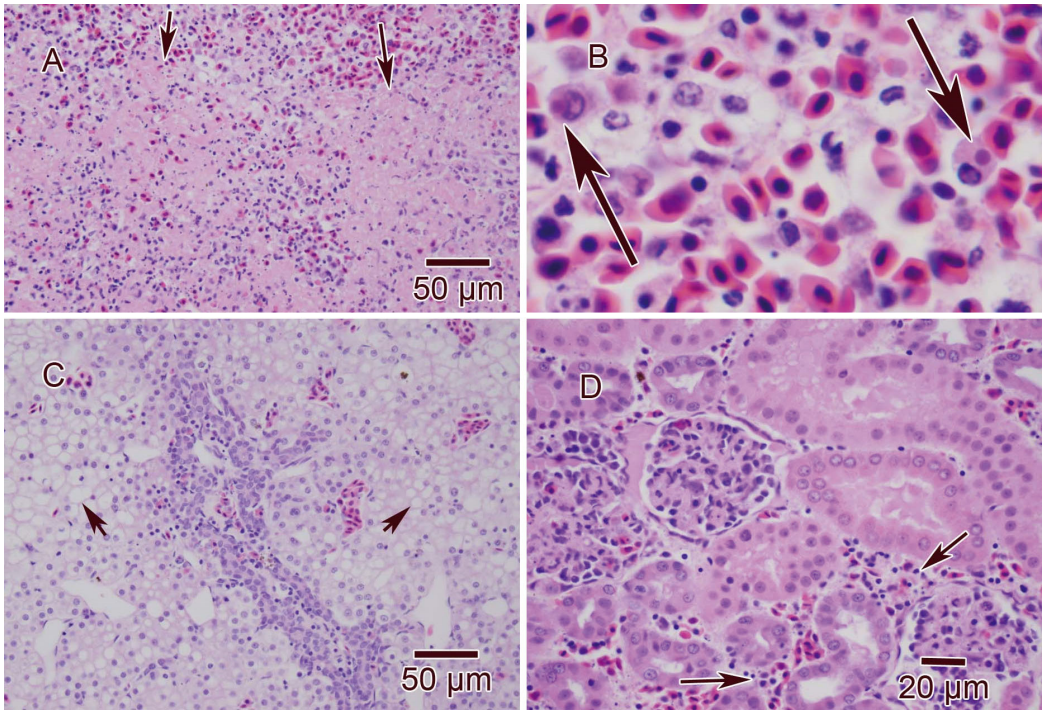

Fig. 1. (A) *Scaphirhynchus albus* spleen with extensive area of necrosis (arrows); H&E stain. (B) Intracytoplasmic inclusion bodies (arrows) in splenic cells. (C) Liver with minimal haematopoietic necrosis noted in portal areas. The livers of all fish had generalized vacuolation (arrows). (D) Kidney with mild haematopoietic necrosis (arrows)

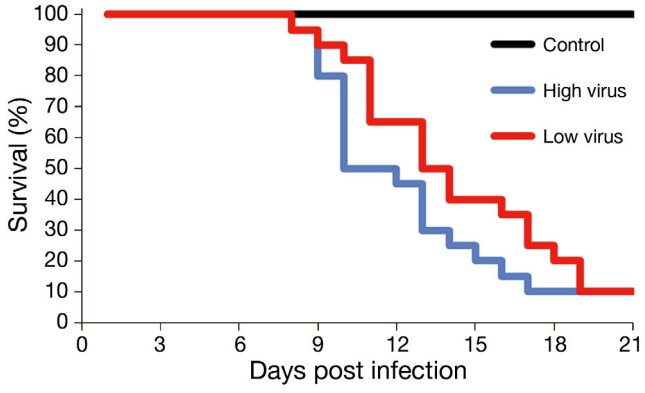

Fig. 2. Cumulative mortality of pallid sturgeon *Scaphirhynchus albus* exposed to 1.3×10^6 $TCID_{50}$ ml^{-1} virus (high virus), 1.3×10^5 $TCID_{50}$ ml^{-1} virus (low virus), or control (no virus) over the 21 d experimental period

Electron microscopy, PCR, and BLASTN

Icosahedral virus particles with a diameter ranging from 145 to 155 nm were observed by electron microscopy following concentration from the culture medium of virus-inoculated EPC cells (Fig. 3).

Sequencing of the PCR amplicon generated 1511 bp of the viral genome that contained the full length MCP sequence (1392 bp; GenBank acc. no. KF646249).

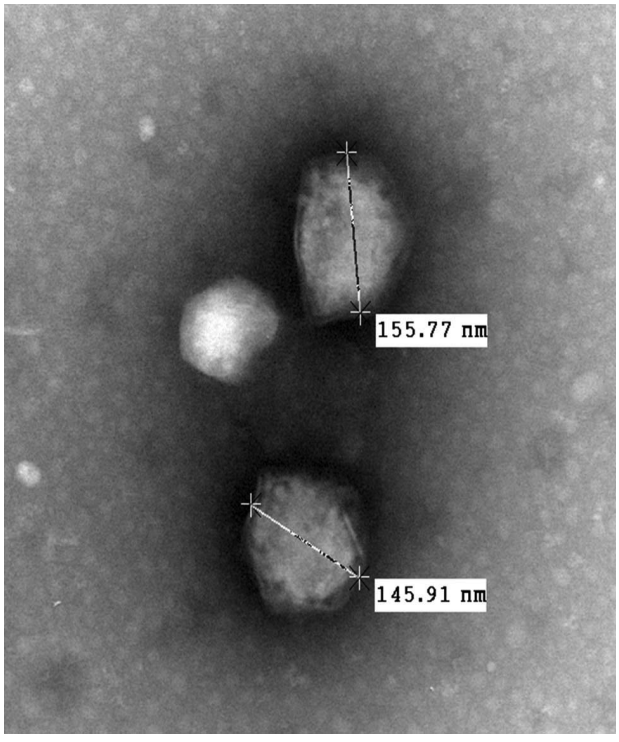

Fig. 3. Negative stain electron photomicrograph of icosahedral virions as isolated from tissues of juvenile pallid sturgeon *Scaphirhynchus albus*

The BLASTN searches for the MCP gene sequence had greatest identity with iridoviruses belonging to the genus *Ranavirus*, including 100% identity with FV3 as originally isolated from northern leopard frog (Table 1).

DISCUSSION

In this report, we confirm that FV3 was responsible for a 2009 mass mortality event of captive juvenile pallid sturgeon in the BPSFH. The virus that we isolated from moribund individuals was indistinguishable by the MCP gene sequence from FV3 as originally isolated from northern leopard frog (Tan et al. 2004, Holopainen et al. 2009). Our data from naturally occurring and experimentally infected hatchery-reared pallid sturgeon support the suspicions of a single prior report in wild threespine stickleback (Mao et al. 1999) that FV3 can cause mortality in fish. Brenes et al. (2014a) recently demonstrated that the FV3 we isolated from pallid sturgeon in our study also is capable of infecting and causing mortality in mosquito fish *Gambusia affinis*. These results collectively suggest that FV3 is among the least host-specific pathogens known to cause lethal disease in poikilothermic vertebrates (Gray et al. 2009, Whittington et al. 2010). The genetic basis of this broad host range and virulence is an area of active investigation among ranavirus researchers. These findings suggest that controlling the global spread of ranaviruses will likely require a better understanding of the transmission dynamics between complex aquatic vertebrate communities as well as the migration patterns or anthropogenic movements of these vertebrate hosts (Gray & Miller 2013).

The original source for the virus found in juvenile pallid sturgeon in the present study remains unknown. To date, vertical transmission of ranavirus is unknown (Gray et al. 2009). Thus, it is unclear whether the adult pallid sturgeon spawned at the BPSFH in 2009 might have served as the source of the virus observed in their offspring. The recovery of virus from 2 of 5 fish sampled at the end of the experimental studies in our report (22 d) may suggest the potential for a longer-term association of the virus with the fish host. Brenes et al. (2014a) reported subclinically infected mosquito fish after a 28 d challenge with our isolate. Longer-term trials, as conducted for MRSIV by Kurobe et al. (2011), are needed to properly assess the potential for older age classes to serve as FV3 carriers and to determine whether vertical transmission is possible. The water supply of the BPSFH as a potential source of virus is suspected in part because it is drawn directly from Blind Pony Lake without treatment. This lake supports numerous aquatic vertebrates that could serve as hosts for FV3. Although no animals were examined directly from Blind Pony Lake, preliminary screening of liver samples collected from 31 adult American bullfrogs *Lithobates catesbeianus* and 4 tadpoles and 26 adult plains leopard frogs *L. blairi* during the fall of 2009 and 2010 in nearby wetlands tested negative for FV3 by PCR and cell culture (J. T. Briggler unpubl. data).

Table 1. Nucleotide comparison of the full length major capsid protein sequence (1392 nt) of the frog virus 3 isolate from pallid sturgeon *Scaphirhynchus albus* (GenBank acc. no. KF646249) to other ranaviruses. Ranavirus species recognized by the International Committee on the Taxonomy of Viruses are underlined. Note: largemouth bass virus, guppy virus 6, and doctor fish virus are all considered synonyms of *Santee-Cooper ranavirus*

Ranavirus	GenBank acc. no.	Identity (%)	Reference
Frog virus 3	FJ459783	100.0	Holopainen et al. (2009)
Rana grylio virus	JQ654586	99.8	Lei et al. (2012)
Soft-shelled turtle iridovirus	EU627010	99.8	Huang et al. (2009)
Bohle iridovirus	AY187046	98.7	Marsh et al. (2002)
Common midwife toad virus	JQ231222	98.6	Mavian et al. (2012a)
Tiger frog virus	AF389451	98.6	He et al. (2002)
Rana esculenta virus	FJ358611	98.3	Holopainen et al. (2009)
Pike-perch iridovirus	FJ358610	98.3	Holopainen et al. (2009)
Andrias davidianus ranavirus	KC865735	98.3	Chen et al. (2013)
Epizootic haematopoietic necrosis virus	FJ433873	97.8	Jancovich et al. (2010)
European catfish virus	FJ358608	97.3	Holopainen et al. (2009)
European sheatfish virus	JQ724856	97.3	Mavian et al. (2012b)
Cod iridovirus	GU391284	97.2	Ariel et al. (2010)
Ranavirus maxima	AY187046	97.1	Ariel et al. (2010)
Ambystoma tigrinum virus	AY150217	96.3	Jancovich et al. (2003)
Short-finned eel ranavirus	FJ358612	94.1	Holopainen et al. (2009)
Guppy virus 6	FR677325	78.1	Unpublished
Doctor fish virus	FR677324	78.1	Unpublished
Largemouth bass virus	FR682503	78.0	Unpublished
Grouper iridovirus	AY666015	70.0	Tsai et al. (2005)
Singapore grouper iridovirus	AY521625	69.4	Song et al. (2004)

A remarkably similar epizootic to that in 2009 occurred at the same time of year in 2001 at the BPSFH, resulting in nearly 100% loss of the entire young of the year pallid sturgeon cohort (B. Drecktrah pers. obs.). Interestingly, a virus was isolated from the moribund sturgeon in 2001, and the full MCP sequence of this virus revealed it was also an FV3 isolate (T. B. Waltzek & R. P. Hedrick unpubl. data). FV3 has also been isolated from dying juvenile Russian sturgeon *Acipenser gueldenstaedtii* at a hatchery in Warm Springs, Georgia (USA), in 2005, and the isolate later proved lethal by injection in both juvenile Russian sturgeon and lake sturgeon *A. fluvescens* (N. Heil & R. Bakal pers. comm.). Finally, we have also isolated a ranavirus related to FV3 (based on preliminary sequencing of the MCP gene) from juvenile white sturgeon *A. transmontanus* while sampling for viruses at a California farm in 1998 (R. P. Hedrick & T. B. Waltzek unpubl. data). Further challenge studies across a wider taxonomic range of fishes are needed to determine whether certain lineages, such as imperiled groups like sturgeon, are predisposed to FV3 as has been determined for certain larval amphibians (Hoverman et al. 2011). Given the lethal nature and frequency of viral outbreaks (e.g. FV3 and MRSIV) among pallid sturgeon hatcheries engaged in restoration efforts (Kurobe et al. 2010, 2011), a better understanding of ecology (e.g. temperature, density dependence) of the agents is warranted.

The BPSFH epizootics reported here share characteristics with other outbreaks in aquaculture facilities around the world rearing amphibians (e.g. bullfrog *L. catesbeianus*, pig frog *L. grylio*, tiger frog *Rana tigrina*, Chinese giant salamander *Andrias davidianus*) and reptiles (Chinese softshell turtle *Pelodiscus sinensis*) for food or as part of species restoration projects (Chen et al. 1999, Zhang et al. 2001, Weng et al. 2002, Majji et al. 2006, Mazzoni et al. 2009, Geng et al. 2011). Although a model characterizing the epidemiology of ranaviruses in captive facilities has not been published, presumably typical density-dependent effects, including host immunosuppression (via stressors associated with crowding such as poor husbandry) and ease of viral transmission, are key factors favoring the pathogen in hatchery conditions (Mazzoni et al. 2009, Gray & Miller 2013). Epizootics involving ranaviruses are typically associated with high mortality (typically >60%) in larval or immature animals that likely lack immunocompetence. Furthermore, pallid sturgeon and Chinese giant salamanders have recently undergone severe population bottlenecks that may further impair their resistance to pathogens as a result of decreased immunogenetic diversity (Pearman & Garner 2005).

The lethal nature of this FV3 isolate in pallid sturgeon offers a stark contrast to recent studies that did not find significant mortality in black bullhead *Ameiurus melas*, common carp *Cyprinus carpio*, goldfish *Carassius auratus*, northern pike *Esox lucius*, and pike-perch *Sander lucioperca* (Bang Jensen et al. 2009, 2011a,b, Gobbo et al. 2010) following exposure to an FV3 isolate from the northern leopard frog. One explanation for this difference could be that genetic variation influencing virulence exists among FV3 isolates, which would have gone undetected by only sequencing a small portion of the MCP gene. We are in the process of exploring genomic variation of FV3 isolates across broad host ranges and temporal-spatial scales.

The absence of significant mortality but the ability to isolate virus from tissues following FV3 challenges of black bullhead, northern pike, and pike-perch indicate that these fish become infected (Bang Jensen et al. 2009, 2011a,b, Gobbo et al. 2010). That FV3 may occur in fish populations without manifesting as overt disease has been shown by recent surveillance efforts targeting viral haemorrhagic septicemia virus (VHSV) in the USA. In these surveys, FV3 was inadvertently isolated from outwardly healthy fathead minnow *Pimephales promelas*, walleye *S. vitreus*, and northern pike (N. Phelps & J. Warg pers. comm.). Brenes et al. (2014b) hypothesized that some fish may function as reservoirs for FV3, whereas amphibian larvae amplify the concentration of ranavirus in water and serve as a catalyst for outbreaks. Water temperature may also be an important factor in determining severity of infections in fish hosts, as shown by the pallid sturgeon outbreaks subsiding as water temperatures decreased. Although mortality subsided, virus was isolated from survivors that appeared overtly healthy in December 2009. For larvae of 3 amphibian species (*Lithobates sylvaticus*, *L. clamitans*, *Ambystoma maculatum*), infection prevalence and mortality due to ranavirus was greater at 25°C compared to 10°C in experimental challenges (M. J. Gray & D. L. Miller unpubl. data).

Understanding whether fish surviving exposure become asymptomatic carriers and how long they are capable of transmitting the disease will be imperative to the development of effective mitigation strategies. Using the FV3 isolated from pallid sturgeon in our study, Brenes et al. (2014b) recently demonstrated that interclass transmission is possible by systemically cohabitating naïve and infected aquatic vertebrates from different classes (e.g. amphibian:

Hyla chrysoscelis; reptile: *Trachemys scripta elegans*; and fish: *Gambusia affinis*). Knowing what differences may exist in the outcomes of the association of FV3 isolates with their diverse amphibian, reptilian, and fish hosts will be needed to properly assess the potential negative impacts of this important virus on both hatchery and naturally occurring populations present in our aquatic ecosystems.

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