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Effect of water temperature on frog virus 3 disease in hatchery-reared pallid sturgeon *Scaphirhynchus albus*

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ABSTRACT: Ranaviruses are large double-stranded DNA viruses within the genus *Ranavirus* (family *Iridoviridae*) that are being detected with increasing frequency among aquacultured and wild fishes. In the USA, multiple sturgeon hatcheries have experienced ranavirus epizootics resulting in significant morbidity and mortality in young-of-year (YOY). Significant economic losses have resulted from repeated outbreaks of frog virus 3 (FV3), the type species for the genus *Ranavirus*, in YOY pallid sturgeon *Scaphirhynchus albus* reared at a hatchery within the Missouri River Basin. Water temperature and stocking density are known to influence the severity of ranavirus disease in ectothermic vertebrates. To determine the effect of water temperature on ranavirus disease in hatchery-raised *S. albus*, we conducted FV3 challenges at 2 temperatures (17 and 23°C) and compared cumulative survival over a 28 d study period. A mean (±SE) survival rate of 57.5 ± 13.2% was observed in replicate tanks of sturgeon maintained at 23°C, whereas no mortality was observed among sturgeon maintained at 17°C. In a second challenge study, we compared the effect of water temperature on disease progression by regularly sampling fish over the study period and evaluating lesions by histopathology and *in situ* hybridization, and by assessing viral titer and load in external and internal tissues using virus isolation and qPCR, respectively. Results suggest that temperature manipulation may be an effective mitigation strategy that sturgeon hatcheries can employ to minimize ranavirus-associated disease.

KEY WORDS: Sturgeon · Frog virus 3 · Ranavirus · Aquaculture

1. INTRODUCTION

Sturgeon belong to the family Acipenseridae, one of the oldest ray-finned fish families with fossils dating back to the Upper Cretaceous period (Gardiner 1966). They are among the largest and longest-lived of all known fishes, with adults reaching maturity late in life (Bemis et al. 1997, Berra 2007). Sturgeon

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have historically been fished for their meat and caviar, and overfishing combined with habitat destruction and pollution have decreased wild stocks of many species (Artyukhin 1997, Debus 1997, Baker & Borgeson 1999). All sturgeon are currently designated CITES Appendix I or II (UNEP-WCMC 2021), and many are listed as Endangered or Critically Endangered on the IUCN Red List of Threatened Species (IUCN 2021). Fishery restrictions have led to decreased pressure on wild sturgeon stocks over the last 2 decades, and aquaculture is steadily increasing to meet the demand for meat and caviar. Production of sturgeon occurs predominantly in China, Europe, Russia, and the USA (Chebanov & Billard 2001, Bronzi et al. 2011). Multiple hatcheries in North America aim to help replenish dwindling wild stocks of native species, including the endangered pallid sturgeon *Scaphirhynchus albus*.

Infectious agents have negatively impacted sturgeon aquaculture and restoration efforts. The melanized fungus *Veronaea botryosa* has been an impediment to production of Siberian sturgeon *Acipenser baerii* in Florida and white sturgeon *A. transmontanus* in California (Steckler et al. 2014, Groff et al. 2021, Yazdi et al. 2021). In particular, viral pathogens (e.g. adenovirus, alloherpesviruses, and iridoviruses) have hindered production of farmed sturgeon (Hedrick et al. 1985, 1990, 1991a,b, Bauer et al. 2002, Kelley et al. 2005, Kurobe et al. 2008, 2010, 2011, Waltzek et al. 2014). Multiple ranavirus epizootics have occurred at 3 separate North American sturgeon hatcheries (Waltzek et al. 2014). In 2001, 2009, 2013, and 2015, young-of-year (YOY) *S. albus* at the Blind Pony State Fish Hatchery (BPSFH) in Sweet Springs, MO, experienced heavy mortalities (up to 90–100%) caused by a strain of frog virus 3 (FV3) (Waltzek et al. 2014, Stilwell 2017). These 4 FV3 epizootics resulted in economic losses of >$400 000 US and have hampered efforts to replenish wild *S. albus* stocks (J. Colehour unpubl. data). Ranavirus epizootics have also caused high mortality in farmed *A. transmontanus* in California in 1998 and Russian sturgeon *A. gueldenstaedtii* in Georgia in 2004. Koch's postulates (as modified for viral diseases; Rivers 1937) were fulfilled in the 2009 *S. albus* epizootic, and intracoelomic injection of the FV3 isolate from the 2004 Russian sturgeon outbreak reproduced disease in both juvenile *A. gueldenstaedtii* and lake sturgeon *A. fulvescens* (R. Bakal unpubl. data).

The severity of ranavirus epizootics in sturgeon hatcheries is likely due to a combination of host, viral, and environmental factors. Hatcheries rearing YOY sturgeon under intensive conditions often experience increased infectious disease outbreaks as environmental conditions deteriorate (e.g. poor water quality, inappropriate water temperature, malnutrition) (Barton & Iwama 1991, Chua et al. 1994, LaPatra et al. 1996, Georgiadis et al. 2000, 2001, Drennan et al. 2005, Savin et al. 2011). High stocking densities facilitate transmission by increasing contact rates between animals, as well as pathogen concentrations within the water (Woodland et al. 2002, Brunner et al. 2007, Brenes et al. 2014, Brunner et al. 2015). Additionally, many sturgeon stocks have undergone severe genetic bottlenecks, potentially resulting in decreased immunogenetic diversity and increased susceptibility to pathogens (LaPatra et al. 1999, Marranca et al. 2015), as has been observed in other species on the brink of extinction (O'Brien & Evermann 1988, Pearman & Garner 2005).

Temperature is a well-known factor influencing ranaviral disease in fish (Wedemeyer et al. 1976). Whittington & Reddacliff (1995) observed that natural outbreaks of epizootic hematopoietic necrosis virus (EHNV) were most severe in juvenile redfin perch *Perca fluviatilis* congregating in shallow warm waters, and experimental infections with EHNV in rainbow trout *Oncorhynchus mykiss* showed highest mortality at temperatures beyond the host's upper threshold (Ariel et al. 2009). Watson et al. (1998) determined that maintaining temperatures either above or below the recommended range for *A. transmontanus* resulted in increased mortality and/or severity of infection with white sturgeon iridovirus. Seasonal FV3 epizootics in 2001, 2009, 2013, and 2015 at the BPSFH were most severe when water temperatures were elevated during summer months (Waltzek et al. 2014). Although temperature tolerance studies have not been extensively performed in *S. albus*, a study using 18 juveniles noted stress at temperatures above 30°C, with mortality beginning at 33°C (Blevins 2011). Shovelnose sturgeon *S. platorynchus*, the closest relative to *S. albus*, grow most efficiently at 22°C, with steadily increasing mortality in individuals raised above that temperature (Kappenman et al. 2009).

In the current study, water temperature was examined for its effect on ranaviral disease following bath challenges of FV3 in YOY *S. albus*. In the first study, cumulative survival was compared among YOY *S. albus* held at 2 water temperatures (17 and 23°C), and in the second study, disease progression was compared among YOY *S. albus* at the 2 temperatures.

2. MATERIALS AND METHODS

2.1. Quarantine and husbandry

The use of juvenile *Scaphirhynchus albus* in these studies was approved by and conducted in compliance with the University of Florida (UF) Institutional Animal Care and Use Committee (protocol 2016-09405). YOY *S. albus* were obtained from the Gavins Point Fish Hatchery in South Dakota with no previous history of ranavirus infection. Before shipment to the UF Wildlife and Aquatic Veterinary Disease Laboratory (WAVDL) in Gainesville, FL, pectoral fin clips from 60 fish were individually tested and confirmed negative for Missouri River sturgeon iridovirus by the US Fish and Wildlife Service Bozeman Fish Health Center in Bozeman, MT, using a conventional PCR assay (Kurobe et al. 2010). At the WAVDL, fish were quarantined for 6 wk prior to experimentation. Fish were arbitrarily assigned to rectangular glass holding tanks ranging from 204 to 455 l in volume. Tanks were supplied with flow-through dechlorinated municipal water at 17°C and aerated with airstones. Flow-through rate was set at 10 volume changes d^{-1}, and fish were stocked at a density of 2.8 fish ft^{-3} (~1 fish per 10 l) of tank space. After a 3 d acclimation period, water temperature in half of the tanks was raised to 23°C at a rate of 1°C d^{-1} for the remainder of the quarantine period. Fish were fed twice daily to satiation with a combination of commercial pellets (Otohime Larval and Grow-out Feed containing 52% crude protein and 12% crude fat), frozen brine shrimp, and frozen bloodworms. Water temperature was recorded twice daily in all tanks, and dissolved oxygen (DO) levels were measured daily using a Hach meter until levels were determined to be stable at ≥85% O_2 saturation, after which DO was measured weekly. Water chemistry analysis was performed every second day (total ammonia nitrogen, nitrite, pH) or every week (total hardness and total alkalinity) using a multi-parameter water quality kit (Model FF-1A, Hach). Fish were monitored twice daily for morbidity and mortality.

2.2. Necropsy

On Day 36 of quarantine, 10 fish were arbitrarily selected and sacrificed for health assessments, which consisted of gross physical examination, microscopic examination of tissue wet mounts, aerobic bacterial culture, and ranavirus screening. Immediately following collection of superficial external tissue (skin, fin, gill) wet mounts, fish were euthanized in 1000 mg l^{-1} tricaine methane sulfonate (MS-222®, Argent Laboratories, Finquel®) buffered in an equal weight of sodium bicarbonate and followed by pithing. Individuals were weighed (g) and measured for fork length (FL, cm), and a postmortem examination was performed to identify gross lesions. Additionally, sterile autoclaved instruments were used to collect posterior kidney cultures, which were inoculated onto Columbia agar with 5% sheep blood and incubated for 72 h at 24°C for bacterial screening. Additional wet mounts of internal organs (stomach, intestine, liver, posterior kidney, and spleen) were collected, and external and internal tissue samples were then assessed for lesions and pathogens (i.e. bacteria, fungi, parasites) via light microscopy at 40, 100, and 200× magnifications. The 10 individuals were screened for ranavirus using a pan-ranavirus TaqMan qPCR assay (Stilwell et al. 2018) and virus isolation in cell culture as described in Section 2.3.

2.3. Virus culture

Sterile autoclaved instruments were used to collect pooled internal tissues (liver, kidney, and spleen) from each individual, which were diluted 1:25 in minimal essential medium (MEM) with 2% fetal bovine serum (MEM-2). Tissue suspensions were then homogenized using a stomacher (Seward Stomacher® 80 Biomaster) on high speed for 2 min. An aliquot (200 µl) of each pooled tissue homogenate was then processed for DNA extraction (described in Section 2.4), and the remainder was further processed for virus isolation. The remaining homogenate was then clarified by centrifugation at 3000 × *g* (10 min at 4°C) to remove cellular debris. An equal volume of clarified tissue homogenate was added to a MEM-2 antibiotic solution, resulting in a final concentration of 500 IU penicillin ml^{-1}, 500 µg streptomycin ml^{-1}, 12.5 µg fungizone ml^{-1}, and 14 mM HEPES (4-(2-hydroxyethyl)-1- piperazine ethane sulfonic acid) buffer. After incubating overnight at 4°C, the samples were again clarified at 3000 × *g* (10 min at 4°C), and 10 µl of undiluted and serially diluted (10^{-1} to 10^{-11}) tissue homogenates were inoculated onto 6 replicate wells of a 96-well plate containing confluent monolayers of epithelioma papulosum cyprini (EPC) cells. After a 1 h virus adsorption period, 190 µl of MEM-2 were added to each well. Two replicate wells per dilution containing only MEM-2 served as negative controls. Cells were incubated at 24°C and observed for cytopathic effect

(CPE) daily for 14 d. The 50% tissue culture infectious dose ($TCID_{50}$) was calculated by endpoint analyses at 14 d post-inoculation (PI) (Ramakrishnan 2016). Samples not displaying CPE on Day 14 were blind passaged and observed for an additional 10 d before being declared negative.

2.4. qPCR

For ranaviral testing via qPCR, 200 µl of each pooled tissue homogenate were transferred to lysis bead tubes (Benchmark Scientific) and processed in a TissueLyser II (Qiagen) at 30 cycles s^{-1} for 1 min. DNA extraction was then performed using a Qiacube (Qiagen) with the DNeasy Blood and Tissue Extraction protocol (Qiagen). TaqMan qPCR samples were tested in triplicate using 4 µl of DNA extract (e.g. 100 ng per reaction) with a single 18S rRNA gene internal control per sample (Stilwell et al. 2018). Threshold of detection (Ct) and number of copies were estimated for samples using the 7500 Fast Real-Time PCR System software program (Applied Biosystems) based on the use of a linearized plasmid standard curve. Samples were considered positive for ranavirus if 2/3 of the wells generated a Ct value of ≤40.

2.5. Study 1: Effect of water temperature on cumulative survival

After the 6 wk quarantine period, 120 sturgeon were arbitrarily selected for the first challenge study. Forty exposed and 20 unexposed fish were used at each of 2 water temperatures, referred to hereafter as cold (17°C) and warm (23°C). Virus for the challenge study was prepared from a frozen stock of an isolate cultured from moribund *S. albus* submitted to the WAVDL from the BPSFH during the facility's summer 2015 epizootic (Stilwell 2017). The PSRV2015 isolate was inoculated into a 75 cm^2 flask and then passaged into 175 cm^2 flasks (n = 4) containing confluent EPC cells with MEM-2 at room temperature (24°C). After 2 d, CPE was complete and the supernatants were combined and clarified at 3000 × *g* (10 min at 4°C). The viral titer was determined by $TCID_{50}$ endpoint analyses at 7 d PI as described in Section 2.3 (Ramakrishnan 2016).

The PSRV2015 isolate or negative control (MEM only) exposures were performed via 1 h bath challenge in static, aerated systems at the 2 experimental temperatures (17 and 23°C). Following bath exposure, fish were arbitrarily placed into 84 l tanks and stocked at a density of 10 fish per tank (3.4 fish ft^{-3} [~1.2 fish per 10 l] of tank space) for a 28 d observation period. Four exposed replicate tanks and 2 control replicate tanks were used at each temperature (Fig. A1 in the Appendix). Tanks were aerated with airstones and supplied with flow-through dechlorinated municipal water at either 17 or 23°C with a turnover rate of 10 volumes d^{-1}. Water quality parameters (total ammonia nitrogen, nitrate, pH, total hardness, and total alkalinity) were measured weekly throughout the study. Fish were monitored twice daily for signs of morbidity and mortality. Dead or moribund fish were processed individually as described above during the quarantine period, including weight and FL measurements; gross and wet mount examination of tissues; bacteriological sampling; and ranavirus testing of pooled liver, spleen, and posterior kidney homogenates via TaqMan qPCR and $TCID_{50}$ endpoint analyses. Moribund fish displaying advanced clinical signs of ranavirus infection were euthanized as described above. Endpoint criteria for euthanasia included the display of 2 or more of the following advanced ranaviral disease signs: external hemorrhage of skin and/or fins, distended coelom, erratic swimming pattern, and abnormal buoyancy and/or orientation. To minimize potential pathogen cross-contamination among tanks and fish, each animal was collected individually with separate nets disinfected in 2% Virkon Aquatic for at least 2 min before use (Bryan et al. 2009) and then euthanized in separate containers. At 28 d post exposure (PE), surviving fish were euthanized. Two fish from each tank (i.e. a total of 8 exposed and 4 unexposed control fish for each water temperature) were processed as described above to assess health status and test for ranaviral infection among survivors.

2.6. Study 2: Effect of water temperature on disease progression

The experimental design of Study 2 was identical to that of Study 1 (Fig. A1). On Days 1, 3, 5, 7, 14, and 28 PE, 1 fish from each tank was euthanized as described above, for a total of 12 fish at each sampling period (i.e. 4 exposed and 2 unexposed fish from each temperature treatment). To minimize potential pathogen cross-contamination among tanks and fish, each animal was collected individually with separate nets disinfected in 2% Virkon Aquatic for at least 2 min prior to use (Bryan et al. 2009) and then euthanized in separate containers as described above.

Dead or moribund fish were processed individually as described above during the quarantine period and Study 1 (unless noted otherwise), including weight and FL measurements and gross and wet mount examination of tissues. Additionally, sterile autoclaved instruments were used to collect pooled samples of skin, fin, gill, and barbel from each fish. After a ventral midline incision was made to access the coelomic cavity, a new set of autoclaved instruments was used to collect samples of liver, spleen, posterior kidney, and heart. Using the methods described for Study 1, pooled internal (liver, kidney, spleen, heart, and pericardial lymphomyeloid tissue) and external (skin, fin, gill, and barbel) tissue homogenates were assessed separately for viral load by qPCR and viral titer by $TCID_{50}$ endpoint analyses.

Samples of the above tissues, along with stomach, spiral colon, head, and transverse body wall at the level of the posterior kidney, were placed in 10% neutral buffered formalin for 24 to 48 h for histological fixation. Calcified tissues (e.g. head, body wall, fin, and gill) underwent an additional 48–96 h decalcification step in 0.5 M ethylenediaminetetraacetic acid (EDTA) buffered to a pH of 8.0 (Fisher BioReagents). Formalin-fixed, paraffin-embedded samples of tissues were sectioned at 3 µm, mounted onto glass slides, and stained with hematoxylin and eosin (H&E). Slides were examined for histopathological changes consistent with ranavirus, including hemorrhage; necrosis of hematopoietic tissues, vascular endothelium, and epithelial cells; and cytoplasmic basophilic to amphophilic inclusions (Reddacliff & Whittington 1996, Waltzek et al. 2014, Miller et al. 2015). To complement histopathological interpretation, a subset of fixed tissues was subjected to a ranavirus-specific *in situ* hybridization (ISH) assay as described below. Histological examination (H&E and ISH) was performed on tissue sections from 11 fish (55 slides per stain technique), consisting of 1 exposed fish per temperature treatment per sampling day. Fish with the highest qPCR ranaviral copy number on each day within their treatment groups were preferentially chosen for ISH, as their tissues had the greatest probability of positive labeling.

2.7. Development of a ranavirus RNAscope ISH assay

The ISH assay utilized RNAscope® technology and ZZ probes (Wang et al. 2012) designed from the 1392-nt coding sequence for the ranavirus major capsid gene (MCP). Previously designed assays suggested that the MCP is a good candidate for ranavirus diagnostics as it exhibits 94–100% nucleotide sequence identity of 1392 nt across all ranaviruses, excluding the highly divergent Santee-Cooper ranavirus and Singapore grouper iridovirus (Jancovich et al. 2015). The probe was designed by Advanced Cell Diagnostics (cat #439991) based on the full MCP nucleotide sequence of an FV3 isolated from the 2009 *S. albus* epizootic (PSRV2009) (Waltzek et al. 2014).

Formalin-fixed, paraffin-embedded tissues were sectioned at 5 µm, mounted onto Superfrost™ Plus glass slides (Fisherbrand™), and prepared for the RNAscope® ISH protocol according to the manufacturer's instructions for the manual RNAscope® technique, consisting of baking slides at 60°C for 1 h within 1 wk prior to the assay. The ISH assay was performed using the HybEZ II oven and RNAscope® 2.5 HD RED Reagent Kit (Advanced Cell Diagnostics) according to the manufacturer's instructions. The following steps were performed sequentially and were performed according to the manual procedure of the manufacturer using the temperatures and durations indicated here. Sample deparaffinization and dehydration were performed at room temperature (RT, 23°C) using xylene for 10 min and 100% ethanol for 2 min, respectively. Next, sequential tissue section pretreatments were performed with RNAscope® hydrogen peroxide for 10 min at RT, RNAscope® target retrieval reagents for 15 min at 99°C, and RNAscope® protease plus for 30 min at 40°C. Probes were then hybridized for 2 h at 40°C, followed by signal amplification and signal development according to the manufacturer's instructions. Lastly, tissue sections were counterstained and coverslips were applied. Known FV3 (PSRV2009)-positive and -negative, formalin-fixed, paraffin-embedded *S. albus* tissue sections from a previous experimental exposure study served as procedural controls (Waltzek et al. 2014). In place of the stock red chromagen supplied with the RNAscope® 2.5 HD RED Reagent Kit assay (Advanced Cell Diagnostics), a permanent red chromagen and hematoxylin (Dako Cytomation) were used for labeling and counterstaining, respectively, to ensure long-term preservation of labeling results. Permanent reagents were integrated into the protocol based on riboprobe-based ISH methods developed by Draghi et al. (2010). Additionally, a 5 min bath with 5 mM tetramisole hydrochloride (Sigma-Aldrich) was performed immediately before red chromagen labeling to reduce endogenous alkaline phosphatase activity.

2.8. Statistical analyses

For Study 1, an unpaired *t*-test was performed to determine significance of mean cumulative survival (proportion of surviving fish to total fish) within and among the 2 temperature treatment groups using replicate tanks as the experimental unit. In Study 2, a linear mixed model with the GLIMMIX procedure was performed to determine the significance of TaqMan qPCR viral copy numbers between temperature treatments accounting for fish sampled at the same time points. A mixed model was used to account for both heterogeneity of variances and correlations across time. Analyses were performed using SAS® version 9.3 (SAS Institute). Numerical data are represented as mean ± SE, and statistical differences were considered significant at $p \leq 0.05$.

3. RESULTS

3.1. Quarantine and husbandry

Water chemistry values were within normal limits during quarantine and both studies. Parameter range values during the studies were as follows: 17°C DO: 7.65–8.81 mg l^{-1}, 17°C oxygen saturation: 78.1–89.9%; 23°C DO: 7.33–9.05 mg l^{-1}, 23°C oxygen saturation: 84.3–100.4%, total ammonia nitrogen: 0–0.2 mg l^{-1}, unionized ammonia: 0–0.006 mg l^{-1}, nitrite: 0 mg l^{-1}, pH: 7.75–8.0, total hardness: 136.8–153.9 mg l^{-1}, total alkalinity: 68.4 mg l^{-1}. Water temperatures were successfully maintained at 17 ± 1°C and 23 ± 1°C in the coldwater and warmwater tanks, respectively.

No abnormalities were identified on gross examination or tissue wet mounts performed as part of the health assessments completed during the quarantine period. The mean weight (n = 10) was 17.7 g (range: 14.3–22.3 g) and mean FL was 24.4 cm (range: 20.0–27.5 cm). Aerobic bacterial cultures yielded no growth, and all 10 fish were ranavirus-negative via virus isolation and TaqMan qPCR.

3.2. Study 1: Effect of water temperature on cumulative survival

The mean weight for the 120 fish used in the cumulative survival study was 28.7 g (range 14.4–50 g) and mean FL was 21.6 cm (range: 16.8–25.5 cm). Infection dose for each temperature was 2.14×10^6 $TCID_{50}$ ml^{-1} water. During the 28 d PE observation period, no morbidity or mortality was observed in the 40 virus-exposed fish held in cold water or in the 20 unexposed control fish at either water temperature (Fig. 1). In contrast, 52.5 ± 13.2% cumulative survival was observed in the replicate tanks housing the exposed warmwater fish. Of the affected fish, 3 fish were found dead, while euthanasia endpoints were utilized for 14 fish. The majority of mortalities occurred on Days 3–6 PE. The most frequently observed abnormalities in affected fish were lethargy (8/17 fish) and hemorrhage of the skin and/or fins (13/17 fish; Fig. 2). Additional findings occurring in less than 25% of sampled, exposed warmwater fish included excess mucus production on the skin and/or fins, sloughing/torn fins, proliferative cotton-like material on external lesions, and abnormal orientation (i.e. lying upside down on the tank bottom).

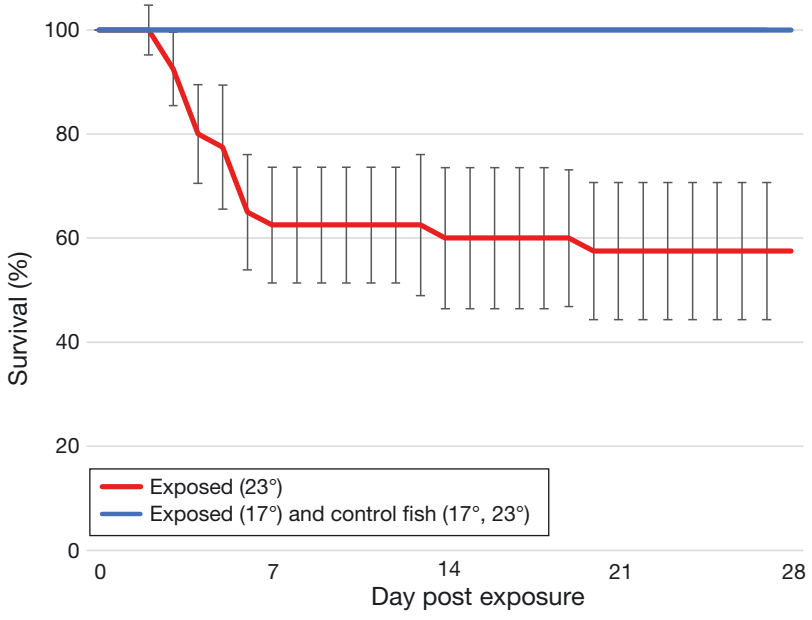

Fig. 1. Study 1 survival curve. Data indicate mean (±SE) survival values in replicate tanks housing young-of-year *Scaphirhynchus albus.* Fish were held at 17°C (coldwater treatment) and 23°C (warmwater treatment) for 28 d after bath exposure to medium containing frog virus 3 (or virus-free medium for controls)

Necropsy, qPCR, and virus isolation were performed on 15/17 exposed warmwater fish, as 2 dead fish were deemed too autolyzed for analysis. Necropsy of the 15 examined individuals revealed several gross lesions consistent with ranavirus infections, including enlarged and/or abnormally pale, dark, or mottled posterior kidney (8 fish), spleen (6), liver (6), and intes-

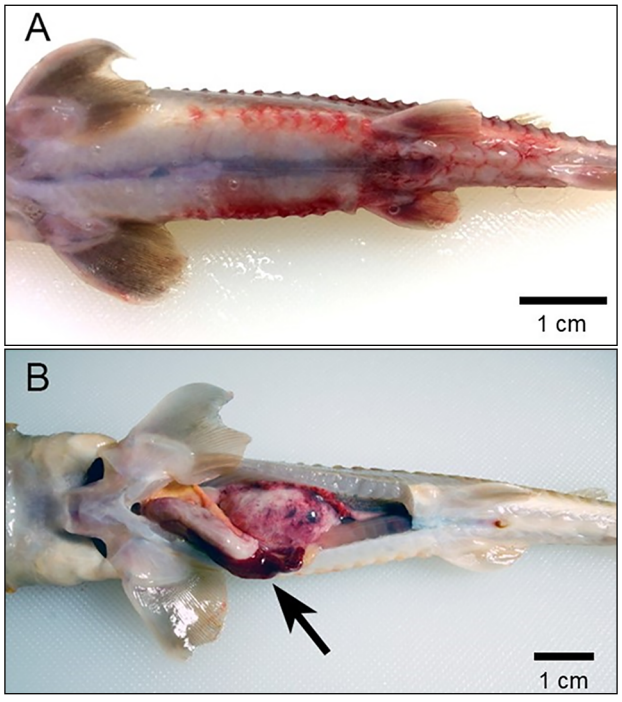

Fig. 2. Photographs of representative gross pathology associated with frog virus 3 disease in *Scaphirhynchus albus* held in warm water (23°C) in Studies 1 and 2. Control fish remained apparently healthy and were not photographed. (A) Regional and severe erythema and hemorrhages of the ventral and peri-cloacal skin and fins, primarily displayed during early infection. (B) Splenomegaly (arrow) and hemorrhages involving coelomic organs, observed during late-stage infection

tine (1); blood within the coelom (5); empty gastrointestinal tract (3); and congested gills (2) (Fig. 2). Bacterial cultures yielded growth in 4/15 fish after 24 h that was identified as belonging to the genus *Aeromonas* via conventional PCR using the universal bacterial primers 27F and 1492R (Yu et al. 2013) followed by Sanger sequencing. The proliferative, cotton-like material on the external lesions of 4 fish was identified microscopically as hypha-like forms (1 fish) and via conventional PCR as oomycetes belonging to the genera *Saprolegnia* (2 fish) or *Aphanomyces* (1 fish).

Fourteen of 15 moribund or deceased virus-exposed warmwater fish tested ranavirus-positive by qPCR, with mean viral loads ranging from $10^{0.7}$ to $10^{6.4}$ viral copies 4 µl^{-1} of extracted internal tissue homogenate DNA (Table 1). Virus isolation was positive for 12/15 fish, with viral titers ranging from $10^{1.8}$ to 10^{6} $TCID_{50}$ ml^{-1} of internal tissue homogenates (Table 1). One additional fish was positive after the blind passage.

The 8 exposed coldwater fish and the 4 control fish at each temperature sampled on Day 28 PE appeared healthy, with no gross or tissue wet mount abnormalities and no bacterial growth observed on aerobic cultures. Additionally, all internal tissue homogenates were negative for ranavirus by TaqMan qPCR and virus isolation (Table 1). In contrast, 5/8 exposed warmwater fish sampled on Day 28 exhibited torn fins, and 4/8 appeared underweight as evidenced by epaxial muscle atrophy and a concave appearance of the ventral coelom. Internally, necropsy revealed that a few fish had undersized livers (3/15 fish) or spleens (1/15) relative to overall body size. Two of 8 fish were ranavirus-positive by qPCR with $10^{1.3}$ to $10^{1.4}$ viral copies 4 µl^{-1}; however, all 8 fish were negative by virus isolation.

3.3. Study 2: Effect of water temperature on disease progression

Mean size of the 120 fish used in Study 2 was 35.6 g (range 18–57 g) and 22.7 cm FL (range 18.4–26.8 cm). Infection dose for each temperature was 4.0×10^{4} $TCID_{50}$ ml^{-1} water. No natural mortality occurred throughout the study. Of the 48 virus-exposed fish, 54% (13/24) of the warmwater fish displayed clinical signs consistent with ranavirus infection, whereas all coldwater fish remained asymptomatic throughout the study (Table 2). By applying the linear mixed model with the GLIMMIX procedure, we showed that internal and external tissue viral loads were significantly higher in the warmwater fish on Days 3, 5, and 7 PE and additionally on Day 14 PE for

Table 1. Study 1 results summary. Survival is reported as mean ± SE for experimental tank replicates and 2 control tank replicates per water temperature; ne: not examined due to lack of mortality

Treatment	Survival (%)	Gross clinical signs	qPCR range (viral copies 4 µl^{-1})	Ranavirus titer ($TCID_{50}$ ml^{-1})	qPCR-positive survivors on Day 28 (%)
23°C virus-exposed	57.5 ± 13.2	Mild to severe	$10^{0.7}$–$10^{6.4}$	$10^{1.8}$–$10^{6.0}$	25 (2/8)
17°C virus-exposed	100	None	ne	ne	0 (0/8)
23°C control	100	None	ne	ne	0 (0/4)
17°C control	100	None	ne	ne	0 (0/4)

Table 2. Study 2 results summary. Ext: pooled external tissues; Int: pooled internal tissues; ne: not examined due to all sampled animals being ranavirus negative via qPCR

Treatment	Gross clinical sign prevalence (%)	100% infection incidence via qPCR	Ranavirus isolation-positive fish (%)	Ranavirus titer ($TCID_{50}$ ml^{-1})
23°C virus-exposed	54 (13/24)	Days 3–14 (Ext, Int), Day 28 (Int)	72 (13/18)	$10^{0.7}$–$10^{4.2}$
17°C virus-exposed	0	Day 7 (Ext), Day 14 (Int)	17 (3/18)	$10^{0.7}$–$10^{1.5}$
23°C control	0	None	ne	ne
17°C control	0	None	ne	ne

internal tissues, compared to viral loads in coldwater fish (Table 3, Fig. 3). Cold- and warmwater virus-exposed fish collected on Days 1 and 28 yielded low viral loads (<10^2 copies 4 μl^{-1} tissue homogenate DNA) and titers (<$10^{1.5}$ $TCID_{50}$ ml^{-1}). Sampled control fish from both water temperatures tested negative for ranavirus via qPCR and virus isolation throughout the duration of the study.

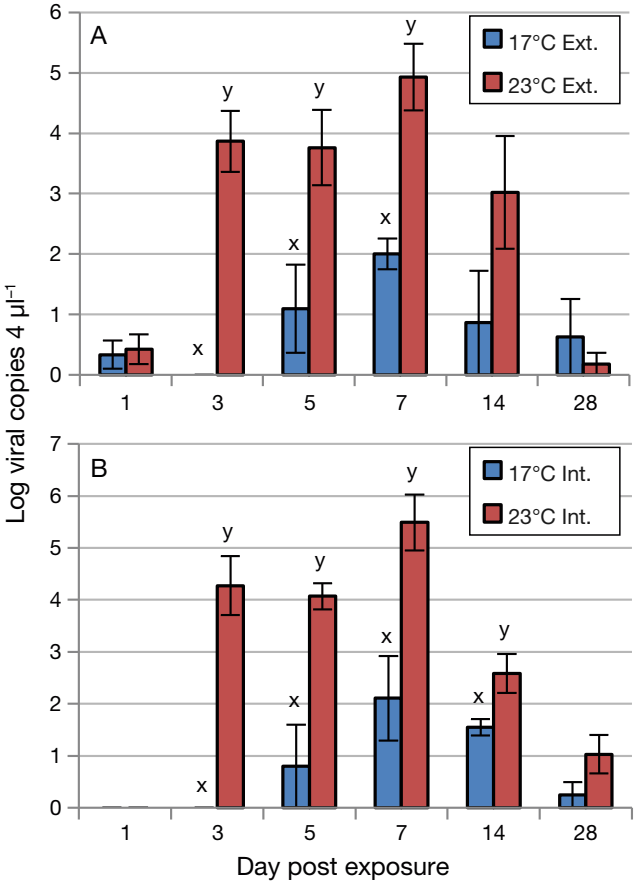

Fig. 3. Comparison of log mean (±SE) ranavirus copies measured via qPCR of tissue homogenates from frog virus 3-exposed *Scaphirhynchus albus* housed in warm water (23°C) and cold water (17°C) during Study 2. Significant differences ($p \leq 0.05$) between the 2 temperatures are denoted by different letters. The qPCR results from sampled negative control fish are not shown, as all sampled control fish tested qPCR-negative for ranavirus. Pooled (A) external (Ext.) and (B) internal (Int.) tissue homogenate qPCR data

Histological examination of slides from 6 warmwater and 5 coldwater virus-exposed fish revealed certain findings consistent with ranavirus infection, including subjectively decreased hematopoietic cell populations in the spleen and pericardial lymphomyeloid tissue, hemorrhage and/or disruption of the lamellar vascular architecture of the gill, and disruption of the epithelium of the skin and fin (Figs. 4–6). These histopathologic findings were more extensive in ranavirus-exposed fish from the warmwater treatment compared to fish from the coldwater treatment, in which microscopic lesions were absent or rare. Cytoplasmic basophilic to amphophilic inclusions consistent with those of ranavirus were not observed in tissues from exposed fish at either temperature. Examined tissue sections from negative control fish were within normal limits with no apparent lesions (Figs. 4–6).

ISH using RNAscope® technology revealed ranaviral nucleic acid within a number of tissues in the

Table 3. Log mean (±SE) qPCR ranaviral copy number for external and internal tissue homogenates in the virus-exposed warmwater and coldwater treatments for Study 2 (n = 4 fish $treatment^{-1}$ d^{-1}). Statistically significant differences ($p \leq 0.05$) between the 2 temperatures are denoted by different superscripts ('x' for external tissue and 'y' for internal tissue)

Day post exposure	External tissue pool 17°C	External tissue pool 23°C	Internal tissue pool 17°C	Internal tissue pool 23°C
1	0.33 (0.23)	0.42 (0.25)	0 (0)	0 (0)
$3^{x,y}$	0 (0)	3.87 (0.51)	0 (0)	4.27 (0.57)
$5^{x,y}$	1.09 (0.73)	3.76 (0.62)	0.80 (0.80)	4.07 (0.25)
$7^{x,y}$	2.00 (0.26)	4.93 (0.55)	2.11 (0.81)	5.49 (0.54)
14^{y}	0.86 (0.86)	3.02 (0.93)	1.55 (0.16)	2.59 (0.37)
28	0.63 (0.63)	0.18 (0.18)	0.25 (0.25)	1.03 (0.37)

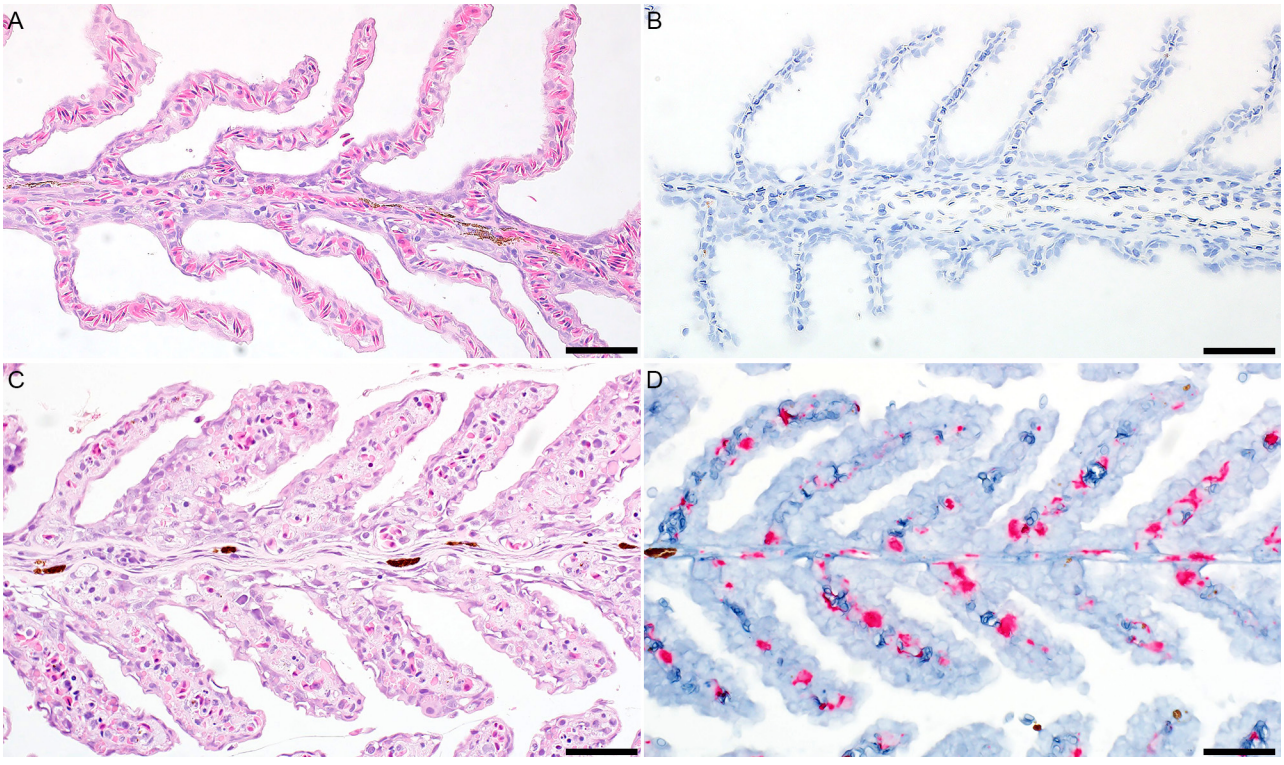

Fig. 4. Representative RNAscope® *in situ* hybridization (ISH) results of gill tissue from warmwater (23°C) negative control and ranavirus-exposed *Scaphirhynchus albus* from Study 2. (A) Negative control fish (H&E stain). (B) Negative control fish. There is an absence of ranavirus labeling (ISH). (C) Exposed fish with foci of necrosis and extravasated erythrocytes along the microvasculature in multiple lamellae (H&E). (D) Exposed fish. ISH shows ranavirus-positive cells within the lamellar interstitium and along the microvasculature. Scale bars = (A–D) 50 µm

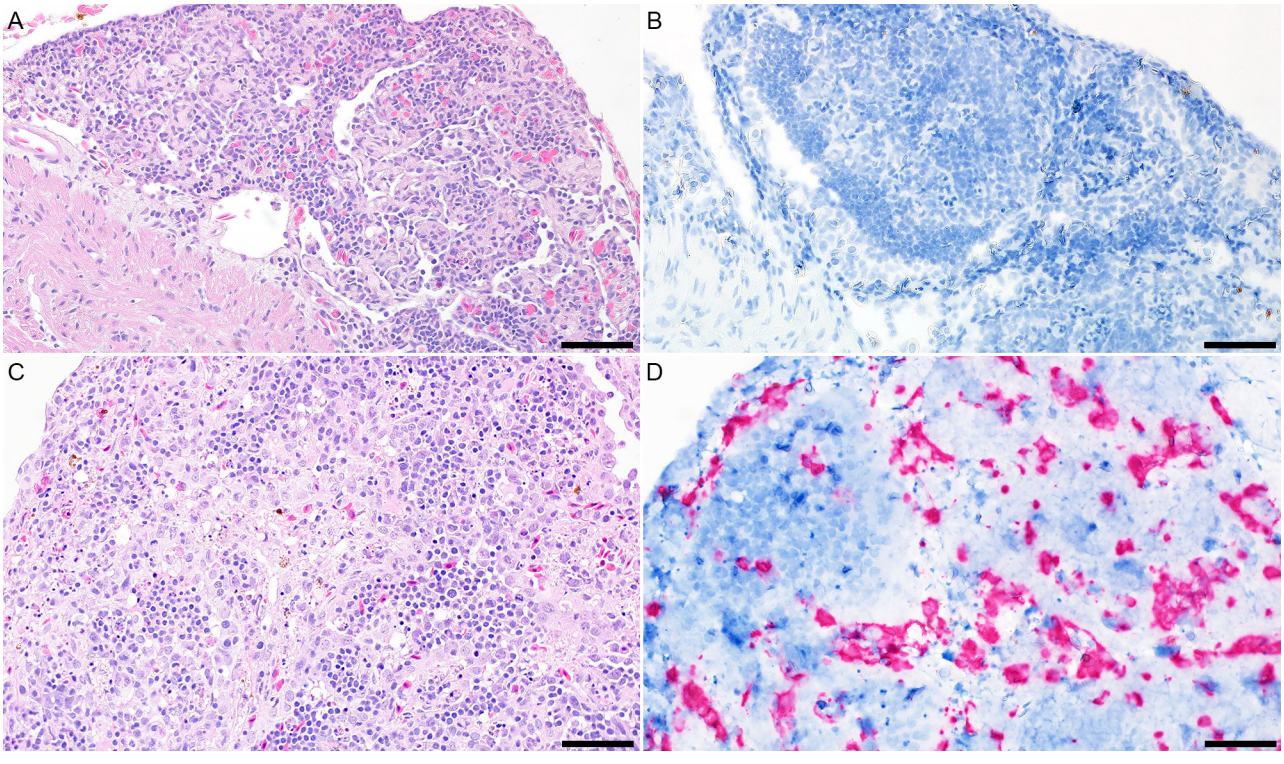

Fig. 5. Representative RNAscope® *in situ* hybridization (ISH) results of pericardial lymphomyeloid tissue from warmwater (23°C) negative control and ranavirus-exposed *Scaphirhynchus albus* from Study 2. (A) Negative control fish (H&E). (B) Negative control fish. There is an absence of ranavirus labeling (ISH). (C) Exposed fish showing depletion of hematopoietic cells from the pericardial lymphomyeloid tissue (H&E). (D) Exposed fish. ISH reveals ranavirus-positive cells scattered within the pericardial lymphomyeloid tissue. Scale bars = (A–D) 50 µm

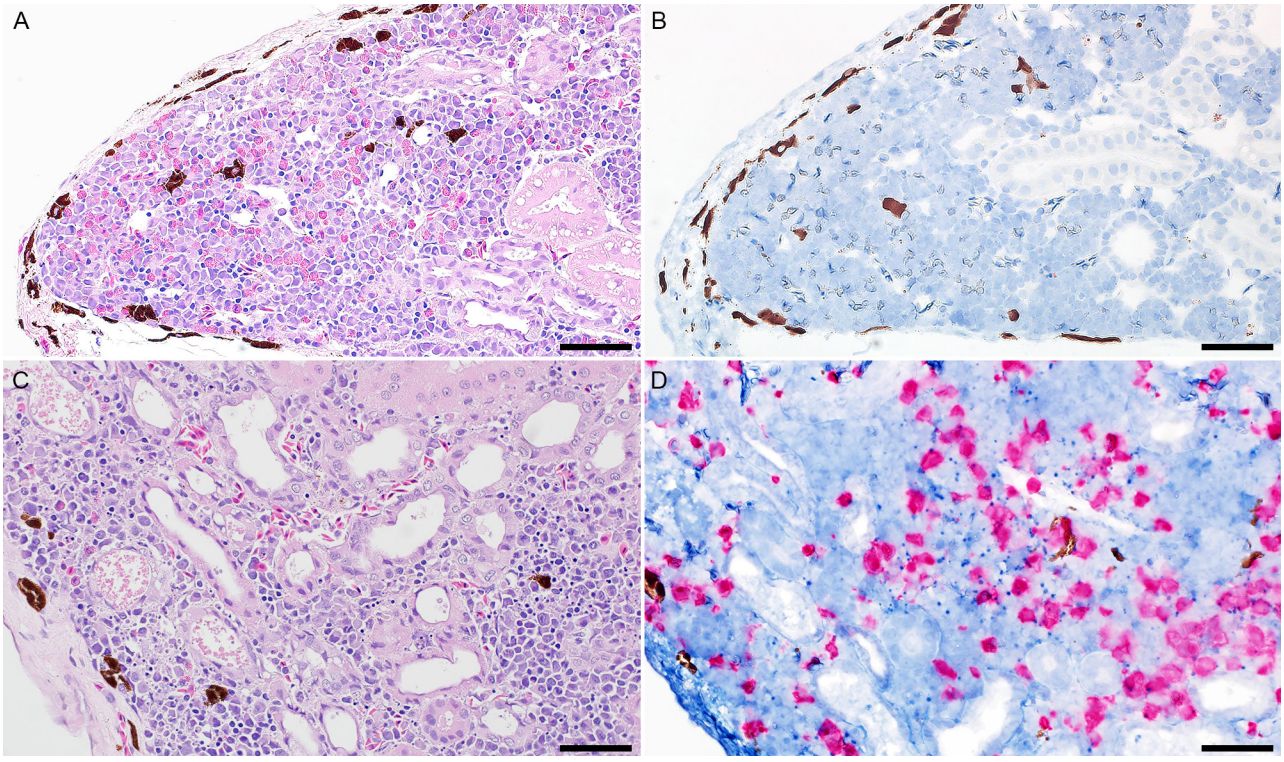

Fig. 6. Representative RNAscope® *in situ* hybridization (ISH) results of kidney tissue from warmwater (23°C) negative control and ranavirus-exposed *Scaphirhynchus albus* from Study 2. (A) Negative control fish (H&E). (B) Negative control fish. There is an absence of ranavirus labeling (ISH). (C) Exposed fish with mild multifocal vacuolation of tubular epithelial cells (H&E). (D) Exposed fish. ISH reveals ranavirus-positive cells scattered within the interstitium. Scale bars = (A–D) 50 µm

warmwater virus-exposed fish, including skin, fin, gill, barbel, olfactory epithelium, heart, meningeal and pericardial lymphomyeloid tissue, liver, spleen, stomach, intestine, and posterior kidney (Table 4, Figs. 4–6). Tissue sections from the exposed coldwater fish and negative control fish (both temperatures) were negative by ISH on all sampling dates.

4. DISCUSSION

Our studies suggest that temperature has a clear effect on FV3 disease in YOY *Scaphirhynchus albus*. Study 1 demonstrated that the severity of disease and mortality rate in virus-exposed fish were significantly greater at a water temperature of 23°C, compared to

Table 4. Study 2 RNAscope® *in situ* hybridization (ISH) results from virus-exposed coldwater (17°C) and warmwater (23°C) fish corresponding with the highest average TaqMan qPCR viral load per day post exposure (PE); ne: not examined due to all sampled animals being ranavirus negative via qPCR on the specified day

Day PE	17°C			23°C		
	ISH positive tissues	qPCR copy no. External	qPCR copy no. Internal	ISH positive tissues	qPCR copy no. External	qPCR copy no. Internal
1	None	10	0	None	9	1
3	ne	0	0	Skin, fin, gill, olfactory epithelium, heart, meningeal and pericardial lymphomyeloid tissue, liver, spleen, intestine, posterior kidney	37 500	351 769
5	None	1214	1592	Skin, spleen, intestine	329 115	35 726
7	None	202	5084	Skin, fin, gill, barbel, olfactory epithelium, heart, meningeal and pericardial lymphomyeloid tissue, liver, spleen, stomach, intestine, posterior kidney	1 178 776	2 308 890
14	None	2807	106	Gill	623 465	4536
28	None	320	0	Olfactory epithelium	0	134

17°C. The mean cumulative survival of 52.5% tanks containing warmwater virus-exposed fish in Study 1 was higher than the 10% previously reported by Waltzek et al. (2014). This difference may be explained by viral factors including the isolate (PSRV2009 vs. PSRV2015) and viral titer used in the bath challenges, host factors including the size and genetic background of the young-of-year *S. albus*, and environmental factors (e.g. tank volume, stocking density, and water flow rate).

A slightly higher viral titer was used in the Study 1 bath challenge than in the challenge study of Waltzek et al. (2014), suggesting that viral titer was unlikely to have resulted in the difference in survival between the studies. Unique genetic differences, often involving genes encoding proteins of unknown functions, were observed in all of the closely related PSRV isolates (Stilwell 2017), hindering interpretation of why the PSRV2015 isolate used in this study might be less pathogenic than the PSRV2009 isolate used previously. The mean weight of the fish used in Study 1 (28.7 g) was slightly lower compared to the those used by Waltzek et al. (2014) (39.8 g), which was unlikely to explain the difference in cumulative survival. Although the YOY *S. albus* in both studies were supplied by the Gavins Point Fish Hatchery in South Dakota, the genetic background of the parents and resulting offspring may have varied between the studies. Finally, environmental factors including tank dimension and material, stocking density, and water flow rate varied between the studies and may have contributed to the observed difference in cumulative survival.

The clinical signs, viral load, and viral titer data presented in Study 2 demonstrated that FV3 disease progressed more rapidly and became more pronounced at the warmer water temperature. Previously, a relatively high FV3 viral titer induced disease in YOY *S. albus* more rapidly than did a lower titer; however, by the end of that study, cumulative survival was identical between the lower and higher titer treatments (Waltzek et al. 2014).

The histological methods used in Study 2, including an ISH method using RNAscope® technology, provide novel insight into FV3 tissue tropism in juvenile *S. albus*. Histological examination revealed a greater tissue distribution in fish held at the elevated water temperature. Gross and microscopic lesions of certain tissues were similar to those previously reported in experimental FV3 sturgeon infections (Waltzek et al. 2014, Miller et al. 2015); however, additional tissue sampling in this study expanded the known tissue distribution of the virus. Although some tissue distributions were already documented for ranaviruses in fish (e.g. hematopoietic tissues), other locations (e.g. gill, skin, sensory tissues) are less well known and may aid in refining future sampling protocols. For example, gill and epidermis were each RNAscope®-positive in 60% (3/5) of the tested warmwater fish during the first 2 wk following infection, suggesting these may be acceptable tissues for cases where non-lethal and/or relatively non-invasive ranavirus screening methods are preferred. The presence of infection in several external tissues (e.g. skin, fin, gill, sensory epithelium) also suggests that one or more of these tissues could serve as a route of infection and/or viral shedding. Further study is warranted to examine tissue distribution across other ranavirus and host taxa, as well as tissue sources for viral shedding.

Study 2 data demonstrated that fish held in cold water did become infected with FV3, despite lacking clinical signs of disease. Viral DNA was detected within tissues by qPCR, and more importantly, virus was recovered in cell culture. This finding is important because it suggests that fish exposed at lower temperatures harbor the virus and could potentially serve as viral reservoirs until conditions (e.g. thermal stress) become favorable for viral replication and transmission. In Study 2, some exposed fish at both temperatures remained infected after 28 d by qPCR and virus isolation as previously reported (Waltzek et al. 2014). Future challenge studies are needed to determine whether survivors harbor the virus over longer periods of time and whether they might pose a risk if restocked into wild populations. Studies are also needed to determine whether survivors mount cellular and humoral immune responses following exposure. Demonstrating that fish exposed at lower temperatures, at which mortality is minimal, eventually clear the virus and/or become protected would be an important step toward developing an effective mitigation strategy. Ultimately, temperature manipulation may be a possible management tool to minimize ranaviral outbreaks in hatcheries rearing endangered sturgeon, whether used alone or in combination with other strategies, including reduced stocking densities, disinfection of incoming water, and vaccination.

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Appendix.

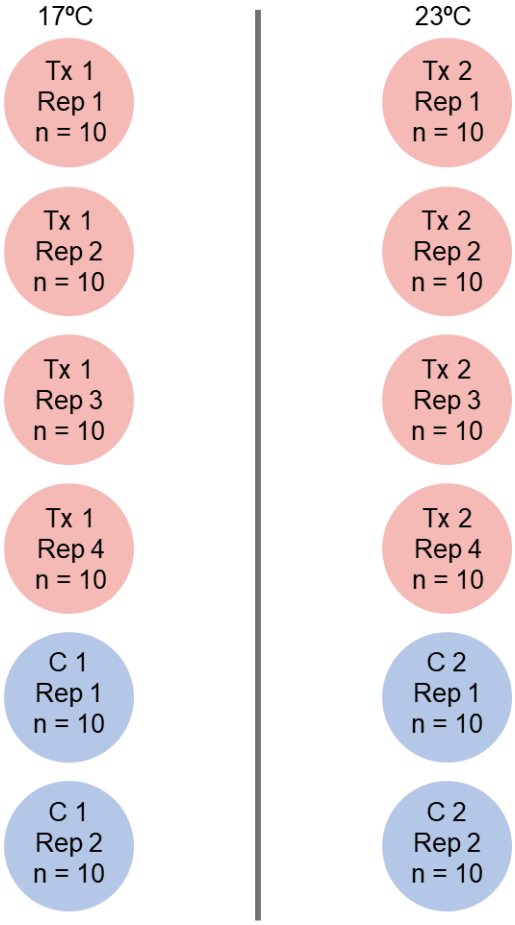

Fig. A1. Experimental tank design for Studies 1 and 2. Sample sizes (n) of *Scaphirhynchus albus* are indicated. Tx: treatment; C: control tank; Rep: replicate

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