Vol. 148: 95–112, 2022
https://doi.org/10.3354/dao03650

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published online March 17

Comparative virulence of spring viremia of carp virus (SVCV) genotypes in two koi varieties

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ABSTRACT: Spring viremia of carp virus (SVCV), is a lethal freshwater pathogen of cyprinid fish, and *Cyprinus carpio koi* is a primary host species. The virus was initially described in the 1960s after outbreaks occurred in Europe, but a global expansion of SVCV has been ongoing since the late 1990s. Genetic typing of SVCV isolates separates them into 4 genotypes that are correlated with geographic origin: Ia (Asia), Ib and Ic (Eastern Europe), and Id (Central Europe). We compared infectivity and virulence of 8 SVCV strains, including 4 uncharacterized Chinese Ia isolates and representatives of genotypes Ia–d in 2 morphologically distinct varieties of koi: long-fin semi-scaled Beni Kikokuryu koi and short-fin fully scaled Sanke koi. Mortality ranged from 4 to 82% in the Beni Kikokuryu koi and 0 to 94% in the Sanke koi following immersion challenge. Genotype Ia isolates of Asian origin had a wide range in virulence (0–94%). Single isolates representing the European genotypes Ib and Ic were moderately virulent (38–56%). Each virus strain produced similar levels of mortality in both koi breeds, with the exception of the SVCV Id strain that appeared to have both moderate and high virulence phenotypes (60% in Beni Kikokuryu koi vs. 87% in Sanke koi). Overall SVCV strain virulence appeared to be a dominant factor in determining disease outcomes, whereas intraspecies variation, based on koi variety, had less of an impact. This study is the first side-by-side comparison of Chinese SVCV isolates and genotype Ia–d strain virulence in a highly susceptible host.

KEY WORDS: SVCV · Genotype · Strain · Virulence · Koi

1. INTRODUCTION

Ornamental fish rearing is one of the most popular hobbies globally and is based on an international market involving the shipment of millions of live ornamental fish worldwide (Monticini 2010, Dey 2016, Stevens et al. 2017). The value of both imported and exported ornamental fish has on average risen over the last 3 decades and serves as an important revenue source for many countries (FAO 2017, Stevens et al. 2017). In the ornamental fish industry, only about 5–10% of saltwater species are commercially propagated, whereas up to 90% of freshwater species are cultured (Ladisa et al. 2017, King 2019). The need for sustainable aquaculture of many ornamental species will increase as more restrictions are

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placed on the collection of aquatic species from the wild (Tlusty et al. 2013, Dey 2016, King 2019).

A popular freshwater ornamental fish that has a long history of cultivation is koi *Cyprinus carpio koi*, which is an inbred strain of common carp *C. carpio* Linneaeus (Sirisidthi et al. 2015, Yanar et al. 2019). Common carp are popular sport and food fish, but in some regions of the world they are considered to be a highly invasive species (Jeney & Jian 2009, Rahman 2015, FAO 2021). The modern variety of koi was initially developed in Japan to enhance fish color, body shape, and swimming movement (Balon 1995, De Kock & Gomelsky 2015). The market value of koi varies greatly, from inexpensive pet-store grade koi to expensive show-grade or fancy koi varieties that can be sold for millions of US dollars (Bassleer 2015, Teletchea 2019). Thus, ornamental koi breeding and propagation continues to be in demand. However, as with many cultured fish species, one of the issues that arise with rearing koi in captivity is disease (Jeney & Jeney 1995, Kelly & Renukdas 2020).

One of the major pathogens infecting common carp and koi is spring viremia of carp virus (SVCV; family *Rhabdoviridae*, genus *Sprivivirus*, species *Carp sprivivirus*). Due to its highly infectious nature in naïve susceptible fish species, SVCV is one of 10 notifiable fish pathogens listed by the World Organization for Animal Health (OIE 2019a). Outbreaks have occurred in wild and farmed fish and in both adults and juveniles, but younger fish less than 1 yr old are typically more susceptible (Veselý et al. 2014, Emmenegger et al. 2016). SVCV is considered endemic in many European countries, with disease incidents primarily occurring in common carp farms during the spring as water temperatures rise after a cold winter (Fijan 1999, Ahne et al. 2002, Sauerwald et al. 2020). In addition to Europe, the virus has also been reported to cause common carp/koi disease outbreaks in Asia and North America (Goodwin 2002, Dikkeboom et al. 2004, Phelps et al. 2012, Shao & Zhao 2017, Godahewa et al. 2018, Zheng et al. 2018). Suspect cases of SVC disease or SVCV detections reported from various non-cyprinid and Indian carp species from countries within South America and the Middle East need further confirmation (Dixon & Stone 2017). Overall, common carp and koi are considered the species most vulnerable to SVCV infections and suffer the highest mortalities (Fijan 1999).

The SVCV genome is 11 kb long with 5 protein-encoding genes, N, P, M, G, and L (Björklund et al. 1996). Genetic typing and phylogenetic analyses of SVCV isolates, based on P and G gene sequences, resolve them into 4 genotypes that are associated with geographic locale (Stone et al. 2003, Miller et al. 2007, Warg et al. 2007). Genotype Ia isolates are of Asian origin, and all the isolates detected in North America in the last 2 decades belong to this genotype. The remaining SVCV genotypes (Ib, Ic and Id) all originate from central and eastern European countries. Since the late 1990s, there has been a global emergence of genotype Ia and Id strains (Padhi & Verghese 2012). The majority of Ia isolates were detected in North America and Asia. Some of the isolates were responsible for epizootics (Goodwin 2002, Dikkeboom et al. 2004, Godahewa et al. 2018, Zheng et al. 2018), whereas other Ia strains were isolated from asymptomatic and apparently healthy fish (Liu et al. 2004, Garver et al. 2007, Teng et al. 2007, Xiao et al. 2014). Based on the numerous reports of both asymptomatic infected fish and outbreaks associated with Ia isolates, it is speculated that SVCV genotype Ia strains may have a wide range of pathogenicity (Dixon & Stone 2017).

The susceptibility of cyprinid fish species, including koi, to the NC2002 (Ia) isolate has been confirmed in multiple studies (Emmenegger & Kurath 2008, Emmenegger et al. 2016, Boonthai et al. 2017). However, an *in vivo* comparative characterization of Chinese SVCV Ia isolates has not been performed. Our goal was to compare the virulence of Asian, North American, Eastern Europe, and Russian SVCV strains, representing genotypes Ia–d, in 2 different koi varieties with different morphological features and breeding histories (De Kock & Gomelsky 2015). The Beni Kikokuryu (also known as and henceforth referred to as Beni Kiko) koi variety have long fins, are semi-scaled, and have body colors that include some combination of black, red, and/or yellow, and a shade of orange is predominantly present (Pawlak 2009, Gomelsky et al. 2011). The Sanke variety is a 3-colored (red, white, and black) koi with short fins and a fully scaled body (Bercovich et al. 2012, Kodama 2018). Study objectives were to determine the susceptibility of the 2 koi varieties to each virus strain and compare inter-genotype and intra-Ia genotype relative virulence of SVCV in both koi types. To our knowledge, this is the first side-by-side challenge comparison of virulence using isolates representing all 4 SVCV genotypes.

2. MATERIALS AND METHODS

2.1. Virus isolates

Eight SVCV strains were tested in this study and are described in Table 1. The Ia isolates from China,

Table 1. Original source features of spring viremia of carp virus isolates used in virulence challenges with koi

Genotype	Isolate identifier	Country	Collection year	Fish species	Host health status	Reference
Ia	20040741	China	2003	Common carp	Symptomatic	Liu et al. (2008)
Ia	20100910	China	2010	Common carp	Asymptomatic	None
Ia	20070165	China	2007	Grass carp	Asymptomatic	None
Ia	20120450	China	2012	Common carp	Asymptomatic	None
Ia	NC2002	USA	2002	Koi	Symptomatic	Goodwin (2002)
Ib	RHV	Ukraine	1989	Rainbow trout	Symptomatic	Stone et al. (2003)
Ic	P4-7 (or P4)	Russia	1983	Common carp	Symptomatic	Stone et al. (2003)
Id	Fijan (or S30)	Yugoslavia	1971	Common carp	Symptomatic	Fijan et al. (1971)

sampled between 2003 and 2012, were collected from 4 Chinese provinces; Jiangsu (isolate 20040741), Chongqing (20100910), Beijing (20070165) and Henan (20120450), and not in association with any disease outbreak. However, the common carp infected with isolate 20040741 displayed clinical signs of disease (P. Jia pers. comm.). The first North American Ia SVCV isolate, NC2002, originated from North Carolina (USA), though it was believed to have been introduced from Asia (Miller et al. 2007). Isolate NC2002 was affiliated with an epidemic on a large koi farm with ponds located in 2 states (North Carolina and Virginia). However geographic isolation of the farm, along with extensive decontamination efforts, suggest that the outbreak was contained (Goodwin 2002, Miller et al. 2007). NC2002 isolate was provided by Dr. Andy Goodwin (US Fish and Wildlife Service, Pacific Region, Portland, Oregon, USA) and genotyped by Emmenegger & Kurath (2008). The other isolates were genotyped and provided by OIE SVCV reference laboratories at the Shenzhen Academy of Inspection and Quarantine Sciences (Chinese isolates) and the Centre for Environment, Fisheries and Aquaculture Science in the UK (European and Russian isolates). The RHV (Ib) and P4-7 (Ic) strains were selected because they represent the eastern European geographic range of SVCV and are repeatedly used in phylogenetic analyses, including sequence data sets containing recent SVCV Ia isolates (Stone et a. 2003, Miller et al. 2007, Padhi & Verghese 2012, Shao & Zhao 2017, Godahewa et al. 2018, Rud et al. 2019). The RHV isolate collected from rainbow trout also serves as a non-cyprinid host SVCV isolate for evaluating susceptibility testing in the primary host species (koi). The Fijan isolate representing the Id genotype is from former Yugoslavia, was the first SVCV isolate characterized, and is considered the reference strain (Fijan et al. 1971). Henceforth, any abbreviated designation listed as 'Ia', 'Ib', 'Ic', or 'Id' refers to the SVCV genotype of an isolate.

Each virus strain was cultured on epithelioma papulosum cyprini (EPC) cell monolayers (Fijan et al. 1983, Winton et al. 2010). The cells were maintained at 20°C in MEM-10. Virus was amplified as previously described (Emmenegger & Kurath 2008). Briefly, virus was harvested at 90% cytopathic effect by centrifugation at 1000 × g for 10 min to pellet cell debris and then frozen as aliquots at −80°C. Virus titer was determined by plaque assay following the procedures developed by Batts & Winton (1989), except that the incubation temperature was 20°C. The limit of detection of the plaque assay for tissues infected with aquatic rhabdoviruses is approximately 100 PFU g^{-1} (Yusuff et al. 2019).

2.2. Koi host

Two Nishikigoi koi varieties, with different morphological characteristics and breeding histories, were used in the virulence challenges. Beni Kiko koi were originally derived from cross breeding of Kumonryu and Kikusui koi strains in 1992 (De Kock & Gomelsky 2015). Sanke koi are considered a Nishikigoi precursor strain originating in 1898 (De Kock & Gomelsky 2015). Domestic stocks of each koi variety were reared separately at the same koi farm by a regional breeder (King County, Washington [WA], USA). The original koi parentals for each variety were imported from Japan after obtaining fish health certifications, which included testing negative for SVCV. When each koi variety was 1.5–2.0 mo of age, they were transferred to the main wet laboratory at the US Geological Survey Western Fisheries Research Center (WFRC) (Seattle, WA). The koi fry were quarantined upon arrival, segregated from other fish stocks at the center, and remained healthy until the initiation of each challenge experiment. They were held in single-pass, sand-filtered, and ultraviolet-treated fresh water at temperatures rang-

ing from 16–19°C. Both koi varieties were ca. 3.5 mo of age on the day of challenge. The Beni Kiko koi had an average weight of 1.9 g and average length of 5.2 cm. The Sanke koi weighed on average 2.9 g with an average length of 6.1 cm. Sanke koi on average weighed 1.0 g more; however, they were also longer compared to the shorter Beni Kiko koi that weighed less. Fulton's Condition (FC) factor is a measure used in fisheries science to compare relative heaviness of fish and serves as a possible indicator of wellness of a fish species/stock that incorporates both fish weight and length (Ricker 1975, Froese 2006). The FC factors (=100 × body weight [g]/length [cm]^3) for the Sanke and Beni Kiko koi were 1.3 and 1.4 g cm^{-3}, respectively. Thus, the 2 koi varieties were proportionally comparable in overall heaviness and of similar age when tested and thus likely at the same stage of immune system development.

The same diet and feeding regimen were used throughout the study and consisted of a mixed feed diet of Hikari Gold mini-pellets (Kyorin Food Industries) and Classic Fry 1.5 mm floating pellets (Skretting) provided on alternate days. Protocols for rearing and experimental use of live animals were approved by the WFRC Institutional Animal Care and Use Committee (IACUC) and Institutional Biosafety Committee of the WFRC under the guidelines provided by the Guide for the Care and Use of Laboratory Animals (NRC 2011).

2.3. Immersion challenge

On the day of challenge, fish were transferred to 30 l tanks in the WFRC aquatic biosafety level 3 (BSL-3) laboratory. Two independent virus challenge experiments were conducted, one for each koi variety. In each experiment, quadruple tanks of koi (n = 30 tank^{-1}) were challenged by immersion with 1 of 8 SVCV isolates or mock challenged and then held for 34 d. Three of the tanks were used to monitor cumulative mortality, and the fourth tank was used to evaluate virus infection kinetics via samples collected 1, 3, 5, 7, 11, 14, 17 or 19 d post-exposure. Water temperature was initially set at 10–11°C at the start of the challenge (Day 0), providing a cold temperature stressor relative to the stock fish conditions of 16–19°C. After initial exposure to the treatments, the temperature was then raised 1°C d^{-1} until the water temperature reached 13–14°C and then was held constant for the remainder of the challenge experiment. This temperature scheme mimics the winter to spring rising temperatures that occur in nature that are associated with SVCV outbreaks and is considered an important factor for SVCV infection initiation that has been successfully applied in previous SVCV challenge experiments (Ahne 1986, Emmenegger & Kurath 2008). Koi were immersed for 2 h in 3 l of static aerated water containing 1.0 × 10^5 PFU ml^{-1} SVCV. Continuous water flow was then restored to each tank. Koi in the mock treatment groups were immersed in water containing an equivalent volume of cell culture media. Any sampled fish were euthanized with a lethal dose of buffered MS-222 solution following the procedures described by Kell et al. (2013). All fish and experimental conditions (e.g. water and air temperatures, water flow) were monitored daily. Dead fish were collected on the day of death, and fish found moribund were euthanized and collected along with mortalities. Survivors were euthanized at the end of the challenge. All whole fish specimens were placed in individual Whirl-Pak bags (Nasco). To minimize SVCV virion/viral RNA degradation, sampled fish were immediately placed in a −20°C freezer and transferred within 5–15 min to a −80°C freezer until processed (Sanders et al. 2003, OIE 2019b).

2.4. Sample processing and virus detection

Single fish samples (euthanized survivors or fish harvested at set timepoints, or fish collected on the day of death) were thawed, diluted in culture media (MEM) containing antibiotics and homogenized following the procedures described by Emmenegger et al. (2016). The homogenate supernatant was further diluted to 1:40 with the same media, and a portion of each suspension was used to evaluated virus presence/absence and titer by reverse transcription conventional polymerase chain reaction (RT-cPCR) and plaque assay, respectively. A subset of all collected fish samples was tested to qualitatively confirm virus detection/viability through the entire course of the 34 d virus challenge. In the context of this study, the 'survivors' from each challenge experiment were fish that were never infected, fish infected but whose disease pathology outcome was unknown at Day 34 post-exposure, and/or fish infected but recovering (convalescent) by Day 34 post-exposure. Testing was conducted on virus-exposed dead fish (n = 3 isolate^{-1}), virus-exposed survivors (n = 3 isolate^{-1}), mock-challenged dead fish (all), mock-challenged survivors (n = 2 replicate tank^{-1}), and fish sampled at designated intervals (n = 3 timepoint^{-1} isolate^{-1}).

Whole fish tissue homogenates (210 µl) were used to prepare RNA for analysis with a G gene-specific

RT-cPCR assay (Shimahara et al. 2016) to diagnose SVCV. RNA was extracted using the Qiagen RNeasy kit following the manufacturer's instructions. The RNA was quantified using the NanoDrop ND1000 spectrophotometer (Thermo Scientific) and then stored at −80°C. The RT-PCR test utilizing the SVCV primers described by Shimahara et al. (2016) was performed as outlined by Emmenegger et al. (2016). A positive and negative control sample were included in each batch of samples to ensure that the test performed as expected. The analytical specificity of the assay is inclusive of isolates from all 4 SVCV genotypes and exclusive of pike fry rhabdovirus. The expected amplicon size is 369 bp. Samples were reported as SVCV positive or negative based on the presence or absence of this fragment in 1.5% agarose gels stained with ethidium bromide (1.0 µg ml^{-1}) solution following electrophoresis and using the UVP BioSpectrum 500 MultiSpectral Imaging System for illumination. The plaque assay was conducted as described earlier for titering virus isolates.

2.5. Analysis of mortality data

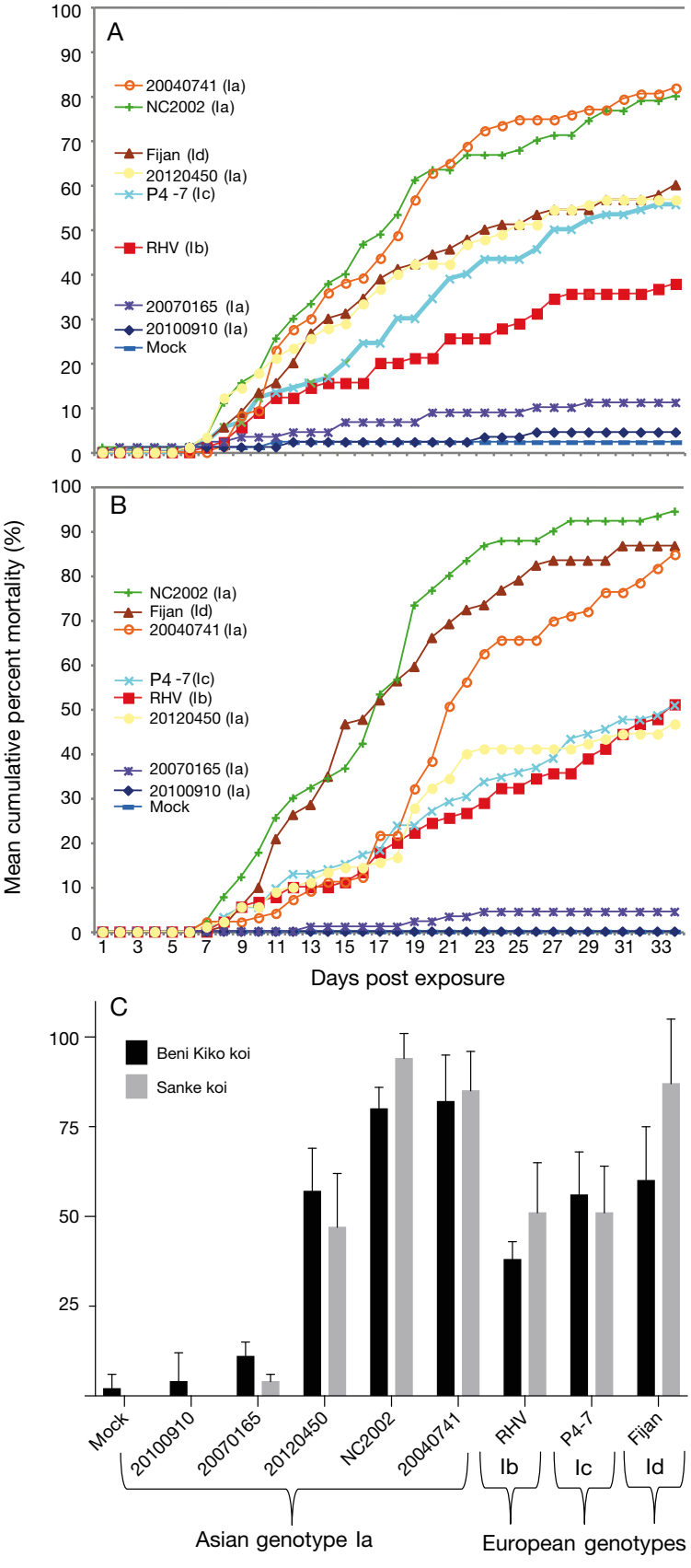
A 1-factor ANOVA (1-way ANOVA) and 2-way ANOVA (simple effect) were performed to assess differences in final mortality between virus strains for each koi type with a Tukey-Kramer post-test if applicable. Two-way ANOVA analyses were performed to compare mortality (virulence) levels between the virus strains (genotypes) for both koi types (main row effect) with a multiple comparison Tukey-Kramer post-test if applicable. The cumulative percent mortality (CPM) of each replicate tank was transformed (arcsine of the square root of the proportion) prior to statistical assessment. A Bonferroni post-hoc test was used to assess the susceptibility differences between the 2 koi varieties. Normality of the residuals was evaluated using Shapiro-Wilk and Kolmogorov-Smirnov tests. Homogeneity of variance was determined by using the Brown-Forsythe test (1-way ANOVA) or Spearman's test for heteroscedasticity (2-way ANOVA). To evaluate virulence on a relative scale, the SVCV strains were assigned to 3 virulence categories defined as low (0–30%), moderate (31–70%), and high (71–100%), based on the final average CPM for each isolate tested, similar to the scale used to assess virulence levels of another fish rhabdovirus (Emmenegger et al. 2013). A significant relationship was designated for all comparisons when p-values were ≤0.05. ANOVA, Tukey post-tests, tests for normality and homogeneity of variances were performed in GraphPad Prism software version 8.2.1 (GraphPad Software).

We also performed analyses with raw mortality data (number of dead and surviving fish at the end of a challenge experiment for each replicate tank per treatment) using generalized linear models (GLMs) for binomial data (Faraway 2016). The probability of mortality was further modeled as a function of the 2 varieties of koi, the 9 treatment groups, which included the 8 strains of the virus and the control (collectively called the strains in the equations), and their interaction. The interaction term provided a way to evaluate whether each virus strain affected each variety of koi uniquely. If the interaction term was found to be significant, then each variety of koi was analyzed separately. If the variation in replicate treatments was found to surpass the level of variation expected under the binomial model, we used an overdispersed binomial GLM that accounted for the extrabinomial variation in the replicates (Williams 1982). Additional details on the binomial and overdispersed binomial GLM methodologies and statistical formulas are available in the Supplement at www.int-res.com/articles/suppl/d148p095_supp.pdf.

Lastly, we examined survival kinetics, which were calculated by Kaplan-Meier analysis, and the Mantel-Cox log rank test was used to compare survival curves. Pairwise comparisons of adjusted p-values from the mortality and survival curve statistical results were carried out for every virus isolate for each of 2 koi types tested to identify pairs of virus strains that did not differ significantly (Wright 1992). Sets of virus strains with no significant differences between their final CPM were listed as 'ns'. Mean day of death (MDD) values were calculated as the sum of days of death divided by the total number of dead fish in each tank, then the average MDD and standard deviation (SD) was determined for the 3 tank replicates for each virus strain per koi type. MDD data for every virus strain within each koi challenge were compared in a 1-way ANOVA with a Tukey post-test if applicable using GraphPad Prism software.

3. RESULTS

The temporal progression of mortality for each koi variety challenged with the SVCV strains is displayed in Fig. 1A,B. The final mean CPM and SD error bars comparing each virus treatment in the 2 koi varieties are displayed in Fig. 1C. Overall, the final mortalities experienced by the 2 koi varieties



were comparable for each virus strain tested with exception of the Fijan (Id) strain (Fig. 1C). Typical external clinical signs of SVC disease, such as hemorrhages on the fins, body, anal vent, and eyes, and distension of the body cavity due to ascites fluid, were present on some of the dead fish from all SVCV genotypes tested. However, many virus-exposed fish also died with no external pathology evident. A summary of the mortality data, the statistical comparisons between the virus strains for both koi types, average mean MDD, and a relative assessment of genotype virulence based on the final average CPM from each experiment are reported in Table 2. No trend was linked to the day the fish died (MDD) among the strains tested for each koi variety. The statistical results for pairwise comparisons of adjusted p-values of each virus strain from mortality (1-way ANOVAs) and survival analyses for each koi variety tested are presented in Table 3. Fish exposed to genotype Ia isolates 2010910 and 20070165 had final mortality levels not significantly different than the mock exposed fish; however, the fish challenged with the other 6 SVCV isolates (representing genotypes Ia, Ib, Ic and Id) all suffered significantly higher mortalities than fish from the negative control treatment group. The residuals (replicate CPMs) passed the normality test for both 1-way and 2-way ANOVAs. Furthermore, the 2-way ANOVA failed the Spearman's homogeneity of variance test, whereas the 1-way ANOVAs passed the Brown-Forsythe homogeneity of variance.

Overall results from statistical analyses utilizing the raw mortality data mirrored the outcomes reported above. The fitted GLM model indicated there was a significant effect of the strain and a significant interaction term (Table S1). Furthermore, tests of model adequacy for the binomial

Fig. 1. Mean cumulative percent mortality (CPM) of (A) Beni Kiko koi and (B) Sanke koi and (C) comparison of final mean CPMs after exposure to SVCV. See Tables 1 & 2 for details. Error bars are ±1 SD

Table 2. Summary results for the SVCV virulence challenge experiments. na: not applicable

Genotype (treatment group)	Mortality result categories[a]	Beni Kiko koi mortality results[b]	Sanke koi mortality results[c]	Mortality difference between koi[d]	Relative virulence classification[e]
Mock (na)	Avg final CPM (Replicate tank CPM) MDD ± SD	2 (0, 0, 7) 7	0 (0, 0, 0) na	ns	na
Ia (20040741)	Avg final CPM (Replicate tank CPM) MDD ± SD	82 (87, 67, 92) 16.9 ± 0.18	85 (77, 81, 97) 21.5 ± 2.19	ns	High
Ia (20100910)	Avg final CPM (Replicate tank CPM) MDD ± SD	4[ns] (0, 13, 0) 17	0[ns] (0, 0, 0) na	ns	Low
Ia (20070165)	Avg final CPM (Replicate tank CPM) MDD ± SD	11[ns] (7, 13, 13) 16.8 ± 7.12	4[ns] (3, 7, 3) 20.0 ± 3.61	ns	Low
Ia (20120450)	Avg final CPM (Replicate tank CPM) MDD ± SD	57 (43, 63, 63) 15.5 ± 0.42	47 (33, 43, 63) 18.7 ± 0.52	ns	Moderate
Ia (NC2002)	Avg final CPM (Replicate tank CPM) MDD ± SD	80 (77, 77, 87) 16.2 ± 0.55	94 (87, 97, 100) 16.5 ± 2.55	ns	High
Ib (RHV)	Avg final CPM (Replicate tank CPM) MDD ± SD	38 (33, 43, 37) 17.1 ± 3.61	51 (40, 47, 67) 20.8 ± 3.28	ns	Moderate
Ic (P4-7)	Avg final CPM (Replicate tank CPM) MDD ± SD	56 (57, 43, 67) 18.6 ± 2.84	51 (63, 37, 53) 20.1 ± 0.62	ns	Moderate
Id (Fijan)	Avg final CPM (Replicate tank CPM) MDD ± SD	60 (73, 63, 43) 16.7 ± 1.07	87 (93, 67, 100) 16.8 ± 1.09	Significant difference ($p < 0.05$)	Moderate–high[f]

[a]Average final cumulative percent mortality (CPM) calculated from individual final CPM of 3 replicate tanks. Mean day to death (MDD) for individual tanks was used to calculate the group average and standard deviation (SD). MDD values with no SD listed indicate that mortality data was from only 1 replicate tank.

[b]For the Beni Kiko koi challenge experiment, final average cumulative mortality from virus strain treatment groups that were not significantly different from the negative control treatment group by mortality analyses indicated with superscript (ns). All MDD comparisons of the virus strains were non-significant.

[c]For the Sanke koi challenge experiment, final average cumulative mortality from virus strain treatment groups that were not significantly different from the negative control treatment group by mortality analyses indicated with superscript (ns). All MDD comparisons of the virus strains were non-significant.

[d]Final Average CPM of each virus strain compared between the 2 koi varieties in a 2-way ANOVA. ns = non-significant result.

[e]Virus strains were assigned to one of 3 relative virulence categories, low (0–30%), moderate (31–70%), and high (71–100%), based the final average CPM of each isolate tested. The Fijan strain was removed from the 2-way ANOVA analyses because it was the only strain demonstrating potential interaction between the 2 koi types. Virulence categories (ns sets) for the remaining virus strains were confirmed by mortality analyses.

[f]The Fijan strain (Genotype Id) was moderately virulent in infected Beni Kiko koi (60 CPM) and highly virulent in infected Sanke koi (87 CPM).

GLM indicated overdispersion in the replicates within treatments. Analysis of the dead and surviving fish (raw mortality data) using a 2-way analysis of deviance with an overdispersed binomial lead to a significant effect of virus strain and a significant interaction between strain and koi variety (Table S2), indicating that each variety should be analyzed separately. Using a 1-way analysis of deviance for each variety of koi indicated that the mortality was significantly different among virus treatments in both varieties (full data summary of the overdispersed binomial GLM analyses is available in the Supplement). Furthermore, relative virulence classification (RVC) levels in Table 2 captured the same variability in mortality as the 9 treatment groups (mock treatment in addition to the 8 virus strains). Note that the RVC level

Table 3. Pairwise comparisons of final mortality and survival analyses. Non-significant adjusted p-values > 0.05 from the mortality analyses listed as 'ns'. Significant adjusted p-values are: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, and ****p ≤ 0.0001. Virus strain pairs in **bold** and boxed were not significantly different by survival analyses. Virulence classification of isolates in vertical columns indicated by color: blue = low virulence; purple = moderate virulence; red = high virulence. CPM: cumulative percent mortality

Beni Kiko koi

	Final CPM	**4**	**11**	**38**	**56**	**57**	**60**	**80**	**82**
	Virulence	LOW	LOW	MODERATE	MODERATE	MODERATE	MODERATE	HIGH	HIGH
	Treatment Group	**20100910**	**20070165**	**RHV**	**P4-7**	**20120450**	**Fijan**	**NC2002**	**20040741**
	20100910 (Ia)	•							
	20070165 (Ia)	**ns**	•						
	RHV (Ib)	**	ns	•					
	P4-7 (Ic)	****	**	ns	•				
	20120450 (Ia)	****	**	ns	**ns**	•			
	Fijan (Id)	****	**	ns	**ns**	**ns**	•		
	NC2002 (Ia)	****	****	*	ns	ns	ns	•	
	20040741 (Ia)	****	****	**	ns	ns	ns	**ns**	•

Sanke koi

	Final CPM	**0**	**4**	**51**	**51**	**47**	**87**	**94**	**85**
	Virulence	LOW	LOW	MODERATE	MODERATE	MODERATE	HIGH	HIGH	HIGH
	Treatment Group	**20100910**	**20070165**	**RHV**	**P4-7**	**20120450**	**Fijan**	**NC2002**	**20040741**
	20100910 (Ia)	•							
	20070165 (Ia)	ns	•						
	RHV (Ib)	***	**	•					
	P4-7 (Ic)	***	**	**ns**	•				
	20120450 (Ia)	***	*	**ns**	**ns**	•			
	Fijan (Id)	****	****	*	*	*	•		
	NC2002 (Ia)	****	****	**	**	**	**ns**	•	
	20040741 (Ia)	****	****	ns	ns	ns	ns	ns	•

for the Fijan virus strain was 'high' for Sanke koi, whereas it was 'moderate' for Beni Kiko koi.

3.1. Koi mortality

Final mortality for the virus-exposed long-fin semi-scaled Beni Kiko koi ranged from 4–82% (Fig. 1A). The highest and lowest mortality levels were observed with genotype Ia strains. Genotype Ib, Ic, and Id strains produced cumulative mortalities of 38, 56, and 60%, respectively. The SVCV strains were grouped into 3 virulence levels, described as low, moderate, and high based on their final CPM (Table 2). Genotype Ia isolates 20100910 and 20070165 from China, were categorized as low virulence strains (Table 2), and the CPM observed with either isolate was not significantly different from the total mortality recorded for the mock-exposed fish. All other virus strains caused significantly higher total mortality relative to the negative control group (Table 2). The European and Russian strains, RHV (Ib), P4-7 (Ic), and Fijan (Id) and a single Chinese isolate 20120450 (Ia) were considered moderately virulent, because they induced mid-range mortalities (38–60%). The classification of genotype Ia isolates NC2002 (80% CPM) and 20040741 (82% CPM) as high virulence strains was supported by both their

survival and mortality results. However, mortality levels for the majority of high (NC2002 and 20040741) and moderate (NC2002, P4-7, and 20120450) virulence strains were not significantly different among one another (Table 3). This result is likely due to variance observed in the CPM of fish from the replicate tanks (Tables 2 & S1, Fig. 1C). Likewise, the 38% CPM for the RHV strain was significantly different by survival analysis from the low virulence strain 20070165 (11% CPM) but was statistically similar by mortality analyses (Table 3). Genotype Ia strains 20070165 and 20100910 produced the lowest mortalities, and their classification as low virulence strains was supported by their mortality and survival results.

In the challenge with short-fin fully scaled Sanke koi, mortalities ranged from 0–94% with similar time course of infection and mortality outcomes demonstrated by the Beni Kiko koi for all SVCV isolates with the exception of the Fijan (Id) strain (Fig. 1B). Genotype Ia isolates 20070165 and 20100910 were again classified as low virulence, because they had final CPMs of ≤4%, and both mortality and survival analyses supported this grouping (Table 3). Isolates 20120450 (Ia), RHV (Ib), or P4-7 (Ic) with mortalities ranging from 47–51% followed the same mortality pattern and clustered within the moderate virulence category. Unlike the Beni Kiko koi (60% CPM), the Sanke koi exposed to the Fijan (Id) strain experienced elevated mortality (87%), which grouped this Id isolate with the Ia strains, 20040741 (85% CPM) and NC2002 (94% CPM) of the high virulence category. The high virulence strains 20040741 (Ia), NC2002 (Ia) and Fijan (Id) were all significantly different from the moderate strains by survival analyses in the Sanke koi. However, mortality analyses (1-way ANOVA) indicated isolate 20040741 was not significantly different from the moderate strains, though a comparison of their adjusted p values indicated they were near the threshold of significance ($p \leq 0.05$, Table 3). The Fijan (Id) and NC2002 (Ia) strain mortality levels in Sanke koi were significantly different from all other moderate and low virulence strains tested both by mortality and survival analyses (Table 3).

Comparisons of susceptibility between Beni Kiko and Sanke koi by 2-way ANOVA mortality analysis indicated a significant difference in final mortality only when koi were infected with Fijan (Id) strain (Table 2, Fig. 1C). Analysis of the mortality data by 2-way ANOVA (with the Fijan strain in the data set) indicated that koi variety may have an effect because of potential interaction between koi types as was also noted in the binomial model analyses (Table S1). In all statistical analyses (with or without the Fijan isolate), virus strain was considered to have an extremely significant effect ($p \leq 0.0001$). When mortality levels of the isolates were compared with koi types in a 2-way ANOVA (without the Fijan (Id) strain), the high (20040741 and NC2002), moderate (RHV, P4-7, and 20120450), and low (20100910 and 20070165) virulence categories were statistically different (Table 2). In this case, removal of the Fijan strain decreased the potential interaction of koi variety from the analyses. All mean day to deaths comparisons of virus strains by 1-way ANOVA were nonsignificant in both koi varieties (Table 2).

3.2. Virus concentrations in dead koi

Mock-exposed Beni Kiko koi (n = 2) that died following challenge tested negative for virus by plaque assay and RT-cPCR. No mortality was observed in mock exposed Sanke koi. Koi that died following virus exposure (n = 3 fish tested virus strain^{-1} challenge^{-1}, total = 45 for both koi varieties) tested positive by plaque assay, with titers ranging from 2.8×10^4 to 1.4×10^8 PFU g^{-1}. These results suggest that koi mortality was due to SVCV infection (Fig. 2). In general, virus titers in dead Sanke koi were 1 log higher relative to those in dead Beni Kiko koi except with Chinese isolate 20120450 (Ia). In this case, Beni Kiko koi, exposed to strain 20120450, had the lowest average virus titer (2.8×10^4 PFU g^{-1}). Interestingly, the highest virus titer (2.7×10^7 to 1.4×10^8 PFU g^{-1}) was observed in koi that died following exposure to low virulence strain, 20070165 (4–11% CPM). This result suggests that virus titer, at the time of death, is not the primary factor influencing SVCV strain virulence.

3.3. Infection in koi sampled at intervals after virus exposure

Mock-exposed Beni Kiko and Sanke koi tested negative by plaque assay and RT-cPCR at all 7 sample timepoints (n = 21 fish koi type^{-1}, total = 42). Mean virus concentrations in virus-exposed koi at each timepoint are presented in Fig. 3. Virus was not detected at the first timepoint (Day 1) with the exception of single Beni Kiko koi exposed to 20070165 (Ia), Fijan (Id), and NC2002 (Ia). These fish tested positive by plaque assay with titers between 1.6×10^3 to 4.4×10^3 PFU g^{-1}, and all 3 fish sampled on Day 1 for each of these strains were positive by RT-cPCR (Fig. 3). Both types of koi at all subsequent timepoints tested

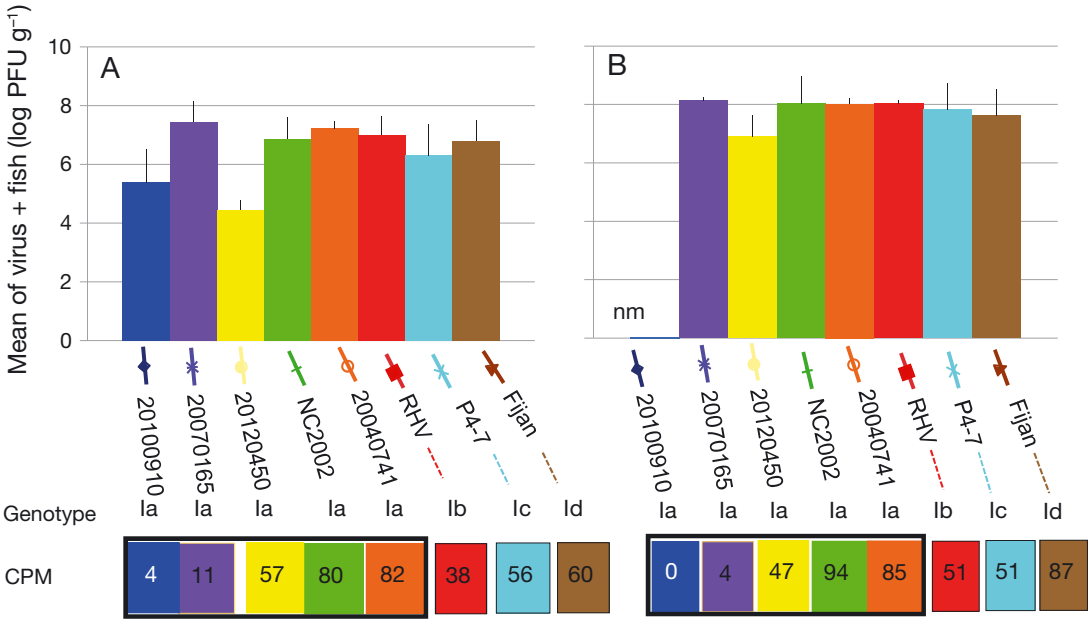


Fig. 2. Virus concentrations in dead fish from the 2 tested koi varieties: (A) Beni Kiko and (B) Sanke. Virus treatment with corresponding colored symbols from the mortality graphs (Fig. 1) are listed on the *x*-axis followed by genotype and final average cumulative percent mortality (CPM) for each treatment group. All virus-exposed dead fish that were tested (n = 3/3 for each treatment group) were positive for virus from both challenges. Sanke koi exposed to isolate 20100910 experienced no mortalities (nm). Limit of detection of the plaque assay is 100 PFU g^{-1}. Error bars are 1 SD around the geometric mean of fish testing positive for virus within each group. All mock-exposed Beni Kiko koi that died in one replicate tank (n = 2) tested negative for virus; no deaths occurred in the mock-exposed Sanke koi treatment group (data not shown)

positive by plaque assay and RT-cPCR except on Day 7. Virus was detected in Beni Kiko koi from Days 3 to 17 following exposure to the virus strains with titers ranging from 2.7×10^3 to 3.3×10^8 PFU g^{-1}. Virus was found in Sanke koi from Days 3 to 19 post-exposure at concentrations between 5.2×10^3 to 4.8×10^7 PFU g^{-1}. At the Day 7 timepoint, Beni Kiko koi infected with strain 20070165 (Ia) tested negative by plaque assay and positive by RT-cPCR (n = 3/3 positive), and Sanke koi on that same sample day, after exposure to the identical strain, were virus-negative by both detection methods. Overall, a temporally associated increase in virus concentration was not observed, and there was no evidence of viral clearance. In general, koi from low virulence strain groups, 20100910 and 20040741, had mean virus titers of $\leq 10^6$ PFU g^{-1}, whereas koi from the moderate and high virulence strain groups had average virus concentrations exceeding 10^6 PFU g^{-1} at one or more sampling timepoints.

3.4. Persistence of virus in surviving koi

Virus prevalence in surviving koi (n = 3–4 fish virus $strain^{-1}$ = 24 or 25 per koi type, 49 fish total) is shown in Fig. 4. No virus was found in mock-exposed survivors (n = 6 fish mock $treatment^{-1}$ $challenge^{-1}$, 12 fish total) (data not shown). Virus was detected by both diagnostic methods in Beni Kiko and Sanke koi survivors for all virus challenges. In general, virus prevalence in koi survivors exposed to the low virulence Ia strains (i.e. 20100910 and 20070165) was slightly lower, compared to all other virus strains. However, virus titers in these koi survivors from the 2 virus treatment groups that experienced little to no mortality (0–11%), were comparable to those found in koi exposed to moderate and high virulence strains (2.6×10^5 to 8.9×10^7 PFU g^{-1}). For example, surviving koi exposed to the low virulence 20070165 strain (4–11% mortality) had titers ranging from 8.2×10^4 to 5.4×10^6 PFU g^{-1}, and one survivor Sanke koi exposed to 20100910 (Ia) isolate (0–4% mortality) had a virus titer of 1.5×10^5 PFU g^{-1} at the end of the 34 d challenge.

4. DISCUSSION

The results of this study provide a framework for increasing our understanding of SVC disease dynamics in koi. Koi mortality was used as an indicator for virus virulence and provided evidence of low, moderate and high virulence categories for Ia–d isolates. Viruses within each virulence subgroup were the same for both varieties of koi, suggesting generally equivalent susceptibility to each SVCV

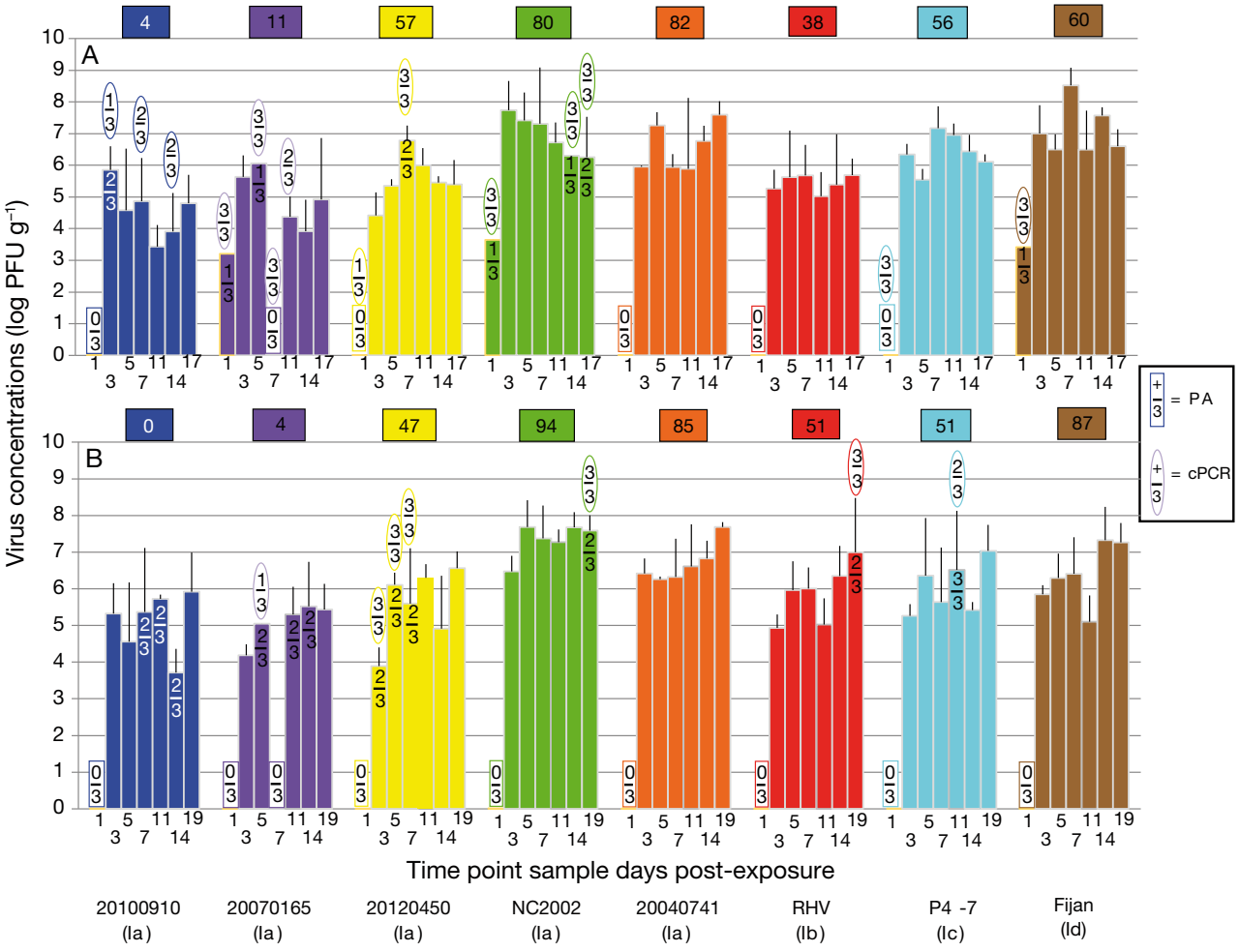


Fig. 3. Virus concentrations in fish sampled at set intervals (1, 3, 5, 7, 11, 14, and 17 or 19 d) after exposure to SVCV strains from the 2 tested koi varieties: (A) Beni Kiko and (B) Sanke. Virus prevalence (n = number of virus-positive fish/number of fish tested) by plaque assay (PA) presented within rectangles/bars; conventional RT-PCR (cPCR) detection prevalence ratios displayed in ovals above time point bars listed on the *x*-axis. Three fish were tested for virus by both methods at each timepoint. Molecular detection cPCR prevalance ratios only shown if they differed from plaque assay prevalence ratios. If no prevalence ratio is listed, then all fish tested for virus from a treatment group at a specific time point were positive (n = 3/3) by both detection methods. Final average cumulative percent mortality (CPM) for each treatment group is displayed at the top of the graphs above each SVCV strain. SVCV genotype (Ia, Ib, Ic, or Id) for each treatment group is listed within parentheses. Error bars are 1 SD around the geometric mean of fish testing positive for virus by plaque assay within each group. Limit of detection of the plaque assay is 100 PFU g^{-1} for tissues. An equivalent number of mock-exposed fish (n = 21, 42 total) screened for virus at each timepoint for each koi type by both methodologies tested negative for virus (data not shown)

strain except for the Id isolate. The Fijan (Id) strain expressed moderate or high virulence phenotypes when infecting a specific koi variety. The virus virulence categories appeared not to be associated with virus genotype, infection prevalence, titer, or persistence. SVCV isolates from genotype Ia displayed a wide virulence range and were found in all 3 virulence categories. The presence of viable virus in koi survivors suggests that SVCV is able to persist, and that these fish may be a source of virus transmission for some period of time following a disease outbreak.

Regarding virus infection of the 2 koi varieties tested, Beni Kiko and Sanke koi displayed comparable susceptibility to 7 out of the 8 SVCV isolates tested. The 27% difference in mortality observed with the Fijan (Id) strain was significant between Beni Kiko and Sanke koi, but additional testing is needed to confirm this virulence phenotype distinction. Overall, our experimental exposures indicated that intraspecies variation (koi variety) was not the key factor in determining SVCV mortality. In contrast, other fish novirhabdoviruses, such as viral hemorrhagic septicemia virus (VHSV) and infectious hematopoietic

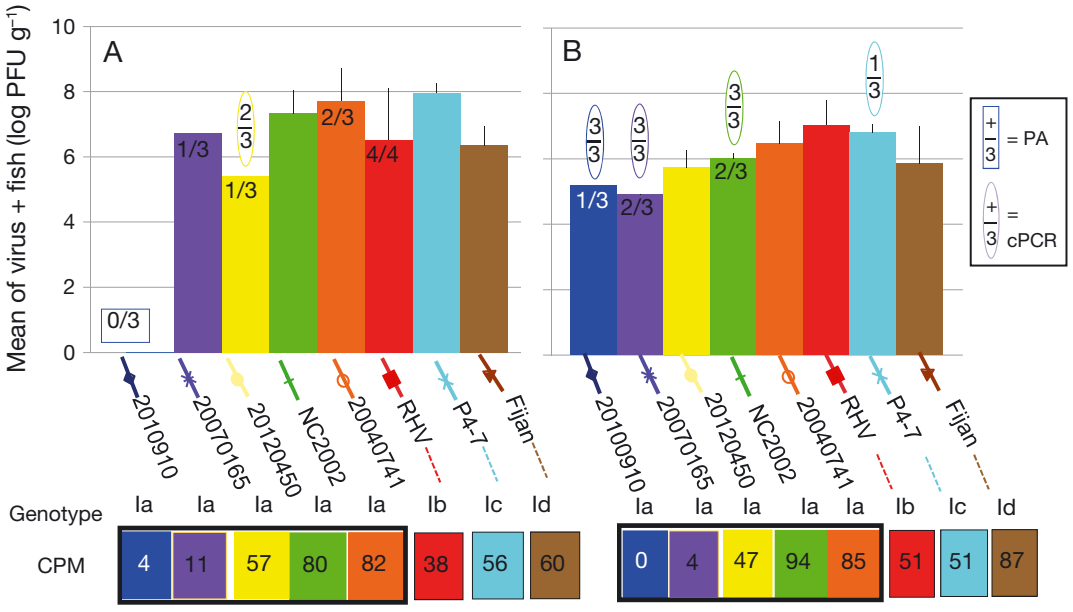


Fig. 4. Virus concentrations in fish that survived the 34 d challenges from the 2 tested koi varieties: (A) Beni Kiko and (B) Sanke. Virus treatment with corresponding colored symbols from the mortality graphs (Fig. 1) are listed on the *x*-axis followed by genotype and final average cumulative percent mortality (CPM) for each treatment group. Virus prevalence (n = number of virus-positive fish/number of fish tested) by plaque assay (PA) displayed within rectangles/bars; conventional RT-PCR (cPCR) detection prevalence ratios displayed in ovals above virus treatment groups listed on the *x*-axis. Three to four survivor fish were tested from each koi type for each virus strain. Molecular detection cPCR prevalance ratios only shown if they differed from plaque assay prevalence ratios. If no prevalence ratio is listed, then all fish tested for a virus treatment group were positive (n = 3/3) by both detection methods. Limit of detection of the plaque assay is 100 PFU g^{-1}. Error bars are 1 SD around the geometric mean of fish testing positive for virus within each group. No virus was detected in the subset of the mock survivors tested (n = 12, data not shown)

necrosis virus (IHNV), have demonstrated intraspecies host variation among their genotypes/genogroups. For example, 3 different broodstock of yellow perch *Perca flavescens* exhibited significantly different levels of mortality upon exposure to the same VHSV IVb genotype isolate (Olson et al. 2013). Similarly, 2 closely related steelhead trout *Oncorhynchus mykiss* populations displayed differential susceptibility (up to 2 orders of magnitude) when experimentally exposed to IHNV genogroup MD strains (Breyta et al. 2014). These and other studies found that novirhabdovirus intraspecies host variation can be a primary factor influencing disease severity in fish (LaPatra et al. 1993, Garver et al. 2006, Peñaranda et al. 2009, Breyta et al. 2013, 2016, Emmenegger et al. 2013, Olson et al. 2013). In our study, intraspecific host species variation did not appear to play a key role in SVCV mortality outcomes.

Different susceptibilities to SVCV Ia isolates may be observed between wild carp and ornamental koi. Phylogenetic analysis of the SVCV P gene revealed 2 Ia subgroups that appeared to correlate with feral common carp (Iai) and farmed/imported koi (Iaii) (Miller et al. 2007). Phylogenetic analyses of the P, M, and G genes of all SVCV isolates from the USA up to 2004 also indicated higher similar nucleotide identity levels among common carp isolates or among the koi isolates between carp and koi strains (Warg et al. 2007). However, more recent phylogenetic analysis of extant SVCV isolates may not support the proposed relationship (Padhi & Verghese 2012, Godahewa et al. 2018). Common carp are considered a highly invasive species in the USA and no are longer propagated (NPS 2019); thus, we selected domestic bred ornamental koi varieties with a known SVCV-free health history that could be imported into our research facility for testing. However, common carp production in other regions of the world, particularly in Asia and Europe, continues, and various strains of common carp from farmed, feral, and wild populations are present worldwide (Xu et al. 2014, 2019a, Rahman 2015, Nedoluzhko et al. 2020, Tóth et al. 2020, Zhao et al. 2020). Because we only examined susceptibility differences between 2 ornamental koi types, additional challenges with other koi varieties or experimental comparisons between imported koi and common carp would further clarify the role intraspecies host variation contributes to SVC disease epidemiology.

Persistence of the virus in fish that survived virus exposure is another component of SVCV epidemiol-

ogy. Carp and koi are long-lived fish species. In the wild, the estimated life span of common carp is typically up to 20 yr (Hecker 1993). Ornamental koi, especially those reared in ponds under excellent environmental conditions, can live as long as 60 yr (James 1985). Thus, common carp and carp hybrids that have survived an SVCV infection may potentially serve as lifelong carriers of the virus, though not much is known about SVCV persistence in survivors (Dixon & Stone 2017, OIE 2019b). Historical evaluations of virus persistence in fish surviving SVCV infections all occurred within Europe, prior to the delineation of SVCV genotypes and the first report of Ia genotype strain in the UK in 1997 (Stone et al. 2003, Miller et al. 2007). Therefore, it is presumed this earlier knowledge of SVCV persistence relates only to European genotypes (Ib, Ic, and Id). Fijan (1984, 1999) postulated that virus shed from farm-reared carp survivors flowed out to open water systems via draining of farm ponds and that the virus circulated back, potentially along with small wild fish and farm escapees, when the ponds were refilled. French scientists performing an experimental bath exposure of common carp (50 g) fingerlings that were held for 180 d, while mimicking the fall to spring water temperature fluctuations, noted that the majority of fish were infected by Day 120, the highest amount of virus shedding occurred between Days 60 to 120, and that most fish died by Day 160 (Baudouy et al. 1980, Wolf 1988, p. 191–216). German scientists conducting another series of SVCV exposure experiments with common carp, weighing 25–30 g, reported the virus was first detected on the gills just 2 h after initial bath exposure but only reappeared 3–4 d later in the internal organs and then persisted for 70 d after which the survivors became virus carriers (Ahne 1977, 1978, Ahne et al. 2002). In a more recent study done by Chinese researchers using a low virulence genotype Ia strain, juvenile koi injected with this SVCV strain showed little to no clinical signs of disease and recovered, but the virus persisted up to 40 d in the asymptomatic survivors (Xiao et al. 2014).

In our bath challenge SVCV experiments, virus prevalence was low in koi Day 1 post-exposure, but by Day 3 all virus strains from each genotype were detected and present at all subsequent timepoints out to Day 17/19 and at end of the 34 d challenge. There was an anomalous absence in detection of viable virus of low virulence (Ia) strain 20070165 on Day 7 in both koi types. Blind sample processing occurred with all collected specimens, and the detection assays for the Day 7 isolate (20070165) samples from the 2 independent koi challenges were performed months apart. It would be interesting if this viral eclipse in detection was a real phenomenon during the infection cycle of a low virulence SVCV strain, but this cannot be elucidated from our study and requires further investigation for confirmation. Overall prevalence of virus was slightly curtailed in fish that were exposed to low virulence strains, but some had virus titers comparable to concentrations seen in the surviving koi infected with moderate and high virulence SVCV strains. We examined virus presence out to 34 d in koi survivors, but longer SVCV challenges, including those that could determine virus longevity in the survivors, duration of virus shedding in water, and the potential of recovered koi to infect introduced naïve cohorts, would further our understanding of SVCV transmission and carrier status.

With reference to virulence levels of SVCV genotype strains, since the late 1990s there has been a reemergence of SVCV detections and outbreaks with genotypes 1a and 1d isolates dominating. The current 1d genotypes reported only from Europe, and the Ia genotypes being globally distributed (Padhi & Verghese 2012). The European Ib and Ic genotypes have not been reported since the late 1980s apart from a 2017–2018 Ib strain detection at common carp farms in eastern Ukraine (Rud et al. 2019). Interestingly, over this 2 yr period, the carp from these farms experienced moderate cumulative mortalities ranging from 47.7–56.7%. In our side-by-side testing, it appears that the European genotype strains Ib and Ic also had moderate virulence. Comparable virulence phenotypes were evident for the Ia, Ib, and Ic genotype strains when tested in the 2 different koi stocks, whereas the Id genotype (Fijan) strain had moderate or high virulence due to the wider range of mortality (60 and 87% CPM) experienced by the 2 koi varieties tested. Basic et al. (2009) performed a phylogenetic G gene sequence fragment analysis of numerous Austrian Id genotype isolates and delineated 3 genetic subgroups (Id1, Id2, and Id3), but they were not linked to any differences in host species or pathology. To our knowledge, there have been no studies documenting differences in virulence from SVCV isolates originating from Europe. We tested only a single representative strain from each of the European genotypes. Therefore, challenges comparing multiple SVCV strains from the Ib, Ic, and Id genotypes are needed to confirm whether the reported virulence phenotype demonstrated in our study is consistent and homogenous within each genotype.

Regarding SVCV genotype Ia variability, the emergence of genotype Ia strains associated with epizootics or sampled from asymptomatic fish hosts has occurred globally since the late 1990s (Stone et al. 2003, Miller et al. 2007). SVCV Ia isolates have been collected from cyprinid fish dying during disease outbreaks in the USA from 2002–2011 in the states of Washington (pet koi pond), North Carolina and Virginia (koi farm), Wisconsin and Minnesota (feral carp), and Missouri (koi distribution facility) (Goodwin 2002, Dikkeboom et al. 2004, Miller et al. 2007, Phelps et al. 2012). Similarly, from 1999 to 2016, SVCV diseased fish or outbreaks due to Ia strains were reported in the UK, Korea, and China (Taylor et al. 2013, Godahewa et al. 2018, Zheng et al. 2018). However, SVCV-Ia infected fish not affiliated with any disease incidents were also found during the same period of time (1997 to 2016) in Austria, Canada, China, Korea, Mexico, Poland, UK, and USA (Stone et al. 2003, Liu et al. 2004, Garver et al. 2007, Miller et al. 2007, Teng et al. 2007, Basic et al. 2009, Gao et al. 2009, Zhang et al. 2009, Phelps et al. 2012, Xiao et al. 2014, Shao et al. 2016, Kim et al. 2018, Maj-Paluch et al. 2019, Ortega et al. 2019). SVCV Ia detections in North America and Europe are presumed to be associated with recent introductions of the virus via aquatic species importations from Asia (Miller et al. 2007, Ip et al. 2016). The variability of pathogenicity documented for SVCV Ia isolates in the field was consistent with the varying virulence levels demonstrated in our side-by-side Ia strain challenge experiments. There was a broad range in mortality (virulence) among the 5 genotype Ia strains we tested, 4–82 and 0–94% in the Beni Kiko koi and Sanke koi, respectively. This wide range of koi mortality elicited from different genotype Ia strain infections indicated that the SVCV virulence phenotype is heterogeneous even in its most susceptible cyprinid host species.

The variability in virulence among the SVCV genotype Ia strains may have a genetic basis. Independent phylogenetic analyses of the G and/or P genes of internationally represented SVCV isolates determined that the Ia genotype could further be subdivided into 2 subtypes (Miller et al. 2007, Zhang et al. 2009). Chinese scientists, after comparing sequences of European isolates with 4 Ia isolates, each sampled from different carp farms in Heilongjiang Province of China, noted higher G gene sequence diversity among Ia strains and suggested that SVCV was evolving independently at Chinese fish farms (Ji et al. 2017). Similarly, Padhi & Verghese (2012), after phylogenetic examination of a large SVCV isolate dataset, determined that Ia and Id genotypes had significantly different mutation rates and that nucleotide substitution rates of the P and G genes for the Ia genotype was 3.5 times higher than Id genotype and attributed the reemergence of SVCV in the late 1990s to increased antigenic variation. The first SVCV Ia isolations reported from China in the early 2000s were not associated with disease incidents, and those subjected to susceptibility testing had low virulence (Liu et al. 2004, Gao et al. 2009, Xiao et al. 2014, Shao et al. 2016). Starting around 2004, there were reports from a few Asian countries of SVCV isolations in association with epizootics, and other isolates, while still not affiliated with an outbreak, were found to be highly pathogenic in subsequent *in vivo* susceptibility experiments (Teng et al. 2007, Shao & Zhao 2017, Godahewa et al. 2018). For example, a 2016 isolate from a grass carp *Ctenopharyngodon idella* discovered during surveillance screening in Shanghai, China, was described as a distinct genetic variant, based on whole genome sequence analysis, and belonged to a new lineage of the genotype Ia strain (Shao & Zhao 2017). Further, this Shanghai strain was phenotypically unusual because it induced severe mortalities (90–100%) in grass carp, common carp, koi, and goldfish *Carassius auratus* at atypical warm water temperatures (16–20°C), and some mortalities (20–25%) occurred even at 26°C during laboratory challenges.

If the Ia genotype strains are becoming increasingly genetically diverse, they may be in the midst of an evolutionary divergence resulting in a myriad of strains with correspondingly variable virulence phenotypes. The higher genetic diversity of Ia strains could be due to prolific virus replication in cyprinid species being reared in high production aquacultural settings within Asian countries (Padhi & Verghese 2012, Kennedy et al. 2016), and then subsequent global dissemination of these rapidly evolving strains occurs via international fish trade. Xiao et al. (2014) concluded, after molecular characterization of a 2013 Ia isolate, that multiple SVCV strains have been co-circulating in China for many years. Similar phylogeographic transmission patterns also occurred for IHNV in China (Jia et al. 2018, Xu et al. 2019b). This may be analogous to the scenario that occurred for rainbow trout intensively cultured in the US, which gave rise to the IHNV M-genogroup with higher nucleotide diversity, and eventually M-genogroup strains evolved that were only highly virulent in trout (Troyer et al. 2000, Troyer & Kurath 2003)

While strain virulence is important, it is not the sole determinant of disease severity, as many environ-

mental and host parameters (e.g. species composition, water temperature, host density, flow dynamics, and host age) can affect infection dynamics. It would be interesting to determine whether the low virulence phenotype strains we identified are maintained during infections with stressors (high host densities, temperature fluctuations, poor water quality) typically known to induce a SVC disease cascade in a susceptible host species. In summary, the variability of virulence of genotype Ia strains documented in global detections/outbreaks of SVCV was further substantiated in our experimental challenges. Additionally, SVCV strain virulence under our experimental conditions appears to be a dominant factor in determining SVCV disease outcomes, whereas intraspecies variation, based on koi variety, had less of an impact.

Data availability. The USGS raw data release for this research study can be found at https://doi.org/10.5066/P9WS6Q0M.

Acknowledgements. Many thanks to Andy Goodwin (US Fish & Wildlife Service), for providing the original North American SVCV isolate (NC2002). He first initiated SVC disease research in the US and has always been willing to share his advice and knowledge. We also thank Keith Way of CEFAS for his generosity and time in sending us the European and Russian isolates. We express gratitude to Zak Schmidt and Charlie Funk for their advice on koi husbandry and tolerance for any silly questions. We thank Rachel Breyta for imparting her expertise on phylogenetic analysis methods and consulting with us on SVCV phylogeny. Dr. E. Ashley Steel provided excellent insights regarding statistical theorems applied to biological data. Dr. Jim Winton was pivotal in facilitating the productive visit by Chinese scientists to our research facility and for initiating the collaborative work between the WFRC and the Shenzhen Academy. This research was funded by the US Geological Survey (USGS) and a grant (JCYJ20170818140641071) provided by the Natural Science Foundation of Shenzhen, PR China. Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

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Editorial responsibility: Thomas B. Waltzek, Gainesville, Florida, USA
Reviewed by: M. Faisal and 1 anonymous referee

Submitted: May 21, 2021
Accepted: December 23, 2021
Proofs received from author(s): March 12, 2022