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Vertical Transmission of Cyprinid Herpesvirus 3 (CyHV-3) in Common Carp *Cyprinus carpio*: Experimental Insights and Implications

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Abstract

Currently, little is known about vertical transmission of cyprinid herpesvirus 3 (CyHV-3). Here, we assessed potentials for (1) vertical transmission of CyHV-3 and (2) viral infection to fertilized eggs via viral-contaminated environmental water. After CyHV-3 exposure, eggs were incubated, and hatchlings were raised to juvenile stage. To assess occurrence of KHVD during the experiment, samplings were performed at various stages, such as fertilized egg, eyed egg, larval, and juvenile stages. The viral DNA was detected only from eggs at 3 h post fertilization, but not from fish at later stages. No mortality associated with CyHV-3 infection was observed in all the groups during the experimental period, or fish became 91 days post hatch. Additionally, those juveniles were re-exposed to CyHV-3 to investigate whether CyHV-3 infection was established at the egg stage. As a result, statistically significant difference was not observed in the cumulative mortality between the larval fish with or without CyHV-3 infection at the egg stage, suggesting that fish exposed to CyHV-3 at the egg stage did not acquire immunity against the viral infection. All our results suggest that it is improbable that KHVD can be transmitted vertically through eggs of common carp that have survived CyHV-3 infection.

Introduction

Koi herpesvirus disease (KHVD) has spread to many countries worldwide since the first mass mortality events associated with the disease were reported in 1998 (Hedrick et al., 2000; WOAH, 2023). The causative agent of KHVD, cyprinid herpesvirus 3 (CyHV-3), has been intensively studied for its etiology, pathogenicity, genome structure, host range, and more (Ilouze et al., 2011; Rakus et al., 2013). The virus is highly virulent to carp and the cumulative mortality rate can exceed 90% in laboratory experiments (Hedrick et al., 2000; Ito et al., 2014). The virus has been impacting carp populations in natural waters as well. Devastating mass mortality events associated with KHVD were reported in the early 2000s throughout Japan (Sano et al., 2004; Hara et al., 2006; Uchii et al., 2009). These mass mortality events continued for years; however, the frequency and amplitude of KHVD outbreaks have decreased over time after the implementation of regulations that restrict 1) the movement of live fish between natural waters, and 2) the release of hatchery-cultured CyHV-3-free fish (Mie Prefectural Government, 2023).

In Japan, there are requests to release common carp into natural environments for stock enhancement because common carp is a major fishing target for anglers. Common carp is indigenous to the country and is also consumed as a protein source. Currently, releases

of common carp to KHVD-occurring natural waters have ceased due to the concern of mass mortality events associated with KHVD. This is because if (1) vertical transmission has occurred in wild common carp populations and/or (2) CyHV-3 infected fish (i.e., carrier fish) remain present in natural environments, the release of hatchery cultured KHVD-free fish into the natural waters can trigger another massive outbreak of the disease. Although both concerns must be addressed for the successful stock enhancement of common carp, we are particularly interested in the vertical transmission of CyHV-3 in natural waters because we believe that it has greater implications. The occurrence of vertical transmission of CyHV-3 indicates that CyHV-3 can be inherited across generations. Despite its importance, little is currently known about the vertical transmission of KHVD. The only available report was published by Kobayashi et al. (2010), who reported that CyHV-3 DNA was not detected among unfertilized eggs (n = 3), fertilized eggs (n = 3), ovarian fluid (n = 2), or milt (n = 7) of koi carp broodstock which had survived KHVD. The authors provided important findings that vertical transmission of the virus was unlikely; however, additional experiments remain critical since the sample sizes examined in that study were relatively small. In addition, the report did not provide any data regarding the consequence of offspring that were exposed to the infectious viral particles at the egg stage. There are other publications which focus on early developmental stages of carp to CyHV-3 infection. For example, Ronsmans et al. (2014) performed challenge tests with embryonic and larval stages of common carp. The authors provided critical information regarding importance of skin mucus against viral infection, however their aim was not testing vertical transmission.

In the present study, we assessed potentials for (1) vertical transmission of CyHV-3 and (2) CyHV-3 infection to fertilized eggs via viral-contaminated environmental water. For testing vertical transmission of CyHV-3, unfertilized eggs were exposed to CyHV-3 first, and then used for fertilization with virus-free milt. In contrast, for testing CyHV-3 infection to fertilized eggs via viral-contaminated environmental water, CyHV-3 free-eggs and milt were used for artificial fertilization, and then fertilized eggs were exposed to CyHV-3. After fertilizations, occurrence of CyHV-3 was assessed at the early life stages of fish, from egg through juvenile stages for both scenarios. Additionally, juveniles were re-exposed to CyHV-3 to investigate whether CyHV-3 infection was established at the egg stage; we assumed that fish that survived CyHV-3 infection at the egg stage would acquire immunity against CyHV-3 and show higher survival rates compared with fish without CyHV-3 infection. Furthermore, an additional side experiment was conducted to assess the efficacy of disinfection by iodophor treatment to take advantage of the experimental setup. Disinfection is unrealistic in natural waters, but such information might be beneficial to controlling KHVD in a different situation.

Methods

Cell Line and Virus

The virus was propagated using a common carp brain (CCB) cell line (Neukirch et al., 1999). The cell line was maintained in the minimum essential medium (Mediatech, Inc. Manassas, VA, USA) supplemented with 10% FBS (Equitech-Bio, Inc., Kerrville, TX, USA) and antibiotics (penicillin and streptomycin). The CCB cells were maintained at 25°C. The virus used for the present study was a CyHV-3 isolate, KHV 0301 (Sano et al., 2004). This isolate was propagated 5 times in CCB cells in 75 cm^2 flasks at 25°C. Aliquots of the cell culture supernatant containing CyHV-3 were placed in 3.6 mL cryotubes and stored at −85°C until utilized. A $TCID_{50}$ titration of the viral stock solution was performed.

Collecting Unfertilized Eggs and Milt

CyHV-3 free common carp broodstock has been maintained at the Tamaki Field Station of the Fisheries Technology Institute (FTI). Fish were kept in groundwater at 16°C until unfertilized eggs and milt were collected. A single broodstock pair was moved to a 2,000 L tank with water temperature maintained at 24°C and kept together to facilitate the final maturation of eggs and milt. After observation of initial spawning behaviors early the next morning (i.e., the male chasing the female), the broodstock pair was separated into individual 2,000 L tanks. Milt was artificially collected from the male using a 2.5 mL syringe. From the female, after washing the genital atrium surface with Ringer's solution, unfertilized eggs were collected by stripping them into a dried stainless-steel bowl. The milt and unfertilized eggs were then used in the following experiments.

Experimental Fish for the Co-rearing Test

To facilitate recognition of transmission of CyHV-3 between fish, colored fish (Taisho Sanshoku, a variety of koi carp) were used as co-reared fish along with the common carp which were raised from CyHV-3-exposed eggs. The koi carp were bred from broodstock kept at the Tamaki Field Station of the FTI. Before the co-rearing test, the susceptibility of the koi carp to CyHV-3 was confirmed by a challenge test and the result was reported as koi B in experiment #1 of Ito et al. (2014).

Infection Experiment #1: Exposing eggs to CyHV-3

Three experimental groups of eggs were prepared as shown in Figure 1. The unfertilized eggs were divided into 3 portions of 30 g each, with approximately 5,000 eggs per group. These eggs were used to prepare the following groups: (1) the “Virus-exposed eggs pre-fertilization group”, (2) the “Virus-exposed eggs post-fertilization group”, and (3) the “Negative control”. The

eggs in each group were further split into 2 subgroups: with or without disinfection with iodophor (Figure 1).

The first group, the "Virus-exposed eggs pre-fertilization group", was prepared as follows. A portion of unfertilized eggs was mixed with 1.0 mL of CyHV-3 culture supernatant ($10^{4.6}$ $TCID_{50}$ mL^{-1}) for 5 min. For this group, we used undiluted CyHV-3 solution to mimic a situation that a female fish is heavily infected with CyHV-3. Then, the eggs were fertilized by adding 1 mL of milt diluted at 1:100 using modified Kurokura's Extender 2 (Magyary et al., 1996). The fertilized eggs were sprinkled and attached to a plastic net. Additionally, these eggs were divided into two subgroups (15 g each). Eggs in one subgroup were disinfected with iodophor (200 mg L^{-1}, 15 min) while those in the other subgroup were not. To prepare the second group of fish, eggs from the "Virus-exposed eggs post-fertilization group" were fertilized as described above, and then were immersed in CyHV-3 working solution (estimated 40$TCID_{50}$ mL^{-1}) for 1 h at 24°C. The CyHV-3 working solution was prepared by diluting stock solution ($10^{4.6}$ $TCID_{50}$ mL^{-1}) at 1:1,000 with groundwater. For this group, we used diluted CyHV-3 solution and extended CyHV-3 exposure time to reproduce an environmentally relevant condition. Similar to the first group described above, the eggs were divided into two subgroups; one subgroup received disinfection with iodophor and the other did not. In the negative control, eggs were not exposed to CyHV-3. The fertilized eggs in the negative control were also divided into two subgroups and treated as described earlier.

All the eggs were kept in 120 L tanks continuously receiving temperature-controlled groundwater (0.6 L min^{-1}) and maintained at 24.0°C. To evaluate the hatching rate, 100 fertilized eggs of each subgroup were kept in a separate box which was attached to the inside of each subgroup's tank and monitored for 1 week after fertilization. All the larvae were fed with brine shrimp (*Artemia salina*) until 14 days post hatch (dph) and were subsequently switched to commercial diets consisting of a mixture of crumble diets (Ayutech, Marinetech) and pellets (Saki-Hikari®, Kyorin). At 50 dph, to reduce the density, fish were randomly removed down to 100 fish in each tank. Subsequently, all the fish were observed for up to 91 dph. These juveniles were later used for the viral challenge experiment (Infection Experiment #2, n = 30 per each subgroup) and co-rearing test with naïve koi carp (n = 70 per each subgroup) as described below.

For detection of CyHV-3 DNA by polymerase chain reaction (PCR), samples (n = 5) were collected from different fish life stages from each subgroup as follows: eggs at 3 h post fertilization, eyed eggs at 2 days post fertilization, larvae at 1 dph, and juveniles at 1 month post hatching. At each sampling, 5 eggs or larvae were collected and pooled together and treated as one sample for analysis due to their small sizes. For juveniles, whole fish were individually analyzed.

Infection Experiment #2: Viral Challenge Experiment on Juveniles

To investigate whether CyHV-3 infection was established during the egg stage, a challenge test was performed for all the subgroups at 91 dph. Thirty fish were randomly selected from each subgroup (average body weights: 2.06 – 2.53 g) and were immersed for 1 h in a viral solution for 1 h at 24°C (estimated 40$TCID_{50}$ mL^{-1}). The viral solution was prepared by diluting stock solution ($10^{4.6}$ $TCID_{50}$ mL^{-1}) at 1:1,000 with groundwater.

After CyHV-3 exposure, all the fish were kept in 60 L tanks at 24.0°C with continuous water flow (0.6L min^{-1}) and fed a commercial diet once daily (Saki-Hikari®, Kyorin). Fish were observed daily, and dead fish were removed from a tank and kept at −85°C until further processed. On day 56, the experiment was terminated;

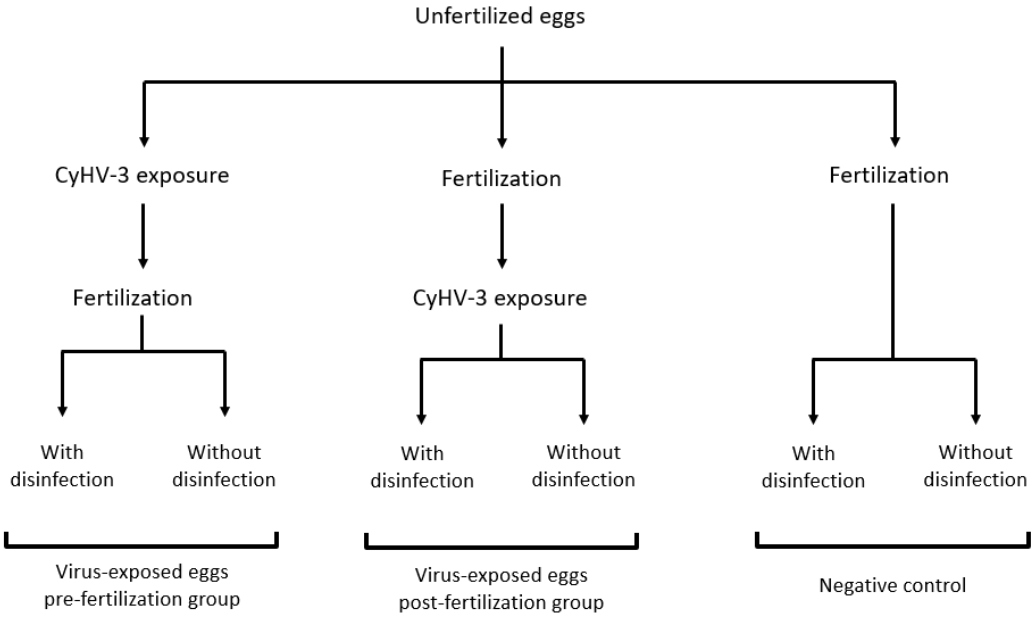

Figure 1. Experimental design of Infection Experiment #1: Exposing eggs to CyHV-3.

all surviving fish were euthanized using 2-phenoxyethanol diluted at 1:500 with groundwater. For detecting CyHV-3 DNA by PCR, a portion of the caudal fin and gills was collected from all dead fish. These tissues were pooled together for analysis. In contrast, the brain was also collected along with the caudal fin and gills from survivors. These tissues were also pooled. The samples were used for viral DNA detection by PCR (Gilad et al., 2002) as described below.

Co-rearing Test with Naïve Koi Carp

The experimental fish were co-reared with naïve koi carp to investigate whether the common carp from CyHV-3-exposed eggs had become viral carriers. Juveniles at 91 dph (n=70, average body weights ranging between 2.02–2.53 g) from each subgroup were used for the test and placed in a fish tank with naïve fish (n=5, average body weight 3.22 g). All the fish were kept in 120 L tanks at 24.0°C with continuous water flow (0.6L min^{-1}), fed a commercial diet (Saki-Hikari®, Kyorin) once daily. On day 56, the experiment was terminated, and all surviving fish were euthanized using 2-phenoxyethanol diluted at 1:500 with groundwater. The caudal fins, gills, and brains were collected from 10 surviving fish that were randomly selected from each subgroup and the tissues were pooled together for analysis. Similarly, samples from all five co-reared naïve koi carp were pooled. These samples were used for PCR detection of viral DNA (Gilad et al., 2002) as described below.

Detection of CyHV-3 DNA by Conventional PCR

For the detection of CyHV-3, the conventional PCR method described by Gilad et al. (2002) was utilized. Genomic DNA was extracted from tissues using the Gentra Puregene Tissue Kit (Qiagen, Hilden, Germany). The PCR reagent, *TaKaRa Ex Taq*® Hot Start Version (TaKaRa Bio Inc., Kusatsu, Japan), was used and reactions were performed according to the manufacturer's protocol (Gilad et al., 2002).

Statistical Analyses

Student's t test was used to compare means of hatching rates between with and without iodophore disinfection. In contrast, the statistical differences in the mortality rates between each infected group and negative control group were determined using Fisher's exact test. Values of $P<0.05$ were considered significant.

Results

A summary of Infection Experiment #1, exposing eggs to CyHV-3, is shown in Table 1. The average hatching rate of the disinfected treatment group (M=15.7, SD=2.5) was lower than the non-disinfected treatment group (M=37.0, SD=15.0); however the difference was not statistically significant, $t(4)=2.6$, $P=0.057$. CyHV-3 DNA was detected only from one of five samples of both "Virus-exposed eggs post-fertilization; Disinfected group" and "Virus-exposed eggs post-fertilization; Non-disinfected group" in the samples collected at 3 h after fertilization.

The results for the detection of CyHV-3 DNA and cumulative mortality curves from Infection Experiment #2 are shown in Table 2 and Figure 2, respectively. In Infection Experiment #2, the cumulative mortality rates among all the subgroups were high (67%–87%). Among individual subgroups, the mortality rate for the subgroup, "Virus-exposed eggs pre-fertilization, Disinfected", was higher than the others (87%). However, the differences were not statistically significant ($P>0.05$). As expected, CyHV-3 DNA was detected among all the dead and surviving fish from all the CyHV-3 re-infected groups (Table 2).

CyHV-3 DNA was not detected in any fish tested in the experiment "Co-rearing test with naïve koi carp". This includes the juveniles that received CyHV-3 exposure at the egg stage (from Infection Experiment #1) and naïve fish co-reared with the juveniles for 56 days (data not shown). Additionally, mortality was not observed.

Discussion

In piscine viral infections, such as infectious hematopoietic necrosis virus and nodavirus infections, there are indications that vertical transmissions may occur by contamination of egg surfaces (Bovo et al., 2005). However, little is known about the vertical transmission of KHVD. Kobayashi et al. (2010) reported

Table 1. Results for hatching rate, detection of CyHV-3 DNA from fish at different life stages (eggs, larvae, and juveniles), and cumulative mortality in each group until 91 days post hatch (dph) after CyHV-3 exposure at the egg stage. For detection of CyHV-3, a PCR assay was used

Group	Hatching rate (%)	PCR detection for CyHV-3*1				Cumulative mortality (%)*2
		Eggs	Eyed eggs	Larvae	Juveniles	
Virus-exposed eggs pre-fertilization; Disinfected	13	0/5	0/5	0/5	0/5	0
Virus-exposed eggs pre-fertilization; Non-disinfected	52	0/5	0/5	0/5	0/5	0
Virus-exposed eggs post-fertilization; Disinfected	16	1/5	0/5	0/5	0/5	0
Virus-exposed eggs post-fertilization; Non-disinfected	22	1/5	0/5	0/5	0/5	0
Negative control; Disinfected	18	0/5	0/5	0/5	0/5	0
Negative control; Non-disinfected	37	0/5	0/5	0/5	0/5	0

*1 The results are given as number of positive fish/number of fish tested. *2 Cumulative mortality over 91 days in experiment#1

that CyHV-3 DNA was not detected in unfertilized eggs, fertilized eggs, ovarian fluid, or milt from koi carp broodstock that had survived KHVD. However, the sample number in the study was relatively small, and therefore the data are insufficient for a reliable evaluation. In addition, the report does not provide any data regarding the consequence of offspring that were exposed to the infectious viral particles at the egg stage. To fill this knowledge gap, exposure experiments were performed with a larger sample size. Again, the objective of this study was to assess the potential for the transmission of KHVD via eggs or milt by using unfertilized and fertilized eggs of common carp exposed to CyHV-3. In this study, two scenarios of KHVD transmission were simulated (Figure 3). The first scenario involved CyHV-3 transmission via ovarian fluid from the CyHV-3 carrier broodstock (Figure 3A). The likelihood of this scenario was assessed using the "Virus-exposed eggs pre-fertilization group". The second scenario involved the presence of CyHV-3 in environmental water during spawning (Figure 3B). The likelihood of the second scenario was assessed using the "Virus-exposed eggs post-fertilization group". In this study, there was no significant mortality associated with CyHV-3 infections from the egg to juvenile stages among the virus-exposed eggs in both scenarios. Although speculative, the virions might have inactivated before the fish hatched. This possibility is supported by PCR results in which viral DNA was detected from the virus-exposed eggs at 3 h after fertilization, but not from later

Table 2. Results of cumulative mortality among common carp which hatched from CyHV-3 exposed eggs and later experimentally infected with the virus as well as detection of CyHV-3 DNA by PCR for the experimentally challenged fish

Group	Cumulative mortality (%)*1	PCR detection of viral DNA*2	
		Dead fish	Surviving fish
Virus-exposed eggs pre-fertilization; Disinfected	87	26/26	4/4
Virus-exposed eggs pre-fertilization; Non-disinfected	73	22/22	8/8
Virus-exposed eggs post-fertilization; Disinfected	67	20/20	10/10
Virus-exposed eggs post-fertilization; Non-disinfected	73	22/22	8/8
Negative control; Disinfected	67	20/20	10/10
Negative control; Non-disinfected	73	22/22	8/8

*1 Cumulative mortality over 56 days in experiment#2. *2 The PCR results are given as number of positive fish/number of fish tested

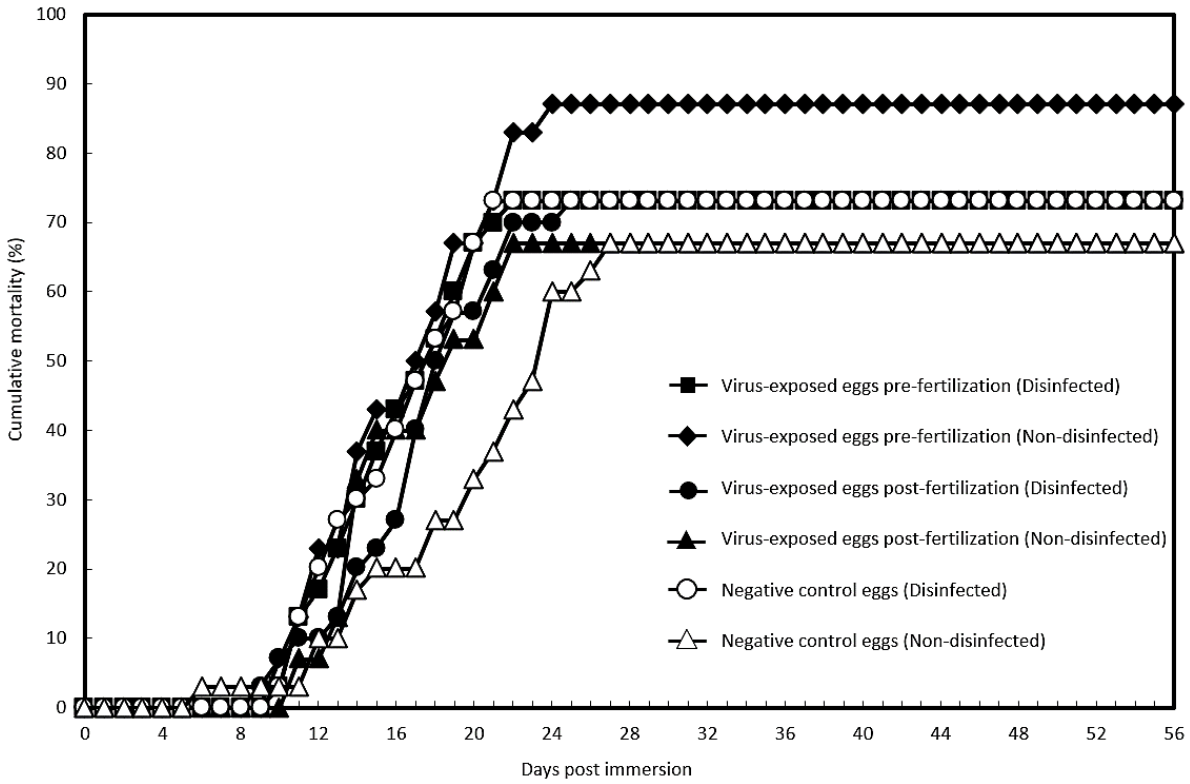

Figure 2. Cumulative mortality curves for common carp which hatched from CyHV-3 exposed eggs experimentally infected with the virus. Virus-exposed eggs pre-fertilization (Disinfected); (■), Virus-exposed eggs pre-fertilization (Non-disinfected); (◆), Virus-exposed eggs post-fertilization (Disinfected); (●), Virus-exposed eggs post-fertilization (Non-disinfected); (▲), Negative control eggs (Disinfected); (○), Negative control eggs (Non-disinfected); (△)

stages (Table 1). Another possibility is the low affinity of CyHV-3 virions to fish eggs. Among the eggs exposed to CyHV-3 without disinfection (both pre- and post-fertilization), only 1 out of 10 samples was found to be positive for CyHV-3 DNA. A higher positivity rate can be expected if CyHV-3 virions attach to the surface of fish eggs.

The common carp that hatched from virus-exposed eggs were re-exposed to CyHV-3 to investigate whether CyHV-3 infection was established during the egg stage. Ronen et al. (2003) reported that live attenuated virus is effective for protection against KHVD, suggesting that differences in survival rates can be used as an indicator for previous viral infection. If the surviving juvenile fish from virus-exposed eggs show some degree of resistance against CyHV-3, the cumulative mortality rates of such juveniles would be lower than the negative control. However, no such trend was observed and there was no significant difference seen between the two groups. Additionally, no mortality was observed in naïve koi carp that were co-reared with juveniles that were exposed to CyHV-3. These results suggest that the juvenile fish raised from the virus-exposed eggs did not possess immunity against KHV, indicating that KHV infection was not established when exposed at the egg stage.

Vertical transmission of CyHV-3 is unlikely when considering the stability of CyHV-3 in environmental water, incubation time of common carp embryos, and insusceptibility of carp larvae to CyHV-3. CyHV-3 can lose its infectivity before fish become susceptible to the virus. Carp embryos require 4.5 days of incubation time

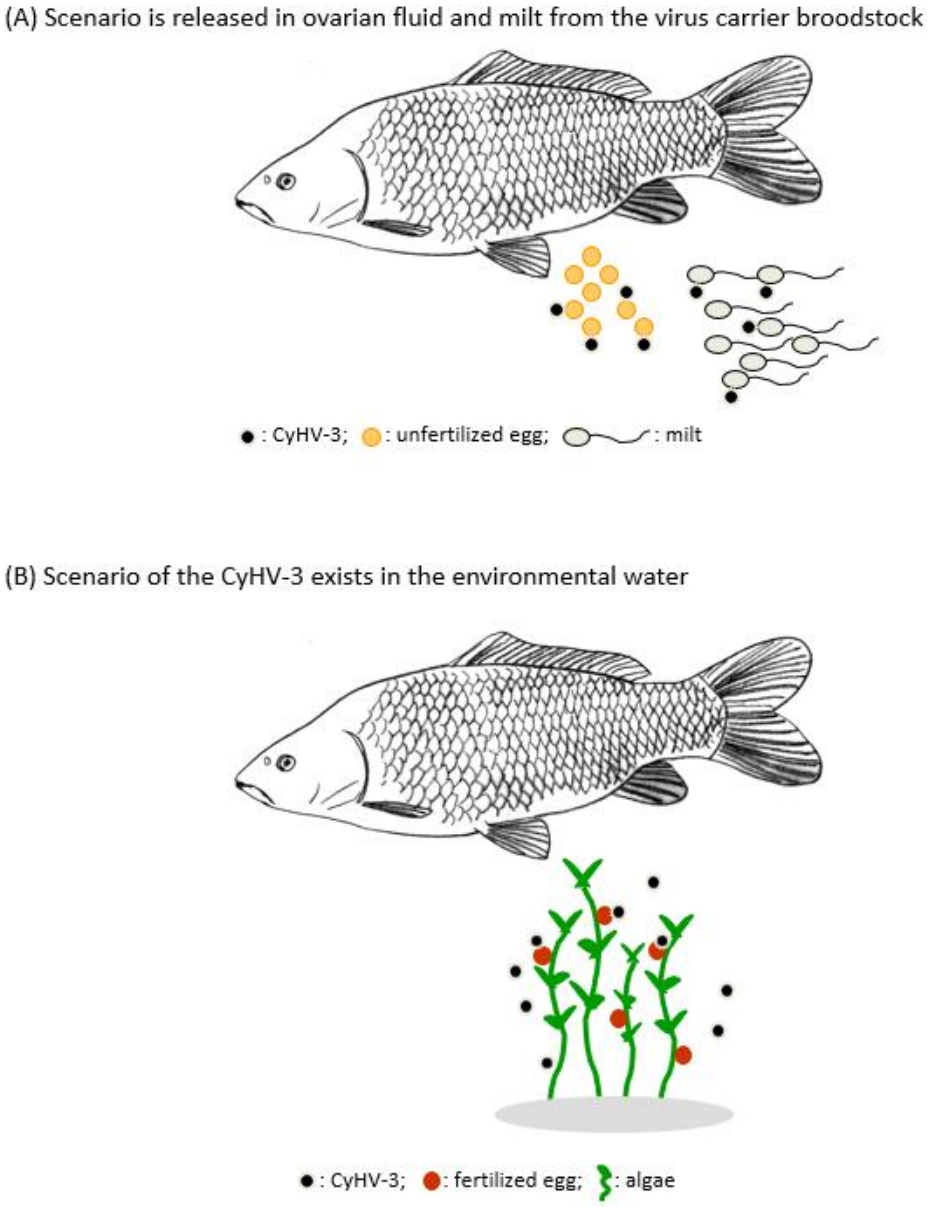

Figure 3. Transmission scenarios assumed in this study. The first scenario is that CyHV-3 is released in ovarian fluid and milt from the viral carrier adlts. The possibility of vertical transmission in scenario (A) was simulated by the "Virus-exposed eggs pre-fertilization group". Scenario (B) suggests that CyHV-3 exists in the environmental water during spawning (transmission via environmental water). The possibility of scenario (B) was simulated by the "Virus-exposed eggs post-fertilization group"

until hatching when maintained at 22°C (Linhart et al., 2005). Even after hatching, carp are not susceptible to CyHV-3 immediately, and CyHV-3 infection was not established during the carp's larval stage as demonstrated by exposure experiments in a laboratory setting (Ito et al., 2007; Ronsmans et al., 2014). In contrast, the infectivity of CyHV-3 in environmental water can be lost in 3 days at temperatures higher than 15°C (Shimizu et al., 2006). Therefore, even if egg surfaces are contaminated with CyHV-3, the virus can be inactivated before carp become susceptible to it. Additionally, CyHV-3 in ovarian fluid, and milt may not infect fertilized eggs as shown in this study.

As a side experiment, we performed a test to assess the efficacy of disinfection by iodophor treatment to take advantage of the experimental setup although the aim of this experiment was not relevant to the main scientific questions that were trying to address in this study. Unfortunately, the data from this study are not informative regarding the efficacy of iodophor for KHV disinfection because CyHV-3 infection was not established at the egg stage. However, there was a trend that hatching rates were lower in the iodophor treatment groups. The disinfection method used in the study (200 mg L^{-1} for 15 min) may be too harsh for common carp.

In Lake Biwa, mass mortalities of wild common carp due to CyHV-3 infection occurred in the spring of 2004. Interestingly, the antibody against CyHV-3 was not found in the offspring of the surviving fish of the mass mortality event (<300 mm in length and captured in the summer of 2006) (Uchii et al., 2009). Such data strongly suggest that the juvenile carp were not infected with CyHV-3 even in natural water, supporting the findings in this study.

Conclusion

Based on the findings of this study and previous reports, it is improbable that KHVD can be transmitted vertically through eggs and milt of common carp that have survived CyHV-3 infection. Therefore, it is presumed that the number of CyHV-3 infected fish in natural aquatic environments will decline over time in the absence of further disease outbreaks (i.e., through horizontal transmission). Further research employing mathematical modeling is necessary to estimate the 'clearance time' of KHVD in natural water bodies. Moreover, it is crucial to investigate the viral kinetics and conditions under which CyHV-3 can reactivate in carrier fish. This investigation is essential for predicting the frequency of mass mortality events associated with KHVD in the future.

Ethical Statement

This experimental infection was performed in accordance with the 'Guidelines for Animal Experimentation' of the FTI's animal welfare regulations.

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Author Contribution

Takafumi Ito and Kei Yuasa contributed to the design of the study. Takafumi Ito performed the experimental infection and virological study. Tomofumi Kurobe contributed to the statistical analyses and improvement the manuscript. All authors read and approved the final manuscript.

Conflict of Interest

The authors have declared that no competing interests exist.

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