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Viral haemorrhagic septicaemia virus (VHSV) remains viable for several days but at low levels in the water flea *Moina macrocopa*

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ABSTRACT: Viral haemorrhagic septicaemia virus (VHSV) Genotype IVb has been isolated from amphipods belonging to the genus *Diporeia*, but it has yet to be established whether crustacean zooplankton act as vectors of this virus for fish species. Therefore, we evaluated the viability of infectious VHSV in the water flea *Moina macrocopa*. VHSV was re-isolated from replicate groups of *M. macrocopa* that had been immersed with $10^{8.0}$, $10^{7.0}$, and $10^{5.0}$ $TCID_{50}$ ml^{-1} of VHSV (DK-3592B, Genotype Ia). Furthermore, 40 *M. macrocopa* that had been immersed with $10^{8.0}$ $TCID_{50}$ ml^{-1} of VHSV for 72 h had VHSV titers of $10^{2.7}$–$10^{4.3}$ $TCID_{50}$. Thus, VHSV was clearly taken up by *M. macrocopa* and remained viable in this crustacean for several days. However, no mortality was observed over a 28 d period in rainbow trout *Oncorhynchus mykiss* that were fed VHSV-contaminated *M. macrocopa* for 14 d, and we found that the virus titer significantly decreased after a 4 h incubation with pyloric caecal extracts from rainbow trout, indicating that passage through the gut is likely to result in a significant decrease in viral titer. This may explain why consumption of prey containing low levels of VHSV did not result in clinical VHS.

KEY WORDS: Viral haemorrhagic septicaemia virus · Viability · *Moina macrocopa* · Rainbow trout *Oncorhynchus mykiss* · Vector

INTRODUCTION

Viral haemorrhagic septicaemia virus (VHSV) is a negative-sense single-stranded RNA virus that belongs to the genus *Novirhabdovirus* within the family *Rhabdoviridae* (Dietzgen et al. 2012). VHSV is the etiological agent of serious disease outbreaks that occur in wild and farmed fish in the Northern Hemisphere (OIE 2016), and its isolates can be divided into 4 major genotypes and a number of subtypes that have relatively distinct geographical distributions (Einer-Jensen et al. 2004, 2005, Lumsden et al. 2007): in general, Genotypes I–III are distributed in European waters, while Genotype IV is distributed in North American and Asian waterbodies (Einer-Jensen et al. 2004, 2005, Lumsden et al. 2007).

Until the beginning of the 1990s, it was thought that VHSV only caused severe mortalities in farmed rainbow trout *Oncorhynchus mykiss* in continental Europe. However, over the last 3 decades, this disease has been isolated from more than 80 freshwater and marine fish species in North America, Northeast Asia, and Europe (OIE 2016), and has caused mortalities in a range of marine and freshwater fish species irrespective of whether they are cultured or wild, although the genotypes of the causative agents differ (Munro et al. 2015, OIE 2016). Furthermore, VHSV is also transmitted horizontally regardless of the genotype (Skall et al. 2004, Dale et al. 2009, Goodwin & Merry 2011, Ito & Olesen 2013). Thus, VHSV represents one of the most important viral fish pathogens, as it results in extensive economic losses to

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the aquaculture industry and also impacts natural resources.

VHSV was recently isolated from amphipods belonging to the genus *Diporeia* in the Laurentian Great Lakes in North America, leading to the suggestion that infected crustacean zooplankton may expose predators to the risk of VHSV infection (Faisal & Winters 2011). *Diporeia* spp. are a suitable food for wild fish species in the Great Lakes Basin (Scharold et al. 2004), and the consumption of infected baitfish or other virus-contaminated foods may be a risk factor for the introduction of VHSV (Schönherz et al. 2012, Getchell et al. 2013). However, despite the importance of understanding the transmission routes of this disease to prevent its spread, it has yet to be determined whether crustaceans can act as a vector for the spread of this viral disease to fish species.

Therefore, the aim of this study was to assess whether infectious VHSV can remain viable in crustacean zooplankton and can be transferred to and remain viable in a fish host. Since *Diporeia* spp. are difficult to cultivate under laboratory conditions, the water flea *Moina macrocopa* was used as a model zooplankton, while rainbow trout was used as the host.

MATERIALS AND METHODS

Cell line and virus

The epithelioma papulosum cyprini (EPC) cell line (Fijan et al. 1983) was used for propagation, re-isolation, and titration of VHSV. The cells were maintained in minimum essential medium (MEM; Mediatech) supplemented with 10% fetal bovine serum (FBS; Equitech-Bio) and antibiotics (100 U penicillin ml^{-1} and 100 µg streptomycin ml^{-1}). Stock cultures were incubated at 25°C, and cultures for virus propagation and re-isolation were incubated at 15°C. VHSV isolate DK-3592B (Lorenzen et al. 1993) was used for the exposure experiment with *Moina macrocopa* and the experimental infection of rainbow trout.

Uptake and viability of VHSV in *Moina macrocopa*

Cultivation of *M. macrocopa*

Clones of the water flea *M. macrocopa* were produced by obtaining a single individual from a pond at the Tamaki Laboratory of the National Research Institute of Aquaculture (NRIA), Japan Fisheries Research and Education Agency, and pre-culturing it in 80 ml of Rice Straw and Mukuriwa-ku (RSM) medium in a ventilated 75 cm2 cell culture flask (Greiner Bio-One, 658175). The RSM medium was made by supplementing 1 l of tap water with 5 g of cut rice straw and 0.5 g of Mukuriwa-ku (Takuyousuisan-kougyou), which is a powder made from crab shell and yeast that is used for culturing water fleas. This solution was then autoclaved, and the supernatant was decanted into another sterilized beaker and used as a culture medium for the water fleas. The original water flea was identified as *M. macrocopa* based on its morphological characteristics.

The water fleas were fed live *Chilomonas* spp. (Cryptophyceae). The *Chilomonas* spp. were obtained from the pond at the Tamaki Laboratory of the NRIA, pre-cultured in RSM medium in a 24-well plate (Corning, 3526) and cloned by 2 rounds of cloning by limiting dilution. The cloned *Chilomonas* spp. were then cultured in 1 l of RSM medium, which was kept for approximately 4–5 d at 20–25°C (room temperature, RT). *M. macrocopa* were cultured in 80 ml of the RSM medium containing *Chilomonas* sp. in a ventilated 75 cm2 cell culture flask at RT. Subculture of *M. macrocopa* was carried out once every 3–4 d at a 1:2 split ratio.

Expt 1: exposure of *M. macrocopa* to VHSV

Cultivated *M. macrocopa* were filtered through REED Healthy-Cooking Paper (Lion), which had been folded to form a funnel and autoclaved. The trapped *M. macrocopa* were then resuspended in RSM medium and 400 adults were selected for use in Expt 1. The adult *M. macrocopa* were divided equally across 20 plastic dishes (50 mm diameter; Nunc, 150326), each containing 5 ml of RSM medium. Five dishes were then assigned to each treatment group.

VHSV culture ($10^{8.5}$ 50% tissue culture infectious dose [$TCID_{50}$] ml^{-1}) was added to each dish in 3 of the groups (i.e. a total of 15 dishes) to give final doses of $10^{7.0}$, $10^{5.0}$, and $10^{3.0}$ $TCID_{50}$ ml^{-1}, respectively. The remaining group (i.e. 5 dishes) was left untreated as a negative control. All dishes were kept at 15°C to investigate the presence of VHSV in the cultured *M. macrocopa* following virus exposure. *M. macrocopa* in 1 dish from each group were sampled at 3, 24, 48, 72, and 144 h following viral exposure, and tested by virological examination on cell culture and reverse transcription polymerase chain reaction (RT-PCR), as described below.

Expt 2: evaluation of viral titer in VHSV-exposed *M. macrocopa*

Cultivated *M. macrocopa* were collected using the same procedure as in Expt 1, and 360 adults were selected for use in Expt 2. These adults were distributed equally across 9 plastic dishes (50 mm, Nunc, 150326), each containing 5 ml of RSM medium. VHSV culture was then added to each dish to give a final concentration of $10^{8.0}$ $TCID_{50}$ ml^{-1}, and the dishes were incubated at 15°C. All *M. macrocopa* in each of 3 replicate dishes were then collected at 24, 48, and 72 h following viral exposure for viral examination and titration.

Virological examination of *M. macrocopa*

The procedures that were used for virus isolation and treatment of *M. macrocopa* in Expt 1 are outlined in Fig. 1. VHSV-exposed *M. macrocopa* were collected by filtration through REED Healthy-Cooking Paper (Lion), and the immersion solution that passed through the paper was then passed through a 0.45 µm filter (DISMIC-13CP, Advantec) and examined for VHSV (Fig. 1, Steps 1 and 2). *M. macrocopa* on the filter were then each washed 7 times with 4 ml MEM to remove the virus from the body surface (Fig. 1, Step 3), after which the body surface was disinfected 3 times with 4 ml of 99.5% ethanol (EtOH; Nacalai Tesque; Fig. 1, Step 4). Finally, *M. macrocopa* were washed 3 times in 4 ml MEM to reduce the likelihood that EtOH would affect the EPC cells. Following the third wash, the MEM was passed through a 0.45 µm filter and tested for VHSV to determine whether all of the virus that had not been taken up had been removed by the procedure (Fig. 1, Step 5). The collected *M. macrocopa* were resuspended in 2 ml MEM (Fig. 1, Step 6) and homogenized using a glass homogenizer on ice. The homogenized *M. macrocopa* were then placed into a sterile 2 ml centrifuge tube and centrifuged at 250 × *g* for 10 min (Fig. 1, Step 7). The supernatant was passed through a 0.45 µm filter and used for virus isolation (Fig. 1, Step 8).

Fig. 1. Outline of the virus isolation procedure. EPC: epithelioma papulosum cyprini cell line; EtOH: ethanol; MEM: minimal essential medium; VHSV: viral haemorrhagic septicaemia virus

One day old cultures of EPC cells were drained and inoculated with 1 ml of the medium from Steps 2, 5, or 7 and adsorbed at 15°C for 1 h. Fresh medium supplemented with 10% FBS was then added and the flasks were incubated at 15°C for 14 d, over which time they were regularly checked for a cytopathic effect (CPE). At the end of the 14 d period, a blind passage was conducted and the cells were observed for a further 14 d. Total RNA was extracted from the second-passage supernatant using TRIzol® LS Reagent (Life Technologies) and tested by conventional RT-PCR.

In Expt 2, the samples were prepared as in Expt 1. They were then titrated on 96-well microplates with 1 d old EPC cells and incubated at 15°C for 21 d.

Exposure of rainbow trout to VHSV through feeding

Rainbow trout culture

Rainbow trout were bred from broodstock that were free from VHSV, infectious hematopoietic necrosis virus, and infectious pancreatic necrosis virus at the Tamaki Laboratory of the NRIA. The obtained eggs were disinfected with iodophor (200 mg l^{-1}, 15 min) after fertilization. Rainbow trout fry were fed commercial crumble diets (Ayutech, Marinetech) until 14–21 d after hatching and were then fed commercial pellets (Saki-Hikari®; Kyorin). All stages were maintained in well water at approximately 15–16°C to prevent any infection.

Expt 3: Feeding trial using VHSV-contaminated *M. macrocopa*

In total, 240 rainbow trout (mean +SD weight, 0.4 ± 0.16 g; length, 3.2 ± 0.3 cm) were used in the experimental infection, evenly divided into 8 treatment groups. Two groups (i.e. 60 fish) were fed 40 VHSV-contaminated *M. macrocopa* once daily for 14 d. Forty *M. macrocopa* were prepared each day during the feeding experiment using the same procedure as outlined for Expt 2. Briefly, *M. macrocopa* were immersed with DK-3592B (108.0 $TCID_{50}$ ml^{-1}) at 15°C for 1 d and then collected by filtration using cooking paper, washed with MEM, and disinfected with EtOH. The collected *M. macrocopa* were then resuspended in MEM. After the 14 d feeding period, the fish were fed a commercial diet once daily until the end of the experiment.

A further 2 groups of fish were challenged by immersing them in a 1:1000 dilution of the washing medium that was used after the *M. macrocopa* had been disinfected (Fig. 1, Step 5) for 1 h at 13°C, while 2 more groups were immersed in a 1:1000 dilution of the medium that was used to infect the *M. macrocopa* (actual viral titer, $10^{7.3}$ $TCID_{50}$ ml^{-1}) at 13°C for 1 h. In addition, 1 group was immersed in a 1:1000 dilution of VHSV DK-3592B ($10^{8.5}$ $TCID_{50}$ ml^{-1}) at 13°C for 1 h as a positive control. All of the fish in these 5 groups were fed a commercial diet once a day. The remaining group of rainbow trout was fed 40 normal *M. macrocopa* once a day for 14 d as a negative control, after which they were fed a commercial diet until the end of the experiment.

All 8 groups of rainbow trout were kept in 60 l tanks at an average of 13.8°C (range, 12.8–14.0°C). Mortality was observed over 28 d, after which samples were collected from all dead and surviving fish, inoculated on cell cultures, and tested by RT-PCR. This experimental infection was performed in accordance with the 'Guidelines for Animal Experimentation' of the NRIA's animal welfare regulations under license 28006.

Virological examination of rainbow trout

All fish that died during the experiment were examined virologically using EPC cell cultures. In addition, samples for virological examination were obtained from all surviving fish at the end of the trials. Fish were kept at –85°C until further processing. Kidney homogenates were suspended in approximately 50× the volume of MEM and filtered through a 0.45 µm filter. Aliquots of 100 µl and 10 µl of each filtrate were then inoculated onto subconfluent 1 d old EPC cells in 24-well culture plates and incubated at 15°C. The cells were observed for CPE over a period of 14 d.

Examination by RT-PCR

Total RNA was extracted from the supernatant of second-passage samples in Expt 1 and from the kidney of each fish in Expt 3 using TRIzol® Reagent (Life Technologies) following the recommended protocol. The total RNA was dissolved in 100 µl DNase/RNase-free distilled water (Life Technologies) and stored at –85°C until further processing. RT-PCR was performed using the SuperScript® One-Step RT-PCR System with Platinum® Taq (Life Technologies) and

the specific primers and thermocycling profile for VHSV described in the Manual of Diagnostic Tests for Aquatic Animals (OIE 2016), except for the volume, which was scaled down from 50 to 20 µl.

Influence of stomach and pyloric caecal contents on viral titer

The contents of the stomach and the pyloric caeca were removed from a rainbow trout 24 h after it had been fed a commercial diet. The contents of the stomach, the pyloric caeca, and the commercial diet were each homogenized, suspended in approximately 20× the volume of MEM, and centrifuged at 1800 × *g* (10 min, 4°C). The supernatant (900 µl) from each sample was then collected into 15 individual microtubes, and 100 µl of VHSV DK-3592B ($10^{7.5}$ $TCID_{50}$ ml^{-1}) were added. The mixture of MEM and VHSV was placed into 15 individual microtubes as a positive control. These tubes (n = 60) were incubated at 15°C for 0 (initial), 1, 2, 4, and 6 h, and then the incubated supernatants and MEM were passed through a 0.45 µm filter and the virus titers of the samples were measured using the EPC cell line at 15°C. Each treatment group at each incubation duration was analyzed in triplicate, and the mean values were then calculated.

To evaluate the influence of the stomach and pyloric caecal extracts of rainbow trout on the VHSV titer, the statistical difference between the titer values for each group at each incubation time was determined using Student's *t*-test; $p < 0.05$ was considered significant.

RESULTS

Expt 1: exposure of *Moina macrocopa* to VHSV

No mortality was observed in any of the groups of *M. macrocopa* during exposure to VHSV for 3, 24, 48, or 72 h. However, approximately 60% of the individuals in each group, including the negative control, had died by 144 h after immersion, likely due to the natural life span or starvation.

The results of the virus isolation and confirmatory RT-PCR are shown in Table 1. VHSV was re-isolated from the immersion medium and from all individuals when *M. macrocopa* were exposed to 10^7 $TCID_{50}$ ml^{-1}, regardless of the immersion period. Similarly, when *M. macrocopa* were exposed to 10^5 $TCID_{50}$ ml^{-1}, VHSV was re-isolated from all samples of immersion medium except that which was obtained 144 h after immersion and was found in individuals that had been immersed for 24–72 h. By contrast, although VHSV was re-isolated from the immersion medium 3 and 24 h after exposure to 10^3 $TCID_{50}$ ml^{-1}, it was not isolated from any individuals. VHSV was not isolated from the washing medium following disinfection with EtOH for any of the groups. The presence of VHSV was confirmed by RT-PCR in all samples that exhibited CPE in the cell cultures. All non-immersed *M. macrocopa* tested negative for VHSV.

Table 1. Results of virus isolation from *Moina macrocopa* immersed with viral haemorrhagic septicaemia virus (VHSV). VHSV was detected by reverse transcription polymerase chain reaction on second-passage cell culture supernatant. Each sample group consisted of 20 *M. macrocopa* individuals. Detection in *M. macrocopa* was carried out following disinfection of the body surface with ethanol

Immersion duration (h)	Virus concentration ($TCID_{50}$ ml^{-1})	Detection of VHSV in samples: Immersion medium	*M. macrocopa* with ethanol
3	10^7	+	+
	10^5	+	–
	10^3	+	–
	0 (negative control)	–	–
24	10^7	+	+
	10^5	+	+
	10^3	+	–
	0 (negative control)	–	–
48	10^7	+	+
	10^5	+	+
	10^3	–	–
	0 (negative control)	–	–
72	10^7	+	+
	10^5	+	+
	10^3	–	–
	0 (negative control)	–	–
144	10^7	+	+
	10^5	–	–
	10^3	–	–
	0 (negative control)	–	–

Expt 2: evaluation of viral titer in VHSV-exposed *M. macrocopa*

The sequential viral titers from *M. macrocopa* following immersion with VHSV DK-3592B are shown in Table 2. VHSV was detected in all *M. macrocopa* that had been immersed with $10^{8.0}$ $TCID_{50}$ ml^{-1} for 24–72 h, with the VHSV titers ranging from $10^{1.3}$ to

Table 2. Titers of viral haemorrhagic septicaemia virus (VHSV) in *Moina macrocopa* that had been immersed with VHSV for various lengths of time. Each sample group consisted of 40 *M. macrocopa* individuals

Immersion duration (h)	Virus titer ($TCID_{50}$ ml^{-1} or $sample^{-1}$) Immersion medium	*M. macrocopa*
24	$10^{7.3}$–$10^{7.8}$	$10^{1.5}$–$10^{4.7}$
48	$10^{7.8}$–$10^{7.9}$	$10^{1.3}$–$10^{3.1}$
72	$10^{7.5}$–$10^{7.7}$	$10^{3.8}$–$10^{4.7}$

$10^{4.7}$ $TCID_{50}$ $sample^{-1}$ (mean titer from 3 *M. macrocopa* samples = $10^{4.2}$, $10^{2.7}$, and $10^{4.3}$ $TCID_{50}$ $sample^{-1}$ at 24, 48, and 72 h after exposure, respectively). The VHSV titers obtained from the immersion medium ranged from $10^{7.3}$ to $10^{7.9}$ $TCID_{50}$ ml^{-1}. No virus was detected in any of the washing medium samples that were obtained after disinfection with EtOH.

Expt 3: Feeding trial using VHSV-contaminated *M. macrocopa*

The cumulative mortalities and results of RT-PCR and virus isolation from rainbow trout that were fed VHSV-contaminated *M. macrocopa* are shown in Table 3. No mortalities were observed over the 28 d experimental period in any of the fish that were fed VHSV-contaminated *M. macrocopa* for 14 d. Furthermore, VHSV was not isolated from any of the fish that were fed VHSV-contaminated *M. macrocopa*, and the VHSV genome was not detected by RT-PCR in any of the samples from these fish.

The cumulative mortalities of the rainbow trout that were challenged with immersion medium and VHSV DK-3592B isolate were 20 and 93%, respectively, whereas no mortality was observed in the fish that were challenged with washing medium after surface disinfection of *M. macrocopa* or in the negative control group. VHSV was isolated from all dead fish and a few of the surviving fish in the infected groups, and the VHSV genome was detected by RT-PCR in all of the samples that were obtained from fish that died during the experiment and from 1 surviving fish in the infected groups. None of the samples from fish that survived infection tested positive for VHS, and all non-infected fish tested negative. VHSV was not detected in the negative control group.

Influence of stomach and pyloric caecal contents on viral titer

To assess the reduction in VHSV in the digestive system of rainbow trout after feeding, the VHSV titers were assessed after up to 6 h incubation in MEM, diet extracts, and extracts of the stomach and pyloric caecal contents (Fig. 2). VHSV titers in the MEM, diet extracts, and stomach contents did not significantly decrease over the 6 h incubation period at 15°C, with titers ranging from $10^{5.8}$ to $10^{6.5}$ $TCID_{50}$ ml^{-1}. However, the VHSV titer in the pyloric caecal

Table 3. Cumulative mortality rates in the infection trials and detection of viral haemorrhagic septicaemia virus (VHSV) in the tested rainbow trout *Oncorhynchus mykiss*. The fish were tested individually for VHSV by cell culture, and the findings were confirmed by reverse transcription polymerase chain reaction (RT-PCR) of kidney material

Group	Tank ID	Cumulative mortality (%)	Dead fish (positive/tested)		Surviving fish (positive/tested)	
			Cell culture	RT-PCR	Cell culture	RT-PCR
Fed VHSV-contaminated	1	0			0/30	0/30
Moina macrocopa	2	0			0/30	0/30
	Mean	0				
Washing medium after disinfection	1	0			0/30	0/30
with ethanol	2	0			0/30	0/30
	Mean	0				
Immersion medium	1	13	4/4	4/4	2/26	1/26
	2	27	8/8	8/8	1/22	0/22
	Mean	20				
DK-3592B (positive control)		93	28/28	28/28	0/2	0/2
Fed non-treated *M. macrocopa*		0			0/30	0/30

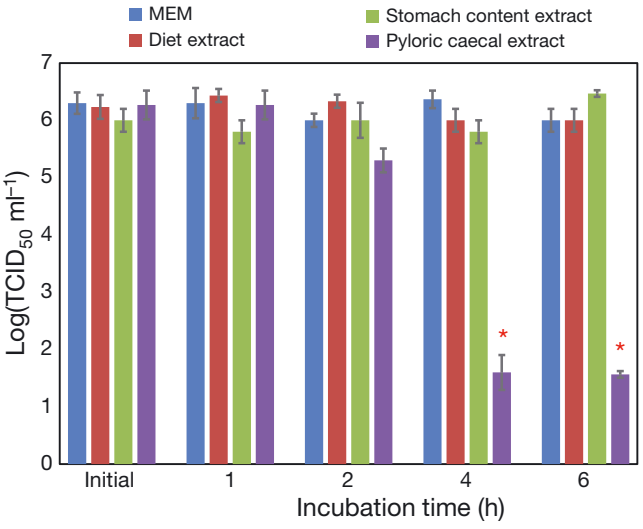

Fig. 2. Changes in viral haemorrhagic septicaemia virus titers after incubation with minimum essential medium (MEM), diet extract, and extracts of the stomach or pyloric caecal contents from rainbow trout *Oncorhynchus mykiss*. The error bars indicate the standard deviations of the means (n = 3). Asterisks indicate that the virus titers were significantly different from those of the other groups at a particular incubation time ($p < 0.05$, Student's *t*-test)

contents significantly decreased from $10^{6.3}$ to $10^{1.6}$ $TCID_{50}$ ml^{-1} after 4 and 6 h incubations ($p < 0.05$, Student's *t*-test).

DISCUSSION

Faisal & Winters (2011) successfully isolated VHSV from amphipods belonging to the genus *Diporeia* in the Laurentian Great Lakes. However, it remains unknown whether crustacean zooplankton can act as vectors of VHSV for fish species (OIE 2016), and whether VHSV is incorporated into and can multiply in zooplankton. Therefore, in this study we examined whether infectious VHSV remains viable in crustacean zooplankton by immersing *Moina macrocopa* that had been cultured under virus-free conditions in a solution containing VHSV and measuring the viral titers. In addition, to examine whether *M. macrocopa* could act as a vector of this disease, rainbow trout were fed VHSV-contaminated *M. macrocopa* and the occurrence of VHS in the fish was determined. Finally, the viability of VHSV following incubation with the contents of the rainbow trout gut system was measured to determine whether it could cause VHS.

VHSV was re-isolated from *M. macrocopa* that had been immersed with $10^{7.0}$ and $10^{5.0}$ $TCID_{50}$ ml^{-1} following disinfection of the outside of the body with EtOH. The detection period of VHSV in *M. macrocopa* depended on the virus concentration in the immersion solution (Table 1). If the virus had replicated in *M. macrocopa*, a gradual increase in the viral titer would be expected even when exposed to a low titer. Therefore, these results indicate that VHSV did not multiply in *M. macrocopa*.

Titers of $10^{2.7}$–$10^{4.3}$ $TCID_{50}$ $sample^{-1}$ were re-isolated from *M. macrocopa* that had been immersed in 10^{8} $TCID_{50}$ ml^{-1} of VHSV for 72 h, suggesting that high concentrations of VHSV in the environment could be incorporated into *M. macrocopa* and remain viable for several days. However, rainbow trout that were fed VHSV-contaminated *M. macrocopa* for 14 d did not suffer from VHS over a 28 d observation period. Furthermore, the titer of VHSV significantly decreased from $10^{6.3}$ to $10^{1.6}$ $TCID_{50}$ ml^{-1} after a 4 h incubation in pyloric caecal extract at 15°C. Windell et al. (1969) previously reported that rainbow trout require 36 h to digest a pellet diet at 15°C. Therefore, it is possible that VHS did not occur in the rainbow trout because the virus that was carried by *M. macrocopa* was inactivated in the pyloric caeca.

Infection of fish with VHSV through feeding is possible, however, as demonstrated by Schönherz et al. (2012), who found that 6 out of 25 rainbow trout died after intubation of a feed homogenate containing a recombinant strain of VHSV Genotype Ia (1.3×10^{8} $TCID_{50}$ ml^{-1}), and demonstrated that viral replication occurred in the stomach and kidney tissues. Sequential measurement of the VHSV titer of *M. macrocopa* in the present study (Table 2) indicated that the rainbow trout received approximately $10^{2–3}$ $TCID_{50}$ *M. macrocopa* $sample^{-1}$. Consequently, the different findings of these studies were likely due to differences in the viral titers and diet materials. Getchell et al. (2013) also reported that there were no mortalities in tiger muskellunge (male northern pike *Esox lucius* × female muskellunge *E. masquinongy*) following the consumption of VHSV IVb-infected fathead minnows *Pimephales notatus*, despite the VHSV N-gene copy number per 50 ng total RNA from pooled organ samples ranging from 9.1×10^{4} to 5.5×10^{8} in the fed group. Moreover, Snow et al. (2009) found that VHS did not occur in Atlantic cod *Gadus morhua* that had been fed pellets containing high levels of VHSV III (1×10^{7} $TCID_{50}$ g^{-1} feed). Neither of these reports explained why VHS did not occur in the fish species, but it is possible that VHSV in the feed may have been inactivated during the digestive process in these fish species. Digestibility varies according to food type, fish size, and water temperature (Fänge & Grove 1979), and so the viral titer that allows oral

transmission of VHSV may also depend on these parameters.

This study demonstrated that VHSV can be taken up from the environmental water by *M. macrocopa* and can remain viable within *M. macrocopa* for several days. However, sequential titration of VHSV and clinical observation indicated that *M. macrocopa* individuals were not affected by VHSV, making it unlikely that this crustacean would act as a vector of VHSV for rainbow trout due to inactivation during digestion. This study also highlights the value of *M. macrocopa* as a model for examining the relationship between zooplankton and piscine pathogenic viruses.

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