

Effects of experimentally induced infections of goldfish *Carassius auratus* with cyprinid herpesvirus 2 (CyHV-2) at various water temperatures

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ABSTRACT: In this study, we examined the influence of water temperature on the development of herpesviral haematopoietic necrosis (HVHN) in goldfish *Carassius auratus* after experimentally induced infection with cyprinid herpesvirus 2 (CyHV-2). In Expt 1, Ryukin goldfish were infected with CyHV-2 by intraperitoneal injection and maintained at 4 different water temperatures. Cumulative mortalities of the 15, 20, 25 and 30°C groups were 10, 90, 90 and 60%, respectively. Therefore, the temperature range of 20–25°C is considered highly permissive for HVHN. One of 6 surviving fish of the 15°C group died after a rapid temperature increase to 25°C at 30 d post infection. All 3 Edonishiki goldfish, co-reared with the surviving Ryukin in tanks where the water temperature was increased from 15 to 25°C, died. In Expt 2, Edonishiki goldfish were exposed to CyHV-2 by bath immersion at 13 or 24°C, resulting in cumulative mortalities of 0 and 87%, respectively, at 28 d post-exposure. No mortality of the surviving Edonishiki in the 13°C treatment was observed when the water temperature was increased to 24°C. In addition, in Expt 2, no mortality was observed in any Ranchu co-reared with CyHV-2-immersed Edonishiki in the group where water temperature was increased from 13 to 24°C, even after re-immersion challenge with CyHV-2. It is interesting to note that CyHV-2 DNA was detected in the kidneys of 4 of the 5 surviving Ranchu co-reared with the CyHV-2-immersed Edonishiki group where the water temperature was increased from 13 to 24°C. Therefore, it is likely that the surviving Edonishiki of the 13°C group were virus carriers. This study indicates that most fish infected with CyHV-2 at 13–15°C acquire resistance to HVHN, but as carriers they are able to infect naïve fish.

KEY WORDS: *Cyprinid herpesvirus 2* · CyHV-2 · Resistance · *Carassius auratus*

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INTRODUCTION

The fish disease herpesviral haematopoietic necrosis (HVHN), caused by infection with cyprinid herpesvirus 2 (CyHV-2), has led to mortalities of goldfish *Carassius auratus* in Asia, North America, Oceania and Europe (Jung & Miyazaki 1995, Groff et al. 1998, Chang et al. 1999, Stephens et al. 2004, Goodwin et al. 2006, Hine et al. 2006, Jeffery et al. 2007). In June 2013, a major fish kill of adult goldfish occurred in

Runnemede Lake, NJ, USA: an estimated 3000–5000 fish died within ~5 d, and the etiological agent was confirmed as CyHV-2 (Lovy & Friend 2014). Moreover, other recent studies have reported that mass mortalities caused by CyHV-2 infection occurred in Prussian carp *Carassius gibelio* in China (Wang et al. 2012, Xu et al. 2013) and the Czech Republic (Daněk et al. 2012). Furthermore, Fichi et al. (2013) detected this virus from crucian carp *Carassius carassius* in Italy. These recent reports suggest

that this virus has been spread by movement of diseased or carrier fish and that CyHV-2 has the potential to infect species other than goldfish of the genus *Carassius*.

In order to prevent further spread of the disease to new watersheds, basic studies on the characteristics of the virus and the disease are essential. However, there are no reports on the influence of water temperature on the development of HVHN in goldfish since the ability to isolate virus from diseased fish and propagation of the virus in cell culture have been problematic (Jung & Miyazaki 1995, Li & Fukuda 2003). Recently, a method has been developed to enable virus isolation and replication of CyHV-2 using a cell line established from goldfish fin (GFF) (Ito et al. 2013). Furthermore, experimental infections of goldfish by immersion as well as intraperitoneal (IP) injection with the cultured virus were successful (Ito et al. 2013). Subsequently, Ito & Ootake (2013) reported on the potential efficacy of vaccines derived from formalin-inactivated cell culture supernatant of CyHV-2-infected GFF cells against HVHN in goldfish. Furthermore, we demonstrated that sensitivity of indigenous Cyprininae fish species in Japan to CyHV-2 is much lower than that of goldfish (Ito & Maeno 2014).

In this report, we describe the influence of water temperature on the development of HVHN in goldfish after experimentally induced infection. In addition, this study investigated possible of release of the virus to the environment from the surviving host fish which had acquired resistance to CyHV-2.

MATERIALS AND METHODS

Cell line

The GFF cell line (Li & Fukuda 2003, Ito et al. 2013) from the fin of the Ryukin variety of goldfish *Carassius auratus* was used for this study. Cultures of the cell line 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 mg streptomycin ml⁻¹) at 25°C, according to standard procedures (Gravell & Malsberger 1965)

Virus

The virus used for the present study was the CyHV-2 Saitama-1 (SaT-1) isolate, which was isolated in GFF cells from diseased goldfish, calico variety, in

Saitama Prefecture, Japan, in 1999 (Ito et al. 2013). The CyHV-2 SaT-1 isolate was sub-cultured 5 times according to Ito et al. (2013) in GFF cells in 75 cm² flasks (Greiner Bio-one) at 25°C. Aliquots of the cell culture supernatant containing CyHV-2 were placed in 3.6 ml cryo tubes (Nunc®, Thermo Fisher Scientific) and stored at –85°C until use. One aliquot was thawed, and each of the 8 well columns of 96-well microplates, seeded with newly sub-cultured GFF cell suspension, were inoculated serially with 10-fold dilutions of the viral stock and incubated at 25°C to obtain the TCID₅₀ titre of the viral stock used in the subsequent experiments. This titre was calculated according to Reed & Muench (1938).

Fish

Three varieties of *C. auratus*, Ryukin, Edonishiki and Ranchu, were used for the experimentally induced infections. The different varieties of goldfish were used to distinguish originally stocked fish and co-reared fish. All varieties of goldfish were bred from CyHV-2-free broodstock at the Tamaki laboratory, National Research Institute of Aquaculture (NRIA). Eggs of all 3 varieties were disinfected with iodophor (200 mg l⁻¹, 15 min) immediately after fertilization. Fry of all 3 varieties of goldfish were fed water fleas (*Daphnia* sp.) until 14–21 d after hatching and were fed a commercial pellet (Saki-Hikari®, Kyorin) from 21 d onwards. All stages were maintained in well water to prevent any infection.

Infection experiment 1: determination of the optimal temperature for HVHN

The experimental design and fish size of each experimental group are summarized in Table 1. We used 92 goldfish: 80 Ryukin and 12 Edonishiki individuals. Ryukin were divided into 8 groups of 10 fish each. Two groups each of Ryukin were kept at 15.5 (range, 15.0 to 15.8°C), 20.0 (19.5 to 20.5°C), 24.6 (24.0 to 25.0°C) and 30.0°C (29.4 to 30.5°C) as the designated groups 15, 20, 25 and 30°C, respectively. Fish of each group were then acclimated to the experimental water temperature for 10 d before infection. Fish from 4 different temperature groups were IP injected with 0.1 ml supernatant from CyHV-2-infected cell culture of the GFF cell line with a titre of 10^{3.0} TCID₅₀ ml⁻¹. Fish from the other 4 different temperature groups were injected with cell culture medium without virus as the negative controls. All groups of

Table 1. Experimental design and mean \pm SD total length and body weight of goldfish used in this study. CyHV-2: cyprinid herpesvirus 2; IP: intraperitoneal; dpi: days post infection

Expt	Variety	Treatment	Water temperature group	n	Total length (cm)	Body weight (g)			
1	Ryukin	IP CyHV-2	15°C (change to 25°C from 30 dpi)	10	7.70 \pm 0.70	10.5 \pm 2.52			
			Co-reared Edonishiki (from 30 dpi)	3	7.90 \pm 0.69	15.0 \pm 2.91			
			20°C	10	7.75 \pm 0.80	10.6 \pm 2.83			
			25°C	10	7.61 \pm 0.55	11.7 \pm 2.01			
			30°C (change to 25°C from 30 dpi)	10	7.50 \pm 0.77	10.4 \pm 2.22			
			Co-reared Edonishiki (from 30 dpi)	3	7.13 \pm 0.40	17.5 \pm 3.27			
			IP cell culture medium (negative control)	15°C (change to 25°C from 30 dpi)	10	7.83 \pm 0.58	10.2 \pm 1.46		
			Co-reared Edonishiki (from 30 dpi)	3	7.20 \pm 0.69	15.9 \pm 2.64			
			20°C	10	7.30 \pm 0.36	10.7 \pm 1.50			
			25°C	10	7.10 \pm 0.36	11.4 \pm 2.38			
			30°C (change to 25°C from 30 dpi)	10	7.23 \pm 1.05	11.6 \pm 3.47			
			Co-reared Edonishiki (from 30 dpi)	3	7.17 \pm 0.76	13.9 \pm 2.56			
			2	Edonishiki	Immersion + re-immersion CyHV-2 (42 dpi)	13°C (change to 24°C from 28 dpi)	15	7.50 \pm 0.46	24.4 \pm 4.62
						Co-reared Ranchu (from 28 dpi)	5	7.44 \pm 0.65	22.7 \pm 3.08
24°C	15	8.12 \pm 0.71				23.4 \pm 5.04			
Co-reared Ranchu (from 28 dpi)	5	8.26 \pm 0.46				29.4 \pm 8.17			
Immersion	13°C (change to 24°C from 28 dpi)	15				7.30 \pm 0.72	23.5 \pm 4.97		
Cell culture medium (negative control)	Co-reared Ranchu (from 28 dpi)	5				7.96 \pm 0.51	30.3 \pm 4.75		
24°C	15	7.45 \pm 1.21				21.6 \pm 6.69			
Co-reared Ranchu (from 28 dpi)	5	7.62 \pm 0.44				28.9 \pm 6.04			

Ryukin were kept in 60 l tanks at the 4 different water temperatures, fed a commercial diet (Saki-Hikari[®], Kyorin) once a day and observed over a 30 d period. At 30 d post infection (dpi), the experiment was terminated with: 3 of 9 surviving Ryukin from the infected 15°C group, 2 of 4 surviving Ryukin from the infected 30°C group and 5 of 10 surviving Ryukin from the negative control groups of 15 and 30°C. These fish were euthanized by 2-phenoxyethanol diluted at 1:500 with well water and used for PCR analysis and virus isolation. The others, 6 of 9 surviving Ryukin from the infected 15°C group, 2 of 4 surviving Ryukin from the infected 30°C group and 5 of 10 surviving Ryukin from the negative control groups of 15 and 30°C, were used to investigate the influence of water temperature change for HVHN. Water temperatures of the 15 and 30°C groups were rapidly changed to 25°C within 3 h to simulate possible temperature fluctuation during transport for auction and retail sale. Additionally, to investigate whether the surviving Ryukin from these 2 groups became virus carriers, the 12 naïve Edonishiki were divided into 4 groups of 3 fish each and were co-reared with the surviving Ryukin from the IP injected groups and the negative control groups at the adjusted water temperature. The kidneys of all dead and surviving fish were tested for the presence of CyHV-2 DNA according to Waltzek et al. (2009) and for infectious virus by virus re-isolation according to Ito et al. (2013).

Infection experiment 2: investigation of resistance acquisition by surviving host fish after CyHV-2 infection

The experimental design and fish size of each experimental group are summarized in Table 1. We used 80 goldfish: 60 Edonishiki and 20 Ranchu individuals. Edonishiki were divided into 4 groups of 15 fish each. Two groups of Edonishiki were each kept at 12.8 (range, 12.1 to 13.0°C) and at 24.3°C (23.8 to 24.5°C) as the designated groups 13 and 24°C, respectively. Fish of each group were then acclimated to the experimental water temperature for 10 d before infection. One group of each of the 2 water temperature fish groups was immersed for 1 h in viral culture supernatant ($10^{3.0}$ TCID₅₀ ml⁻¹) diluted at 1:1000 with well water at the respective water temperatures. The other groups from the 2 different water temperatures were immersed similarly in diluted cell culture medium without the virus as a negative control. All groups were kept in 60 l tanks and fed a commercial diet (Saki-Hikari[®], Kyorin) once a day and their cumulative mortalities were scored for 28 d. At 28 dpi, the water temperature of the 13°C groups was increased to 24°C within 3 h, to simulate possible temperature fluctuations during transport and temporary holding of fish at auction. Subsequently, the 20 Ranchu were divided into 4 groups of 5 fish each and were co-reared with the surviving Edonishiki of each

of the 4 experimental groups. Furthermore, to investigate whether the surviving fish had acquired resistance to CyHV-2, a re-immersion challenge using the same preparation of virus as the first infection was carried out for both infected groups including the co-reared Ranchu at 42 dpi. Fish of both re-challenged groups and the negative control group were observed for cumulative mortality for 14 d after re-immersion. At Day 56, the experiment was finished: all surviving fish were euthanized using 2-phenoxyethanol diluted at 1:500 in well water. The kidneys of all dead and surviving fish were tested for the presence of CyHV-2 DNA according to Waltzek et al. (2009) and for infectious virus by virus re-isolation according to Ito et al. (2013).

Detection of viral DNA from fish kidney by PCR

For the specific detection of CyHV-2, the PCR technique described by Waltzek et al. (2009) was employed. The DNA template for positive control was extracted from the supernatant of virus-infected cell culture using InstraGene™ Matrix (Bio-Rad Laboratories). For samples from experimentally infected fish, total DNA was extracted from the kidney of all fish using Gentra Puregene Tissue Kit (Qiagen). The PCR reaction mixture used was *TaKaRa Ex Taq*® Hot Start Version (TaKaRa) according to the manufacturer's protocol. The thermocycling profile was performed according to Waltzek et al. (2009).

Virus re-isolation from experimentally infected fish

The virus re-isolation method described by Ito et al. (2013) was employed. Briefly, homogenates of the kidney of all individual experimentally infected fish were prepared using an approximately 50-times volume of MEM, after which these were filtered (0.45 µm). One day after seeding GFF cells in a 25 cm² flask (Greiner Bio-one), the culture medium was removed and 500 µl of the kidney preparation was added and absorbed at 25°C for 1 h, after which fresh medium supplemented with 10% FBS was added and the cultures were incubated at 25°C. The cells were observed each day for 14 d for cytopathic effect.

Statistical analysis

The statistical difference between each infected group and negative control group was determined by

Fisher's exact test. Differences of $p < 0.01$ were considered significant.

RESULTS

Infection experiment 1

Cumulative mortality curves and results of detection of CyHV-2 DNA by PCR and re-isolation of virus from Expt 1 fish are shown in Fig. 1 and Tables 2 & 3, respectively. Cumulative mortalities of Ryukin after IP injection of the group of 15, 20, 25 and 30°C were 10, 90, 90 and 60%, respectively (Fig. 1A). Mortality of fish in the 30°C group was observed from 1 dpi but ceased after 5 dpi. The period in which the 25°C group fish died was from 5 to 12 dpi. The onset of fish mortality in the 20°C group was later than in the 25°C group, i.e. from 8 to 16 dpi. One fish of the 15°C group died at 18 dpi. The mortality rates in the infected 20, 25 and 30°C groups were significantly higher than those of the parallel negative control groups (0% mortality).

In goldfish from Expt 1, CyHV-2 DNA was detected at 30 dpi from the kidneys of all surviving fish as well as all dead fish that had been experimentally infected with CyHV-2 in all temperature groups (Table 2). The virus was re-isolated from the kidneys of dead experimentally infected fish, in most cases, from all water temperature groups (Table 2). No cytopathic effect was observed in attempted virus isolations from the surviving fish from Expt 1 at 30 dpi. No CyHV-2 viral DNA was detected from the kidneys of the negative control fish.

One of 6 surviving Ryukin of the 15°C group died 3 d after the water temperature was changed to 25°C. All 3 naïve Edonishiki co-reared with the surviving fish of the 15°C group died within ~12 d of co-rearing. However, 5 of 6 surviving Ryukin of the 15°C group showed no mortality 21 d after the water temperature change. Two surviving Ryukin of the 30°C group and 3 Edonishiki co-reared with the surviving fish did not show any clinical signs, and no mortality was observed during 21 d (Fig. 1B). At 21 d after the water temperature change, CyHV-2 DNA was detected from the kidneys of a dead experimentally infected Ryukin and the 3 dead co-reared Edonishiki in the group in which the water temperature was changed from 15 to 25°C (Table 3). The virus was re-isolated from the kidneys of the dead Ryukin and Edonishiki. Furthermore, CyHV-2 DNA was detected from all surviving fish in the experimentally infected groups in which the water temperature had been changed from 15 to 25°C

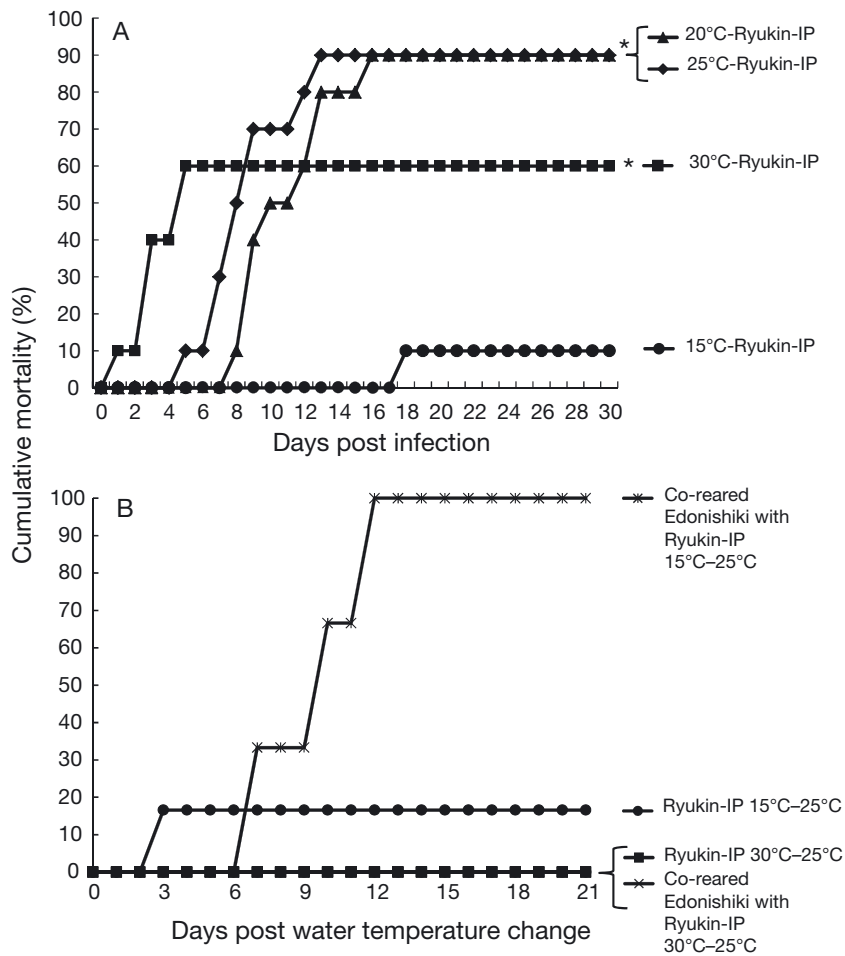


Fig. 1. Cumulative mortality curves for goldfish experimentally infected with cyprinid herpesvirus 2 (CyHV-2) in Expt 1. (A) Intraperitoneal (IP) injection at different water temperatures (●: infected at 15°C; ▲: infected at 20°C; ◆: infected at 25°C; ■: infected at 30°C). (B) Mortality of infected Ryukin and co-reared Edonishiki after change in water temperature (●: infected Ryukin from 15 to 25°C; ×: co-reared Edonishiki with infected Ryukin from 15 to 25°C; ■: infected Ryukin from 30 to 25°C; ×: co-reared Edonishiki with infected Ryukin from 30 to 25°C). No mortality was observed in any of the negative control groups. Note that some symbols are hidden behind others. * in (A) indicate that the mortality rate at the end of the experiment was significantly different from that of the negative control ($p < 0.01$, Fisher's exact test)

and 30 to 25°C, although the virus was not re-isolated from these surviving fish (Table 3). No CyHV-2 viral DNA was detected from the kidneys of the negative control fish. By the end of the experiment, no mortality was observed in any of the non-infected fish groups.

Infection experiment 2

Cumulative mortality curves and results of the detection of CyHV-2 DNA by PCR and re-isolation of

the virus from Expt 2 fish are shown in Fig. 2 and Table 4, respectively. In Expt 2, using the Edonishiki variety that were experimentally infected by bath immersion at 13 and 24°C, cumulative mortalities were 0 and 87%, respectively (Fig. 2). The mortality rates in the experimentally infected 24°C group were significantly higher than those in the respective negative controls. From 28 dpi, at which the water temperature of the 13°C group was increased to 24°C, no mortality of any of the surviving fish was observed during 14 d. Furthermore, naïve Ranchu co-reared with all groups where the temperature was rapidly changed did not show any clinical signs during 14 d. Following re-immersion with CyHV-2 at 42 dpi, all 5 Ranchu co-reared with the infected Edonishiki 24°C group died within 9 d post exposure. However, no Edonishiki or co-reared Ranchu in the experimentally infected group in which the water temperature was changed from 13 to 24°C showed any clinical signs. By the end of the experiment, no mortality was observed in any of the non-infected fish groups.

CyHV-2 DNA was detected and the virus was re-isolated from the kidneys of all dead Edonishiki in the 24°C group. CyHV-2 DNA was detected from the kidneys of all surviving Edonishiki from the 24°C group. However, the virus isolations from the kidney of these survivors were negative. CyHV-2 viral DNA was detected in dead Ranchu co-reared with surviving Edonishiki of the 24°C group, and the virus was re-isolated from the kidney of all 5 individuals. For the surviving Edonishiki and co-reared Ranchu of the group in which the water temperature was changed from 13 to 24°C, CyHV-2 DNA was detected from 5 of the 15 surviving fish and 4 of the 5 surviving co-reared Ranchu at 42 dpi, although the virus was not re-isolated from the kidney of these fish (Table 4). CyHV-2 viral DNA was not detected from the kidney of the negative control fish.

Table 2. Results of cumulative mortality in each group and detection of cyprinid herpesvirus 2 (CyHV-2) DNA and re-isolation of the virus from the kidneys of experimental Ryukin from Expt 1. Values are given as number of positive fish/number of fish tested. NT: not tested; IP: intraperitoneal

Treatment	Water temperature group	Cumulative mortality (%)	Dead fish		Surviving fish	
			PCR	Virus re-isolation	PCR	Virus re-isolation
IP CyHV-2	15°C	10	1/1	1/1	3/3 ^a	0/3 ^a
	20°C	90	9/9	8/9	1/1	0/1
	25°C	90	9/9	9/9	1/1	0/1
	30°C	60	6/6	4/6	2/2 ^a	0/2 ^a
IP cell culture medium (negative control)	15°C	0	–	–	0/5 ^a	NT
	20°C	0	–	–	0/10	NT
	25°C	0	–	–	0/10	NT
	30°C	0	–	–	0/5 ^a	NT

^aSurviving fish were used for the water temperature change experiment and co-reared with naïve Edonishiki

Table 3. Results of detection of cyprinid herpesvirus 2 (CyHV-2) DNA and re-isolation of the virus from the kidneys of Ryukin from Expt 1 (water temperature change), which were co-reared with naïve Edonishiki. Values are given as the number of positive fish/number of fish tested. NT: not tested; IP: intraperitoneal

Treatment	Water temperature group	Cumulative mortality (%)	Dead fish		Surviving fish	
			PCR	Virus re-isolation	PCR	Virus re-isolation
IP CyHV-2	25°C from 15°C (6 fish)	17	1/1	1/1	5/5	0/5
	Co-reared Edonishiki (3 fish)	100	3/3	3/3	–	–
	25°C from 30°C (2 fish)	0	–	–	2/2	0/2
	Co-reared Edonishiki (3 fish)	0	–	–	0/3	0/3
IP cell culture medium (negative control)	25°C from 15°C (5 fish)	0	–	–	0/5	NT
	Co-reared Edonishiki (3 fish)	0	–	–	0/3	NT
	25°C from 30°C (5 fish)	0	–	–	0/5	NT
	Co-reared Edonishiki (5 fish)	0	–	–	0/3	NT

DISCUSSION

This paper is the first report to detail the influence of water temperature on HVHN of goldfish after experimentally induced infection. In Expt 1, fish were CyHV-2 infected by IP injection at various water temperatures to estimate the optimal water temperature for CyHV-2 infection. In Expt 2, the effects of increasing water temperature on the survival of fish after CyHV-2 infection by bath were studied under simulated culture conditions.

The first death occurred earlier in the 25°C group than in the 20°C group. This result is consistent with the study of Ito et al. (2013), in which the progression of disease appeared to be more rapid at 25°C than at 20°C. Although fish death in the 30°C group occurred more rapidly than in the other groups, fish mortality reached a plateau of 60% at 5 dpi. The results demonstrated that water temperatures of 20–25°C are most permissive for HVHN.

In Expt 1, the mortality of Ryukin in the 15°C group after IP injection was 10%. After the water tempera-

ture had been changed to 25°C, 5 of the 6 fish survived. Moreover, in Expt 2, no mortality of Edonishiki of the 13°C group after bath immersion with CyHV-2 (at a dose that caused 87% mortality at 24°C) was observed and all individuals survived an increase in water temperature to 24°C after re-immersion challenge with CyHV-2. CyHV-2 DNA was detected from 5 of the 15 surviving Edonishiki. These results indicate that most fish challenged with CyHV-2 at 13–15°C were resistant to infection, and fish infected at the low temperature were able to survive at 24°C. In contrast to our findings in the present study with CyHV-2, carp infected with the closely related cyprinid herpesvirus 3 (CyHV-3) at 12°C exhibited no mortality for 30 wk, but began to die after the water temperature reached 21–23°C (St-Hilaire et al. 2005). In addition, the most permissive water temperatures for koi herpesvirus disease caused by CyHV-3 infection are in the range of 18–23°C (Gilad et al. 2003), similar to the temperature range for HVHN.

Ito et al. (2013) reported that CyHV-2 DNA was detected from infected fish at 8 d post exposure.

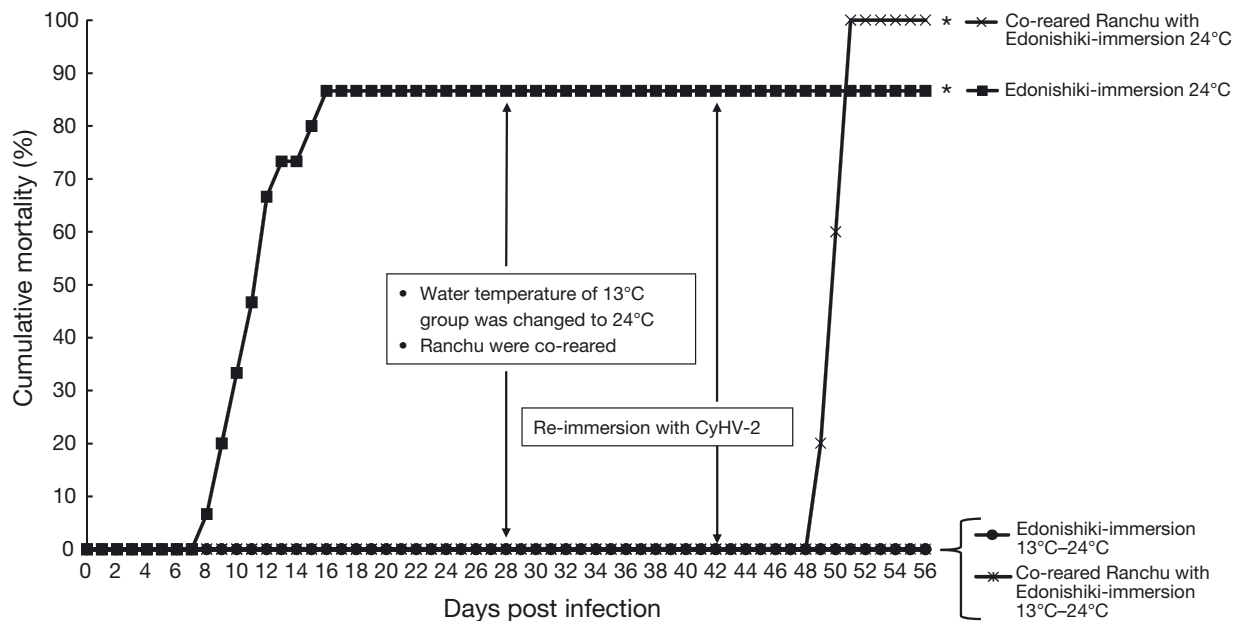


Fig. 2. Cumulative mortality curves for goldfish (Ryukin and Edonishiki) experimentally infected with cyprinid herpesvirus 2 (CyHV-2) in Expt 2. Bathing immersion under different water temperatures: (●: infected Edonishiki at 13°C; ■: infected Edonishiki at 24°C; ×: co-reared Ranchu with infected Edonishiki from 13°C to 24°C; ×: co-reared Ranchu with infected Edonishiki at 24°C). No mortality was observed in any of the negative control groups. Note that some symbols are hidden behind others. Asterisks (*) indicate that the mortality rate at the end of the experiment was significantly different from that of the negative control ($p < 0.01$, Fisher's exact test)

Table 4. Results of detection of cyprinid herpesvirus 2 (CyHV-2) DNA and re-isolation of the virus from the kidneys of Edonishiki from Expt 2. Values are given as the number of positive fish/number of fish tested. NT: not tested; dpi: days post infection

Treatment	Water temperature group	Cumulative mortality (%)		Dead fish		Surviving fish (at 42 dpi)	
		First immersion (by 28 dpi)	Re-immersion (from 42 dpi)	PCR	Virus re-isolation	PCR	Virus re-isolation
Immersion CyHV-2	13°C–Edonishiki (change to 24°C from 28 dpi)	0	0	–	–	5/15	0/15
	Co-reared Ranchu (from 28 dpi)	–	0	–	–	4/5	0/5
	24°C–Edonishiki	87	0	13/13	13/13	2/2	0/2
	Co-reared Ranchu (from 28 dpi)	–	100	5/5	5/5	–	–
Immersion	13°C–Edonishiki (change to 24°C from 28 dpi)	0	0	–	–	0/15	NT
Cell culture medium (negative control)	Co-reared Ranchu (from 28 dpi)	–	0	–	–	0/5	NT
	24°C–Edonishiki	0	0	–	–	0/15	NT
	Co-reared Ranchu (from 28 dpi)	–	0	–	–	0/5	NT

However, in another study, CyHV-3 DNA was detected from infected fish at 1 d post exposure (Gilad et al. 2004). These results indicate that the viral kinetics of CyHV-3 in carp differ significantly from those of CyHV-2 in goldfish. Moreover, a formalin-inactivated vaccine was effective against CyHV-2 infection in goldfish (Ito & Ootake 2013), although a simple inactivated vaccine against CyHV-3 infection in carp was not effective (Ilouze et al. 2011). Taken to-

gether with previous studies, the difference in development of resistance at low water temperatures between CyHV-2 infection in goldfish and CyHV-3 infection in carp indicates a fundamental difference in the interactions between these fish species and the respective pathogenic viruses.

The mechanism by which most goldfish infected with CyHV-2 at 13–15°C acquire resistance to the virus might be triggered on-farm, in auction ponds, in

markets and in retail sites. In Expt 1, 1 of 6 surviving Ryukin of the group of 15°C died after the water temperature was changed to 24°C, and mortality of Edonishiki that were co-reared with fish of this group was observed (Table 3). In addition, in Expt 2, no mortality was observed in any Rancho co-reared with CyHV-2-immersed Edonishiki in the group where water temperature was increased from 13 to 24°C, even after re-immersion challenge with CyHV-2. In contrast, all 5 Rancho co-reared with the 24°C Edonishiki group died (Table 4). It is interesting to note that CyHV-2 DNA was detected in the kidneys of 4 of the 5 surviving Rancho co-reared with the CyHV-2-immersed Edonishiki group where the water temperature was increased from 13 to 24°C. Detection of CyHV-2 DNA from the surviving Rancho strongly suggested virus transmission occurred from Edonishiki immersed with CyHV-2 at 13°C, although the infection might be very weak. Therefore, these results indicate that the virus could be transmitted by surviving fish that have acquired resistance to HVHN via infection CyHV-2 under low water temperature conditions. This feature of the disease may be one of the reasons for the spread of CyHV-2, since goldfish are harvested and transported to auction ponds in the winter season. Furthermore, the spread of the virus might cause mass mortalities of goldfish when any one or more of several stress factors occur, such as spawning or sudden temperature changes, e.g. during transport for auction and purchase.

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LITERATURE CITED

- Chang PH, Lee SH, Chiang HC, Jong MH (1999) Epizootic of herpes-like virus infection in goldfish, *Carassius auratus* in Taiwan. *Fish Pathol* 34:209–210
- Daněk T, Kalous L, Veselý T, Krásová E and others (2012) Massive mortality of Prussian carp *Carassius gibelio* in the upper Elbe basin associated with herpesviral hematopoietic necrosis (CyHV-2). *Dis Aquat Org* 102:87–95
- Fichi G, Cardeti G, Cocumelli C, Vendramin N and others (2013) Detection of cyprinid herpesvirus 2 in association with an *Aeromonas sobria* infection of *Carassius carassius* (L.), in Italy. *J Fish Dis* 36:823–830
- Gilad O, Yun S, Adkison MA, Way K, Willits NK, Bercovier H, Hedrick RP (2003) Molecular comparison of isolates of an emerging fish pathogen, the koi herpesvirus, and the effect of water temperature on mortality of experimentally infected koi. *J Gen Virol* 84:2661–2668
- Gilad O, Yun S, Zagmutt-Vergara FJ, Leutenegger CM, Bercovier H, Hedrick RP and others (2004) Concentrations of a koi herpesvirus (KHV) in tissues of experimentally infected *Cyprinus carpio koi* as assessed by real-time TaqMan PCR. *Dis Aquat Org* 60:179–187
- Goodwin AE, Khoo L, LaPatra SE, Bonar C and others (2006) Goldfish hematopoietic necrosis herpesvirus (cyprinid herpesvirus 2) in the USA: molecular confirmation of isolates from diseased fish. *J Aquat Anim Health* 18:11–18
- Gravell M, Malsberger RG (1965) A permanent cell line from the fathead minnow (*Pimephales promelas*). *Ann NY Acad Sci* 126:555–565
- Groff JM, LaPatra SE, Munn RJ, Zinkl JG (1998) A viral epizootic in cultured populations of juvenile goldfish due to a putative herpesvirus etiology. *J Vet Diagn Invest* 10:375–378
- Hine PM, Tham KM, Morrison R (2006) Cyprinid herpesvirus 2 in New Zealand goldfish. *Surveillance* 33:3–5
- Ilouze M, Davidovich M, Diamant A, Kotler M, Dishon A (2011) The outbreak of carp disease caused by CyHV-3 as a model for new emerging viral diseases in aquaculture: a review. *Ecol Res* 26:885–892
- Ito T, Maeno Y (2014) Susceptibility of Japanese Cyprininae fish species to cyprinid herpesvirus 2 (CyHV-2). *Vet Microbiol* 169:128–134
- Ito T, Ototake M (2013) Vaccination against cyprinid herpesvirus 2 (CyHV-2) infection in goldfish *Carassius auratus*. *Bull Eur Assoc Fish Pathol* 33:158–164
- Ito T, Kurita J, Ozaki A, Sano M, Fukuda H, Ototake M (2013) Growth of cyprinid herpesvirus 2 (CyHV-2) in cell culture and experimental infection of goldfish *Carassius auratus*. *Dis Aquat Org* 105:193–202
- Jeffery KR, Bateman K, Bayley A, Feist SW and others (2007) Isolation of a cyprinid herpesvirus 2 from goldfish, *Carassius auratus* (L.), in the UK. *J Fish Dis* 30:649–656
- Jung SJ, Miyazaki T (1995) Herpesviral haematopoietic necrosis of goldfish, *Carassius auratus* (L.). *J Fish Dis* 18:211–220
- Li X, Fukuda H (2003) *In vitro* culture of goldfish cell sensitive to goldfish herpesvirus. *J Shanghai Fish Univ* 12:12–18 (Shanghai Shuichan Daxue Xuebao)
- Lovy J, Friend SE (2014) Cyprinid herpesvirus-2 causing mass mortality in goldfish: applying electron microscopy to histological samples for diagnostic virology. *Dis Aquat Org* 108:1–9
- Reed LJ, Muench H (1938) A simple method of estimating fifty per cent endpoints. *Am J Hyg* 27:493–497
- St-Hilaire S, Beevers N, Way K, Le Deuff RM, Martin P, Joiner C (2005) Reactivation of koi herpesvirus infections in common carp *Cyprinus carpio*. *Dis Aquat Org* 67:15–23
- Stephens FJ, Raidal SR, Jones B (2004) Haematopoietic necrosis in a goldfish (*Carassius auratus*) associated with an agent morphologically similar to herpesvirus. *Aust Vet J* 82:167–169
- Waltzek TB, Kurobe T, Goodwin AE, Hedrick RP (2009) Development of a polymerase chain reaction assay to detect cyprinid herpesvirus 2 in goldfish. *J Aquat Anim Health* 21:60–67
- Wang L, He J, Liang L, Zheng X, and others (2012) Mass mortality caused by cyprinid herpesvirus 2 (CyHV-2) in Prussian carp (*Carassius gibelio*) in China. *Bull Eur Assoc Fish Pathol* 32:164–173
- Xu J, Zeng L, Zhang H, Zhou Y, Ma J, Fan Y (2013) Cyprinid herpesvirus 2 infection emerged in cultured gibel carp, *Carassius auratus gibelio* in China. *Vet Microbiol* 166:138–144