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Passive immunisation of goldfish with the serum of those surviving a cyprinid herpesvirus 2 infection after high temperature water treatment

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Abstract

Herpesviral haematopoietic necrosis of goldfish caused by cyprinid herpesvirus 2 (CyHV-2) can be controlled by raising water temperature to a virus non-permissive temperature of 34°C. Consequently, the goldfish can survive and acquire resistance to the disease; the underlying mechanism of acquired resistance, however, remains unclear. In this study, we investigated serological changes in the surviving goldfish, with a focus on their humoral immunity, and examined whether sera of the surviving goldfish conferred passive immunity to naïve goldfish. Levels of anti-CyHV-2 antibodies in 8 of the 9 survivors measured via ELISA were higher than those in control fish. Neutralising antibodies were detected in the sera of 2 survivors, but no direct correlation was observed between ELISA optical density value and neutralising antibody titer. Passive immunisation tests showed that recipients injected with the serum containing neutralising antibodies showed higher survival rates than the control group. The sera from 6 other survivors showed no effect on the recipient's mortality regardless of anti-CyHV-2 antibody levels. These results suggest that neutralising antibodies can contribute to acquired immunity in survivors, and other protective factors, including cell-mediated immunity, may work in the survivors that show no detectable neutralising antibodies.

Introduction

Herpesviral haematopoietic necrosis (HVHN), caused by cyprinid herpesvirus 2 (CyHV-2), has led to significant losses to goldfish (*Carassius auratus*) and Prussian carp (*C. gibelio*) farms worldwide (Jung and Miyazaki, 1995; Haenen et al., 2016; Nanjo et al., 2016). The disease manifests with severe necrosis of haematopoietic tissues (Jung and Miyazaki, 1995; Xu et al., 2013).

CyHV-2 infection can be controlled by maintaining the water temperature at 33–35°C for goldfish (Tanaka, 2005; Ito and Maeno, 2014) and 32°C for Prussian carp (Liang et al., 2015). Shibata et al. (2015) reported that temperatures 34°C and above are non-permissive to CyHV-2. The surviving goldfish can acquire resistance to the disease (Tanaka, 2005; Nanjo et al., 2016). Understanding the mechanism underlying this acquired resistance can help elucidate how the goldfish becomes immune to the disease

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and can help in developing efficient control measures; however, the resistance mechanism remains unclear. Such immunity may involve cell-mediated or humoral immune responses. In this study, we investigated changes in anti-CyHV-2 antibody levels, with a focus on humoral immunity, and the presence of neutralising antibodies in the sera of surviving fish after increasing the rearing water temperature. We also assessed the effect of passive immunisation in naïve goldfish to examine whether the antibodies in the survivors can protect the recipients.

Materials and methods

Fish and virus

Goldfish reared in the Yoshida Station, Tokyo University of Marine Science and Technology, Japan, were used in this study. CyHV-2 Sat-1 isolate (Ito et al., 2013) was propagated in a goldfish fin cell line, RyuF-2 (Shibata et al., 2015), at 25°C using medium 199 (M199; Sigma-Aldrich) supplemented with 5% fetal bovine serum (Gibco) and 0.2% kidney extract from healthy goldfish as described by Shibata et al. (2015). The infectious titer of the virus culture for fish inoculation was $10^{3.8}$ $TCID_{50}$ mL^{-1}. For ELISA, partially purified virus was used: virus culture, after clarifying by low centrifugation, was concentrated with a centrifugation at 25,000 × *g* (No. 4N rotor; Tomy RS-205 centrifuge) for 30 min at 4°C. The virus pellet was suspended in TNE buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA 2Na) and layered onto a (5%) sucrose gradient from 25% to 60% in TNE. After centrifugation at 77,600 × *g* (P40ST rotor; Hitachi CP70MX centrifuge) for 17 h at 4°C, a visible band interface between 50% and 55% sucrose were collected, diluted in TNE, and centrifuged at 111,700 × *g* (P40ST rotor) for 30 min at 4°C. The partially purified virus was re-suspended in TNE and stored at −80°C for further use. Protein concentration of the virus solution was measured using the Bradford protein assay reagent (Wako Pure Chemicals).

Selection of fish surviving CyHV-2 infection as sera donors for passive immunisation test

Fifteen goldfish (T1–15) (average body weight, 83.9 g; body length, 123.7 mm) were intraperitoneally (i.p.) injected with 0.05 mL of diluted virus culture ($10^{0.8}$ $TCID_{50}$ mL^{-1}) to prepare surviving fish. Six goldfish (C1–6) were injected with M199 medium as a negative control group. All fish were kept at 25°C for 18 h after injection, and the water temperature was increased to and maintained at 34°C for 6 days. It was then reduced to 25°C for 2 days, increased to 34°C for 6 days, and finally maintained at 25°C. At 36 days post-inoculation (dpi), the fish were infected with CyHV-2, as described above, to confirm their resistance to the virus challenge. Control fish received the same amount of M199 medium. For sampling, peripheral blood from the infected fish was drawn from caudal blood vessels 18, 31, and 55 (19 days after the second i.p. virus challenge) dpi, and the serum was separated by centrifugation at 3,000 × *g* for 10 min (AR015-24 rotor; Tomy MX-301 centrifuge). Serum from the control fish was collected 31 and 55 dpi. Prior to further use, the donor sera were inactivated at 45°C for 30 min.

ELISA for measurement of anti-CyHV-2 antibodies

Levels of anti-CyHV-2 antibodies in individual donor sera were measured by ELISA O.D. values. Test sera were isolated from 3 control fish and the surviving fish. Concentration of each reagent for ELISA was set considering the

maximum positive to negative ratio by combination of individual reagent concentrations. Wells of 96-well ELISA microplates (Sumilon) were coated with 50 µL well^{-1} of partially purified CyHV-2 (1 µg mL^{-1}) in 0.1M carbonate buffer (pH 9.6) for 15 h at 4°C. After washing the wells 5 times with phosphate buffered saline containing 0.05% Tween 20 (PBS-T), the wells were blocked with 1 % bovine serum albumin in PBS-T for 1 h at 25°C, followed by the addition of 50 µL of test goldfish serum diluted 1:400 with PBS-T and incubated for 2 h at 25°C. After washing with PBS-T, the wells were incubated for 1 h at 25°C with anti-ginbuna IgM mouse monoclonal antibody B12 (Toda et al., 2011) diluted 1:20 with PBS-T, which can react with goldfish IgM. The plates were washed and incubated for 1 h at 25°C with anti-mouse IgG (H+L) goat Ig conjugated with peroxidase (Kirkegaard & Perry Laboratories [KPL]) diluted 1:5000 with PBS-T. After washing, colour reaction was initiated by the addition of 100 µL well^{-1} of SureBlue (KPL) for 15 min at 25°C. Colour development was terminated by adding an equal volume of 1 N HCl, and optical density was estimated at 450 nm ($O.D._{450}$) using a MPR-A4i micro-plate reader (Tosoh).

Serum neutralisation test

One-half serial diluted goldfish serum from each surviving fish (T1, 6-8, 11, 13) and a pooled serum sample from control fish (C1-3) collected at 55 dpi were incubated with an equal volume of CyHV-2 supernatant adjusted to $10^{3.1}$ $TCID_{50}$ mL^{-1} for 1 h at 25°C. Then, 50 µL of the mixture was inoculated in 96-well plates seeded with RyuF-2 cells and was incubated for 14 days at 25°C to observe development of cytopathic effect under an inverted light microscope (Nikon TS100).

Passive immunisation of naïve goldfish with the serum from donors

A passive immunisation test was performed using the individual sera of 6 surviving donors and a pooled serum sample from 3 controls (uninfected donors; C1–3) isolated at 55 dpi (Table 1). The transferred sera of the 6 surviving donors were selected considering the level of ELISA O.D. value; high (T6-8), middle (T1, 13), and low (T11). T1 and T8 sera contained the neutralising antibody titer. The serum from fish T12 at 55 dpi showed a considerably higher ELISA O.D. value than those of the others, therefore, it was omitted from this test. Inactivated surviving donor sera after diluting 1:3 or 1:30 with M199 medium were i.p. injected into 5 recipient naïve goldfish (average body weight, 6.9 g; body length, 41.7 mm) for each donor serum, and a pool of negative control fish serum was injected into 5 recipient fish in the same way. The recipients were kept at 25°C for 24 h after serum injection and were subsequently immersed in water containing $10^{0.8}$ $TCID_{50}$ mL^{-1} of CyHV-2. As uninfected controls for checking the toxicity of the sera, 6 recipients were injected with the pooled sera from 3 surviving donors (T6, 7, 13) or control donors (C1-3).

Results

Fish surviving CyHV-2 infection after high temperature treatment as donors for passive immunisation test

Nine of the 15 fish survived after first inoculation and second challenge with the virus. Two goldfish (T14 and 15) died during the high temperature water treatment, and 2 (T3 and 4) died on days 9 and 16 after reducing the temperature to 25°C. Two fish (T9 and 10) died after the second virus challenge at 36 dpi. Causes of all deaths were confirmed to be CyHV-2

Table 1. Results of the ELISA, neutralisation test, and passive immunisation test.

Donor serum			Passive immunisation			
	$O.D_{450}$ in ELISA	ND_{50}	Recipient group	Injection with	Survival rate (%)	
					1/3 serum	1/30 serum
C1, 2, 3 pool	0.11	<10	Uninfected	M199	100	-
T 6, 7, 13 pool	0.64	-	Uninfected	M199	100	-
C 1, 2, 3 pool*	0.11	<10	Infected	CyHV-2	0	0
T 1	0.35	14	Infected	CyHV-2	40[a]	0
T 6	0.75	<10	Infected	CyHV-2	20	0
T 7	0.68	<10	Infected	CyHV-2	20	0
T 8	0.72	28	Infected	CyHV-2	60[a,b]	0
T 11	0.16	<10	Infected	CyHV-2	0	0
T 13	0.48	<10	Infected	CyHV-2	0	0

[a,b] Respectively denote significant differences between the experimental and infected control groups ($p < 0.05$), as assessed via Kaplan-Meier analysis with log-rank test and Fisher's exact test.
* Infected control.

infection by indirect immunofluorescence test (IFAT) on the kidney smear using anti-CyHV-2 mouse monoclonal antibody 3D3 (Nanjo et al., 2016). By contrast, none of the uninfected control fish died.

ELISA for measurement of anti-CyHV-2

Sera of the 12 surviving fish (T1, 2, 4–13) and 3 control fish (C1–3) were used for ELISA. Sera of C1-3 negative control fish showed low O.D. values of 0.053-0.214 and no remarkable change after second injection with culture media (Figure 1). At 18 dpi, sera of two individuals (T7 and 11) showed low O.D. values (0.056 and 0.111), while those of 8 fish (T1, 2, 5, 6, 8, 9, 12 and 13) showed high values 0.340-1.178. At 55 dpi, O.D. of the sera of 4 surviving fish (T6–8, 12) increased, whereas those of 5 surviving fish showed no marked change (T1, 11 and 13) or a decrease (T2 and 5).

Serum neutralisation test

Sera of the surviving fish (T1, 6–8, 11, and 13) and a pooled serum sample of negative control fish (C1–3) collected at 55 dpi were used. Neutralising antibody titer (ND_{50}) was detected in the sera T1 and T8 at 1:14 and 1:28, respectively (Table 1).

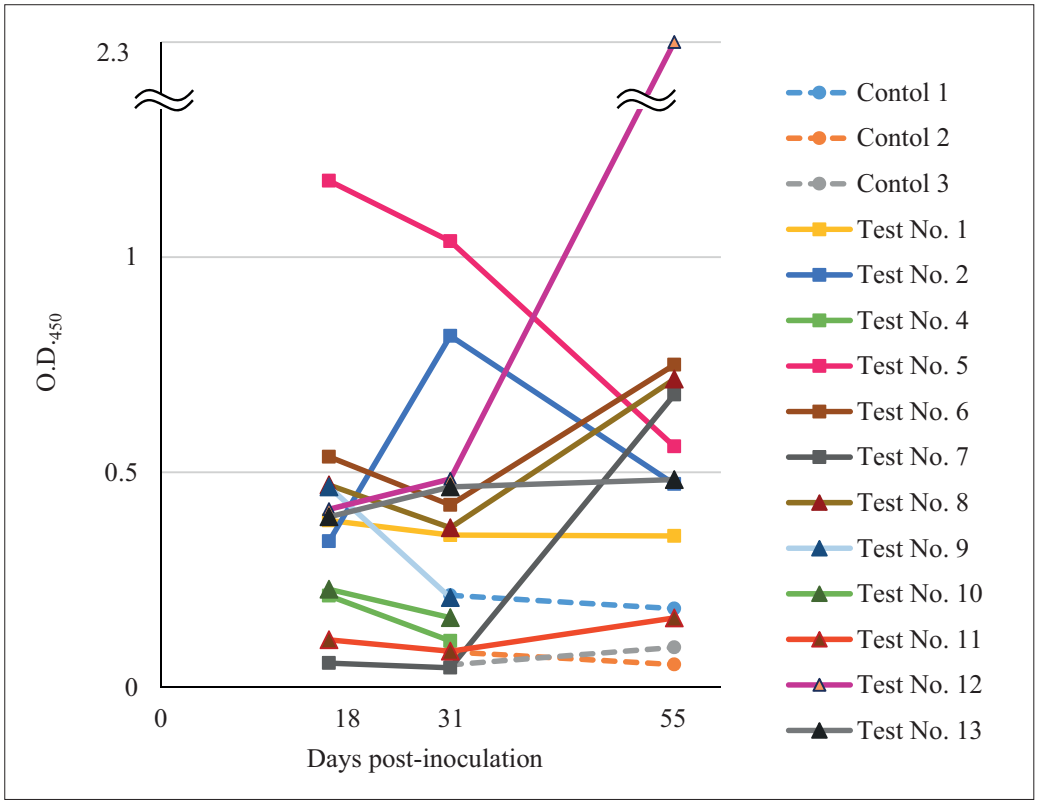

Figure 1. Enzyme-linked immunosorbent assay for measurement of anti-CyHV-2 antibodies. ELISA O.D. values in the sera of fish injected with CyHV-2 (Test) or M199 medium (Control). T3, T14, and T15 fish died before serum sampling.

Passive immunisation test

Sera of the 6 surviving fish (T1, 6–8, 11 and 13) and a pooled serum of control fish (C1–3) were used. Uninfected recipient groups (uninfected controls) that received the pooled control serum and serum from the surviving fish had no mortality. All infected recipient groups that received a control-pool serum (infected control) or surviving-fish serum diluted 1:30 died. Survival rates were no more than 20% in the infected groups that received surviving fish serum diluted 1:3, except T1 and T8, which respectively showed a survival rate of 40% and 60% (Table 1). Both T1 and T8 groups showed statistically significant differences ($p < 0.05$) with respect to the infected control group, as determined via Kaplan-Meier analysis with log-rank test (Figure 2). There was a significant difference ($p < 0.05$) in cumulative mortality between T8 and the infected control groups, as estimated using Fisher's exact test (Table 1).

Discussion

Two fish dying after the second virus challenge at 36 dpi seemed not to have sufficient immunological protection. The other fish showed resistance to the disease, indicating that the survivors used as donors in the passive immunisation test acquired immunity to the disease. These survivors (donors) showed different levels and patterns of change in anti-CyHV-2 antibodies by ELISA; T4 and 10-11 showed O.D. values identical to those of the control fish (C1-3), while others showed higher values than those of the control. In some fish (T6-7, 12), the second virus challenge likely worked

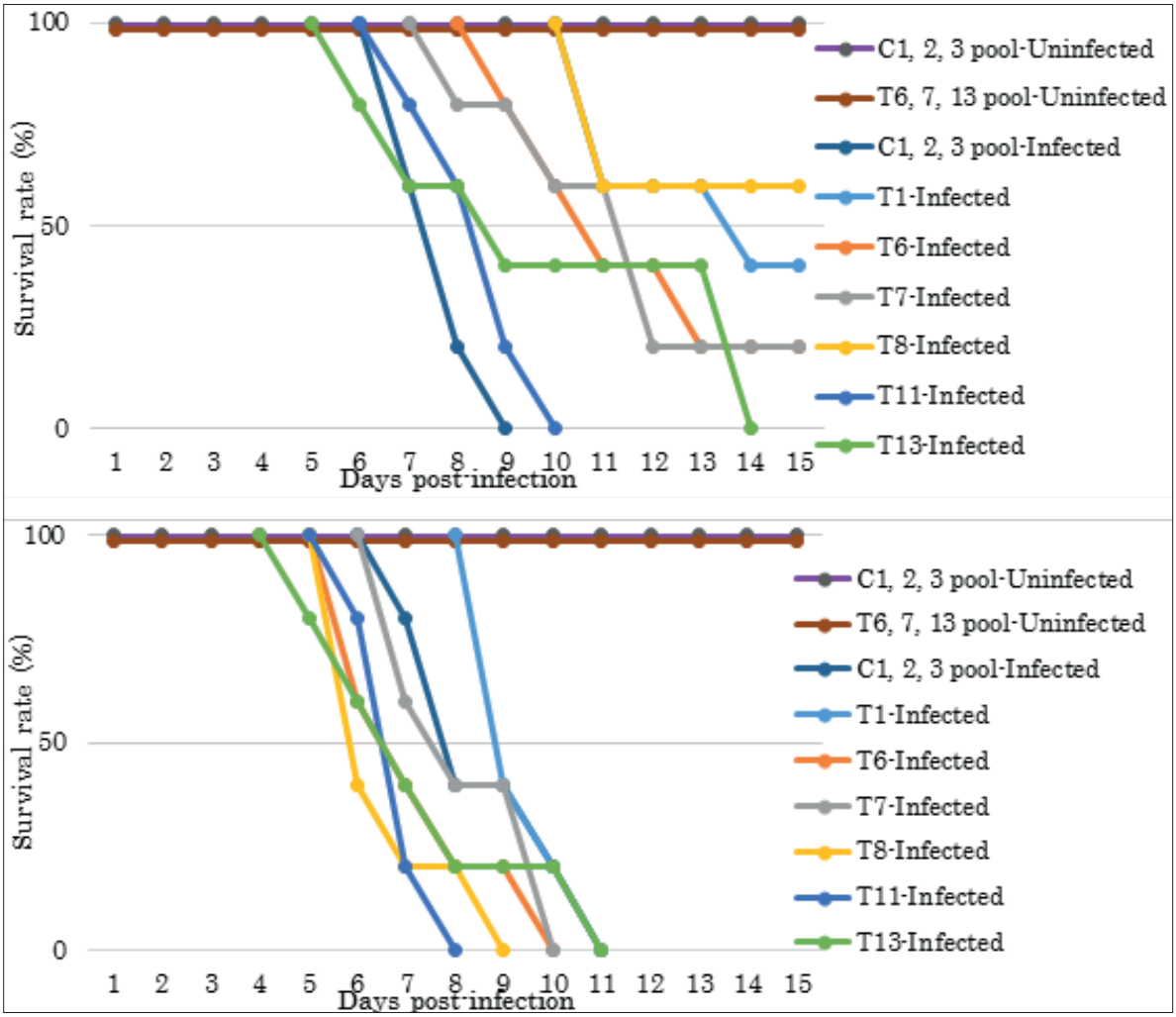

Figure 2. Survival rates for the passive immunisation test. Five naïve goldfish as recipients for each group were injected with inactivated donor sera diluted 1:3 (A) and 1:30 times (B). Group names are shown in Table 1. The recipients were reared at 25°C for 24 h after serum injection and were then exposed to CyHV-2.

as a booster, resulting in an increase in the O.D. value at 55 dpi.

Neutralising antibodies were detected in two sera out of 9 from surviving goldfish (ND_{50} = 14 and 28) at 55 dpi, which showed an O.D. value in ELISA of 0.36 (middle) and 0.73 (high), respectively. However, 2 other fish (T6 and 7) showing high levels of sera O.D. (0.75 and 0.68) did not possess detectable neutralising antibodies in their sera, indicating that there was no direct correlation between the amount of neutralising antibodies and anti-CyHV-2 antibodies (O.D. value in ELISA) in this study. In the passive immunisation test, recipient groups injected with the serum (diluted 1:3) containing neutralising antibodies demonstrated higher survival rates than controls. Recipient groups receiving these sera diluted 1:30 showed 100% mortality due to the concentration of neutralising antibody in the serum being low (1:14 and 1:28), thereby likely establishing a dose dependency of the neutralising antibody. The results suggest that neutralising antibodies can contribute to the protection of the survivors against the disease. Ito and Ototake (2013) and

Zhang et al. (2016) reported that an inactivated CyHV-2 vaccine was effective, and that the vaccine induced high levels of circulating neutralising antibodies. The present study demonstrating the importance of the neutralising antibodies further agrees with these findings. However, the reproducibility of our results must be confirmed using higher numbers of recipient fish.

A surviving donor (T11) showed anti-CyHV-2 antibody levels identical to those of the control, but neutralising antibodies were undetectable in its serum. The donor, however, showed resistance during the second virus challenge. Passive immunisation with this serum failed to protect the recipient goldfish. Perhaps other protective factors, including cell-mediated immunity, may be at work. Adkison et al. (2005) reported that a passive immunisation test using pooled anti-CyHV-3 serum showed little protection to CyHV-3 challenge, suggesting that development of effective resistance to the disease will most likely involve stimulation of cell-mediated immune responses.

Increasing the water temperature to virus non-permissive 34°C following a virus infection can help the surviving fish acquire resistance against a subsequent virus challenge (Tanaka, 2005; Nanjo et al., 2016). However, the protection factors, neutralising antibody, cell-mediated immunity, and other influences seemed different for each surviving fish in this study. CyHV-2 reactivated in the surviving fish after the water temperature was reduced from 34°C to 25°C, but the number of reactivated virus particles may depend on virus propagation during the first stage of infection at 25°C until 18 h after virus injection (Nanjo et al., 2016). The present study showed that two infected fish died after reducing the water temperature from 34°C to 25°C. Therefore, virus propagation may influence protective factors consequently induced. Further studies are necessary to elucidate in detail the importance of all the various factors.

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References

Adkison MA, Gilad O and Hedrick RP (2005). An enzyme linked immunosorbent assay (ELISA) for detection of antibodies to the koi herpesvirus (KHV) in the serum of koi *Cyprinus carpio*. *Fish Pathology* **40**, 53-62.

Haenen O, Way K, Gorgoglione B, Ito T, Paley R, Bigarré L and Waltzek T (2016). Workshop "Novel viral infections threatening Cyprinid fish." *Bulletin of the European Association of Fish Pathologists* **36,** 11-23.

Ito T and Ototake M (2013). Vaccination against cyprinid herpesvirus 2 (CyHV-2) infection in goldfish *Carassius auratus*. *Bulletin of the European Association of Fish Pathologists* **33**, 158–164.

Ito T and Maeno Y (2014). Effects of experimentally induced infections of goldfish *Carassius auratu*s with cyprinid herpesvirus 2 (CyHV-2) at various water temperatures. *Diseases of Aquatic Organisms* **110**, 193–200.

Ito T, Kurita J, Ozaki A, Sano M, Fukuda H and Ototake M (2013). Growth of cyprinid herpesvirus 2 (CyHV-2) in cell culture and experimental infection of goldfish *Carassius auratus*. *Diseases of Aquatic Organisms* **102**, 193-202.

Jung SJ and Miyazaki T (1995). Herpesviral haematopoietic necrosis of goldfish, *Carassius auratus* (L.). *Journal of Fish Diseases* **18**, 211-220.

Liang LG, Xie J, Chen K and Bing XW (2015). Pathogenicity and biological characteristics of CyHV-2. *Bulletin of the European Association of Fish Pathologists* **35**, 85–93.

Nanjo A, Shibata T, Saito M, Yoshii K, Tanaka M, Nakanishi T, Fukuda H, Sakamoto T, Kato G and Sano M (2016). Susceptibility of isogeneic ginbuna *Carassius auratus langsdorfii* Temminck et Schlegel to cyprinid herpesvirus-2 (CyHV-2) as a model species. *Journal of Fish Diseases* [doi:10.1111/jfd.12500].

Shibata T, Nanjo A, Saito M, Yoshii K, Ito T, Nakanishi T, Sakamoto T and Sano M (2015). *In vitro* characteristics of cyprinid herpesvirus 2: effect of kidney extract supplementation on growth. *Diseases of Aquatic Organisms* **115**, 223–232.

Tanaka M (2005). Elevating water temperature for control of herpesviral hematopoietic necrosis of goldfish, *Carassius auratus*. *Bulletin of the Saitama Prefectural Agriculture and Forestry Research Center* **5**, 37-43. In Japanese.

Toda H, Saito Y, Koike T, Takizawa F, Araki K, Yabu T, Somamoto T, Suetake H, Suzuki Y, Ototake M, Moritomo T and Nakanishi T (2011). Conservation of characteristics and functions of CD4 positive lymphocytes in a teleost fish. *Developmental and Comparative Immunology* **35**, 650–660.

Xu J, Zeng L, Zhang H, Zhou Y, Ma J and Fan Y (2013). Cyprinid herpesvirus 2 infection emerged in cultured gibelio carp, *Carassius auratus gibelio* in China. *Veterinary Microbiology* **166**, 138–144.

Zhang L, Ma J, Fan Y, Zhou Y, Xu J, Liu W, Gu Z and Zeng L (2016). Immune response and protection in gibel carp, *Carassius Gibel*, after vaccination with β-propiolactone inactivated cyprinid herpesvirus 2. *Fish and Shellfish Immunology* **49**, 344–350.