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Short communication

Rock bream iridovirus (RBIV) replication in rock bream (*Oplegnathus fasciatus*) exposed for different time periods to susceptible water temperatures

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ABSTRACT

Rock bream iridovirus (RBIV) is a member of the *Megalocytivirus* genus that causes severe mortality to rock bream. Water temperature is known to affect the immune system and susceptibility of fish to RBIV infection. In this study, we evaluated the time dependent virus replication pattern and time required to completely eliminate virus from the rock bream body against RBIV infection at different water temperature conditions. The rock bream was exposed to the virus and held at 7 (group A1), 4 (group A2) and 2 days (group A3) at 23 °C before the water temperature was reduced to 17 °C. A total of 28% mortality was observed 24–35 days post infection (dpi) in only the 7 day exposure group at 23 °C. In all 23 °C exposure groups, virus replication peaked at 20 to 22 dpi (10^6–10^7/μl). In recovery stages (30–100 dpi), the virus copy number was gradually reduced, from 10^6 to 10^1 with faster decreases in the shorter exposure period group at 23 °C. When the water temperature was increased in surviving fish from 17 to 26 °C at 70 dpi, they did not show any mortality or signs of disease and had low virus copy numbers (below 10^2/μl). Thus, fish need at least 50 days from peaked RBIV levels (approximately 20–25 dpi) to inhibit the virus. This indicates that maintaining the fish at low water temperature (17 °C) for 70 days is sufficient to eradicate RBIV from fish body. Thus, RBIV could be eliminated slowly from the fish body and the virus may be completely eliminated under the threshold of causing mortality.

1. Introduction

Iridoviridae is a family of large double-stranded DNA virus (120–300 nm) with an icosahedral morphology [1]. The family includes five genera: *Iridovirus*, *Chloriridovirus*, *Ranavirus*, *Lymphocystivirus* and *Megalocytivirus*. *Megalocytiviruses* have been the cause of disease in >50 fish species and currently threaten the aquaculture industry, causing great economic losses in Korea, Japan, China and Southeast Asia [2–10]. Rock bream iridovirus (RBIV), which belongs to the genus *Megalocytivirus* [11] remains an important health problem in rock bream (*Oplegnathus fasciatus*).

Water temperature is known to affect the immune system and susceptibility of fish to virus infection [12–20]. In Korea, RBIV outbreaks typically occur in cultured rock bream from August to September, when water temperature between 23 and 27 °C [10]. Recently, several studies have demonstrated a clear correlation between mortality and the water temperature against RBIV infection in rock bream; i) rock bream injected with RBIV and held at 29, 26, 25, 23, 21, 20 or 18 °C had 100% mortality, but no mortality at 17 or 13 °C [21–25], ii) mortality due to RBIV in rock bream at 23 °C could be controlled by reducing water temperature to 17 °C during very early stages of infection [21]. Furthermore, it was observed that the survivors of high virus dose injections in rock bream gained protective immunity. Thus, water temperature plays a critical role not only for mortality, but also to obtain the protective immunity in rock bream against RBIV infection.

Several other experimental studies on fish viral diseases have demonstrated the effect of water temperature regulations on protective immunity of survivors against infectious haematopoietic necrosis virus (IHNV), koi herpesvirus (KHV), viral haemorrhagic septicaemia virus (VHSV) and nervous necrosis virus (NNV) [12,18,19,26,27]. Recent studies reported the long-term kinetics of NNV infectivity in sevenband grouper, injected with a live NNV at

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17.3 °C and reared at natural seawater temperature [28]. The NNV infectivity titer was detected from 5 days post injection and peaked on 10 days at $10^{7.43\pm0.35}$TCID$_{50}$/g. However, NNV was under the detection limit ($\leq 10^{2.8}$TCID$_{50}$/g) between 42 and 128 days; hence, it was suggested that fish need at least 42 days from initial infection to inhibit the active virus, thus completely eliminating it from their body. Therefore, identifying a time period in which the virus has been completely eliminated from the fish's body to prevent the mortality due to viral infection is essential. However, the time-dependent virus replication pattern and time required to completely eliminate the virus from the fish's body has not been demonstrated in rock bream against RBIV infection.

In the present study, RBIV was artificially infected to rock bream at different water temperature conditions, and the virus replication pattern was evaluated in the rock bream from 2 to 100 days post infection (dpi) to determine the influence of water temperature on RBIV replication. Additionally, time points/period in which the virus was completely eliminated from survivors due to RBIV infection were investigated.

2. Materials and methods

2.1. Experimental fish

RBIV free rock bream were obtained from a local farm and reared at the Fisheries Science Institute at Chonnam National University. Approximately 500 fish (7.4 ± 1.0 cm/9.1 ± 1.3 g) were maintained in large tanks (10 ton) with a continuous seawater supply with aeration. The required number of fish for the experiments was transferred to the experimental infection facility.

2.2. Source of RBIV

The virus used in the present study was originally isolated from RBIV infected rock bream in 2010 as explained earlier [29].

2.3. Determination of viral copy number in the spleen and spleen index

For the analysis of the RBIV copy number, the spleen was obtained from RBIV infected fish at several sampling points. Genomic DNA was isolated from the whole spleen (20–150 mg) of each fish using an AccuPrep®Genomic DNA extraction kit (Bioneer, Korea) according to the manufacturer's instructions. Real-time polymerase chain reaction (PCR) was carried out in an Exicycler 96 Real-Time Quantitative Thermal Block (Bioneer, Korea) using an AccuPre®2× Greenstar qPCR Master mix (Bioneer, Korea) as described previously [29]. A standard curve was generated to determine the RBIV major capsid protein (MCP) gene copy number by qRT-PCR as described previously [29]. RBIV MCP gene specific primer set (F 5′ tgcacaatctagttgaggaggtg 3′ and R 5′ aggcgttccaaaagtcaagg 3′) was used to yield 90 bp product, and reaction conditions were similar to those previously described [29]. The virus copy number was determined from 1 μl of DNA from 100 μl of total DNA that was extracted from a whole spleen. The detection limit of RBIV MCP copy number was 1.0×10^1/μl. The spleen indexes were calculated according to the following formula:

Spleen index = (Spleen weight (g)/ Fish weight (g)) × 100.

2.4. Experimental infection at shifted water temperature (group A)

Fish (11.7 ± 1.7 cm/34.1 ± 2.1 g) were intraperitoneally (i.p.) injected with 100 μl/fish containing 1.1×10^7 MCP gene copies. The control group was i.p. injected with 100 μl/fish of phosphate-buffered saline (PBS). Each group of fish at 23 °C for 7 (60 fish of group A1), 4 (80 fish of group A2) and 2 days (80 fish of group A3) was reduced to 17 °C and maintained in aquaria containing 250 L of UV-treated seawater. The cumulative mortality of group A1 has previously published [29]. The overall daily water exchange rate was 10% of system volume per day (25 L/day).

To determine RBIV replication pattern in water temperature shifting group, spleens were collected from 5 virus injected fish and 5 control fish at 2, 4, 7, 10, 15, 20, 22, 25, 28, 30, 40, 50, 60, 70 and 100 days post infection (dpi). Spleens were stored at −80 °C after being flash frozen in liquid nitrogen. Table 1 summarizes the experimental conditions.

2.5. Experimental infection at 17 °C (group B)

To evaluate virus copy number at different time points of RBIV infected fish at 17 °C, 50 fish in each group (12.0 ± 1.2 cm/35.0 ± 2.8 g) was i.p. injected in the same manner as the water temperature shifting group. Control group was i.p. injected with 100 μl/fish of PBS. The fish were maintained in aquaria containing 250 L of UV-treated seawater. The overall daily water exchange rate was 10% of system volume per day (25 L/day). Spleens were collected from 5 virus-injected fish and 5 control fish at 2, 4, 7, 10, 15, 20, 25, 30 and 40 dpi. Spleens were stored at −80 °C after being flash frozen in liquid nitrogen. Table 1 summarizes the experimental conditions.

2.6. Influence of increased water temperature on mortality

At 70 dpi, survivors were randomly selected from group A1 (5 fish), A2 (10 fish) and A3 (10 fish), then the water temperature was increased again to the highly susceptible water temperature of 26 °C (1 °C/day) to determine whether the virus remaining the can be re-activated and cause fish mortality, or whether immunity can suppress virus replication in the fish body. Those fish were observed for 50 more days (until 120 dpi), and MCP gene copy number of all survived fish was analyzed at the end of the experiment at 120 dpi. All fish were maintained in aquaria containing 30 L of UV-treated seawater. The overall daily water exchange rate was 50% of system volume per day (15 L/day).

Table 1
Experimental details of artificial infection.

Group	Infection dose /fish	No. of fish	Sampling point	Mortality (%)
A1 (23 °C reduced to 17 °C at 7 dpi[a])	1.1×10^7/fish	60	2, 4, 7, 10, 15, 20, 22, 25, 28 and 30 dpi[a]	28
A2 (23 °C reduced to 17 °C at 4 dpi)	1.1×10^7/fish	80	7, 10, 15, 20, 25, 30, 40, 50, 60, 70 and 100 dpi	0
A3 (23 °C reduced to 17 °C at 2 dpi)	1.1×10^7/fish	80	7, 10, 15, 20, 25, 30, 40, 50, 60, 70 and 100 dpi	0
B (17 °C)	1.1×10^7/fish	50	2, 4, 7, 10, 15, 20, 25, 30 and 40 dpi	0

[a] dpi: days post infection.

3. Results and discussion

In fixed water temperatures of higher than 23 °C, rock bream mortality was extreme at 100% [21–25]. For this reason, shifting the water temperature from 23 °C to 17 °C was attempted to obtain survivors determine the RBIV replication effect on water

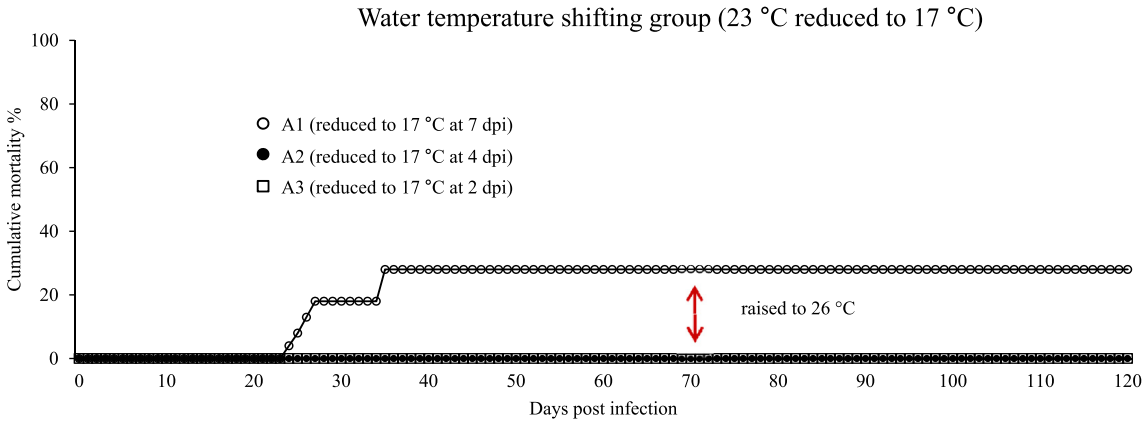

Fig. 1. Cumulative mortality of rock bream intraperitoneally (i.p.) injected with 1.1 × 10^7 MCP gene copy/fish of RBIV at 23 °C, with the water temperature reduced to 17 °C at 7, 4 and 2 dpi (2 °C/day). Cumulative mortality was calculated using the formula [number of dead fish/fish population parameter × 100]. All surviving fish were subjected to water temperature increase (1 °C/day) up to 26 °C at 70 dpi (arrows). The cumulative mortality of group A1 (7 day) previously published [29] is shown for reference.

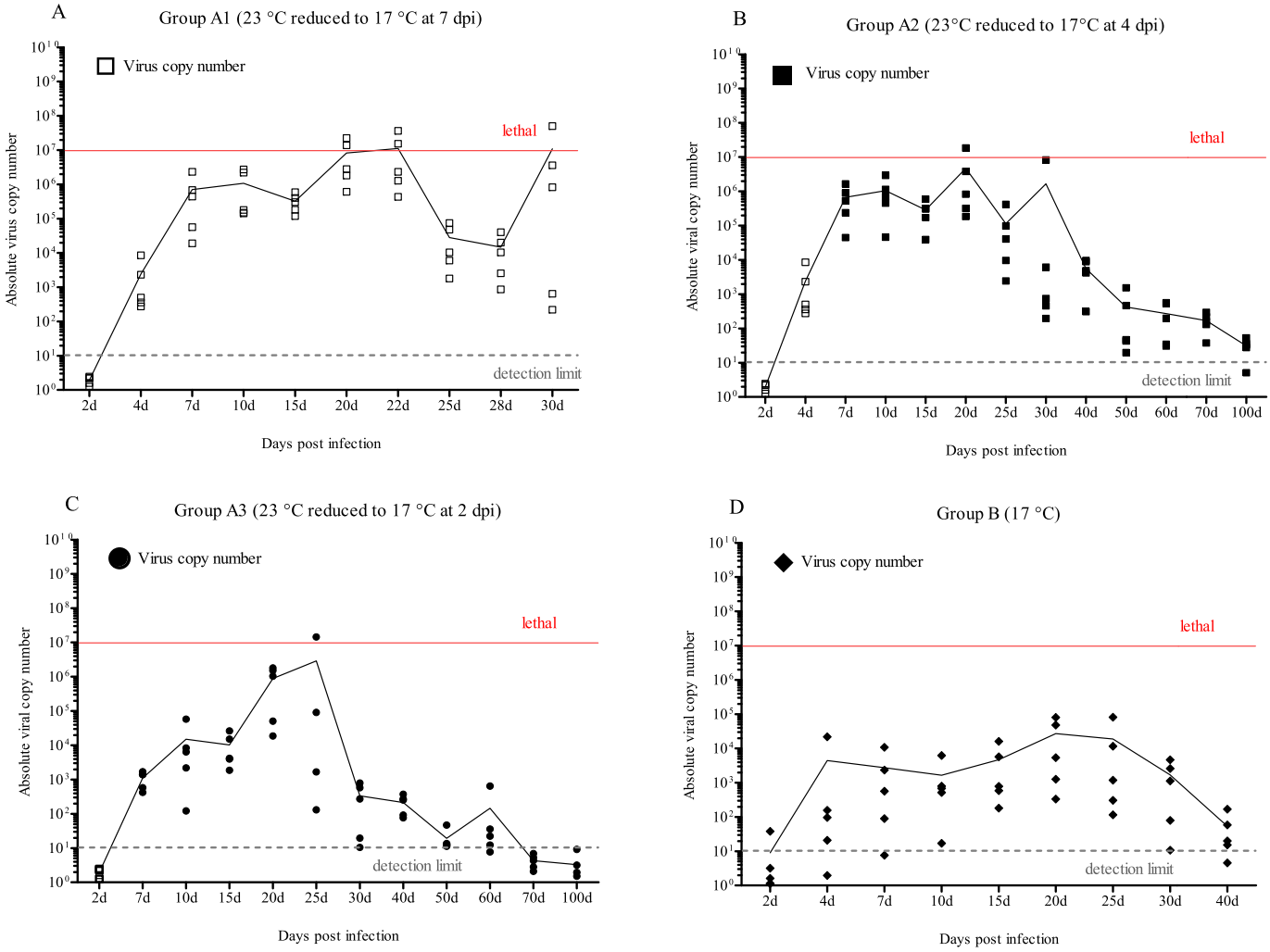

Fig. 2. Absolute virus copy number of rock bream by different water temperature condition after 1.1 × 10^7 MCP gene copy/fish of RBIV was injected intraperitoneally (i.p.). (A) Fish were injected at 23 °C, and the water temperature was reduced to 17 °C at 7 dpi (2 °C/day). (B) Fish were injected at 23 °C, and the water temperature was reduced to 17 °C at 4 dpi (2 °C/day). Virus copy number and spleen index for 2 and 4 dpi were plotted using the Fig. 2A. (C) Fish were injected at 23 °C, and the water temperature was reduced to 17 °C at 2 dpi (2 °C/day). Virus copy number and spleen index for 2 dpi were plotted using the Fig. 2A. (D) Fish were injected at 17 °C.

temperature, and evaluate the virus elimination for a recovery stage from RBIV infection. Rock bream infected with RBIV and held for 7, 4 and 2 days at 23 °C before the water temperature was reduced to 17 °C had mortality rates of 28% (group A1), 0% (group A2) and 0% (group A3), respectively (Fig. 1). This indicates that the fish mortality by RBIV infection was highly dependent on period of exposure days into susceptible water temperature. Although fish mortality was observed in only group A1, with 1 fish dying at 24, 25, 26, 27 and 35 dpi (virus copy number was 10^7–10^8/μl) (Figs. 1 and 3), the fish of groups A2 and A3 had a similar virus copy ranges to group A1 (Fig. 2A, B and 2C). In groups A1, A2 and A3, the acute stage of RBIV infection occurred from 7 to 22 dpi, when virus replication reached its peak at 20 to 22 dpi (average range of 10^5–10^7/μl), then reduced at 25 dpi. Individual differences were high between 25 and 30 dpi, with 20 fish exhibiting low virus copy numbers (<10^3) and 5 fish exhibiting high virus copy numbers (>10^6) (Fig. 2A, B and 2C; Supplementary Table 1, 2 and 3). Between 20 and 25 dpi, important immune responses occurred that determined fish survival or death; most of the fish could drastically eliminate the virus from the host body, with a few fish that failed to eliminate the virus succumbing to death. Therefore, immune responses at approximately 20–25 dpi may be important in evaluating factor(s) for survival.

Furthermore, no mortality occurred from 30 to 100 dpi in group A2 and A3, and these fish showed no clinical signs of RBIV and gradual decrease of virus copy numbers (average 10^7 reduced to under detection limit level as below 10^1/μl) (Fig. 2B and C). Moreover, our results further suggest that reduced virus replication was responsible for the alterations of major clinical signs of RBIV infection in fish (i.e. enlarged spleen size) (Supplementary Tables 1, 2, 3 and 4). Therefore, this time period (30–100 dpi) was regarded as a recovery stage from infection. RBIV could be eliminated slowly from the fish body, and the virus was completely eliminated under the threshold (>10^1) of causing mortality in group A2 from 100 dpi and in group A3 from 70 dpi (Fig. 2B and C). This observation suggests that the once RBIV is replicate at highly susceptible water temperature (over 23 °C); it is difficult to inactivate in fish body. However, we found that the virus was inhibited in the fish body when fish were first maintained at 23 °C (highly susceptible condition), and then the reduced water temperature to 17 °C (non-susceptible condition). Similar to this results, VHSV and hirame rhabdovirus (HIRRV)-infected olive flounder (*Paralichthys olivaceus*) at 20 °C (non-susceptible condition) can reduce viral loads in the fish body [30,31]. They observed early and effective apoptosis, interferon and inflammatory cytokines-related immune responses at 20 °C compared to the fish infected at 10 or 15 °C (highly susceptible condition). Thus, we hypothesised that rock beam immune responses also regulated by water temperature-dependent and activate some unknown factor(s) for inhibition of virus replication. Therefore, for the further study, evaluation of antiviral immune responses to inhibit virus replication may be necessary to identify important factor(s) for survival and water temperature/virus replication-dependent immune defence mechanism in rock bream that recover from RBIV.

This study showed that when the virus copy levels were stable at 70 dpi in group A2 and A3 (1.68 × 10^2 and 4.28 × 10^0/μl, respectively) (Fig. 2B and C), all surviving fish (5 fish of group A1, and 10 fish of group A2 and A3, respectively) had been exposed to non-permissive water temperature of 17 °C, which was then increased from 17 °C to 26 °C, and they did not show any mortality or clinical signs of disease (Fig. 1) and had low virus copy numbers (below 10^2 at 120 dpi) (Fig. 3). Our previous study reported that almost all surviving RBIV-infected fish obtained by water temperature shifting (23 °C–17 °C) that were re-exposed at the highly susceptible water temperature of 26 °C at 100 dpi (average virus copy number at 2.7 × 10^2/μl) survived until 50 days post increasing water temperature [21]. However, the risk of reoccurrence and mortality remained, although the possibility is very low. Thus, in this study, to determine whether the remaining virus in the fish can be re-activated and cause fish mortality, a time point 30 days earlier than that of the previous experiment was chosen. Therefore, at 70 dpi, the virus copy number at 1.68 × 10^2 and 4.28 × 10^0/μl in group A2 and A3, respectively, was a minimal and once the number of virus decrease below the threshold to cause mortality, there is a lower possibility of fish mortality occurring in susceptible water temperatures of 26 °C. This suggests that 70 days is sufficient to eradicate RBIV from the fish body. Further, it could suggest that fish need at least 50 days from the peak virus level (approximately 20–25 dpi) to avoid re-activation of the virus, thus completely eliminating it from the fish's body. Hereby we suggest monitoring of the viral load in the fish body post recovery from RBIV infection in rock bream farms, as the RBIV may exist in latent form in the fish's body for more than 50 days.

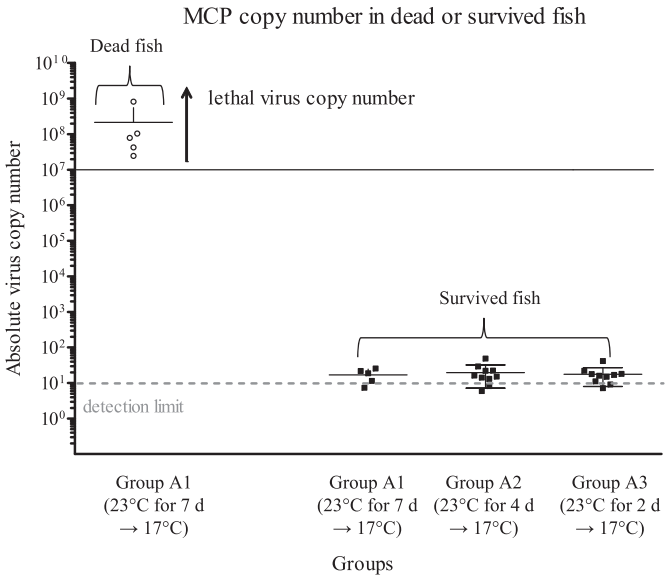

Fig. 3. The MCP gene copies of all dead fish from the group A1 (23 °C reduced to 17 °C at 7 dpi) and the MCP gene copies in all rock bream survivors from group A1 (23 °C reduced to 17 °C at 7 dpi), A2 (23 °C reduced to 17 °C at 4 dpi) and A3 (23 °C reduced to 17 °C at 2 dpi) are shown. RBIV MCP copy number was 1.0 × 10^1/μl can be regarded as negative.

On the other hand, fish exposed to virus at the susceptible water temperature of 23 °C for a short period (7, 4 and 2 days) followed by exposure at 17 °C for a longer period in modulated water temperature groups, high virus replication (around 10^5–10^7/μl) was maintained until 20 to 25 dpi because RBIV can replicate slowly in fish body at the non-susceptible water temperature of 17 °C. At 17 °C, the virus copy number was still relatively high at 20, 25 and 30 dpi (2.72 × 10^4, 1.89 × 10^4 and 1.69 × 10^3/μl, respectively) then reduced to minimum numbers at 40 dpi (average 5.34 × 10^1/μl) (Fig. 2D). This was evident from the previous report in which rock bream that did not die at non-susceptible water temperature of 13 °C, all fish died as a result of RBIV when the water temperature raised from 13 °C to 25 °C, after maintaining for 30 dpi at 13 °C [23]. This observation suggest that even though rock bream did not die at 13 °C, the virus load is still high at 30 dpi and the remaining virus may re-active, causing mortality. This indicates that the RBIV could replicate slowly in fish body even at low water temperature including non-mortality conditions (13 and 17 °C). This might be the main reason for the mass mortality in rock bream aquaculture industry due to RBIV infection.

4. Conclusions

In the present study, elimination of the virus from fish body by shifting the water temperature at 23 °C to a non-susceptible water temperature at 17 °C after RBIV infection was shown. However, fish need at least 50 days from peaked RBIV level (around 20 to 25 dpi) to not to reactivate the virus. This suggests that RBIV could be eliminated slowly from the fish body and virus will be completely eliminated below the threshold of causing mortality. Additionally, previously activated RBIV was not easily inactivated in the fish body because it has very strong pathogenicity against host fish, as evidenced by this long experimental schedule.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fsi.2017.09.038.

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