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Comparison of Viral Replication, Antiviral Gene Expression and Antioxidant Enzyme Activity in Gibel Carp (*Carassius auratus gibelio*) Infected with Cyprinid Herpesvirus 2 at Susceptible and Less-susceptible Temperatures

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Abstract

Cyprinid herpesvirus 2 is a DNA virus that is a concern for the aquaculture worldwide. Here, the challenge of CyHV-2 infection was conducted in gibel carp at a host susceptible temperature (25 ℃) and a less-susceptible temperature (15 ℃). The viral replication and expression of antiviral genes were analyzed by real time PCR, and the activities of antioxidative enzymes in serum were also examined. Results showed that CyHV-2 was in normal proliferative in 25 ℃ group, while the growth was inhibited in 15 ℃ group. Moreover, IFNϕ1 and Mx were highly expressed from 12 hpi to 48 hpi in both groups, but the 15 ℃ group showed quicker and stronger response. TNF-α and IL-1β showed early upregulation at 12 hpi followed by a sharp decline in both groups. Fatty acid synthase (FAS) and Caspase-8 showed a higher expression level in 15 ℃ group than that in 25 ℃ group during 48-96 hpi. Furthermore, the activities of antioxidant enzymes (SOD and CAT) in 15 ℃ group were higher than those in 25 ℃ group at late infection. Thus, water temperature affected the immune response against CyHV-2 infection, which may ultimately lead to host survival in the less-susceptible temperatures.

Introduction

Cyprinid herpesvirus 2 (CyHV-2) is an emerging pathogen that has caused huge economic losses. It is a large DNA virus that belongs to the genus Cyprinivirus within the family Alloherpesviridae (Liu et al., 2018). At first, the disease caused by CyHV-2 infection was reported in the cultured goldfish *Carassius auratus* in Japan in 1995(Jung & Miyazaki, 1995). Subsequently, it has spread rapidly to many countries including China (Xu et al., 2013), Turkey (Kalayci et al., 2018) and some European countries (Fichi et al., 2013). Owing to its high morbidity and mortality, CyHV-2 has become a major concern to the culture industry of goldfish and gibel carp throughout the world. Unfortunately, there are lack of efficient drugs or measures to control CyHV-2 infection until now.

Fish is typically poikilothermic animal whose life activity extremely affected by water temperature. In recent years, the impact of water temperature on the disease resistance of fish has drawn an increasing interest worldwide, especially focusing on the aquatic virus infection. There exists a close relationship between water temperature and virus infection. For example,

viral haemorrhagic septicaemia virus (VHSV) infection often occurs at the temperature below 15 ℃, causing 50-70% mortalities in fish but no mortality is observed at temperature above 20 ℃ (Kim et al., 2009). Besides, it was reported that warm-water culture conditions could inhibit the replication of WSSV in *Litopenaeus vannamei* (Piamsomboon et al., 2016). As for CyHV-2, typical symptoms like hyperaemia in the jaws and reddening gill by CyHV-2 infection often occurred at water temperature above 20 ℃ (Ito & Maeno, 2014). Understanding why temperature could affect the disease course is necessary for establishing the epidemic prevention and control measures of CyHV-2 infection.

It is well-known that the immune system of fish plays a key role in defense against various pathogens' invasion. However, the defensive capacity of the host immune system is susceptible to many environment factors including water temperature, and it may increase the risk of disease outbreak when the host immune is weakened or inhibited. Recently, it has been demonstrated that there may exist somewhat relationship between the temperature and the immune system of fish. As reported, the expression levels of several antiviral molecules tend to be temperature-dependent after virus infection (Avunje et al., 2012). Besides, fish survived from virus infection at a less-susceptible water temperature can acquire an effective prevention against the virus reinfection (Chai et al., 2020).

Nowadays, gibel carp has become one of the most cultured fishes in the region of Yangtze River in China (Ren et al., 2021). They can grow well between 4 ℃~30 ℃ with the optimum growth temperature of 15 ℃~25 ℃ (Li et al., 2016). However, the development of gibel carp culture is challenged by the emergence of gibel carp haematopoietic necrosis caused by CyHV-2 infection (Zhu et al., 2015). The objective of this study was to investigate the difference of viral replication, antiviral gene expression and antioxidant enzyme activity in gibel carp infected with CyHV-2 at susceptible and less-susceptible temperatures. We chose a host susceptible temperature (25 ℃) and a less-susceptible temperature (15 ℃). Gibel carp were intraperitoneally injected with CyHV-2 and reared at 15 ℃ and at 25 ℃, respectively. The viral load kinetics and the expression profiles of several antiviral genes in the spleen were examined by real-time PCR in both groups. Moreover, the activities of two antioxidant enzymes (SOD and CAT) in serum were also measured to evaluate the stress response under the two temperatures.

Materials and Methods

Fish and Virus

Healthy gibel carp (average body weight of 30 ± 0.5g) were obtained from a local farm in Yancheng city, Jiangsu province, China. The fish were confirmed to be initially free from CyHV-2 by the PCR detection (Wang et al., 2015). Then the fish were kept in fiberglass tanks supplied with aerated water and fed with the commercial diet before the experiment. No fish died or displayed symptoms like anorexia, apathy, or necrotic spots on the jaws during the period.

CyHV-2 strain used here was isolated from a moribund gibel carp in a farm in Yancheng, Jiangsu Province (Zhang et al., 2020). The diseased fish exhibited typical symptoms of CyHV-2 infection, with a cumulative mortality of approximate 85% within 1 week. The spleen was pooled, homogenized in PBS, and centrifuged. Then the supernatant containing CyHV-2 virions was stored at -80 ℃ until use.

Experimental Infection

Firstly, a total of 200 gibel carp were randomly divided into two groups (100 fish per group) and maintained at 15 ± 0.5 ℃ and 25 ± 0.5 ℃, respectively. For each temperature group, the fish were randomly distributed into the infection group (n=50) and control group (n=50) after acclimation for 7 days. Fish in the infection group were intraperitoneally injected with 100 μL of virus suspension (5×10^6 copies/fish), while those in the control group were intraperitoneally injected with 100 μL of PBS. Afterwards, five fish were randomly chosen per tank at 0, 12, 24, 48, 72, 96 and 120 h post-infection (hpi). Fish were aseptically dissected, and the spleen were sampled for DNA and RNA extraction. Alongside, the blood from each individual fish was sampled and the serum were isolated. All procedures were carried out according to the Chinese legislation for animal experimentation guidelines.

Virus Detection

Quantitative real-time PCR was performed to determine the virus replication. Firstly, the total DNA was extracted from the spleen samples using Ezup Column Animal Genomic DNA Purification Kit (Sangon Biotech, China) according to the manufacturer's protocols. The concentration of extracted DNA was adjusted to 50 ng/μL and used for the template. Using the specific primers targeting CyHV-2 (Table 1), the reaction was performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Germany). The reaction mixtures in a final volume of 20 μL contained 2 μL of diluted DNA, 5 μmol of each primer, and 10 μL of PrimeScript RT Enzyme Mix (TaKaRa, Japan). The reaction was run in triplicates for each sample. Finally, CyHV-2 DNA loads were determined by extrapolating Ct values from the standard curve and expressed as mean $\log_{10}$ copies/100 ng DNA (Qiao et al., 2018).

Total RNA Extraction and Reverse Transcription

Total RNA was extracted from the spleen samples using the RNAiso reagent (TaKaRa, Japan) following the manufacturer's instructions. The RNA quality was

Table 1. Detailed information of the primers used for real time PCR

Primers	Primer sequences (5′-3′)	Reference
Mx-F	ACAAAGCAAGAAACGATTAACCTGG	Mou, C. Y. *et al.* (2018)
Mx-R	TCTACAACTGTCTGTTCAGTGCCCTT	
IFNϕ1-F	GCTTCGGGAAATGAGTGGACAAT	Mou, C. Y. *et al.* (2018)
IFNϕ1-F	TTCACTTTTGTTAGATTCCATTGCG	
TNFα-F	CATTCCTACGGATGGCATTTACTT	Grayfer, L. *et al.* (2009)
TNFα-R	CCTCAGGAATGTCAGTCTTGCAT	
IL-1β-F	GCGCTGCTCAACTTCATCTTG	Grayfer, L. *et al.* (2009)
IL-1β-R	GTGACACATTAAGCGGCTTCAC	
Caspase 8-F	CTGTTTTGGGCGTGGATG	Lu, J. *et al.* (2018)
Caspase 8-R	CCTTGGCAGGCTTGAATG	
FAS-F	CCAAAATAACACCACCCACAACTA	Liu, M. *et al.* (2018)
FAS-R	ATGCCAAAGAGCCTGAAAACC	
β-actin-F	CTCCCCTCAATCCCAAAGCCAA	Liu, M. *et al.* (2018)
β-actin-R	ACACCATCACCAGAATCCATCA	
CyHV2-F	TTAGCGTCAGGTCCATAG	Xu, L. *et al.* (2014)
CyHV2-R	GGCGTGTAGAAATCAAACT	

checked by 1% agarose gel electrophoresis and the concentration was determined using a Nanodrop ND-2000 spectrophotometer (Quawell, USA). Then 1 μg of total RNA was reversely transcribed into cDNA using PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa, Japan). The resulting cDNA was used to determine the expression profiles of antiviral genes after CyHV-2 infection under different water temperatures.

Expression Profiles of Antiviral Genes

We further investigate the expression profiles of antiviral genes after CyHV-2 infection at host susceptible and less-susceptible temperatures. Using the resulting cDNA as templates and β-actin was chosen as a reference gene, the expression levels of Mx, IFNϕ1, TNF-α, IL-1β, FAS and Caspase-8 in spleens were examined with relative real-time PCR. All primers were listed in Table 1. The reaction mix contained 10 μL of PrimeScript RT Enzyme Mix, 2 μL of diluted cDNA, 0.4 μL of each primer (10 mM), and RNase-free water to a volume of 20 μL. Each reaction was run in triplicates to minimize errors. Finally, the melting curve analysis from 60 ℃ to 95 ℃ was conducted to confirm the amplified product was single. The relative expression level of each gene was normalized to β-actin and analyzed by $2^{-\triangle\triangle Ct}$ method.

Determination of Antioxidant Enzymes Activities

For antioxidant enzymes activities analysis, the activities of superoxide dismutase (SOD) and catalase (CAT) in serum were measured using the corresponding test kits (NanJing JianCheng Bio Inst, China) according to the manufacturer's protocols. The SOD activity was measured spectrophotometrically at 550 nm. One unit of SOD activity was defined as the amount of enzyme that caused a 50% inhibition of nitro blue tetrazolium. The CAT activity was measured spectrophotometrically at 240 nm. One unit of CAT activity was defined as the activity required to destroy 1μ mol H_2O_2 at 25 ℃ in 0.2 mol/L phosphate buffer for 1 min. All enzyme assays were carried out on ice and repeated for 3 times. The changes in absorbance were monitored to determine the enzyme activities using a microplate reader (KHB, China).

Statistical Analysis

Results were subjected to the statistical analyses using SPSS 21.0 software. The data are presented as mean ± SE (n), where n is the number of samples. Differences between control and treatment groups were assessed by one-way ANOVA. Statistical significance was accepted at $P<0.05$ and Tukey's multiple comparison post hoc tests to determine differences.

Results

CyHV-2 Loads in Spleen Under 15 ℃ and 25 ℃

Here, we investigated the dynamic changes of viral load in gibel carp both in the 15 ℃ group and the 25 ℃ group as an indicator of viral replication. Gibel carp were infected artificially with CyHV-2 and the viral DNA loads in spleens were examined by qPCR. In our previous study, gibel carp were infected with CyHV-2 at 15 ℃ and 25 ℃, and the survival rate was 5% and 100%, respectively (Data not shown). That was in accordance with the epidemiological surveys for the disease. As shown in Figure 1, the viral DNA copies in the spleen of gibel carp was detected as early as 12 hpi in both groups. With the extension of the infection time, the virus loads in the 15 ℃-group were maintained at 10^5 copies/100 ng DNA, while that of the 25 ℃-group reached above $10^{8.4}$ copies/100 ng DNA, suggesting that the virus growth was inhibited in the 15 ℃ group.

Expression Kinetics of Antiviral Genes Under 15 ℃ and 25 ℃

Expression kinetics of diverse classes of antiviral genes in the spleen of virus-infected gibel carp under 15 ℃ and 25 ℃ were investigated (Figure 2). For the interferon-related genes (IFNϕ1 and Mx), the genes were highly expressed from 12 hpi to 48 hpi in both groups with a tendency of increase first and then decline later. Particularly, the 15 ℃ group showed rapid and strong expression of interferon-related genes in contrast with the delayed response in the 25 ℃ group. For inflammatory cytokines (TNF-α and IL-1β), the genes in the 15 ℃ group were up-regulated from at 12 hpi and 24 hpi and subsequently maintained at a low level by the end of the trial. As for the 25 ℃ group, the expression profiles of two genes had a transient upregulation at 12 hpi and decreased drastically later. For apoptosis-related genes (FAS and Caspase-8), the expression levels were very low at 12 hpi and 24 hpi in both the experimental groups. However, the two genes showed a relatively higher expression level in the 15 ℃ group than that in the 25 ℃ group during 48-96 hpi.

Antioxidant Enzyme Activities Under 15 ℃ and 25 ℃

The activity of two antioxidant enzymes (SOD and CAT) were examined to evaluate the stress response at the two temperatures. As shown in Figure 3, a significant reduction in antioxidant enzyme activities was found during the CyHV-2 infection in both groups. At the late stage of CyHV-2 infection, it was observed that the levels of the antioxidant enzymes in the 15 ℃ group were relatively higher than those in the 25 ℃ group.

Discussion

Recently, the development of gibel carp culture is challenged by the emergence of gibel carp hematopoietic necrosis caused by CyHV-2 infection. Previous study showed that the mortality caused by some aquatic viruses was closely related to water temperature, which was described as the abiotic master factor for fishes. Thus, it is necessary to explore the relationship between the CyHV-2 infection in the host and water temperature. As for CyHV-2, high mortality caused by infection is often recorded at temperatures above 20 ℃ by the epidemiological survey but hardly happens at temperatures below 15 ℃ (Ouyang et al., 2020). For this reason, we conducted the CyHV-2 infection artificially under 15 ℃ and 25 ℃, to clarify the difference of CyHV-2 infection in gibel carp under susceptible and less-susceptible temperatures. In the present study, we investigated the viral DNA replication, the antiviral genes expression and antioxidant enzyme response in gibel carp after CyHV-2 infection at the two temperatures.

Previous studies had showed that there were close relationships between temperature and virus replication (Chung et al., 2015). As for CyHV-2, Ito et al. found that cumulative mortalities of Ryukin goldfish infected with CyHV-2 at 15 ℃ and 25 ℃ were 10% and 90% (Ito et al., 2013). However, the virus could grow well at both 15 ℃ and 25 ℃ in RyuF-2 cells, indicating that the replication of CyHV-2 was not inhibited at 15 ℃ in vitro (Ito et al., 2013). In this study, we found that there was obvious difference in viral loads of virus-infected gibel carp between 15 ℃ and 25 ℃. The viral DNA copies in the 25 ℃ group rapidly increased and reached their

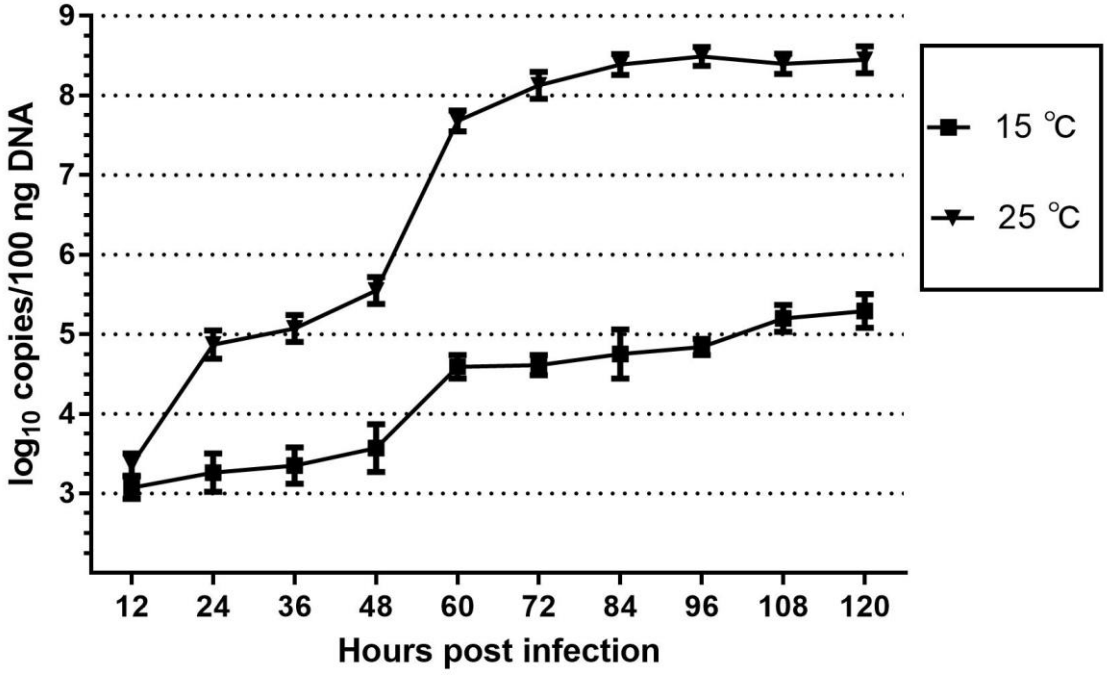

Figure 1. Viral DNA copies of CyHV-2 in the spleen of gibel carp maintained at 15 ℃ and 25 ℃.

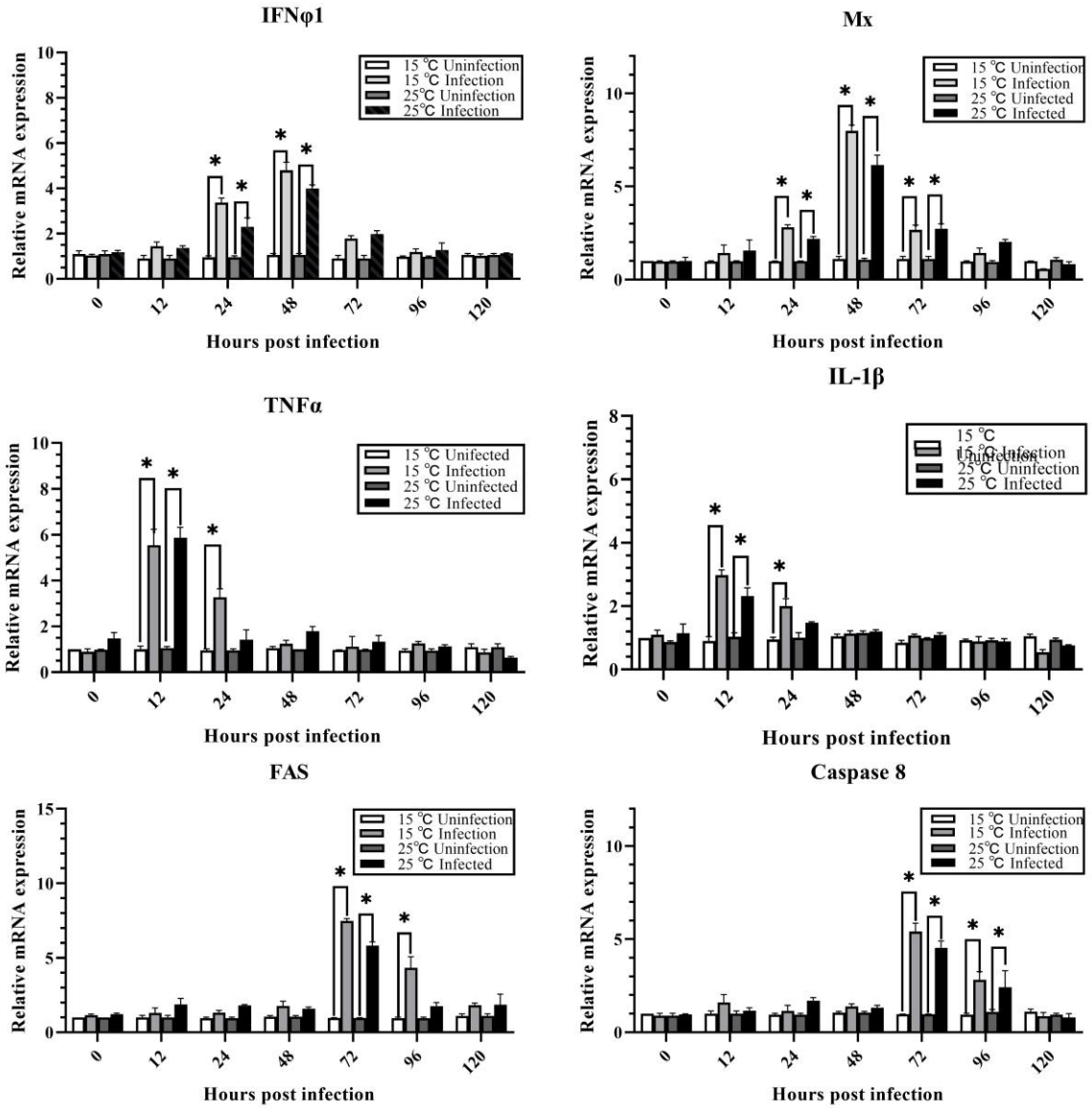

Figure 2. Expressions profiles of antiviral genes in spleen of gibel carp maintained at 15 ℃ and 25 ℃. Expression levels were normalized against the β-actin. The relative expression was plotted with standard error against the time after infection.

peaks by 72 hpi, while those in the 15 ℃ group increased slowly to a stable value by the end of the trial (shown in Figure 1). In gibel carp, the viral DNA replication was inhibited when infected with CyHV-2 at 15 ℃, which may lead to host survival.

The influence of water temperature on virus infection involves a set of complex interactions between the virus and the host immune system (Kayansamruaj et al., 2014). In fish, several studies have demonstrated that there were temperature-dependent immune responses during virus infection (Zhang et al., 2017). Among them, interferon-mediated immune responses are key to establish an antiviral defense and inhibit viral replication (Mou et al., 2018). In this study, two interferon-related genes (IFNϕ1 and Mx) were highly expressed from 12 hpi to 48 hpi in both groups, with a tendency of increase first and then decline later. Particularly, the 15 ℃ group showed quicker and stronger expression of interferon-related genes in contrast. Thus, we can predict that the early activation of interferon-related genes in gibel carp infected with CyHV-2 at 15 ℃ may be correlated with the disease resistance, which is well supported by the previous report, pointing out that the quicker induction of interferon-related genes was essential to overcome viral pathogenicity (Poynter & DeWitte-Orr, 2016).

Inflammation could prevent spread of infection as a protected mechanism against the invading pathogens (Nuriev & Johansson, 2019). In most cases, virus infection could trigger an intracellular inflammatory response that includes increasing expression of proinflammatory cytokines, including TNF-α and IL-1β. The expression of these cytokines plays essential roles involved in activating the IFN and other immune-related pathways. In this study, when gibel carp infected with CyHV-2 at 15 ℃, the two proinflammatory cytokines

(TNF-α and IL-1β) in the spleens were up-regulated from at 12 hpi and 24 hpi and subsequently maintained at a low level by the end of the trial. In the 25 ℃ group, the expression profiles of two genes exhibited a similar trend. However, the genes were immediately up-regulated at 12 hpi with slight extension compared to those in the 15 ℃ group. Similarly, the upregulation of the proinflammatory cytokines at the early phase of virus infection was also described in hirame rhabdovirus-infected flounder (Zhang et al., 2017). However, it was noted that the duration of upregulation of the proinflammatory cytokines was longer after CyHV-2 infection at 15 ℃. This suggested that virus failed to depress the inflammatory response in the host at the adaptation period at the less-susceptible temperature.

Previous study reported that CyHV-2 could induce apoptosis in host cells during their infection cycles (lu & Lu, 2018). Apoptosis, the programmed cell death process of damaged cells, plays a vital role in the normal development and steady state of host cells. FAS is a transmembrane receptor located on the cell membrane, which can trigger the activation of death receptor signal pathway, regulate the secretion of Caspase 8 and lead to apoptosis (Song et al., 2021). In our study, FAS and Caspase-8 was very low at 12 hpi and 24 hpi in both the experimental groups, implying that the apoptosis induced by CyHV-2 infection was inhibited at an early stage. This is consistent with the previous study which reported that VHSV suppresses apoptotic genes at early stage of viral infection (Kole et al., 2019). It is worth noting that the two genes showed a relatively higher expression level in the 15 ℃ group than that in the 25 ℃ group during 48–96 hpi. Similarly, the higher expression of apoptosis-related genes at the late infection stage was also observed in the VHSV-infected fish at the less-susceptible temperature(Kole et al., 2019).

The activity of antioxidative enzyme in serum is often used to evaluate the immune state of a fish (Meng et al., 2017). Increasing evidence have shown that virus infection can induce overproduction of reactive oxygen species (ROS), which leads to oxidative stress within host cells (Kim et al., 2012). SOD and CAT enzymes, the main antioxidant enzymes in fish, are significant defenses against the ROS produced in cells. Here, we examined the activities of SOD and CAT in the serum. As shown in Figure 3, a significant reduction in SOD and CAT activities in CyHV-2 infected gibel carp was observed at both 15 ℃ and 25 ℃. It agrees with the previous report that virus infection can stimulate ROS production and inhibit antioxidant enzyme levels (Wang et al., 2012). Besides, it is noteworthy that the levels of the antioxidant enzymes in the 15 ℃ group were relatively higher than those in the 25 ℃ group at the late stage of virus

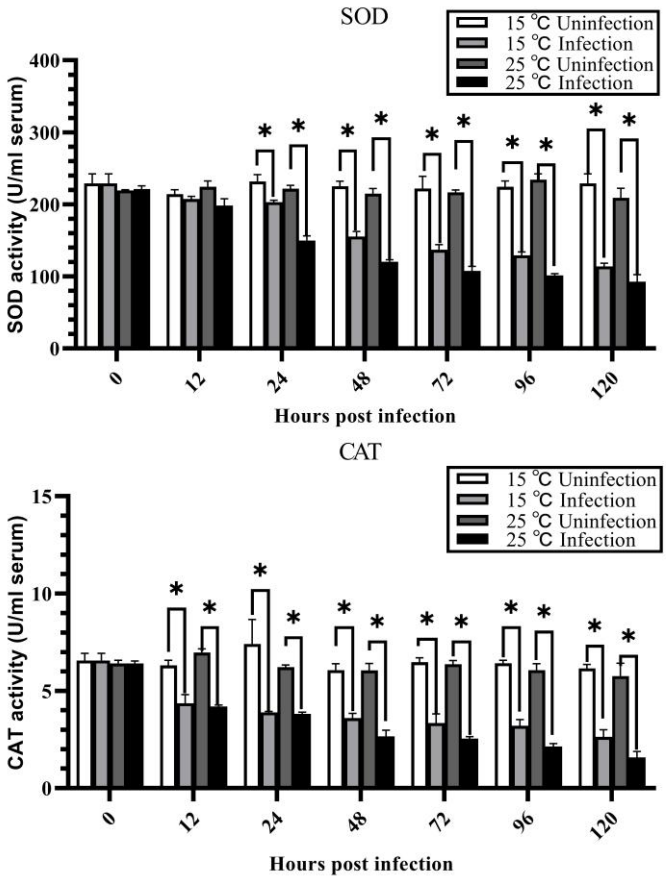

Figure 3. Activities of SOD and CAT in the serum of gibel carp maintained at 15℃ and 25℃.

infection, suggesting that severity of depletion in enzymatic antioxidant defenses may be alleviated when CyHV-2 infected at less-susceptible temperature.

Conclusion

In conclusion, we investigated the differences of viral replication, antiviral gene expression and antioxidant enzyme activity in gibel carp infected with CyHV-2 under 15 ℃ and 25 ℃. Compared with the 25 ℃ group, fish in the 15 ℃ group showed a stronger and quicker induction of interferon-related genes and proinflammatory genes at the early infection stage, and more powerful apoptotic response and antioxidant defense at the late infection stage. Taken together, water temperature affected the immune response against CyHV-2 infection, which may ultimately lead to host survival in the less-susceptible temperatures. The study would help us to gain an insight on the relationship between temperature and CyHV-2 infection, providing a certain value in developing the control measures for gibel carp hematopoietic necrosis.

Ethical Statement

Authors declare no ethical statement.

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Author Contribution

Jialin Zhang: Conceptualization, Writing -review and editing; Shaodong Meng: Methodology, Visualization and Writing -original draft; Heng Ge: Writing -review and editing; Shanling Sun: Writing - review and editing; Xinran Yu: Methodology, Visualization; Guo Qiao: Writing - review and editing; Qiang Li: Funding Acquisition, Project Administration, Resources.

Conflict of Interest

The author(s) declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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