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Temperature-dependent viral replication and antiviral apoptotic response in viral haemorrhagic septicaemia virus (VHSV)-infected olive flounder (*Paralichthys olivaceus*)

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ABSTRACT

The olive flounder (*Paralichthys olivaceus*) shows a high rate of mortality to viral haemorrhagic septicaemia virus (VHSV) in the winter and spring but has zero mortality over 20 °C. In this experiment, we studied the effect of rearing temperature on viral replication, viral transcription and antiviral apoptotic immune response in VHSV-infected olive flounder by real-time polymerase chain reaction. Olive flounder were given intra-peritoneal injections of VHSV ($10^{7.8}$ $TCID_{50}$/ml) and were reared at 15 °C or 20 °C. Five fish were randomly sampled for head kidney at 3, 6 and 12 h post-infection (hpi) and 1, 2, 4 and 7 days post-infection (dpi). Total RNA extracted from the tissue was reverse transcribed and used as template for real-time PCR. In the 15 °C group, the number of viral gRNA copies peaked after 2 dpi and remained high through 7 dpi, while in the 20 °C group, the copy number was at the highest at 1 dpi but drastically declined at later stages. Viral mRNA levels in the 15 °C group gradually increased starting at 3 hpi to reach their maximum value at 12 hpi and remained high until 2 dpi, whereas the other group showed much lower copy numbers that were undetectably low at 4 and 7 dpi. Type II IFN expression increased as the viral copies increased and the 20 °C group showed quicker and stronger expression than the 15 °C group. The MHC class I and CD8 expression was high in both the groups at early stage of infection (3–6 hpi) but at later stages (2–7 dpi) in 15 °C group expression reduced below control levels, while they expressed higher to control in 20 °C group. The expression of granzyme in 15 °C fish showed a single peak at 2 dpi, but was consistently expressing in 20 °C fish. Individuals expressed very high levels of perforin expressed very high levels of caspase 3. In 15 °C fish, TNFα, FasL and p53 expressed significantly higher than 20 °C only at initial stages of infection (3–6 hpi). Caspase 3 expression found to be low in 15 °C fish whereas it was significantly elevated in 20 °C group. Interestingly individual fish with high caspase 3 expression contained very low viral RNA. Thus, from our experiment, we can conclude that an effective apoptotic immune response in VHSV-infected olive flounder plays a crucial role in the survival of the host at higher temperatures.

1. Introduction

The olive flounder (*Paralichthys olivaceus*), a temperate marine fish that is distributed along the Western Pacific coast, is a major aquaculture species for the Korea and Japan [1,2]. However, since 2001 Korean olive flounder aquaculture industry has been experiencing wide-spread mortality due to viral haemorrhagic septicaemia virus (VHSV) infection during the winter and spring, but shows no mortality in the summer season [3,4], indicating a relationship between antiviral immunity and rearing temperature. Sano et al. [5] demonstrated the effect of water temperature on the mortality of VHSV-infected olive flounder. They found that the fish do not die at 20 °C, but the same group succumbs to the virus at 15 °C. This phenomenon of temperature-dependent pathogenicity has been reported for infectious haematopoietic necrosis virus (IHNV) or VHSV-infected salmonids [6–8], VHSV-infected Japanese eel (*Anguilla japonica*) [9], VHSV-infected bluegills (*Lepomis macrochirus*) [10] and spring viraemia of carp virus (SVCV)-infected common carp (*Cyprinus carpio*) [11].

Upon viral infection, the host induces expression of interferon (IFN) to stimulate antiviral immune responses. Both type I and type II IFNs are effective activators of major histocompatibility complex

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(MHC) class I, which presents processed antigen to CD8+ T cells (cytotoxic T lymphocyte – CTL) [12,13]. Activated CTLs kill the infected cell by releasing cytolytic granules (such as granzyme and perforin) or activating death receptors to initiate apoptosis of the target cell [14,15].

An immediate apoptotic effect upon viral infection should limit viral replication and stop or reduce viral spreading. However, some viruses have developed mechanisms to block or delay early apoptosis so that they can achieve maximum replication [14,16]. Though the recombinant Mx protein was very efficient in controlling the VHSV in in vitro condition [17], VHSV-infected olive flounder containing high viral copies at 15 °C expressed high level of Mx transcript but toll-like receptor 7 (TLR-7) and TLR-2 were under-expressed [18]. The non-virion (NV) protein of VHSV is reported to inhibit apoptosis at an early infectious stage to allow effective viral recruitment [19]. A VHSV NV knockout mutant failed to cause disease in olive flounder at 15 °C [20,21] confirming that VHSV has an inhibitory mechanism on the host immune system.

Based on our previous results [18] and the importance of an apoptotic immune defence mechanism, we designed this study to investigate the expression kinetics of apoptosis-associated genes in VHSV-infected olive flounder maintained at two different temperatures. We developed VHSV infection in olive flounder at 15 °C and 20 °C to study the apoptotic immune response and the viral replication at early infectious as well as later recovery period.

2. Materials and methods

2.1. Experimental infection

VHSV (F1Wa05 strain) cultured in fathead minnow (FHM) cells was stored at −80 °C. Olive flounder (18–22 g) collected from a local farm were divided into two groups and were maintained at 15 ± 0.5 °C and 20 ± 0.5 °C in a controlled indoor rearing facility. The fish were acclimatised to their respective rearing condition for 7 days prior to infection. A nested polymerase chain reaction (PCR) of 10 randomly selected individuals using VG1-VD3 and VD5-VD3 primers (VG1: 5′-atggaatggaacacttttttc-3′ VD5: 5′-tcccgctatcagtcaccag-3′and VD3: 5′-tgtgatcatgggtcctggtg-3′) [22] confirmed that the fish were initially free of VHSV. Sixty fish per group were intraperitoneally injected with 100 μl of viral suspension ($10^{7.8}$ $TCID_{50}$/fish). A similar mock injection was given to control fish for both the 15 °C and 20 °C groups. We randomly selected five fish/sample at 3, 6 and 12 h post-infection (hpi), and 1, 2, 4 and 7 day post-infection (dpi) from both the 15 °C and 20 °C groups. The head kidneys of fish were aseptically dissected, flash frozen and stored at −80 °C until they were used for total RNA extraction.

2.2. Total RNA extraction and reverse transcription

Total RNA was extracted using RNAiso Plus (Takara Bio Inc, Japan), following standard protocols. The dried RNA pellet was dissolved in RNase-DNase free water (Sigma–Aldrich, USA) and was aliquoted and stored at −20 °C until further use. The RNA was checked for quality by 1% agarose gel electrophoresis and was quantified using a NanoDrop™ 1000 spectrophotometer (Thermo Scientific, USA). One microgram of total RNA treated with DNase I (Amplification grade; Invitrogen Life Technologies, USA) was reverse transcribed using moloney murine leukaemia virus (M-MLV) reverse transcriptase (Bioneer, Korea) using an oligo dT primer (Invitrogen Life Technologies, USA) as recommended by the manufacturer. The resulting cDNA was stored at −20 °C.

The cDNA of VHSV genomic RNA (gRNA) was synthesised by reverse transcribing 1 μg of total RNA with specific sense primer for the VHSV N gene (5′-gtatcataaaagatgatgagttatgttacagggg-3′). This cDNA was used for absolute quantification of mature virus (negative strand genome). To quantify the viral N gene transcript (positive sense mRNA), cDNA was synthesised using oligo dT primers (the same mentioned above).

2.3. Primers

Primers for partial sequencing of caspase 3 (sense primer- 5′-*gtcccacagcttcagatacagc*-3′, antisense primer 5′-*gacgtggagacgacatcag*-3′; product size: 712 bp) were designed from our cDNA library following earlier described methods [18]. The deduced amino acid sequence showed close homology with the caspase 3 from the fire clownfish (*Amphiprion melanopus*) (92%), mandarin fish (*Siniperca chuatsi*) (91%), Atlantic salmon (*Salmo salar*) (73%), chicken (*Gallus gallus*) (66%) and human (*Homo sapiens*) (65%), confirming that the obtained sequence is caspase 3 of the olive flounder. Primers for real-time PCR were designed using Primer3-Plus. The primer efficiency was determined as previously described [18]. The details of the primers used for real-time PCR expression study are given in Table 1.

2.4. Quantitative expression of immune genes and viral RNA

Real-time PCR was carried out in an *Exicycler*™ 96 Real-Time Quantitative Thermal Block (Bioneer, Korea) using SYBR Green *AccuPower*® PCR PreMix (Bioneer, Korea). One microlitre of five-fold diluted cDNA was used as the PCR template (performed in duplicate). The reaction program was set according to the supplied manual. The relative expression level was calculated based on Pfaffl's normalization method [23] using the equation

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

Target gene	GenBank acc. no	Product length	Sense primer	Antisense primer	Ta[a]	PCR efficiency
β actin	HQ386788	131	cctcttccagccttcattc	tggttcctccagatagcac	56	2.0979
VHSV N RNA	EF079895	138	atctggaggcaaagtgcaag	ccatgaggttgtcgttgttg	62	2.0221
Type II IFN	AB435093	126	ctacaagcggcgatatgatg	ggaggttctggatggttttg	64	2.0807
MHC Class I	AB126921	148	tctccctcctctccagtcagc	gctcatctggaaggtcccgtcat	58	1.9694
CD8	AB082957	108	taagggcaacactaacacagg	atgaggaggaggagaaggag	56	2.0980
TNFα	AB040449	113	aaacacctcacgtccatca	gcgtcctcctgactcttct	56	1.9888
Perforin	AB084905	100	aacaacaacaacaacccacac	Tgtccctgtcccacactt	56	1.9325
Granzyme	AB191196	121	attccaggcagctcataaa	gtcttggtttccttcacaga	58	1.9217
FasL	AB206381	128	gagcaaatcgagggacgaga	ttcctttctccagcgtgacc	64	1.9674
p53	EF564441	159	cgaggaaagcagcaccaaga	ccccgaccgacaataggaag	58	1.9558
Caspase 3	JQ394697[b]	115	acatcatgacacgggtgaac	tccttcgtcagcattgacac	58	1.9146

[a] Ta: annealing temperature.
[b] Sequenced in this study.

$$\text{Expression ratio} = E_t^{\Delta Ct\ (\text{control}-\text{experiment})} / E_c^{\Delta Ct\ (\text{control}-\text{experiment})}$$

where, E_t is the real-time PCR efficiency of the target gene transcript and E_c is the real-time PCR efficiency of the internal control (β-actin) gene transcript.

We targeted the VHSV N gene to determine the absolute copy number of the viral genome and to assess its transcription rate. The method for developing a standard curve was described earlier [18].

2.5. Statistical analysis

The statistical analysis was performed by using SAS version 9.2 (SAS Institute Inc. USA). The gene expression at different time points was compared by one-way analysis of variance (ANOVA), using Duncan's multiple range test. An unpaired *t*-test was used to ascertain statistical significance for differences in gene expression between the 15 °C and 20 °C fish groups. Pearson's correlation was performed to understand the relation between the expression levels of different gene transcripts. A value of $p < 0.05$ was considered to indicate the statistical significance. The mean expression values and the standard error at each time point were determined.

3. Results

3.1. Experimental infection

Olive flounder reared at 15 °C were observed for visible dropsy starting at 4 dpi. The fish mortality between 14 and 20 dpi resulted in a final mortality of 24%. Neither dropsy nor mortality was observed in the 20 °C group or the control groups. The dead fish exhibited ascites and severe haemorrhage in their internal organs and muscles.

3.2. Time-dependent replication of VHSV

Viral gRNA and mRNA copies were quantified by an absolute quantification method. Neither pre-challenged nor mock-injected fish tested positive for VHSV. We analysed gRNA and mRNA copies of VHSV at the above-mentioned sampling times in both the 15 °C and 20 °C fish groups.

The virus-infected olive flounder maintained at 15 °C tested positive for viral gRNA and mRNA as early as 3 hpi. The viral transcription gradually increased and reached its peak value by 12 hpi and remained at that level until 2 dpi, while the gRNA copy number increased from 2 dpi (Table 2A). Although the gRNA levels at 4 dpi were still high, surprisingly, the mRNA copy number was much lower; two out of five fish studied had no detectable viral transcripts. One fish had ascites at this time (not included in the relative expression study), exhibited a large amount of viral gRNA (15392) and mRNA (2364). On 7 dpi, we observed a high degree of variation in gRNA copy number among the infected fish of the group. At the same time, the viral transcription was much lower or undetectable in all fish except one fish with ascites, which had high gRNA and mRNA.

Fish reared at 20 °C contained similar levels of gRNA but had higher levels of mRNA at 3 hpi compared to the 15 °C group (Table 2B). The gRNA copies were at the highest level at 1 dpi in 20 °C, whereas it was at the highest level at 2 dpi in 15 °C (Fig. 1A). However, the level of viral mRNA at 20 °C remained constant from 3 hpi to 2 dpi and was 10-fold lower than that seen in 15 °C (Fig. 1B). A drastic difference in the viral copy between the two temperature groups was noted at 2 dpi, due to a sharp decline in the gRNA copies in the 20 °C fish group. By 4 dpi, the levels of gRNA and mRNA in most of the 20 °C group declined further; one out of five fish still contained extremely high gRNA but had no detectable mRNA. The decline in viral copies in the later recovery phase was much stronger and quicker in the 20 °C group than in the 15 °C group. By 7 dpi, all fish at 20 °C had much fewer viral genomes, while the 15 °C fish had higher copies of viral genome.

3.3. Relative gene expression kinetics

The study included relative expression of type II IFN, MHC class I, CD8 and apoptotic genes in fish reared at 15 °C and 20 °C (Tables 2A and 2B). Type II IFN expression increased with the viral transcription level. When viral transcription was at its peak, type II IFN expression was at its maximum; type II IFN expression was lower at early and late stages when the viral copy numbers were low. At 12 hpi, the 15 °C fish group had a high Pearson's correlation coefficient ($p < 0.05$) of 0.94 between type II IFN and viral gRNA. The 20 °C fish group expressed quicker and more robust type II IFN from 6 hpi to 1 dpi, which gradually returned to normal levels by 7 dpi (Fig. 2A). The type II IFN expressed by fish at 20 °C positively correlated ($p < 0.05$) with viral copy number at 6 hpi ($r = 0.88$) and 12 hpi ($r = 0.99$). Expression of MHC class I and CD8 were elevated in the 15 °C fish group until 1 dpi, reaching their maximum expression at 6 hpi. However, expression levels were reduced to under control levels at 4 and 7 dpi. Fish in the 20 °C group expressed two-fold higher levels of MHC class I than the control, which remained at similar levels throughout the experiment and expressed significantly higher ($p < 0.05$) than 15 °C group at 7 dpi (Fig. 2B). In 20 °C, CD8 was highly expressed at 3 and 6 hpi, and showed reduced but still elevated expression at later stages, expressing considerably higher to 15 °C at 4 dpi (Fig. 2C).

The apoptotic genes studied included granzyme, perforin, tumour necrosis factor α (TNFα), Fas ligand (FasL), p53 and caspase 3 (Tables 2A and 2B). In the 15 °C fish, granzyme and perforin showed elevated expression at early stages (3–6 hpi), but expression declined at 12 hpi and 1 dpi at the same time the viral transcription reached maximum. The 15 °C group expressed higher levels of granzyme compared to the 20 °C fish at 2 dpi, before reducing the expression at 4 and 7 dpi (Fig. 2D). Perforin was highly expressed at 4 and 7 dpi in 15 °C when the fish showed a drastic reduction in viral copies (Fig. 2E). The 20 °C group expressed granzyme at two- to five-fold higher levels than control throughout the experimental period while, perforin exhibited enhanced expression at 6 hpi and 2 to 4 dpi. In both the groups, fish with high perforin expression had higher caspase 3 expression (in the 15 °C group, fish nos. 3, 26 and 31; in the 20 °C group, fish nos. 6, 23 and 30) (Tables 2A and 2B).

The 15 °C group expressed the apoptosis inducers FasL and p53 at elevated levels only for 3–6 hpi, while elevated TNFα expression extended until 12 hpi. Transcription levels of most of these genes were reduced to near-control levels in most of the 15 °C fish analysed from 1 to 7 dpi; p53 was the exception; it recovered high expression levels at 7 dpi (Table 2A). The expression of granzyme, perforin, FasL, p53, and caspase 3 showed high correlation at 3 hpi. An increase in expression (6–12 hpi) of TNFα and FasL was noted in the 20 °C fish group, while p53 had higher expression at 3 hpi and again at 1–4 dpi (Table 2B). TNFα expression was significantly higher in 15 °C group compared to 20 °C at 3 hpi but found similar in later part of the experiment (Fig. 2F). The difference in the expression levels of FasL and p53 between the 15 °C and 20 °C groups was statistically significant ($p < 0.05$) at 6 hpi, where the expression levels in the former group were higher than in the latter group. However, at later stages, expression of FasL was reduced to near-control levels in both groups (Fig. 2G) while p53 showed expression peak at 2 dpi in 20 °C and 7 dpi in 15 °C (Fig. 2H).

Table 2A
Absolute copy numbers of viral mRNA and gRNA, relative expression of apoptotic transcripts in the head kidney of VHSV-infected olive flounder in the 15 °C group at different times after infection. Five fish were analysed per time point. The values are also depicted as the mean ± standard error (m ± se).

Time	Fish no	gRNA[§]	mRNA[*,§]	Type II IFN	MHC class I	CD8	Granzyme	Perforin	TNFα	FasL	p53	Caspase 3
3 h	1	292.9	68.9	1.7	4.4	3.1	1.6	2.1	4.5	2.2	1.2	2.1
	2	194.7	60.8	5.3	6.9	5.7	7.0	4.0	12.7	1.8	6.4	3.1
	3	787.2	4.5	1.9	1.1	2.0	3.5	3.8	2.2	3.6	1.1	1.2
	4	466.6	–	27.1	1.3	1.7	43.7	82.9	5.0	18.1	43.9	61.6
	5	244.9	–	–	1.2	1.6	1.6	3.4	3.4	0.9	1.5	12.9
m ± se		397.2 ± 107.7	27.2 ± 34.5	7.2 ± 11.3	3.0 ± 2.6^{b}	2.8 ± 1.7^{bc}	11.5 ± 8.1^{ab}	19.2 ± 15.9	5.6 ± 1.9^{a}	5.3 ± 3.2 ^{ab}	10.8 ± 8.3	16.2 ± 11.5
6 h	6	186.5	1313.8	–	14.1	10.4	5.3	8.2	4.7	7.3	14.7	1.7
	7	37.7	160.5	11.5	3.2	20.6	16.3	2.4	5.4	7.5	15.1	2.1
	8	208.4	78.4	1.5	14.2	22.6	4.0	13.0	2.9	6.3	8.8	2.0
	9	92.8	18.2	5.5	2.1	6.2	3.1	9.0	4.4	7.8	6.4	0.9
	10	13.4	6.0	5.9	5.5	22.8	6.7	10.9	7.9	13.2	3.7	1.9
m ± se		107.8 ± 39.0	315.4 ± 561.5	4.9 ± 4.5	7.8 ± 5.9^{a}	16.5 ± 7.7^{a}	7.1 ± 2.4^{ab}	8.7 ± 1.8	5.1 ± 0.8^{ab}	8.4 ± 1.2^{a}	9.7 ± 2.2	1.7 ± 0.2
12 h	11	166.9	5427.5	70.0	1.8	0.5	1.5	0.5	10.6	0.8	0.8	2.3
	12	69.4	504.9	31.1	2.7	2.4	1.5	4.2	5.2	3.0	1.2	1.8
	13	37.6	53.0	11.1	2.6	1.1	0.7	1.0	2.0	0.2	1.0	2.9
	14	12.0	30.7	1.1	1.3	4.0	3.5	3.8	3.0	4.2	0.4	1.1
	15	7.9	3.2	0.2	0.5	2.1	3.1	0.5	4.0	1.1	0.4	3.7
m ± se		58.8 ± 29.2	1203.8 ± 2370.1	22.7 ± 29.2	1.8 ± 0.9^{b}	2.0 ± 1.3^{bc}	2.1 ± 0.5^{b}	2.0 ± 0.8	4.9 ± 1.5 ^{ab}	1.9 ± 0.7^{b}	0.8 ± 0.2	2.4 ± 0.4
1 d	16	105.5	4441.0	120.3	11.3	2.3	1.6	–	1.3	0.1	0.9	1.2
	17	217.6	601.7	94.8	5.3	7.7	1.4	1.7	0.6	0.5	1.9	0.8
	18	1245.1	813.0	5.2	2.4	3.0	3.5	1.8	1.4	0.1	1.6	1.0
	19	49.4	55.6	10.1	1.9	1.7	1.6	0.6	1.8	0.1	0.6	0.7
	20	11.2	19.3	15.3	0.7	21.3	6.2	8.2	0.3	10.7	0.3	0.6
m ± se		325.8 ± 232.5	1186.1 ± 1851.7	49.1 ± 54.2	4.3 ± 4.2^{ab}	7.2 ± 8.2^{b}	2.9 ± 0.9^{b}	2.5 ± 1.5	1.1 ± 0.3^{c}	2.3 ± 2.1^{b}	1.1 ± 0.3	0.8 ± 0.1
2 d	21	1337.6	3092.8	246.5	2.7	0.5	11.6	2.4	2.9	6.3	2.2	1.7
	22	4505.1	2468.0	24.4	0.9	0.9	13.6	4.1	0.6	1.7	0.7	1.0
	23	75.9	104.0	0.0	2.1	0.8	20.9	1.1	0.4	0.2	1.5	3.4
	24	169.9	100.0	16.5	0.8	0.4	20.4	4.2	1.0	0.1	1.1	2.2
	25	250.2	83.9	1.3	0.8	0.8	11.5	1.7	0.6	0.2	1.2	2.0
m ± se		1267.7 ± 841.0	1169.7 ± 1486.8	57.7 ± 106.0	1.5 ± 0.9^{b}	0.7 ± 0.2^{c}	15.6 ± 2.1^{a}	2.7 ± 0.6	1.1 ± 0.5$_{c}$	1.7 ± 1.2 ^{b}	1.4 ± 0.2	2.1 ± 0.4
4 d	26	4669.4	9.9	1.1	0.4	0.4	8.6	52.4	3.6	0.6	0.3	40.1
	27	308.0	4.6	3.8	0.2	0.3	4.5	35.0	2.1	8.1	0.2	3.8
	28	181.2	–	1.5	0.2	0.0	1.0	5.1	2.9	0.2	0.2	3.8
	29	22.1	–	4.4	0.7	0.2	0.9	3.7	2.5	1.4	0.2	1.7
	30	670.0	38.2	0.5	0.6	0.1	2.0	3.0	0.1	0.0	1.0	5.1
m ± se		1170.1 ± 881.3	10.9 ± 15.6	2.2 ± 1.7	0.4 ± 0.2^{b}	0.2 ± 0.2^{c}	3.4 ± 1.4^{b}	19.8 ± 10.1	2.3 ± 0.6 ^{bc}	2.1 ± 1.5$_{b}$	0.4 ± 0.2	10.9 ± 7.3
7 d	31	2.2	–	1.7	0.0	0.1	3.1	72.9	0.3	2.0	35.0	68.3
	32	560.1	–	1.6	0.1	1.5	2.0	20.7	3.7	0.4	2.6	9.3
	33	152.6	2.0	1.7	0.6	0.3	1.0	3.8	1.1	2.4	12.5	0.5
	34	8.4	2.3	0.7	0.2	0.3	0.9	2.1	1.2	0.2	7.7	2.9
	35[**]	3917.7	512.1	0.6	0.2	0.0	0.6	0.9	0.6	0.5	7.3	0.3
m ± se		926.2 ± 754.2	103.7 ± 228.3	1.3 ± 0.6	0.3 ± 0.2^{b}	0.4 ± 0.6^{c}	1.5 ± 0.4^{b}	20.1 ± 13.7	1.4 ± 0.6^{c}	1.1 ± 0.5^{b}	13.0 ± 5.7	16.3 ± 13.1

–: Not detected.
Superscript lower case letters denote significant differences among groups according to the Duncan's multiple range test ($\alpha = 0.05$).
[*] Data of mRNA copies is reported from our published article [18].
[§] Absolute copy number is per 50 ng total RNA.
[**] Fish with ascites.

Table 2B
Absolute copy numbers of viral mRNA and gRNA, relative expression of apoptotic transcripts in the head kidney of VHSV-infected olive flounder in the 20 °C group at different times after infection. Five fish were analysed per sample. The values are also depicted as the mean ± standard error (m ± se).

Time	Fish No	gRNA§	mRNA§	Type II IFN	MHC class I	CD8	Granzyme	Perforin	TNFα	FasL	p53	Caspase 3
3 h	1	185.2	244.9	0.7	1.4	–	3.0	1.7	1.7	2.0	3.6	2.3
	2	196.1	129.4	0.3	2.7	2.4	2.1	2.1	0.9	1.3	4.0	1.0
	3	475.0	24.6	0.3	1.6	4.4	2.7	0.7	0.7	1.3	5.5	0.7
	4	175.5	14.9	0.4	2.0	8.1	1.3	1.4	0.8	1.9	2.7	0.2
	5	648.7	–	0.8	2.5	5.0	3.6	0.3	1.0	0.6	7.5	1.4
m ± se		336.1 ± 96.2	83.0 ± 103.8	0.5 ± 0.2	2.0 ± 0.6	4.0 ± 3.0^{b}	2.5 ± 0.4	1.2 ± 0.3	1.0 ± 0.2^{b}	1.4 ± 0.2	4.7 ± 0.9^{ab}	1.1 ± 0.4
6 h	6	16.9	358.0	62.4	3.7	6.0	2.1	62.6	6.5	–	–	126.2
	7	776.0	59.1	32.3	2.0	8.6	1.6	0.9	15.8	3.1	2.7	1.2
	8	56.2	59.5	36.1	3.2	9.5	4.6	4.3	2.6	4.9	0.6	5.3
	9	672.4	–	7.9	0.7	8.6	2.9	3.5	1.5	6.2	1.9	0.9
	10	519.5	–	–	1.0	10.8	2.4	1.8	4.6	2.3	2.2	1.6
m ± se		408.2 ± 157.2	95.7 ± 149.5	27.7 ± 24.8	2.1 ± 1.3	8.7 ± 1.7^{a}	2.7 ± 0.5	14.6 ± 12.0	6.2 ± 2.5^{a}	4.1 ± 0.8	1.9 ± 0.4^{b}	27.0 ± 24.8
12 h	11	302.5	152.1	699.8	3.3	2.5	4.4	0.1	13.1	0.1	0.5	9.4
	12	79.2	13.8	52.9	2.6	1.0	2.2	1.9	3.8	1.8	1.5	1.7
	13	72.2	23.0	8.1	0.9	0.6	2.9	0.9	1.6	0.3	0.4	1.7
	14	1082.8	5.2	7.9	1.0	1.0	3.5	2.7	3.0	14.8	0.6	3.8
	15	9.3	4.9	2.4	0.5	1.5	2.8	8.6	2.7	0.3	1.4	14.5
m ± se		309.2 ± 199.7	39.8 ± 63.2	154.2 ± 305.7	1.7 ± 1.2	1.3 ± 0.7^{c}	3.1 ± 0.4	2.9 ± 1.5	4.9 ± 2.1^{ab}	3.4 ± 2.8	0.9 ± 0.2^{b}	6.2 ± 2.5
1 d	16	68.9	231.2	22.1	1.7	5.0	0.3	1.5	0.9	0.1	1.8	11.5
	17	993.6	83.9	58.4	3.2	4.6	4.5	2.9	0.4	3.0	5.1	2.2
	18	568.2	66.7	12.8	0.8	3.3	4.7	2.3	0.2	0.1	1.3	6.7
	19	132.2	29.4	0.9	0.0	2.2	4.0	2.9	0.2	0.0	0.1	12.5
	20	4521.2	4.2	20.1	0.4	1.6	3.5	5.1	0.2	3.9	1.7	1.1
m ± se		1256.8 ± 832.9	83.1 ± 88.5	20.1 ± 22.8	1.2 ± 1.3	3.3 ± 1.5^{bc}	3.4 ± 0.8	2.9 ± 0.6	0.4 ± 0.1^{b}	1.4 ± 0.8	2.0 ± 0.8^{ab}	6.8 ± 2.3
2 d	21	198.2	301.4	5.1	1.6	0.9	2.3	4.4	0.8	1.2	0.7	1.1
	22	367.0	275.6	32.2	1.1	1.6	3.2	10.8	0.5	1.1	5.3	0.9
	23	15.2	11.3	6.2	0.0	0.6	1.3	447.8	2.2	0.2	–	411.1
	24	16.0	5.9	3.2	0.8	4.6	0.4	3.4	0.8	–	1.6	49.6
	25	53.4	–	1.2	0.3	0.8	21.4	1.0	0.5	0.3	17.9	48.1
m ± se		130.0 ± 68.1	119.0 ± 155.0	9.6 ± 12.8	0.8 ± 0.6	1.7 ± 1.7^{bc}	5.7 ± 3.9	93.5 ± 88.6	1.0 ± 0.3^{b}	0.7 ± 0.2	6.4 ± 3.5^{a}	102.2 ± 78.0
4 d	26	8.3	18.9	8.3	3.7	4.3	2.5	1.5	2.6	0.1	0.3	23.6
	27	33.1	2.2	14.0	3.4	3.4	4.4	6.2	3.8	6.3	4.3	1.9
	28	4753.8	–	7.9	1.4	1.1	8.9	1.6	0.4	0.3	3.3	20.6
	29	–	–	2.3	0.1	0.7	1.4	3.2	0.8	1.6	3.0	1.1
	30	29.7	–	–	1.2	4.6	3.7	143.2	8.5	0.6	–	614.6
m ± se		965.0 ± 947.2	4.8 ± 7.9	6.5 ± 5.5	1.9 ± 1.5	2.8 ± 1.8^{bc}	4.2 ± 1.3	31.1 ± 28.0	3.2 ± 1.4^{ab}	1.8 ± 1.2	2.7 ± 0.8^{ab}	132.4 ± 120.7
7 d	31	84.5	3.3	2.0	3.7	2.2	2.3	3.8	0.6	1.6	2.2	0.8
	32	1.9	–	2.9	1.8	1.5	4.3	2.3	0.5	0.0	0.7	19.5
	33	20.5	–	0.7	2.2	1.5	2.1	2.5	0.9	0.7	2.7	1.0
	34	10.8	–	0.9	0.9	0.8	7.2	0.1	0.3	0.0	1.2	20.6
	35	–	–	1.7	0.2	0.6	2.6	1.7	0.6	0.0	1.7	11.2
m ± se		23.5 ± 15.7	1.5 ± 1.0	1.6 ± 0.9	1.8 ± 1.3	1.3 ± 0.6^{c}	3.7 ± 1.0	2.1 ± 0.6	0.6 ± 0.1^{b}	0.5 ± 0.3	1.7 ± 0.4^{b}	10.6 ± 1.3

§: absolute copy number is per 50 ng total RNA.
–: Not detected Superscript lower case letters denote significant differences among groups according to the Duncan's multiple range test ($\alpha = 0.05$).

Among the apoptotic genes, caspase 3 showed the most drastic differences in expression. In the 15 °C group, caspase 3 expression was significantly higher in two fish at 3 hpi, one fish at 4 dpi and two fish at 7 dpi; apart from these fish, the rest of the cohort expressed very low or near-control caspase 3 levels. Interestingly the 15 °C group, fish, which expressed high levels of caspase 3, contained very low or undetectable viral transcripts (Table 2A). Conversely, in the 20 °C fish group, the gene expression was near-

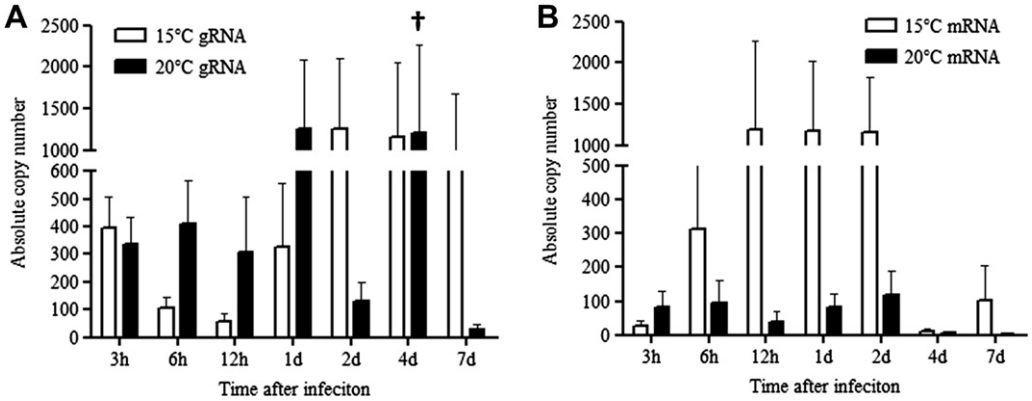

Fig. 1. Absolute viral copy number of gRNA (A) and mRNA (B) of VHSV in 50 ng total RNA of virus-infected olive flounder maintained at 15 °C and 20 °C. Mean copy number with standard error bar plotted against time after viral infection. (†: The average value of gRNA of 20 °C group at 4 dpi was high in one out of five individuals sampled, resulting in higher mean with high standard error). Details of individual experimental fish are given in Tables 2A and 2B.

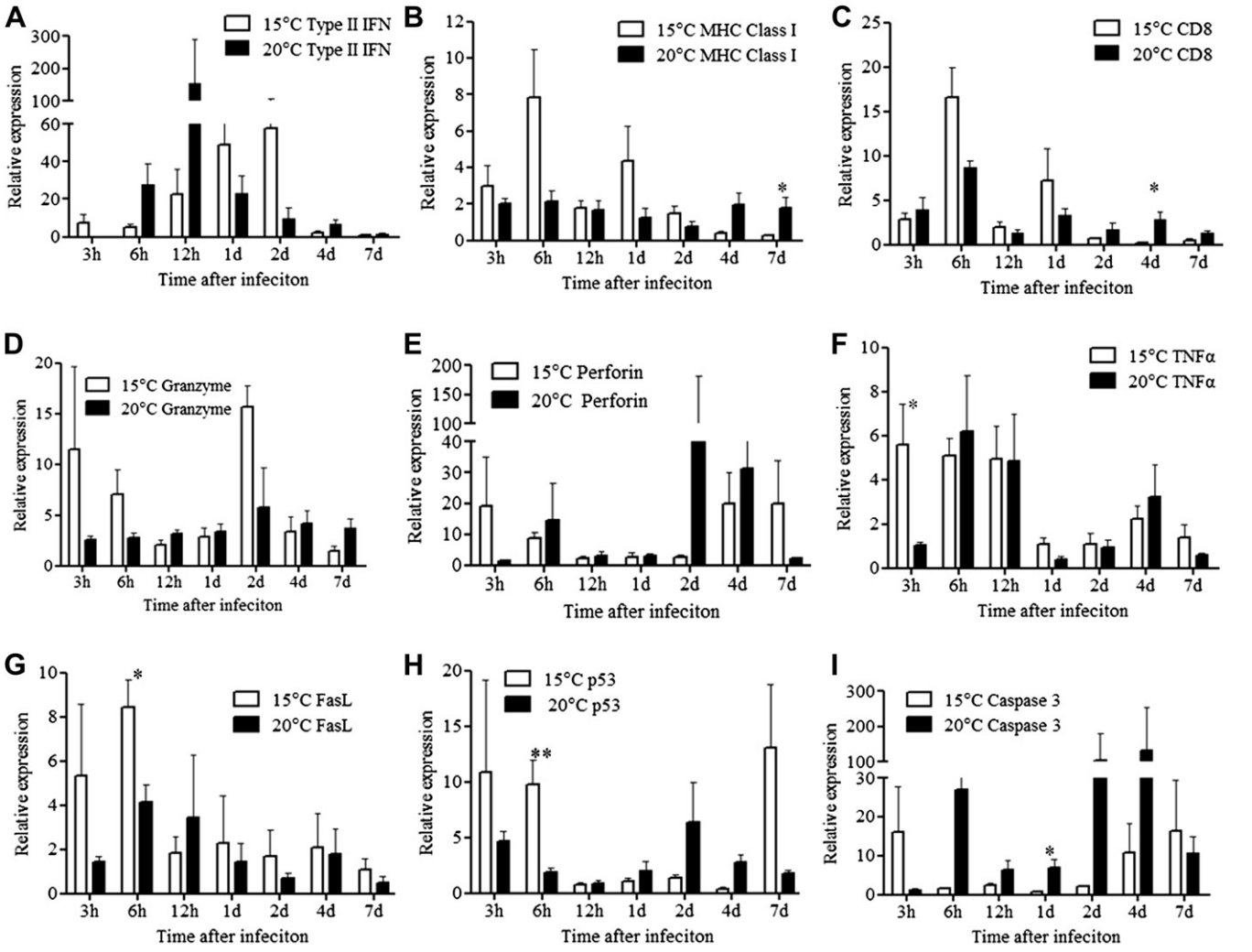

Fig. 2. Relative immune-related gene expression in kidney tissue of VHSV ($10^{7.8}$ $TCID_{50}$/fish)-infected olive flounder reared at 15 °C and 20 °C. Expression levels were normalized against the internal control, β actin. Expression levels between the two groups were compared by real-time PCR, and the mean relative expression of type II IFN (A), MHC class I (B), CD8 (C), granzyme (D), perforin (E) TNFα (F), FasL (G), p53 (H) and caspase 3 (I) were plotted with standard error against the time after infection. An unpaired *t*-test ($p < 0.05$ indicated by asterisks * and $p < 0.01$ by **) was used to determine statistical significance between the two groups at each time point. Details of individual experimental fish are given in Tables 2A and 2B.

control levels at 3 hpi, but showed elevated expression starting at 6 hpi and remaining high until 7 dpi (Table 2B). An unpaired *t*-test ($p < 0.05$) showed a significant difference in the expression of caspase 3 between the two groups at 1 dpi. At this time point, caspase 3 expression in the 20 °C group was 6–7 times higher than controls, while it was below control levels in the 15 °C group (Fig. 2I). More interestingly, at 2 dpi the VHSV copy numbers drastically declined in the 20 °C fish when caspase 3 expression was at peak, while the 15 °C group showed a rapid increase in viral copy numbers with low caspase 3 expression. In 15 °C fish, the expression of caspase 3 increased at 4–7 dpi during which viral copies showed a decline.

4. Discussion

VHSV infection of the olive flounder is temperature-dependent, resulting in a maximum mortality rate at temperatures approximately 8–15 °C [4]. Although the virus does not develop clinical sign in olive flounder at 20 °C, the viral growth at this temperature was higher when tested in a cell line [4]. Furthermore, the susceptibility of VHSV-infected olive flounder depends on the rearing temperature; no mortality occurs at 20 °C but large-scale mortality of the fish was observed at or below 15 °C [5,24,25]. Sano et al. [5] showed that VHSV-infected fish reduced their mortality to 20% when the temperature was shifted from 14 °C to 20 °C at 2 dpi, whereas fish kept at 14 °C had an 80% mortality rate. However, shifting the culture temperature of VHSV-infected olive flounder from 20 °C to 15 °C at 2 dpi caused a cumulative mortality of 13% compared to a mortality of 93% when they were immediately transferred after intra-peritoneal injection. Although these observations confirmed that VHSV infection in olive flounder is strictly temperature-dependent, the host immune factors responsible for host survival at higher temperatures or susceptibility at lower temperatures are not known. In previous experiments, we analysed the relative expression of TLR- and IFN-related genes in VHSV-infected olive flounder maintained at 15 °C at different time intervals from 3 hpi to 7 dpi [18]. In the study, we found that TLR-2 and TLR-7 were downregulated in olive flounder, but the host was not deficient in pro-inflammatory cytokines or antiviral proteins. These findings led us to investigate other possible reasons that could account for the varied immune response at the two temperatures in VHSV-infected olive flounder.

The first step of replication cycle of rhabdovirus, a negative sense ssRNA virus, inside the host cell is transcription of viral genome. The RNA polymerase of rhabdovirus exhibits a gradient of transcription of the viral genes [26]. Hence, inside the host cell the transcript for N gene is the prevailing mRNA of VHSV. When abundant newly produced nucleoprotein is available, the viral transcription is shut off and replication is initiated [26,27]. Thus, nuclear protein of the virus plays important role in switch over between viral mRNA synthesis and gRNA synthesis. For these reasons, we targeted quantification of mRNA as well as gRNA of viral N gene. Use of oligo dT as a primer for reverse transcription resulted in synthesis of cDNA of viral transcript (positive sense RNA) which has poly A tail while, specific sense primer against viral N gene reverse transcribed viral negative sense gRNA. Respective cDNA were used as template for absolute quantification of viral mRNA and gRNA. A similar methodology was followed by Vester et al. [28] to quantify viral transcript (positive sense mRNA), negative sense RNA (gRNA) and complementary gRNA (positive sense) in influenza A virus, a negative sense ssRNA virus.

At 3 hpi, the copy number of VHSV gRNA was similar in both groups, which could be due to the injected viral copies, while viral transcription was much higher in the 20 °C group than in the 15 °C fish. In vitro, the VHSV in olive flounder can grow faster at 20 °C than at 15 °C [4,24]. In our experiment, the VHSV cultured in FHM cells grew much faster at 20 °C than at 15 °C but both conditions had similar final titre values of $10^{8.8}$ $TCID_{50}$/ml (data not shown). The gRNA in fish from the 20 °C group greatly increased at 1 dpi but drastically declined at 2 dpi (discussed more in later part), whereas the gRNA in fish from the 15 °C group significantly increased at 2 dpi. This indicates that the virus can produce a significant amount of progeny by 2 dpi in the host at 15 °C, while at 20 °C, the host either kills or controls the viral replication in the same infectious period. This replication difference could explain low level of mortality of VHSV-infected olive flounder when they were shifted from 20 °C to 15 °C 2 days after infection rather than being transferred immediately (93%) [5]. The group of 20 °C fish contained low to undetectable levels of viral gRNA or mRNA by 7 dpi, explaining why Sano et al. [5] did not observe any mortality in the group shifted from 20 °C to 15 °C at 9 dpi. Our experiment aimed at a less lethal viral infection (24% cumulative mortality in 15 °C group) in the olive flounder, which explains the reduced viral copies in the 15 °C fish at later recovery stages (4 and 7 dpi), indicating the host immune response can control the virus. We found that the olive flounder at 15 °C could express interferon-stimulated genes, such as ISG 15 and Mx at 1 and 2 dpi [18] that could have reduced the viral copy numbers at later recovery stages. The hosts, which had high gRNA and mRNA copies at 4–7 dpi, may succumb to the viral infection at a later stage of experiment. Fish from the 15 °C group with high gRNA copy numbers also contained high viral mRNA, but interestingly, a few showed considerably lower viral transcription, despite high viral gRNA content. One of the possible causes for this difference may be related to caspase 3 expression, which is discussed later.

Entry of virus results in the secretion of type II IFN by antigen-presenting cells, Natural killer (NK) cells and T lymphocytes to up-regulate antigen presentation, attract leukocytes to the site of action, enhance NK cell activity and regulate B cell functions [12,29]. The type II IFN response in both groups increased when viral copy numbers peaked, but its expression was much quicker and stronger in the 20 °C fish than in the 15 °C group. Similar result was reported on VHSV-infected olive flounder though the experimental conditions were different [30,31]. In addition, type I IFN expression at 15 °C after VHSV infection was highest when the viral copies were high in our previous study [18]. Both type I and type II IFNs evolved to complement each other, showing redundancies that create a more robust host defence system [29].

VHSV infection in olive flounder resulted in elevated expression of MHC class I and CD8 in the 15 °C fish group at early stages of infection but surprisingly, as the virus number increased, the expression of these two genes was reduced; furthermore, these genes were expressed below control levels during the later stages of the experiment. Transcription of MHC class I remained unchanged, two-fold higher than control levels, in the 20 °C fish because of viral infection, while the CD8 expression was highest at early stage, and remained higher than control level in the recovery phase. Atlantic salmon infected by infectious salmon anaemia virus (ISAV) and IHNV showed only slightly elevated expression of MHC class I [32–34]. Although the olive flounder MHC class I and CD8 are sequenced, their responses to VHSV infection at the two temperatures are not well understood. Microarray experiments in vaccinated olive flounder showed slightly increased expression of MHC class I and CD8 [35,36]. Thus, from the experiment, we could notice a low level but early enhancement in expression of MHC class I and CD8 transcripts in VHSV-infected olive flounder in both the groups, but a reduction in expression at later stages in 15 °C fish. Viral peptides that are presented by MHC class I are recognised by naive CD8 T cells, which then proliferate to become effector CTLs. CTLs that recognise the virus-infected cells secrete a set of target cell death inducers, such as granzyme, perforin and FasL, to initiate apoptosis [15,16].

Apoptosis is a process caused by the activation of cellular death pathways that activate the proteolytic digestion of regulatory proteins leading to metabolic instability, breakdown of the cellular infrastructure and disintegration of the cytoskeleton and genomic fragmentation [14,16]. Cytotoxic T lymphocytes and natural killer cells secrete granzyme and perforin to initiate granule-mediated apoptosis. Granzyme enters the target cell with the help of perforin to activate caspases [37,38]. Granzyme and perforin have been sequenced in different fishes, including the olive flounder; revealing closer homology with their mammalian counterparts [39–45]. Granzyme expressed at consistent levels in the 20 °C group; whereas in the 15 °C fish, it expressed at higher levels at very early and late stages of viral infection, but showed reduced expression at the peak of viral replication. Perforin expression found to be highly elevated in few individuals in both the groups (higher in number in 20 °C group than in 15 °C group), and interestingly the individuals with high perforin level expressed very high caspase 3. Viruses reportedly manipulate the host's apoptosis system to regulate cell death and allow the propagation of viral progeny [46], which may be the reason for an inadequate or delayed apoptotic response in the virus-susceptible group.

Fas ligand and TNFα belong to the TNF superfamily. The two death inducers bind to their respective receptors, Fas receptor and TNF receptor, to activate death domains in the infected/targeted host cell [14–16]. The sequence and functional properties in olive flounder and other fishes were analysed because of the importance of these two death initiators [47–53]. The olive flounder in the 15 °C group expressed TNFα at an early stage but downregulated it to control levels in most of the later sampled fish, while the 20 °C fish expressed TNFα at elevated levels for a much shorter period of time. Turbot infected with VHSV showed elevated TNFα expression within 1 dpi that was later reduced to normal levels [53]. Although there has been some work on fishes' response to recombinant TNFα, their response during apoptosis induction is not well characterized. Kurobe et al. [47] sequenced and verified the cell destructive properties of FasL in olive flounder. Our 15 °C experimental fish group expressed FasL during the initial stage of infection but did not maintain the expression level once the viral copies increased in the host. The 20 °C fish group showed increased levels at early stages but the levels later returned to normal. The short transcription and translation times for FasL could be a reason for

controlled apoptosis. Further experiments on this protein could reveal some promising applications.

p53, an anti-tumour protein, induces apoptosis when the host cell is under stress [54]. Although this gene has been sequenced and studied in several fishes as an apoptosis inducer during toxin stress [55–62], to our knowledge, there have not been any studies on the possible role of p53 in apoptosis induction during viral infection in fishes. Our efforts were focused on finding the gene expression changes due to VHSV infection at the two rearing temperatures. Transcription of p53 was up-regulated for a shorter period at the initial as well as at later recovery stage of infection in both fish groups. From our experiment, we found a possible apoptotic role of p53 in VHSV infection; however, detailed in vitro studies may provide more in-depth knowledge on its role in the cell death mechanism.

Apoptosis inducers activate zymogen caspases, and the subsequent signalling cascade leads to the activation of caspase 3, which performs the final steps in causing host cell death [63,64]. An early and effective apoptotic response, with caspase 3 as an important effector, can shut down viral replication and disease development in the host [14,16]. The transcript of caspase 3 has been sequenced and showed closer homology with the mammalian caspase 3 [65–69]. Considering the importance of caspase 3 in execution of apoptosis, olive flounder caspase 3 was partially sequenced. This sequence showed close homology with other fishes and mammalian counterparts. From the experiment, we found a possible role of caspase 3 in effectively controlling viral replication. In the 20 °C fish, viral copies were higher than in the 15 °C fish at the early stage of infection. However, 20 °C fish expressed very high levels of caspase 3, which could be a major cause for the fishes' effective control over viral transcription on 2–4 dpi. This expression difference could be the reason why one fish sampled from the 20 °C group at 4 dpi showed very high viral gRNA but had undetectable viral transcription. Caspase 3 expression in the 15 °C group was significantly lower except in a few fish sampled and the fish with high caspase 3 levels contained very low or undetectable levels of viral mRNA. One fish from this group sampled at 4 dpi showed high gRNA levels but very low mRNA levels. The same fish expressed 40-fold higher caspase 3, while another fish with ascites sampled on 7 dpi carried significantly higher numbers of viral gRNA and mRNA but caspase 3 was expressed below control. Thus, the expression pattern of caspase 3 and its direct relation with viral transcription allows us to predict a significant role for this apoptotic gene in effectively controlling VHSV replication.

Apoptosis, predominantly mediated by caspases, is known to effectively control viral replication. A number of viruses have shown different strategies for successfully evading host apoptosis [12,70]. As mentioned earlier, caspase 3 was downregulated in a VHSV-infected cell line [19]. Furthermore, the inability of VHSV to induce disease in olive flounder upon removal of the NV gene [20,21] indicating the NV gene has a key role in causing the VHSV pathogenicity, while apoptosis plays a major role in host survival. Thus, the viral anti-apoptotic response could be strong enough to override the host defence mechanism in olive flounder reared at 15 °C water temperature and could replicate effectively for a longer time to produce large numbers of progeny. This observation could explain why olive flounder at 15 °C contained high numbers of viral copies even though they expressed high levels of Mx [18]. Conversely, at a 20 °C culture temperature, the host apoptotic response might be quick enough to suppress viral replication and dominate the virus' inhibitory action.

In conclusion, our in vivo data on VHSV-infected olive flounder support the importance of the apoptotic host immune system in antiviral defence mechanisms. The experiment clearly showed the influence of temperature on the differential expression pattern of apoptotic immune genes and its effect on viral replication in the same batch of olive flounder. The viral replication rate was faster in fish reared at 20 °C compared to fish reared at 15 °C during the initial infection stage. Though the apoptosis inducers were immediately elevated after initiating viral infection in the 15 °C fish group, they under-expressed these genes when viral copies were high; conversely, the 20 °C group expressed the same genes at a higher level. Substantial expression of caspase 3 in the 20 °C fish group controlled viral replication more efficiently than the 15 °C fish that had very low levels of caspase 3. Interestingly, fish that expressed high levels of caspase 3 had low or undetectable viral transcription. From this experiment, we can conclude that the apoptotic immune system is one of the most important host survival mechanisms in defending the olive flounder against VHSV infection. Hence, apoptosis could play a critical role in survival of VHSV-infected olive flounder in the summer but susceptibility in the winter and spring.

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References

[1] Bai SC. The Republic of Korea. In: Lovatelli A, Phillips MJ, Arthur JR, Yamamoto K, editors. FAO/NACA regional workshop on the future of mariculture: a regional approach for responsible development in the Asia-Pacific region. Guangzhou, China, 7–11 March 2006. FAO Fisheries Proceedings. No. 11. Rome, FAO; 2008. p. 199–206.
[2] Froese R, Pauly D, editors. FishBase. World Wide Web Electronic Publication. version, www.fishbase.org; 2011.
[3] Kim WS, Kim SR, Kim D, Kim JO, Park MA, Kitamura SI, et al. An outbreak of VHSV (viral hemorrhagic septicemia virus) infection in farmed olive flounder *Paralichthys olivaceus* in Korea. Aquaculture 2009;296:165–8.
[4] Isshik T, Nishizawa T, Kobayashi T, Nagano T, Miyazaki T. An outbreak of VHSV (viral hemorrhagic septicemia virus) infection in farmed Japanese flounder *Paralichthys olivaceus* in Japan. Dis Aquat Organ 2001;47:87–99.
[5] Sano M, Itoa T, Matsuyamaa T, Nakayasua C, Kuritaa J. Effect of water temperature shifting on mortality of Japanese flounder *Paralichthys olivaceus* experimentally infected with viral hemorrhagic septicemia virus. Aquaculture 2009;286:254–8.
[6] Amend FD. Control of infectious hematopoietic necrosis virus disease by elevating the water temperature. J Fish Res Board Can 1970;27:265–70.
[7] Hbtrick FM, Fryer JL, Knittel MD. Effect of water temperature on the infection of rainbow trout *Salmo gairdneri* Richardson with infectious haematopoietic necrosis virus. J Fish Dis 1979;2:253–7.
[8] Jørgensen VPE. Egtved virus: temperature-dependent immune response of trout to infection with low-virulence virus. J Fish Dis 1982;5:47–55.
[9] Kobayashi T, Shiino T, Miyazaki T. The effect of water temperature on rhabdoviral dermatitis in the Japanese eel, *Anguilla japonica* Temminck and Schlegel. Aquaculture 1999;170:7–15.
[10] Goodwin AE, Merry GE. Mortality and carrier status of bluegills exposed to viral hemorrhagic septicemia virus genotype IVb at different temperatures. J Aquat Anim Health 2011;23:85–91.
[11] Ahne W, Bjorklund HV, Essbauer S, Fijan N, Kurath G, Winton JR. Spring viremia of carp (SVC). Dis Aquat Organ 2002;52:261–72.
[12] Boehm U, Klamp T, Groot M, Howard JC. Cellular responses to interferon-gamma. Annu Rev Immunol 1997;15:749–95.
[13] Vyas JM, Van der Veen AG, Ploegh HL. The known unknowns of antigen processing and presentation. Nat Rev Immunol 2008;8:607–18.
[14] Barber GN. Host defense, viruses and apoptosis. Cell Death Differ 2001;8:113–26.
[15] Chávez-Galán L, Arenas-Del Angel MC, Zenteno E, Chávez R, Lascurain R. Cell death mechanisms induced by cytotoxic lymphocytes. Cell Mol Immunol 2009;6:15–25.
[16] Schultz DR, Harrington Jr WJ. Apoptosis: programmed cell death at a molecular level. Semin Arthritis Rheum 2003;32:345–69.
[17] Caipang CM, Hirono I, Aoki T. In vitro inhibition of fish rhabdoviruses by Japanese flounder, *Paralichthys olivaceus* Mx. Virology 2003;317:373–82.
[18] Avunje S, Kim WS, Park CS, Oh MJ, Jung SJ. Toll-like receptors and interferon associated immune factors in viral haemorrhagic septicaemia virus-infected olive flounder (*Paralichthys olivaceus*). Fish Shellfish Immunol 2011;31:407–14.

[19] Ammayappan A, Vakharia VN. Nonvirion protein of novirhabdovirus suppresses apoptosis at the early stage of virus infection. J Virol 2011;85: 8393–402.
[20] Kim MS, Kim KH. Protection of olive flounder, *Paralichthys olivaceus*, against viral hemorrhagic septicemia virus (VHSV) by immunization with NV gene-knockout recombinant VHSV. Aquaculture 2011;314:39–43.
[21] Kim MS, Kim DS, Kim KH. Oral immunization of olive flounder (*Paralichthys olivaceus*) with recombinant live viral hemorrhagic septicemia virus (VHSV) induces protection against VHSV infection. Fish Shellfish Immunol 2011;31: 212–6.
[22] Miller TA, Rapp J, Wastlhuber U, Hoffmann RW, Enzmann PJ. Rapid and sensitive reverse transcriptase-polymerase chain reaction based detection and differential diagnosis of fish pathogenic rhabdoviruses in organ samples and cultured cells. Dis Aquat Organ 1998;34:13–20.
[23] Pfaffl MW. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res 2001;29:2002–7.
[24] Mori K, Iida H, Nishizawa T, Arimoto M, Nakajima K, Muroga K. Properties of viral hemorrhagic septicemia virus (VHSV) isolated from Japanese flounder *Paralichthys olivaceus*. Fish Pathol 2002;37:169–74.
[25] Isshiki T, Nagano T, Miyazaki T. Effect of water temperature on pathological states of Japanese flounder experimentally infected with haemorrhagic septicaemia virus, a flounder isolate KRRV-9601. Fish Pathol 2002;37:95–7.
[26] Albertini AA, Schoehn G, Weissenhorn W, Ruigrok RW. Structural aspects of rabies virus replication. Cell Mol Life Sci 2008;65:282–94.
[27] Flint SJ, Enquist LW, Krug RM, Racaniello VR, Skalka AM. Principles of virology: molecular biology, pathogenesis, and control. Washington, D.C.: American Society Microbiology; 1999. pp. 162–196.
[28] Vester D, Lagoda A, Hoffmann D, Seitz C, Heldt S, Bettenbrock K, et al. Real-time RT-qPCR assay for the analysis of human influenza A virus transcription and replication dynamics. J Virol Methods 2010;168:63–71.
[29] Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-gamma: an overview of signals, mechanisms and functions. J Leukoc Biol 2004;75:163–89.
[30] Suzuki Y, Yasuike M, Kondo H, Aoki T, Hirono I. Molecular cloning and expression analysis of interferon regulatory factor 10 (IRF10) in Japanese flounder. *Paralichthys Olivaceus* Fish Shellfish Immunol 2011;30:67–76.
[31] Matsuyama T, Fujiwara A, Sakai T, Nakayasu C. Molecular cloning and expression analysis of interferon gamma gene in Japanese flounder *Paralichthys olivaceus*. Fisheries Sci 2009;75:253–5.
[32] Jørgensen SM, Syvertsen BL, Lukacs M, Grimholt U, Gjøen T. Expression of MHC class I pathway genes in response to infectious salmon anaemia virus in Atlantic salmon (*Salmo salar* L.) cells. Fish Shellfish Immunol 2006;21:548–60.
[33] Jørgensen SM, Hetland DL, Press CM, Grimholt U, Gjøen T. Effect of early infectious salmon anaemia virus (ISAV) infection on expression of MHC pathway genes and type I and II interferon in Atlantic salmon (*Salmo salar* L.) tissues. Fish Shellfish Immunol 2007;23:576–88.
[34] Hansen JD, La Patra S. Induction of the rainbow trout MHC class I pathway during acute IHNV infection. Immunogenetics 2002;54:654–61.
[35] Byon JY, Ohira T, Hirono I, Aoki T. Use of a cDNA microarray to study immunity against viral hemorrhagic septicemia (VHS) in Japanese flounder (*Paralichthys olivaceus*) following DNA vaccination. Fish Shellfish Immunol 2005;18: 135–47.
[36] Byon JY, Ohira T, Hirono I, Aoki T. Comparative immune responses in Japanese flounder, *Paralichthys olivaceus* after vaccination with viral hemorrhagic septicemia virus (VHSV) recombinant glycoprotein and DNA vaccine using a microarray analysis. Vaccine 2006;24:921–30.
[37] Voskoboinik I, Smyth MJ, Trapani JA. Perforin-mediated target-cell death and immune homeostasis. Nat Rev Immunol 2006;6:940–52.
[38] Granville DJ. Granzymes in disease: bench to bedside. Cell Death Differ 2010; 17:565–6.
[39] Hwang JY, Ohira T, Hirono I, Aoki T. A pore-forming protein, perforin, from a non-mammalian organism, Japanese flounder, *Paralichthys olivaceus*. Immunogenetics 2004;56:360–7.
[40] Athanasopoulou S, Marioli D, Mikrou A, Papanastasiou AD, Zarkadis IK. Cloning and characterization of the trout perforin. Fish Shellfish Immunol 2009;26:908–12.
[41] Toda H, Araki K, Moritomo T, Nakanishi T. Perforin-dependent cytotoxic mechanism in killing by CD8 positive T cells in ginbuna crucian carp, *Carassius auratus* langsdorfii. Dev Comp Immunol 2011;35:88–93.
[42] Wernersson S, Reimer JM, Poorafshar M, Karlson U, Wermenstam N, Bengtén E, et al. Granzyme-like sequences in bony fish shed light on the emergence of hematopoietic serine proteases during vertebrate evolution. Dev Comp Immunol 2006;30:901–18.
[43] Praveen K, Evans DL, Jaso-Friedmann L. Evidence for the existence of granzyme-like serine proteases in teleost cytotoxic cells. J Mol Evol 2004;58: 449–59.
[44] Praveen K, Leary 3rd JH, Evans DL, Jaso-Friedmann L. Molecular characterization and expression of a granzyme of an ectothermic vertebrate with chymase-like activity expressed in the cytotoxic cells of Nile tilapia (*Oreochromis niloticus*). Immunogenetics 2006;58:41–55.
[45] Ordás MC, Cuesta A, Mercado L, Bols NC, Tafalla C. Viral hemorrhagic septicaemia virus (VHSV) up-regulates the cytotoxic activity and the perforin/granzyme pathway in the rainbow trout RTS11 cell line. Fish Shellfish Immunol 2011;31:252–9.
[46] Hay S, Kannourakis G. A time to kill: viral manipulation of the cell death program. J Gen Virol 2002;83:1547–64.
[47] Kurobe T, Hirono I, Kondo H, Saito-Taki T, Aoki T. Molecular cloning, characterization, expression and functional analysis of Japanese flounder *Paralichthys olivaceus* Fas ligand. Dev Comp Immunol 2007;31:687–95.
[48] Kurata O, Iwasaki T, Matsuyama T, Nakayasu C, Wada S, Hatai K. Lymphocytes with T-cell-like properties express the Fas ligand in the Japanese flounder *Paralichthys olivaceus*. Fish Shellfish Immunol 2011;30:509–14.
[49] Long S, Wilson M, Bengtén E, Clem LW, Miller NW, Chinchar VG. Identification and characterization of a FasL-like protein and cDNAs encoding the channel catfish death-inducing signaling complex. Immunogenetics 2004;56:518–30.
[50] Eimon PM, Kratz E, Varfolomeev E, Hymowitz SG, Stern H, Zha J, et al. Delineation of the cell-extrinsic apoptosis pathway in the zebrafish. Cell Death Differ 2006;13:1619–30.
[51] Hirono I, Nam BH, Kurobe T, Aoki T. Molecular cloning, characterization, and expression of TNF cDNA and gene from Japanese flounder *Paralychthys olivaceus*. J Immunol 2000;165:4423–7.
[52] Xiao J, Zhou ZC, Chen C, Huo WL, Yin ZX, Weng SP, et al. Tumor necrosis factor-alpha gene from mandarin fish, *Siniperca chuatsi*: molecular cloning, cytotoxicity analysis and expression profile. Mol Immunol 2007;44:3615–22.
[53] Ordás MC, Costa MM, Roca FJ, López-Castejón G, Mulero V, Meseguer J, et al. Turbot TNF alpha gene: molecular characterization and biological activity of the recombinant protein. Mol Immunol 2007;44:389–400.
[54] Vousden KH, Lane DP. p53 in health and disease. Nat Rev Mol Cell Biol 2007;8: 275–83.
[55] Le Bras M, Bensaad K, Soussi T. Data mining the p53 pathway in the Fugu genome: evidence for strong conservation of the apoptotic pathway. Oncogene 2003;22:5082–90.
[56] Caron de Fromentel C, Pakdel F, Chapus A, Baney C, May P, Soussi T. Rainbow trout p53: cDNA cloning and biochemical characterization. Gene 1992;112: 241–5.
[57] Brzuzan P, Jurczyk L, Luczyński MK, Góra M. Real-time PCR analysis of p53 mRNA levels in tissues of whitefish (*Coregonus lavaretus*) exposed to benzo[a] pyrene. Pol J Vet Sci 2006;9:139–43.
[58] Brzuzan P, Woźny M, Ciesielski S, Łuczyński MK, Góra M, Kuźmiński H, et al. Microcystin-LR induced apoptosis and mRNA expression of p53 and cdkn1a in liver of whitefish (*Coregonus lavaretus* L.). Toxicon 2009;54:170–83.
[59] Krause MK, Rhodes LD, Van Beneden RJ. Cloning of the p53 tumor suppressor gene from the Japanese medaka (*Oryzias latipes*) and evaluation of mutational hotspots in MNNG-exposed fish. Gene 1997;189:101–6.
[60] Cachot J, Galgani F, Vincent F. cDNA cloning and expression analysis of flounder p53 tumour suppressor gene. Comp Biochem Physiol B Biochem Mol Biol 1998;121:235–42.
[61] Bhaskaran A, May D, Rand-Weaver M, Tyler CR. Fish p53 as a possible biomarker for genotoxins in the aquatic environment. Environ Mol Mutagen 1999;33:177–84.
[62] Cols Vidal M, Hoole D, Williams GT. Characterisation of cDNAs of key genes involved in apoptosis in common carp (*Cyprinus carpio* L.). Fish Shellfish Immunol 2008;25:494–507.
[63] Taylor RC, Cullen SP, Martin SJ. Apoptosis: controlled demolition at the cellular level. Nat Rev Mol Cell Biol 2008;9:231–41.
[64] Benedict CA, Norris PS, Ware CF. To kill or be killed: viral evasion of apoptosis. Nat Immunol 2002;3:1013–8.
[65] Yabu T, Kishi S, Okazaki T, Yamashita M. Characterization of zebrafish caspase-3 and induction of apoptosis through ceramide generation in fish fathead minnow tailbud cells and zebrafish embryo. Biochem J 2001;360:39–47.
[66] Takle H, McLeod A, Andersen O. Cloning and characterization of the executioner caspases 3, 6, 7 and Hsp70 in hyperthermic Atlantic salmon (*Salmo salar*) embryos. Comp Biochem Physiol B Biochem Mol Biol 2006;144:188–98.
[67] Yamashita M, Mizusawa N, Hojo M, Yabu T. Extensive apoptosis and abnormal morphogenesis in pro-caspase-3 transgenic zebrafish during development. J Exp Biol 2008;211:1874–81.
[68] Reis MI, Nascimento DS, do Vale A, Silva MT, dos Santos NM. Molecular cloning and characterisation of sea bass (*Dicentrarchus labrax* L.) caspase-3 gene. Mol Immunol 2007;44:774–83.
[69] Li M, Ding Y, Mu Y, Ao J, Chen X. Molecular cloning and characterization of caspase-3 in large yellow croaker (*Pseudosciaena crocea*). Fish Shellfish Immunol 2011;30:910–6.
[70] Skaletskaya A, Bartle LM, Chittenden T, McCormick AL, Mocarski ES, Goldmacher VS. A cytomegalovirus-encoded inhibitor of apoptosis that suppresses caspase-8 activation. Proc Natl Acad Sci U S A 2001;98:7829–34.