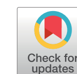
Aquaculture 503 (2019) 51–58

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Differential expression profile of innate immune genes in the liver of olive flounder (*Paralichthys olivaceus*) against viral haemorrhagic septicaemia virus (VHSV) at host susceptible and non-susceptible temperatures



Sajal Kole, Satheesha Avunje[1], Sung-Ju Jung*

Department of Aqualife Medicine, Chonnam National University, Republic of Korea

ARTICLE INFO

Keywords:
Paralichthys olivaceus
VHSV
Liver
Hepcidin
Complement
Apoptosis

ABSTRACT

Viral haemorrhagic septicaemia virus (VHSV) is known to cause high mortality in olive flounder (*Paralichthys olivaceus*) at 15 °C, but no mortality at 20 °C. In order to understand the immune response at 15 °C and 20 °C against VHSV, differential expression kinetics of various innate immune-related genes in the kidney were analyzed in our previous study, and the current study is focused on immune responses in the liver. Fishes were intraperitoneally injected with VHSV ($10^{7.8}$ $TCID_{50}$/fish) and reared at 15 °C and 20 °C. Viral copy number in the 15 °C group peaked at 1 dpi and remained high until 2 dpi; whereas in the 20 °C group, viral copies were much lower than in the 15 °C group. The expression kinetics results revealed significantly high levels of hepcidin expression in the 20 °C group in comparison to the 15 °C group at all time points. Cathepsin-L gene showed early upregulation at 12 hpi followed by a sharp decline at 1 dpi in both the groups; whereas, at 2–4 dpi, only the 20 °C group demonstrated subsequent upregulation of cathepsin-L transcript. The genes of complement pathways, C3 and factor-D showed prolonged downregulation trend in the 15 °C group against the normal expression level in the 20 °C group. Significant upregulation of ISG15, Mx, IL-1β and IL-8 genes were observed in the 20 °C group during 1–2 dpi in comparison with slower and lower responses in the 15 °C group. The MHC-II, MHC-I and CD8 gene transcripts showed significant upregulation only in the 20 °C group in contrast with downregulation in the 15 °C group, whereas, CD4 expression was very high at 12 hpi to 2 dpi in both the groups. The genes related to the apoptotic pathway, p53, HSP70, and caspase-3, also displayed consistent downregulation in the 15 °C group during the entire experimental period in comparison with the other group. In summary, it can be observed that most of the innate immune genes were under-expressed or have little to no response at 15 °C, whereas, the same genes exhibited high level of expression at 20 °C. Thus, it can be inferred that temperature plays a key role in facilitating the host liver to orchestrate a coordinative anti-VHSV immune response ultimately leading to host survival in the 20 °C group. Oppositely, the under-expression of important genes at 15 °C especially the genes related to the apoptotic pathway, cytotoxic T-cell pathway, and the complement system, resulted in failure of the host immune system to combat the viral proliferation.

1. Introduction

Fish being a poikilothermic animal is significantly affected by the temperature in which they live, and their mortality rates vary dramatically due to infection depending on their rearing water temperature. Previously, various attempts have been made in controlling the water temperature in order to combat fish pathogens and reduce fish mortality (Alcorn et al., 2002; Nikoskelainen et al., 2004; Ndong et al., 2007; Xu et al., 2011; Kaneshige et al., 2016). Viral haemorrhagic septicaemia (VHS) is one such serious viral disease affecting a large number of fish species worldwide when the rearing water temperature becomes conducive for infection. The disease is caused by viral haemorrhagic septicaemia virus (VHSV), a (−) ssRNA virus belonging to the genus *Novirhabdovirus* of rhabdoviridae family. The susceptible water temperature for VHS infection in fishes revolves around 8–15 °C. Likewise, in case of olive flounder (*Paralichthys olivaceus*) culture, the VHS disease occurs in late winter and spring season as the temperature drops below 15 °C, causing 50–70% mortalities in all age-groups of the fish but no mortality is observed at higher temperature (≥ 20 °C) during other seasons (Isshiki et al., 2001; Kim et al., 2009). Purposively, by

* Corresponding author at: Department of Aqualife Medicine, Chonnam National University, 50, Daehak-ro, Yeosu 59626, Republic of Korea.
E-mail address: sungju@chonnam.ac.kr (S.-J. Jung).

[1] Present address: Aquatic Animal Health and Environment Division, ICAR-Central Institute of Brackishwater Aquaculture, Chennai 600028, India.

https://doi.org/10.1016/j.aquaculture.2018.12.085
Received 7 August 2018; Received in revised form 29 November 2018; Accepted 28 December 2018
Available online 29 December 2018
0044-8486/ © 2019 Elsevier B.V. All rights reserved.

identifying the differences in the immune response of the olive flounder at mortal and survivor temperature conditions, it is possible to get an insight on important immune-related defence factors against VHSV.

Innate immunity is the first line of defence against infection and is regarded as the primeval form of host defence (Aoki et al., 2008). Teleosts depend primarily on the innate immune system, which is faster but less specific than adaptive immunity (Akira et al., 2006; Magnadottir, 2006). Teleostean liver plays a major role in host defence against pathogens in the external environment and is continually exposed to various gut-derived molecules and foreign antigens. Several studies have demonstrated the capacity of fish liver for scavenging foreign substances, although this activity mainly involves hepatocytes and sinusoidal epithelial cells instead of immune cells (Dalmo et al., 1997). However, recent study elucidated the presence of the resident population of intrahepatic immune cells (IHICs) in teleost liver other than the hepatocytes, which plays an integral part of hepatic immune responses (Möller et al., 2014). Unlike mammalian liver, where the populations of non-hepatocyte immune cells like Kupffer cells, T cells, B cells, NK cells, and NKT cells are much higher, fish liver lack Kupffer cells, and its immune functions mostly depends on the interhepatocytic macrophages and T- and B-lymphocytes (Hampton et al., 1989; Möller et al., 2014). Among various hepatic immune functions, the liver has an essential role in non-specific phagocytosis and cell killing, clearance of pathogens, acute phase reactions and complement production (Wu et al., 2016; Parker and Picut, 2005). Function related to antiviral T-cell responses also comprises a critical part of the hepatic defence system for early viral control and disease prevention (Liang et al., 2016). In addition, the liver possesses pattern recognition receptors (PRRs) for pathogen bindings which play an active role in the receptor-driven pathway of innate immunity as well in the resultant interferon and chemokines regulated downstream pathways (Castro et al., 2014). It can be well understood from the aforementioned studies that liver plays a crucial role in fish immune response but the available knowledge is too fragmentary to support any conclusive statement on the exact functional importance of the liver in disease resistance and host immunity.

Our previous study showed the temperature-dependent expression pattern of various immune genes in the kidney of VHSV-infected olive flounder and their role in host antiviral immune responses against viral pathogenicity at higher temperature (20 °C) compared to 15 °C (Avunje et al., 2011, 2012, 2013). To further our understanding about the host immune response the present study tried to explore the hepatic defence in a similar fashion given the importance of liver in eliciting antiviral immunity in flounder. The study investigated the expression patterns of various immune genes in liver tissue of VHSV-infected olive flounder reared at 15 °C or 20 °C, followed by a comparative analysis of the expression profiles of the immune genes in relation with the viral mRNA copies in a time-dependent manner. The insights gained from the present study will help in better understanding the function of the liver as an important immune organ in antiviral defence mechanism as well as the influence of temperature change on hepatic immune response against VHSV infection in olive flounder.

2. Materials and methods

2.1. Experimental infection

Experimental infection was carried out as previously described (Avunje et al., 2013). Briefly, olive flounder (18–22 g) collected from a local farm were distributed into two groups and were maintained at 15 ± 0.5 °C and 20 ± 0.5 °C in a controlled indoor rearing facility for 7 days prior to infection. Sixty fish per group were intraperitoneally injected with 100 μL of viral suspension ($10^{7.8}$ $TCID_{50}$/fish) of VHSV (F1Wa05 strain) propagated in FHM cells. Alongside, 60 fishes in each temperature (15 °C or 20 °C) were mock infected with $DMEM_0$ and designated as control groups. Afterwards five fish ($n = 5$) were randomly sampled at 12 h post-infection (hpi), and 1, 2, 4, and 7 days post-infection (dpi) from the VHSV-infected and control fish groups reared at both 15 °C and 20 °C temperatures. The liver tissues 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 from the liver tissues using RNAiso Plus (Takara Bio Inc., Japan) as per manufacturer's protocols and quantified by NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, USA). The residual genomic DNA was removed using RNase-free DNase I (Takara Bio Inc., Japan). Total RNA (1 μg) was reverse-transcribed into first-strand cDNA using ReverTra Ace® qPCR RT Kit (Toyobo, Japan) using oligo-dT primer and ReverTra Ace reverse transcriptase in a 10 μL reaction volume as per the manufacturer's protocol. The resulting cDNA was stored at −20 °C.

2.3. Quantitative expression of immune genes and viral RNA

Gene-specific primers targeted for immune-related genes were designed based on available sequences of olive flounder from NCBI database using Primer3Plus. Olive flounder β-actin was selected as a housekeeping gene, and all primers that were used for gene expression are shown in Table 1. Real-time PCR was carried out in an Exicycler™ 96 Real-Time Quantitative Thermal Block (Bioneer, Korea) using SYBR Green AccuPower® PCR PreMix (Bioneer). For relative quantification of each gene, cDNA from liver samples from the two experimental groups as well as from their respective control groups were used as template. The reaction was performed (in duplicate) in a final reaction volume of 20 μL. The reaction program began with a 10 min initial denaturation at 94 °C, followed by 35 cycles of 20 s denaturation (94 °C), annealing (temperature is given in Table 1) and scanning. The threshold cycle (Ct) value was determined using the automatic setting on the Bioneer Exicycler™ 96 Real-Time PCR system. Relative quantification of immune response was estimated using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Viral mRNA was quantified as explained by Avunje et al. (2011).

2.4. Statistical analysis

The data generated for different gene expression were statistically analyzed by using statistical package SPSS version 22 (SPSS Inc., USA) in which data set for each gene were subjected to two-way ANOVA to determine the statistical significance within the group (day wise), between the group (group wise) as well as to evaluate the interaction effect. Duncan's multiple range test and an unpaired *t*-test were used to determine the significant differences in gene expression at different time points within and between the 15 °C and 20 °C fish groups respectively. Comparisons were made at the 5% probability level. The *p*-value below 0.05 was considered statistically significant. The results were expressed as the mean ± standard error.

3. Results and discussion

3.1. Experimental infection and VHSV copy numbers

In the present study, it was noteworthy to see that olive flounder injected with an equal amount of VHSV succumbs to death at 15 °C but clears the virus at 20 °C. There was visible dropsy in the fish reared at 15 °C from 4 dpi which ultimately leads to a cumulative mortality of 24% (mortality was observed from 14 dpi to 20 dpi). However, there were no signs of VHS in the 20 °C group during 30 days of experimental period whereas both the control groups were also devoid of any clinical signs of the disease (Avunje et al., 2012). The mortality observation indicated an effective host immune response against VHSV that only works at 20 °C to overcome viral pathogenicity but failed to do so at

Table 1
Primers used for real-time PCR.

Target gene	GenBank acc. no	Product length	Sense primer	Antisense primer	Ta[a]	References
VHSV N	JF792424	138	atctggaggcaaagtgcaag	ccatgaggttgtcgttgttg	62	(Avunje et al., 2011)
β-actin	HQ386788	131	cctcttccagccttcattc	tggttcctccagatagcac	56	(Avunje et al., 2011)
Hepcidin	AY623817	171	tacaagacgctgtggtgctc	cagagctgtcctgaatgcaa	58	(in this study)
Cathepsin-L	FJ172449	204	acaatgccttccgctacatc	catggatggctacagacacg	58	(in this study)
C3	AB021653	233	ctgcgcacattcctgagtta	tactgctggaccatctgctg	58	(in this study)
CFD	GQ149466	102	ggcaacaagttcaccgccaa	cccagagtccccatcacagc	58	(in this study)
ISG15	AB519717	135	gctgtatgacaacggtcagc	ctcaggaagacctggatgg	60	(Avunje et al., 2011)
Mx	AB110446	159	tcactggatttcccaacctc	tgtcactcaaactgctgctg	62	(Avunje et al., 2011)
IL-1β	AB070835	128	aaagaagcatcacactgtct	ctactcaacaacgccacctt	56	(Avunje et al., 2011)
IL-8	AF216646	200	tcggccgctacattaagagt	ttaaacggcttctgaccccatc	58	(in this study)
CD4	AB643634	177	caagtggacctgtgttgtgg	tcctgcacacccttagcttt	58	(in this study)
CD8	AB082957	108	taagggcaacactaacacagg	atgaggaggaggagaaggag	56	(Avunje et al., 2012)
MHC I	AB126921	148	tctccctcctctccagtcagc	gctcatctggaaggtcccgtcat	58	(Avunje et al., 2012)
MHC II	AY848955	107	gtcgtcaggcttcactctgt	tctctttgccagctcacttt	56	(in this study)
p53	EF564441	159	cgaggaaagcagcaccaaga	ccccgaccgacaataggaag	58	(Avunje et al., 2012)
HSP70	AF053059.1	133	acattgaacgcatggtccag	agcttctcatcctccacagttg	60	(in this study)
Caspase3	JQ394697	115	acatcatgacacgggtgaac	tccttcgtcagcattgacac	58	(Avunje et al., 2012)
TLR2	AB109393	100	gctacatctgcgactctcct	cacagggacacgaacaaatc	58	(Avunje et al., 2013)
TLR7	HQ845984	97	cctgggaaatctggaagaac	tttgagggaggagaaactgc	62	(Avunje et al., 2013)
MDA5	HQ401014	133	acgagcgacctcttgatttg	agcgtcaccacgaagttttg	60	(Avunje et al., 2013)

[a] Ta: annealing temperature.

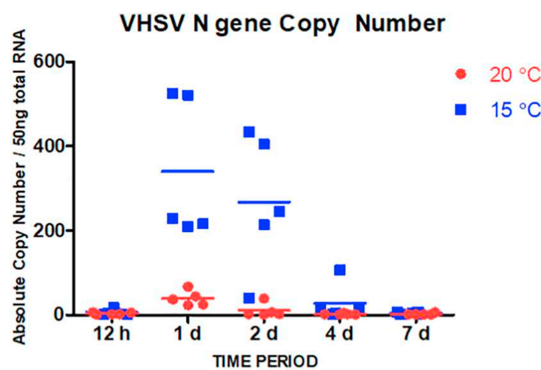


Fig. 1. Absolute viral (VHSV N gene) copy number in liver of viral haemorrhagic septicaemia (VHSV)-infected olive flounder ($10^{7.8}$ $TCID_{50}$/fish) at 15 °C or 20 °C. Mean copy number (n = 5, n is number of fish sampled per time point) per 50 ng total RNA plotted against time after viral infection.

15 °C. In addition, we studied the levels of transcription of the nucleoprotein (N) gene in the liver of the fish (n = 5) for both the 15 °C and 20 °C groups at various time intervals as an indicator of viral replication in the tissue (Fig. 1). From the viral transcript analysis, it was observed that the virus-infected olive flounder in both the groups tested positive for N gene as early as 12 hpi (5.57 and 3.08 copies in the 15 °C and 20 °C respectively). The copy number rapidly increased and reached their peaks by 1 dpi in both the groups, though, the 15 °C group carries tenfold (340.3 copies) more than the 20 °C group (39.3 copies) followed by a declining trend of viral transcription from 2 dpi which significantly reduced by 4 and 7 dpi. Comparing the current highest viral mRNA transcript in the liver (~300 and ~40 copies at 1 dpi in the 15 °C and 20 °C groups, respectively) with that of kidney (~1000 copies and ~100 copies in the 15 °C and 20 °C groups, respectively, during 12 hpi to 2 dpi) from our previous work (Avunje et al., 2011) it was evident that the rate of virus replication in the liver was much lower and slower in liver compared to that of the kidney. The lower viral copy number in the liver corresponds to the fact that liver is a less preferred site for VHSV infection than the kidney as described in earlier studies (Smail and Snow, 2011; Wolf, 1988). Thus a comparative analysis of temperature-dependent hepatic immune responses in olive flounder during VHSV infection will form a basis of immune function in the liver and provide a better immunological knowledge on the immune factors, which are important in controlling viral activity that leads to host survival or pathogen-mediated lethality.

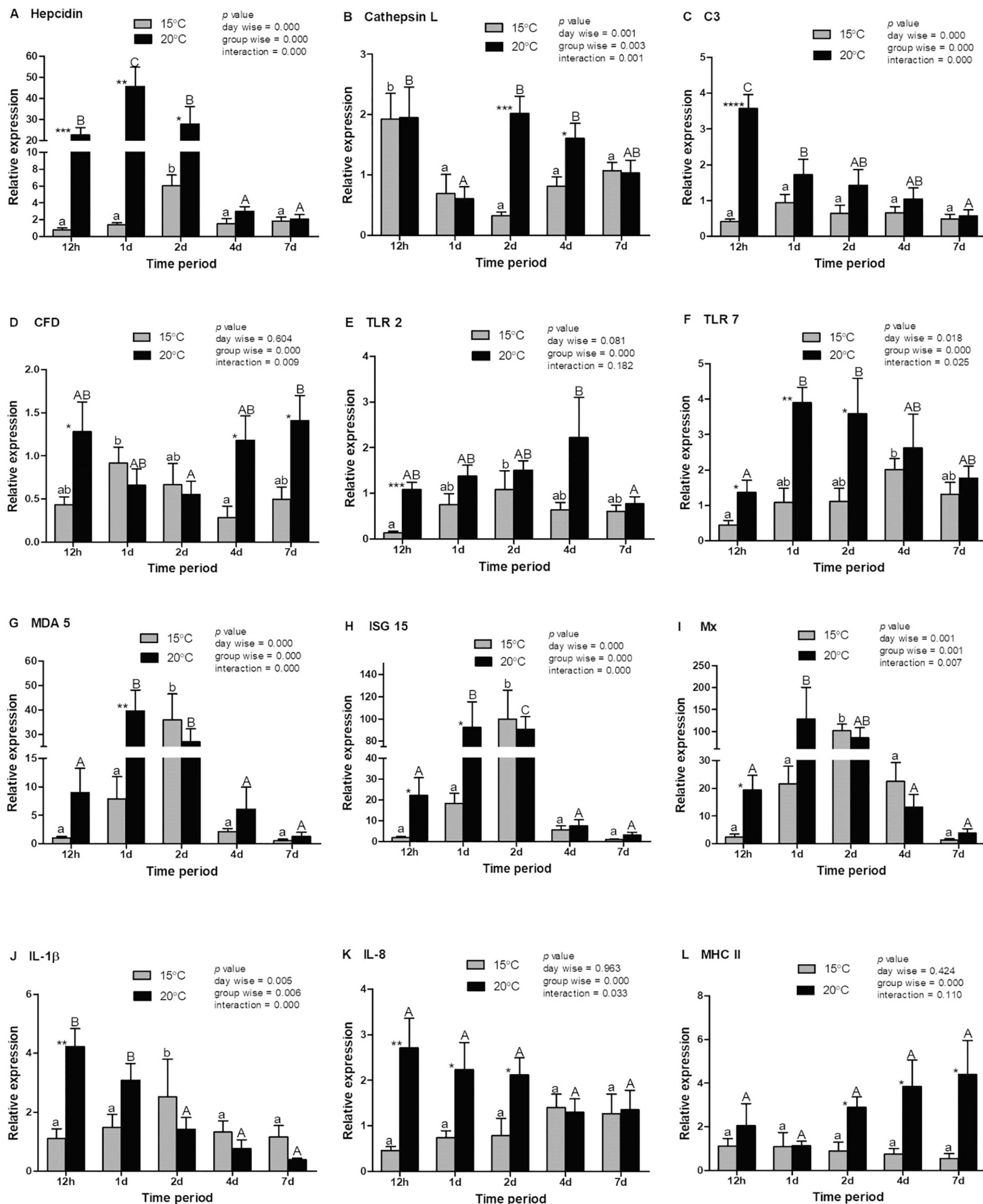
3.2. Expression kinetics of immune-related genes in the liver

The present study included comparative expression kinetics of diverse classes of immune-related genes in the liver which are known for their predominance in the hepatic system as well as important for orchestrating a coordinative defence response in the liver of virus-infected olive flounder at two different temperatures.

3.2.1. Hepcidin and cathepsin L

Among the various immune reactions predominant in the liver, the production of antimicrobial peptides is one of the first lines of hepatic defence against pathogens. Hepcidin, commonly produced antimicrobial peptides in the liver, is involved in the innate immune response of fish against a variety of infectious agents such as viruses, bacteria, and fungi. It also functions as a principal iron-regulatory hormone and known to play a role as an anti-inflammatory molecule (Castro et al., 2014; Domenico et al., 2010; Raida and Buchmann, 2009; Krause et al., 2000). In the present study the expression levels of hepcidin gene transcript differed significantly ($p < .05$) between the two experimental groups with very high level of expression in the 20 °C group at 12 hpi and 2 dpi (~22.83-folds to ~45.77-folds compared to control) against low responses in the 15 °C group (6.02-fold increase at 2 dpi) (Fig. 2A). The significantly higher induction of hepcidin gene can be well supported by several previous findings where significant upregulation of hepcidin gene transcripts in fishes (especially in the liver) on different bacterial infection (Alvarez et al., 2013; Cuesta et al., 2008; Chen et al., 2007; Douglas et al., 2003) as well as viral infection (nervous necrosis virus infection in medaka) (Wang et al., 2010) have been documented.

Next, to hepcidin, we analyzed the expression pattern of cathepsin L (Fig. 2B). It is a lysosomal endopeptidase, a member of the peptidase C1 family (papain-like family) of cysteine proteinases that play an important role in diverse processes including normal lysosome-mediated protein turnover, antigen and proprotein processing, and apoptosis (Cho et al., 2016; Dickinson, 2002). In the study, we observed a significant ($p < .05$) increase in the cathepsin L expression at 12 hpi in both the group followed by a sharp decline at 1 dpi as the virus copy number rises. But interestingly, during 2–7 dpi, only the 20 °C group showed some upregulation while there was no significant change in the



(caption on next page)

Fig. 2. Relative expression analysis of immune genes in liver tissue of olive flounder maintained at 15 °C and 20 °C after viral haemorrhagic septicaemia (VHSV) infection. Expression levels of each gene were compared between the two experimental groups relative to the control and the mean (n = 5) relative expression of Hepcidin (A), Cathepsin L (B), Complement factors, C3 (C), CFD (D), TLR2 (E), TLR7 (F), MDA5 (G) ISG15 (H), Mx (I), IL-1β (J), IL-8 (K), MHC II (L), CD4 (M), MHC I (N), CD8 (O), p53 (P), HSP70 (Q), and caspase-3 (R) were plotted with standard error against time after infection. A two-way ANOVA was performed with the data set for each gene. The *p*-values within the group (day wise), between the group (group wise) and for interaction effect are evaluated to determine statistical significance ($p < .05$). Duncan multiple range test (homogenous subsets for 15 °C and 20 °C groups indicated by lowercase and uppercase alphabets respectively) within the groups (day-wise) and an unpaired *t*-test (indicated by asterisks *) between the groups at different time points were also performed to analyze the statistical differences.

15 °C group compared to control level (2 dpi and 4 dpi). The insignificant change of cathepsin L in the 15 °C group might be an indicator of host's inability of cathepsin L related immune function. Thus it can be inferred that both hepcidin and cathepsin L proved to have an important host immune regulatory function against VHSV in the liver when the temperature was conducive.

3.2.2. Complement factor – C3 and factor D

The fish complement system is an integral part of hepatic immune response and it has a role to play in host defence (Wu et al., 2016; Takahashi et al., 2009). In case of enveloped viruses, the complement system employs various elimination processes viz., direct neutralization of cell-free viruses, lysis of the virion or virus-infected cells, induction of antiviral state and boosting of virus-specific immune responses (Agrawal et al., 2017). Among various complement molecules, complement factor C3 plays a central role in all complement activation pathways. This complement factor not only takes part in the production of membrane attack complex against pathogens but also plays a crucial role in opsonisation, phagocytosis and release of anaphylatoxins C3a, which attract neutrophils to the site of infection (Castro et al., 2014; Boshra et al., 2006). In our study, there was early upregulation of C3 in the 20 °C group (3.5-fold and 1.7-fold increase at 12 hpi and 1 dpi, respectively) in comparison with prolonged downregulation in the 15 °C group (Fig. 2C). In addition, the complement factor D, an important factor for activation of the alternative pathway, showed consistent downregulation in the 15 °C group in contrast with the normal level in the 20 °C group (Fig. 2D). The downregulation of both C3 and factor D at 15 °C, predicts two possibilities i.e., either the virus evades the complement pathway due to which complement system fails to recognize the virus and remains inactivated or the lower temperature negatively influence the host complement activation. However, the reason for the disparity in the expression profiles of C3 and factor D at two differential temperatures cannot be inferred from the present results and a more detailed study is needed to unveil the exact cause. Nevertheless, the differential expression patterns of complement genes at two temperatures suggests that, at 15 °C, the subdued complement system of the host was unable to instigate the lysis of virus ultimately facilitating uninterrupted viral proliferation and disease manifestation which is in contrast to the antiviral complement activation as indicated by early upregulation of both the genes in the 20 °C group.

3.2.3. PRRs pathway genes (TLR2, TLR7, and MDA5)

As it has been reported earlier that the pattern recognition receptors (PRR) pathways mediated immune response are independent of tissue specificity (Kawai and Akira, 2010; Takeda et al., 2003; Akira et al., 2001), we analyzed the expression of PRRs pathway genes TLR2 (Fig. 2E), TLR7 (Fig. 2F) and MDA5 (Fig. 2G). The expression pattern in the liver for all the 3 PRRs gene studied was similar to our previous work on olive flounder kidney (Avunje et al., 2011, 2012, 2013). Stronger expression of TLR2, TLR7 and MDA5 at 12 hpi to 2 dpi in the 20 °C group compared to the 15 °C group enabled proper recognition of viral antigens which in turn helps to stimulate cytokine production that ultimately leads to complete removal of the pathogen and recovery from the infection at 20 °C whereas the lower expression of the PRRs pathway genes at 15 °C can be caused by the adaptation of the virus to evade these receptors, causing disease to the fish.

3.2.4. Interferon-stimulated genes (ISG15, Mx)

Interferon-mediated immune responses involve the stimulation of various antiviral ISGs which are critical in establishing an antiviral state in the host inhibiting viral replication and proliferation (Hu et al., 2011, 2010). In the present study, the 20 °C group showed quicker and stronger expression of ISG15 (Fig. 2H) and Mx (Fig. 2I) genes starting from 12 hpi to 2 dpi in compared with the delayed response in the 15 °C group (at 2 dpi only) can be correlated with the disease resistance in the 20 °C group and VHSV-mediated pathogenicity in the 15 °C group. This finding is well supported by earlier studies (Takami et al., 2010) where they reported a quicker induction of antiviral proteins was essential to overcome viral pathogenicity. From these results, we can interpret that interferon-driven pathway also actively works in the hepatic system for countering viral insults.

3.2.5. Inflammatory cytokines (IL-1β and IL-8)

In addition to the PRRs pathway genes, cytokines and chemokines played essential roles in immune defence by attracting and recruiting various immune cells to infection sites (Cho et al., 2016). Timely response of the pro-inflammatory cytokine (IL-1β) and chemokine (IL-8) (activated by PRRs) proved critical in combating the disease manifestation. They cause inflammatory responses and need to be tightly controlled to have a beneficial immune induction in the host against viral insults (Takeuchi and Akira, 2010; Sims and Smith, 2010). In the VHSV-infected fish maintained at 20 °C, IL-1β (Fig. 2J) showed significant ($p < .05$) upregulation at 12 hpi and 1 dpi that drastically reduced to control levels at 2–4 dpi, indicating an initial inflammation when the viral load was high followed by return to normal at recovery stage as viral copies subside to undetectable levels. In contrast, the 15 °C group showed no significant upregulation throughout the experimental period which resulted in insufficient inflammation reaction to counter viral proliferation. IL-8 (Fig. 2K) expression was significantly higher in the 20 °C group in comparison with the 15 °C group from 12 hpi to 2 dpi. The early upregulation of IL-8 transcripts in the 20 °C group and under-expression in the 15 °C group indicates it is temperature dependent and functions as a chemoattractant for immune cells in the VHSV-infected site. Similar upregulation of chemokine during early viral (VHSV and IPNV) infection was observed in kidney and spleen of rainbow trout (Montero et al., 2009), suggesting that IL-8's induction kinetics are closely oriented with the viral infection. Moreover, the differential expression of IL-1β and IL-8 in the present study indicates their integral role in the hepatic anti-viral immune response against VHSV attack.

3.2.6. Antigen presentation (MHC I, MHC II, CD4, and CD8)

The major histocompatibility complex class I and class II genes along with CD4 and CD8 play key roles in the recognition and presentation of antigens and immune signal transduction after pathogen invasion (Kato et al., 2013; Dan et al., 2013; Raida and Buchmann, 2008). MHC II gene showed no significant difference in the expression in the 15 °C group with slightly high expression at 2 dpi (relative to control). When compared with 15 °C, the 20 °C group displayed significant upregulation ($p < .05$) at 2 dpi to 7 dpi (Fig. 2L). On the contrary, the CD4 expression (Fig. 2M) was interestingly very high at 12 hpi (182.31-fold), 1 dpi (128.62-fold) and 2 dpi (72.25-fold) in the 20 °C group which gradually declines at 4 dpi (18-fold). Similar trends

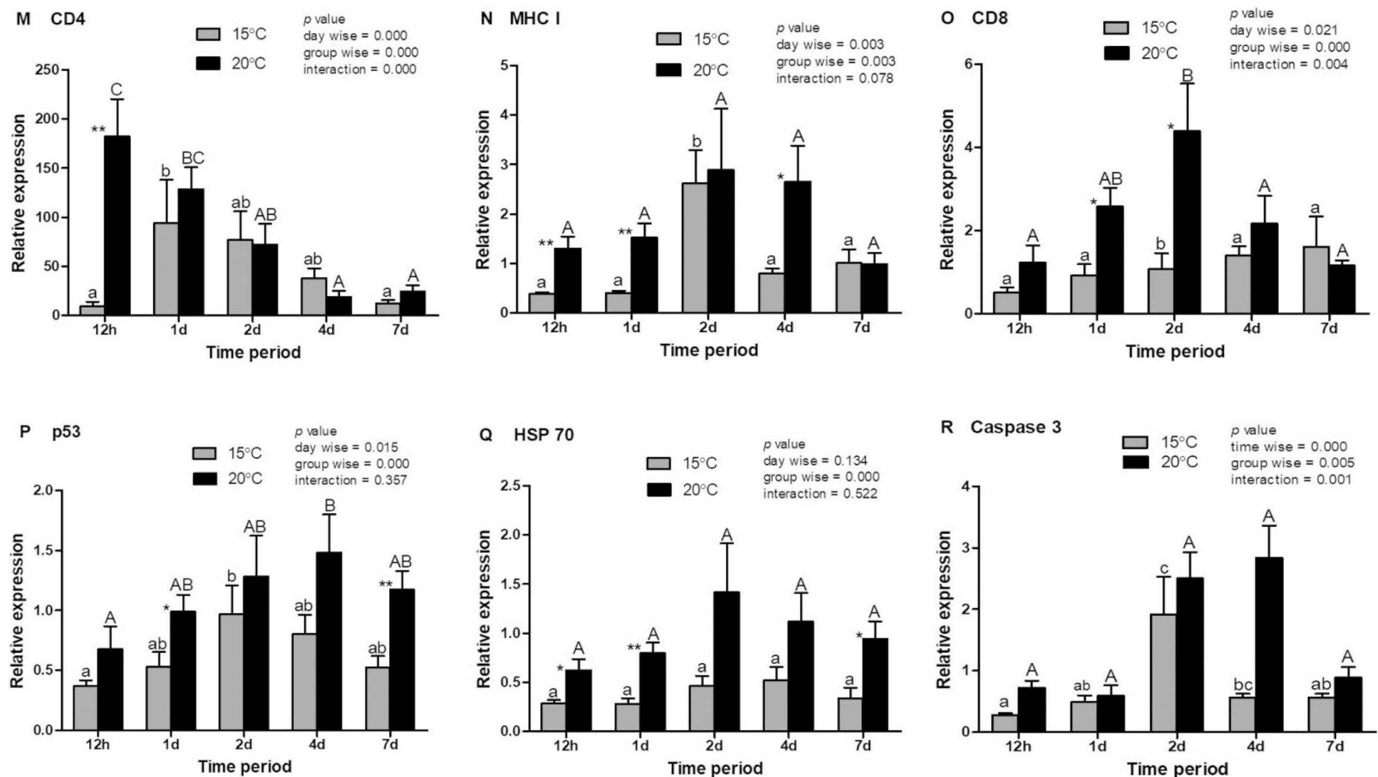


Fig. 2. (*continued*)

in expression were observed in the 15 °C group (93.1-fold at 1 dpi and 76.86-fold at 2 dpi) with an exception at 12 hpi, where there was no significant difference in expression compared to control. The higher expression of MHC II at 20 °C than 15 °C with simultaneous early responses of the CD4 transcript at 20 °C proves the importance of both MHC II and CD4 in viral antigen processing and presentation. The high transcription levels of CD4 molecules observed in olive flounder liver suggested that the liver consists of cells bearing CD4 on the cell surface and its initial response to infection seemed related to a T cell-dependent response. However, the contrasting expression MHC II (late upregulation) with that of CD4 (early upregulation) at 20 °C exhibited no direct relation between them, instead indicating the involvement of other molecules in the antigen presentation steps and suggesting a further detailed investigation.

The MHC I- and CD8-mediated cytotoxic T cell pathway showed contrasting expression kinetics at two different temperatures (Fig. 2N, O). In the 15 °C group, it was observed that the expression of MHC I (except one fish at 2 dpi) and CD8 were significantly declined in the early phase of infection, but normalized after the viral transcript subsides. In comparison, in the 20 °C group, MHC I (at 12 hpi to 4 dpi) and CD8 (at 1 and 2 dpi) genes showed an elevated expression during 1–2 dpi before returning to the normal level by 7 dpi. Thus, in combination with the cathepsin L gene expression which lies upstream in the pathway of an apoptotic cascade it can be concluded that early enhancement of MHC I transcript in the 20 °C group resulted in T cell activation in the hepatic immune system (a hallmark of macrophages at sites of inflammation) followed by a more organized form of apoptosis limiting the ability of the virus to proliferate. This is well supported by previous works where it was documented that viral peptides presented by MHC I are recognized by CD8 T cells which proliferate to become effector cytotoxic T cells that in turn recognize virus-infected cells and induce several cell death inducers of the apoptotic pathway (Chávez-Galán et al., 2009; Schultz and Harrington, 2003).

3.2.7. Stress response and apoptotic response (p53, HSP70, and caspase-3)

Apoptosis, or programmed cell death, is one of the central regulatory features of the immune defence mechanism against pathogens, particularly viruses (Sun and Shi, 2001; Everett and McFadden, 1999). Apoptosis prevents the proliferation of infective agents from uninfected healthy cells by killing infected cells (Jacobson et al., 1997; White and Steller, 1995). The anti-tumor protein p53 is a key molecule in both the extrinsic death receptor pathway (triggering the activation of a caspase cascade) as well as the intrinsic mitochondrial pathway (promoting the formation of the apoptosome), resulting in caspase-mediated apoptosis (Haupt et al., 2003). From our findings, transcriptional profile of p53 gene (Fig. 2P) showed significant downregulation in the 15 °C group in contrast to slight elevation in the 20 °C group. Although, the difference in the expression of p53 in the two experimental groups was statistically significant, still due to the marginal variation, it is difficult to infer about the positive role of p53 molecule in hepatic immune responses against VHSV. Nevertheless, we can predict from the relationship between the higher viral mRNA copies alongwith the downregulation of p53 gene in the 15 °C group that the apoptotic mechanism failed to initiate protection in the 15 °C group which paved the opportunity for the virus to proliferate and cause disease in the host.

HSP70 is another important molecule which undergoes conformational changes in response to stress, making cells resistant to stress-induced cell damage. In the study, prolonged downregulation of HSP70 in the 15 °C group signifies host inability to combat viral stress. However, in the 20 °C group, there was a slight increase in HSP70 transcript from 2 dpi (Fig. 2Q). This enhancement of HSP70 gene transcript during 2–7 dpi indicates the presence of free HSP70 protein in the cytosol resulted after subsequent repair of damaged tissues after viral infection and dissociation from trimeric HSF (heat shock factor) and release of inactive monomeric HSF (Iwama et al., 1998).

The current study also elucidates expression of the apoptosis-related caspase-3 gene, the final executioner of cell death (Taylor et al., 2008; Benedict et al., 2002). Caspase-3 showed a relatively higher expression in the fish reared at 20 °C than at 15 °C during 2–4 dpi (Fig. 2R).

However, caspase-3 transcript was very low at 12 hpi and 1 dpi in both the experimental groups indicating suppression of expression due to VSHV infection at an early stage. This phenomenon is consistent with the study of Ammayappan and Vakharia (2011) where they reported novirhabdovirus like VHSV and IHNV suppresses apoptotic genes like caspase-3 at early stage of viral infection. Similar to the p53 and HSP70 gene expression, the caspase-3 expression dynamics at the two temperatures was not strikingly different to give a conclusive evidence of its role in the hepatic immune responses. Intriguingly, the expression profiles coincided with the viral transcripts suggesting that apoptotic pathway might be potentially more active at 20 °C than at 15 °C. However, further study is needed to better elucidate the role of these apoptotic genes in relation to hepatic immune response against VHSV in olive flounder. In addition, the present study showed lower overall apoptotic activity in the liver when compared with that of the kidney (Avunje et al., 2012) which can be correlated with the differential viral copy number in the two organs i.e., higher copies in kidney than in liver, and henceforth, the resultant apoptotic response was viz-a-viz the viral load.

In summary, our results demonstrate that T lymphocytes play a crucial role in the initial response to VHSV in the liver at 20 °C, as is evident from the upregulation of CD4, CD8, cathepsin L, Mx, and ISG15 genes, which ultimately helps the host to have a coordinated immune response against VHSV leading to host survival. Consequently, a downregulated expression pattern for several important immune-related genes post-infection at 15 °C, especially the genes related to the apoptotic pathway, cytotoxic T-cell pathway, and complement system resulted in the failure of the host immune system to combat the viral proliferation. In addition, the viral infection triggered the activation of several PRRs including TL2, TLR7 and MDA5 along with regulated activation of IL-1β and IL-8 at 20 °C. Regulation of antimicrobial peptides, hepcidin, and complement was also appreciated in the liver at 1–2 dpi in the 20 °C group. Hence, the present study helps us to gain an insights on functional aspects of hepatic defence in orchestrating anti-VHSV immune responses in olive flounder.

Acknowledgments

This research was supported by 'Fish Vaccine Research Center', funded by the Ministry of Oceans and Fisheries, Korea.

References

Agrawal, P., Nawadkar, R., Ojha, H., Kumar, J., Sahu, A., 2017. Complement evasion strategies of viruses: an overview. Front. Microbiol. 8, 1117.

Akira, S., Takeda, K., Kaisho, T., 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat. Immunol. 2, 675–680.

Akira, S., Uematsu, S., Takeuchi, O., 2006. Pathogen recognition and innate immunity. Cell 124, 783–801.

Alcorn, S.W., Murra, A.L., Pascho, R.J., 2002. Effects of rearing temperature on immune functions in sockeye salmon (*Oncorhynchus nerka*). Fish Shellfish Immunol. 12, 303–334.

Ammayappan, A., Vakharia, V.N., 2011. Nonvirion protein of novirhabdovirus suppresses apoptosis at the early stage of virus infection. J. Virol. 85 (16), 8393–8402.

Aoki, T., Takano, T., Santos, M.D., Kondo, H., Hirono, I., 2008. Molecular innate immunity in Teleost fish: review and future perspectives. In: 5th World Fish Congress, pp. 263–276.

Avunje, S., Kim, W.S., Park, C.S., Oh, M.J., Jung, S.J., 2011. Toll-like receptors and interferon associated immune factors in viral haemorrhagic septicaemia virus-infected olive flounder (*Paralichthys olivaceus*). Fish Shellfish Immunol. 31, 407–414.

Avunje, S., Kim, W.S., Oh, M.J., Choi, I., Jung, S.J., 2012. Temperature-dependent viral replication and antiviral apoptotic response in viral haemorrhagic septicaemia virus (VHSV)-infected olive flounder (*Paralichthys olivaceus*). Fish Shellfish Immunol. 32, 1162–1170.

Avunje, S., Oh, M.J., Jung, S.J., 2013. Impaired TLR2 and TLR7 response in olive flounder infected with viral haemorrhagic septicaemia virus at host susceptible 15°C but high at non-susceptible 20°C. Fish Shellfish Immunol. 34, 1236–1243.

Benedict, C.A., Norris, P.S., Ware, C.F., 2002. To kill or be killed: viral evasion of apoptosis. Nat. Immunol. 3, 1013–1018.

Boshra, H., Li, J., Sunyer, J.O., 2006. Recent advances on the complement system of teleost fish. Fish Shellfish Immunol. 20, 239–262.

Castro, R., Abós, B., Pignatelli, J., von Gersdorff Jørgensen, L., González Granja, A., Buchmann, K., et al., 2014. Early immune responses in rainbow trout liver upon viral hemorrhagic septicemia virus (VHSV) Infection. PLoS One 9 (10), e111084.

Chávez-Galán, L., Arenas-Del Angel, M.C., Zenteno, E., Chávez, R., Lascurain, R., 2009. Cell death mechanisms induced by cytotoxic lymphocytes. Cell. Mol. Immunol. 6, 15–25.

Chen, S.L., Li, W., Meng, L., Sha, Z.X., Wang, Z.J., Ren, G.C., 2007. Molecular cloning and expression analysis of a hepcidin antimicrobial peptide gene from turbot (*Scophthalmus maximus*). Fish Shellfish Immunol. 22 (3), 172–181.

Cho, H.K., Kim, J., Moon, J.Y., Nam, B.H., Kim, Y.O., et al., 2016. Microarray analysis of gene expression in olive flounder liver infected with viral haemorrhagic septicaemia virus (VHSV). Fish Shellfish Immunol. 49, 66–78.

Cuesta, A., Meseguer, J., Esteban, M.A., 2008. The antimicrobial peptide hepcidin exerts an important role in the innate immunity against bacteria in the bony fish gilthead seabream. Mol. Immunol. 45 (8), 2333–2342.

Dalmo, R.A., Ingebrigtsen, K., Bøgwald, J., 1997. Non-specific defence mechanisms in fish, with particular reference to the reticuloendothelial system (RES). J. Fish Dis. 20, 241–273.

Dan, X.M., Zhang, T.W., Li, Y.W., Li, A.X., 2013. Immune responses and immune-related gene expression profile in orange-spotted grouper after immunization with *Cryptocaryon irritans* vaccine. Fish Shellfish Immunol. 34, 885–891.

Dickinson, D.P., 2002. Cysteine peptidases of mammals: their biological roles and potential effects in the oral cavity and other tissues in health and disease. Crit. Rev. Oral Biol. Med. 13, 238.

Domenico, I.D., Zhang, T.Y., Koening, C.L., Branch, R.W., London, N., et al., 2010. Hepcidin mediates transcriptional changes that modulate acute cytokine-induced inflammatory responses in mice. J. Clin. Invest. 120, 2395–2405.

Douglas, S.E., Gallant, J.W., Liebscher, R.S., Dacanay, A., Tsoi, S.C.M., 2003. Identification and expression analysis of hepcidin-like antimicrobial peptides in bony fish. Dev. Comp. Immunol. 27, 589–601.

Everett, H., McFadden, G., 1999. Apoptosis: an innate immune response to virus infection. Trends Microbiol. 7, 160–165.

Hampton, J.A., Lantz, R.C., Hinton, D.E., 1989. Functional units in rainbow trout (*Salmo gairdneri*, Richardson) liver: III. Morphometric analysis of parenchyma, stroma, and component cell types. Am. J. Anat. 185, 58–73.

Haupt, S., Berger, M., Goldberg, Z., Haupt, Y., 2003. Apoptosis-the p53 network. J. Cell Sci. 116, 4077–4085.

Hu, G., Yin, X., Xia, J., Dong, X., Zhang, J., Liu, Q., 2010. Molecular cloning and characterization of interferon regulatory factor 7 (IRF-7) in Japanese flounder, *Paralichthys olivaceus*. Fish Shellfish Immunol. 29, 963–971.

Hu, G., Yin, X., Lou, H., Xia, J., Dong, X., Zhang, J., et al., 2011. Interferon regulatory factor 3 (IRF-3) in Japanese flounder, *Paralichthys olivaceus*: sequencing, limited tissue distribution, inducible expression and induction of fish type I interferon promoter. Dev. Comp. Immunol. 35, 164–173.

Isshiki, T., Nishizawa, T., Kobayashi, T., Nagano, T., Miyazaki, T., 2001. An outbreak of VHSV (viral hemorrhagic septicemia virus) infection in farmed Japanese flounder *Paralichthys olivaceus* in Japan. Dis. Aquat. Org. 47, 87–99.

Iwama, G.K., Thomas, P.T., Forsyth, R.B., Vijayan, M.M., 1998. Heat shock protein expression in fish. Rev. Fish Bio. Fisher. 8, 35–56.

Jacobson, M.D., Weil, M., Raff, M.C., 1997. Programmed cell death in animal development. Cell 88, 347–354.

Kaneshige, N., Jirapongpairoj, W., Hirono, I., Kondo, H., 2016. Temperature-dependent regulation of gene expression in Japanese flounder *Paralichthys olivaceus* kidney after *Edwardsiella tarda* formalin-killed cells. Fish Shellfish Immunol. 59, 298–304.

Kato, G., Goto, K., Akune, I., Aoka, S., Kondo, H., Hirono, I., 2013. CD4 and CD8 homologues in Japanese flounder, *Paralichthys olivaceus*: differences in the expressions and localizations of CD4-1, CD4-2, CD8a and CDb. Dev. Comp. Immunol. 39, 293–301.

Kawai, T., Akira, S., 2010. The role of pattern-recognition receptors in innate immunity: update on toll-like receptors. Nat. Immunol. 11, 373–384.

Kim, W.S., Kim, S.R., Kim, D., Kim, J.O., Park, M.A., Kitamura, S.I., Kim, H.Y., Kim, D.H., Han, H.J., Jung, S.J., Oh, M.J., 2009. An outbreak of VHSV (viral hemorrhagic septicemia virus) infection in farmed olive flounder *Paralichthys olivaceus* in Korea. Aquaculture 296 (1–2), 165–168.

Krause, A., Neitz, S., Mägert, H.J., Schulz, A., Forssmann, W.G., Schulz-Knappe, P., Adermann, K., 2000. LEAP-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity. FEBS Lett. 480 (2–3), 127–150.

Liang, Y., Kwota, Z., Sun, J., 2016. Intrahepatic regulation of antiviral T cell responses at initial stages of viral infection. Int. Immunopharmacol. 39, 106–112.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real time quantitative PCR and the 2(−Delta Delta C(T)) method. Methods 25, 402–408.

Magnadottir, B., 2006. Innate immunity of fish (overview). Fish Shellfish Immunol. 20, 137–151.

Möller, A.M., Korytář, T., Köllner, B., Schmidt-Posthaus, H., Segner, H., 2014. The teleostean liver as an immunological organ: intrahepatic immune cells (IHICs) in healthy and benzo [a] pyrene challenged rainbow trout (*Oncorhynchus mykiss*). Dev. Comp. Immunol. 46 (2), 518–529.

Montero, J., Chaves-Pozo, E., Cuesta, A., Tafalla, C., 2009. Chemokine transcription in rainbow trout (*Oncorhynchus mykiss*) is differently modulated in response to viral hemorrhagic septicaemia virus (VHSV) or infectious pancreatic necrosis virus (IPNV). Fish Shellfish Immunol. 27, 661–669.

Ndong, D., Chen, Y.Y., Lin, Y.H., Vaseeharan, B., Chen, J.C., 2007. The immune response of tilapia *Oreochromis mossambicus* and its susceptibility to *Streptococcus iniae* under stress in low and high temperatures. Fish Shellfish Immunol. 22, 686–694.

Nikoskelainen, S., Bylund, G., Lilius, E.M., 2004. Effect of environmental temperature on rainbow trout (*Oncorhynchus mykiss*) innate immunity. Dev. Comp. Immunol. 28, 581–592.

Parker, G.A., Picut, C.A., 2005. Liver immunobiology. Toxicol. Pathol. 33, 52–62.

Raida, M.K., Buchmann, K., 2008. Bath vaccination of rainbow trout (*Oncorhynchus mykiss* Walbaum) against *Yersinia ruckeri*: effects of temperature on protection and gene expression. Vaccine 26, 1050–1062.

Raida, M.K., Buchmann, K., 2009. Innate immune response in rainbow trout (*Oncorhynchus mykiss*) against primary and secondary infections with *Yersinia ruckeri* O1. Dev. Comp. Immunol. 33, 35–45.

Schultz, D.R., Harrington, Jr W.J., 2003. Apoptosis: programmed cell death at a molecular level. Semin. Arthritis Rheum. 32, 345–369.

Sims, J.E., Smith, D.E., 2010. The IL-1 family: regulators of immunity. Nat. Rev. Immunol. 10, 89–102.

Smail, D.A., Snow, M., 2011. Viral haemorrhagic septicaemia. In: Woo, P.T.K., Bruno, D.W. (Eds.), Fish Diseases and Disorders: Viral, Bacterial and Fungal Infections. Vol. 3. CABI, Wallingford, UK, pp. 110–142.

Sun, E.W., Shi, Y.F., 2001. Apoptosis: the quiet death silences the immune system. Pharmacol. Ther. 92, 135–145.

Takahashi, M., Ishida, Y., Iwaki, D., Kanno, K., Suzuki, T., Endo, Y., Homma, Y., Fujita, T., 2009. Essential role of mannose-binding lectin-associated serine protease-1 in activation of the complement factor D. J. Exp. Med. 207 (1), 29–37.

Takami, I., Kwon, S.R., Nishizawa, T., Yoshimizu, M., 2010. Protection of Japanese flounder *Paralichthys olivaceus* from viral hemorrhagic septicemia (VHS) by poly (I:C) immunization. Dis. Aquat. Org. 89, 109–115.

Takeda, K., Kaisho, T., Akira, S., 2003. Toll-like receptors. Annu. Rev. Immunol. 21, 335–376.

Takeuchi, O., Akira, S., 2010. Pattern recognition receptors and inflammation. Cell 140, 805–820.

Taylor, R.C., Cullen, S.P., Martin, S.J., 2008. Apoptosis: controlled demolition at the cellular level. Nat. Rev. Mol. Cell Biol. 9, 231–241.

Wang, Y.D., Kung, C.W., Chen, J.Y., 2010. Antiviral activity by fish antimicrobial peptides of epinecidin-1 and hepcidin 1–5 against nervous necrosis virus in medaka. Peptides 31 (6), 1026–1033.

White, K., Steller, H., 1995. The control of apoptosis in Drosophila. Trends Cell Biol. 5, 74–78.

Wolf, K., 1988. Viral hemorrhagic septicemia. In: Fish Viruses and Fish Viral Diseases. Cornell University Press, Ithaca, New York, USA, pp. 217–249.

Wu, N., Song, Y.L., Wang, B., Zhang, X.Y., Zhang, X.J., Wang, Y.L., Zhang, Y.A., et al., 2016. Fish gut-liver immunity during homeostasis or inflammation revealed by integrative transcriptome and proteome studies. Sci. Rep. 6, 36048.

Xu, G., Sheng, X., Xing, J., Zhan, W., 2011. Effect of temperature on immune response of Japanese flounder (*Paralichthys olivaceus*) to inactivated lymphocystis disease virus (LCDV). Fish Shellfish Immunol. 30, 525–531.