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Different responses in one-year-old and three-year-old grass carp reveal the mechanism of age restriction of GCRV infection

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ABSTRACT

Grass carp is an important fish species in Chinese aquaculture, and can be afflicted by a hemorrhagic disease caused by the grass carp reovirus (GCRV). Interestingly, the affects of GCRV infection of grass carp are age-restricted, meaning that one-year-old grass carp can be infected and can suffer hemorrhagic disease, but three-year-old carp are not so afflicted. In this study, we investigated the mechanism responsible for this age-restricted pathology. We evaluated the relative copy number of GCRV RNA, the expression levels of proteins in blood, and changes in DNA methylation in carp from the two age groups after infection with GCRV. After GCRV infection, the relative copy number of GCRV RNA in three-year-old grass carp was significantly lower than in one-year-old carp. The differences in circulating protein levels mainly occurred in concentrated in complement and coagulation proteins, and the expression levels of these proteins were significantly higher in three-year-old grass carp than in one-year-old carp. Moreover, the expression levels of DNA methylation-related genes in the liver and spleen of one-year-old grass carp were significantly higher than those of three-year-old carp. These results suggested that as age of grass carp increases, faster and more efficient response of the immune system after viral infection, especially the complement system, and differences in DNA methylation may be important factors that affect the age restriction observed in GCRV infection. Our study provides new insights into the mechanisms underlying age restriction of GCRV infection.

1. Introduction

The grass carp (*Ctenopharyngodon idellus*) has been cultivated in China for more than 60 years and is one of the most important aquaculture species in the world. In 2015, grass carp production reached 5.8 million tons and accounted for more than 13% of the world's freshwater aquaculture production [1,2]. However, hemorrhage caused by infection with grass carp reovirus (GCRV) results in substantial economic losses to the grass carp aquaculture industry. GCRV, first isolated in China, is a double-stranded RNA virus that belongs to the genus *Aquareovirus* of the family *Reoviridae* [3]. GCRV infection may trigger apoptosis in grass carp kidney cells [4]. Interestingly, it has been observed that GCRV infection exhibits age restricted pathology in grass carp, in which one-year-old grass carp infected by GCRV may suffer hemorrhage, while three-year-old grass carp do not show symptoms of GCRV infection.

Age restriction in viral infections has been reported other fish species. Rainbow trout that were infected with infectious pancreatic necrosis virus (IPN; Sp type) exhibited decreased disease symptoms with increasing age, and were not sensitive to the disease by 20 weeks of age [5]. Infection of six species of fish in North America with spring viremia of carp virus (SVCV) revealed that younger fish are more susceptible to the disease, and that adult fish are either not infected or can rapidly clear the virus after infection [6].

It has been found that reovirus can replicate in the intestine of mice of all ages, but in the first few weeks of life, the replication efficiency of reovirus and the ability to induce disease are substantially reduced [7]. In the study of the murine retrovirus CasBr, infected newborn mice were ill but mice at ten days of age were totally resistant [8]. Inoculation of acute hepatitis virus in experimental woodchucks revealed that woodchucks around eight weeks of age can be infected, but adult woodchucks are not infected at all [9]. There is a similar phenomenon in humans, wherein rotavirus, a virus in the family of reoviruses, shows the highest infection rate among children under 2-years-old [10], while

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adults are not easily infected [11]. In response to this phenomenon, studies have shown that the expression of innate immune sensor toll-like receptor 3 (TLR3) in the intestine is enhanced with age [12]. TLR3 can recognize the dsRNA carried by rotavirus and can induce activation of IRF3 and NF-κB [13]. IRF3 induces the production of type I interferon [14] leading to antiviral responses. A similar mechanism may be the basis for the age restriction of reovirus. However, the mechanism underlying age restriction of viral infection of teleost fish is largely unknown.

In the past decade, research on disease resistance in grass carp has mainly focused on the identification of immune-related genes [15,16], the development of genomic markers [17,18], the study of gene expression transcriptomes [19–23], and epigenetic studies based solely on DNA methylation modification of immune genes [24,25]. On the other hand, studies on age restriction of GCRV infection are rarely reported. In this study, we investigated viral RNA expression and immune protein expression patterns associated with GCRV infection in grass carp at one year of age and at three years of age. We evaluated relative copy number of viral gene expression in one-year-old and three-year-old grass carp after GCRV infection, analyzed the protein components in the blood, compared the expression level of the immune-related genes, and evaluated differences in expression of DNA methylation-related genes between two group. After GCRV infection, the relative copy number of GCRV RNA in the three-year-old grass carp was significantly lower than that in one-year-old carp. The differentially expressed proteins in the blood were mainly related to complement and blood coagulation. The expression of immune-related genes in three-year-old grass carp was significantly higher than in one-year-old carp, and the expression of DNA methylation genes in each tissue correlated with the copy number of the virus. Our study provides new insights into the mechanisms of age restriction of GCRV infection in grass carp, and may lead to the discovery of better aquaculture methods to prevent viral infection of grass carp populations.

2. Materials and methods

2.1. Ethics statement

All animal experiments were conducted following the Guide for the Care and Use of Laboratory Animals, and the protocol was approved by the Institute of Hydrobiology, Chinese Academy of Sciences. All surgeries were performed under eugenol anesthesia (100 mg/L), and all efforts were made to minimize suffering.

2.2. Experimental animals, virus exposure, and sample collection

Healthy one-year-old grass carp (143 tails, average body weight 8 g) and three-year-old grass carp (64 tails, average body weight 3000 g) were housed in a circulating water system at 26–28 °C for one week, and fed twice daily using commercial feed. The photoperiod was 14 h: 10 h light/dark and the dissolved oxygen in the water was maintained at 8 mg/L. After no abnormal symptoms were observed, the viral challenge experiment was performed. In the one-year-old grass carp group, fish were infected with GCRV (2.97×10^3 RNA copies/μL) by intraperitoneal injection of 160 μL GCRV. In the three-year-old grass carp group, fish were infected by intraperitoneal injection of 60 mL GCRV. Nine fish representing three biological replicates (n = 3 for each biological replicate) were collected before infection and at 1, 2, 3, and 5 days after injection. After euthanasia, gill, intestine, liver, spleen, and kidney were harvested for further analysis. The total mortality of the two groups was determined by counting the number of dead fish every day. After no dead fish were recorded for two consecutive weeks, the experiment was ended, and the total mortality was calculated.

2.3. Protein identification

Four protein mixed samples (n = 3 for each biological replicates) were performed before infection and at 3 days after infection, named UINF1, UIFN3, INF1 and INF3, and three replicates per sample. The reductive alkylation of the protein was as follows: a final concentration of 10 mM dithiothreitol (DTT) was added to reduce the protein, followed by addition of a final concentration of 55 mM ammonium iodoacetate (IAM), and finally 1 μg of Trypsin enzyme was added for overnight hydrolysis for 8 h–16 h. The enzymatically produced polypeptide was desalted by a C18 column, and the demineralized polypeptide was dried and dissolved in 15 μL of Loading Buffer (0.1% formic acid, 3% acetonitrile). The peptide was analyzed by LC-MS/MS (ekspertTMnanoLC; AB Sciex TripleTOF 5600-plus) instrument and the results were evaluated.

2.4. RNA isolation and quantitative real-time PCR (RT-qPCR)

TRizol reagent (Invitrogen, USA) was used to isolate RNA from gill, intestine, liver, spleen, and kidney samples, according to the manufacturer's protocol. The Qubit RNA assay kit (Life Technologies, USA) and RNA Nano 6000 assay kit (Agilent Technologies, USA) were used to measure the concentration and integrity of isolated RNA, respectively. DNase I (Promega, USA)-treated total RNA was used as the template for the first-strand cDNA synthesis.

RT-qPCR was performed using a fluorescence quantitative PCR instrument (Bio-Rad, USA). Primer sequences are shown in Table 1. Each RT-qPCR mixture contained 0.8 μL of each primer, 1 μL template, 10 μL 2 × SYBR Green master mix (TOYOBO), and 7.4 μL ddH2O. The program for RT-qPCR as followed: 95 °C for 10 s, 40 cycles at 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s. Three replicates (n = 3 for each biological replicate) were included for each sample, and the β-actin gene of grass carp served as an internal control to normalize the expression levels. The relative expression level is calculated using the $2^{-\Delta\Delta Ct}$ method [26]. All data represent the mean ± standard deviation of three replicates.

2.5. Statistical analysis

Statistical significance between the experimental group and the control group was determined using one-way ANOVA and Fisher's Least Significant Difference (LSD) posttest. A difference was considered to be significant at $P < 0.05$.

Table 1
Primers used in the study.

Primers	Sequences (5′–3′)	Usage
gcrv	AGCGCAGCAGGCAATTACTATCT ATCTGCTGGTAATGCGGAACG	qPCR of *gcrv*
c3	ATGGTTCGCAAACACTCCTCAG ACAGGTACTTGGCTTCTATGTCAACT	qPCR of *c3*
il-8	ATGAGTCTTAGAGGTCTGGGT ACAGTGAGGGCTAGGAGGG	qPCR of *il-8*
inf-2	TGTTTGATGACTTTGGGATG TCAGGACCCGCAGGAAGAC	qPCR of *inf-2*
mx-1	GTGAAGCCCAGGATGAGATGGC ATCCCGGGAAGGTCGATGAGAG	qPCR of *mx-1*
dnmt1	CCACCGAAATGTGCCGACTG GGTGCCCATGCTTGTCATACAC	qPCR of *dnmt1*
dnmt7	GAGAAGCAAGTGGGCTCCTACG CAGCGATGGAGGTCGTAAGAGTT	qPCR of *dnmt7*
tet3	GCCGCATATCCCTGGTCTTCTA TCGTGTCATCACACCGTCTCGT	qPCR of *tet3*
gnmt	AGTCTGAATGCCCTCTGGTGG ACCAGACTTCAAAGGGACCAAA	qPCR of *gnmt*
β-actin	AGCCATCCTTCTTGGGTATG GGTGGGGCGATGATCTTGAT	qPCR of *β-actin*

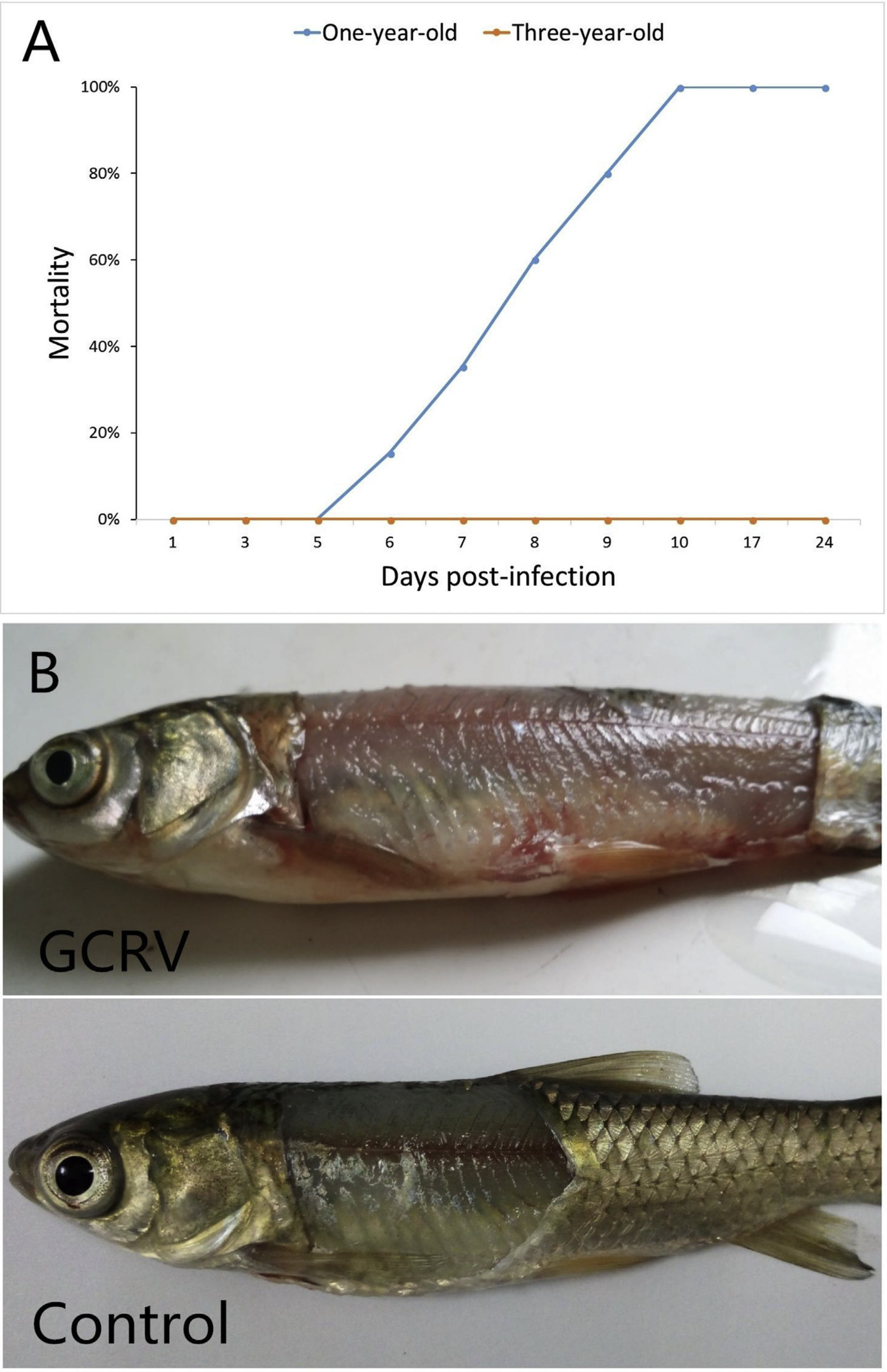

Fig. 1. Mortality of one-year-old and three-year-old grass carp infected with GCRV. (A) The number of dead grass carp in each group was counted every day until no deaths were recorded for two consecutive weeks. (B) The clinical symptom of one-year-old fish after GCRV infection.

3. Results

3.1. *Mortality of grass carp after GCRV infection*

One-year-old and three-year-old grass carp were infected with GCRV virus, and the death of grass carp in the two groups was evaluated after infection. The one-year-old infected grass carp began to die by six days post-infection (dpi), and all infected carp in the one-year-old group had died by 10 dpi (Fig. 1A); all carp exhibited symptoms of hemorrhage (Fig. 1B). During this period, three-year-old GCRV-infected grass carp have no symptoms and did not die. The three-year-old carp were followed for two weeks, and had not died after two weeks post-infection.

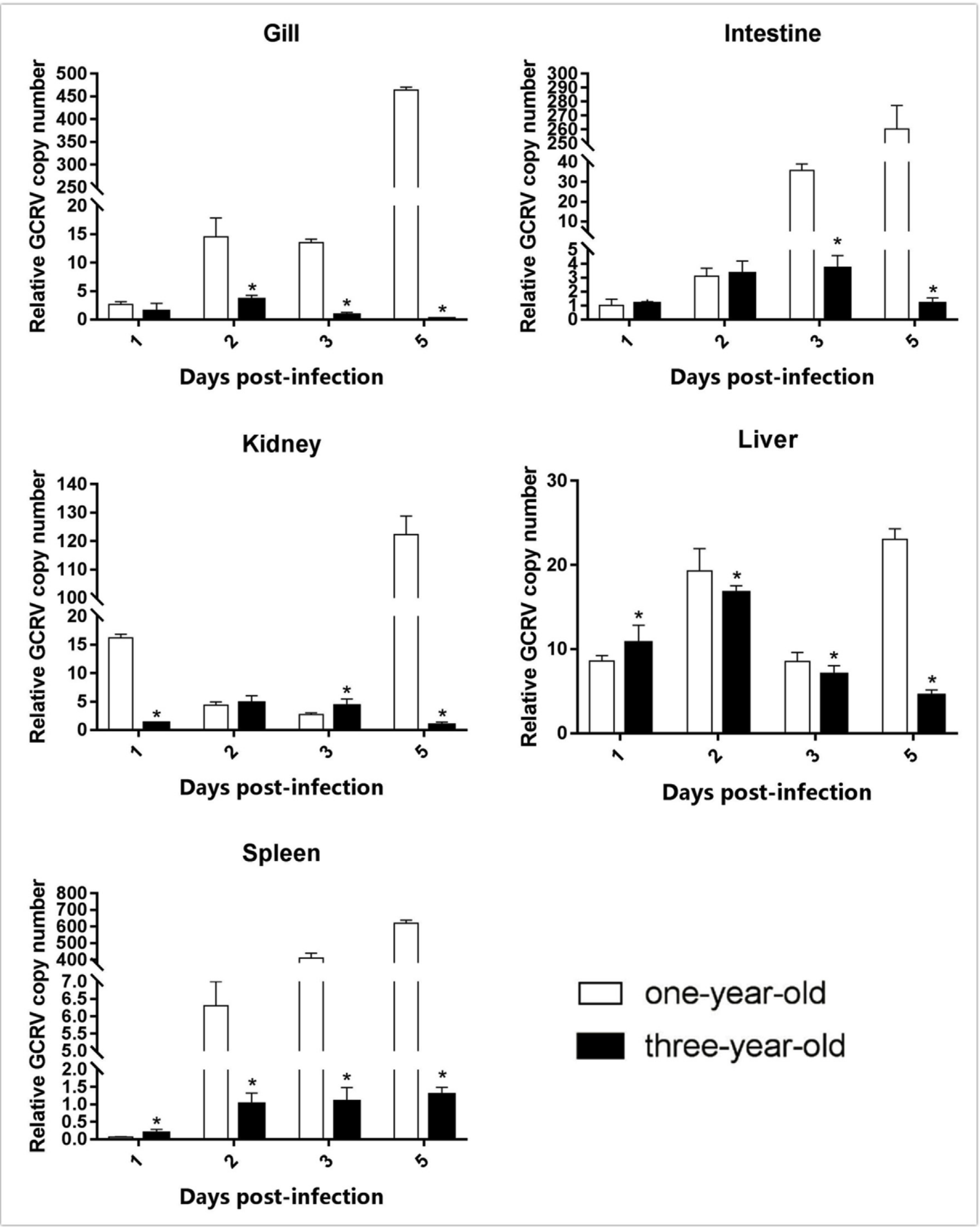

Fig. 2. The relative copy number of GCRV in the infected grass carp. The copy number of GCRV in intestine at 1 dpi in one-year-old grass carp was used as a reference for normalization. All data represent the mean ± S.D. of three replicates. A significant difference ($P < 0.05$) between the two groups is indicated with an asterisk (*).

3.2. Relative copy numbers of GCRV RNA in infected grass carp

The relative copy number of GCRV RNA in five tissues was examined to determine the GCRV infection status in one-year-old and three-year-old grass carp. The virus copy number of the gill and intestine in the three-year-old grass carp is equivalent to that of the one-year-old grass carp on the first day after infection, indicated that the dose we use to infect one-year-old and three-year-old grass carp is appropriate. And the relative copy number of GCRV RNA in the gill, intestine, liver, and spleen of the three-year-old grass carp was consistently lower than in the one-year-old carp after infection, and the GCRV copy number rapidly reduced after 3 dpi (Fig. 2). While the relative copy number of GCRV in the kidney of three-year-old grass carp showed an increasing trend compared with the one-year-old carp, GCRV levels were rapidly down-regulated on 5 dpi. In general, the copy number of GCRV in most tissues of three-year-old grass carp is lower than in one-year-old carp. By 5 dpi, GCRV copy numbers in all tissues from three-year-old grass carp were significantly lower than in one-year-old carp, indicating that the three-year-old grass carp effectively cleared GCRV infection.

Table 2
Summary of protein identification by mass spectrometry.

Group	Total spectra	Identification spectra	Identification peptide	Identification protein
Control-1	25160	12927	2683	302
Control-3	25041	12798	2739	311
Infected-1	25130	12676	2831	296
Infected-3	25413	13003	2550	298

3.3. Mass spectrometry of proteins in the blood of infected grass carp

The proteins in the blood of the one-year-old and three-year-old grass carp before infection and on the third day after infection were subjected to mass spectrometry. Protein identification parameters were set to confidence level conf ≥ 95% and unique peptides ≥ 1, and the number of proteins identified in the pre-infection one-year-old, pre-infection three-year-old, post-infection one-year-old, and post-infection three-year old carp was 302, 311, 296, and 298, respectively (Table 2).

The proteins identified in each group were compared and analyzed (Fig. 3). Amont the four groups, 199 proteins were in common, accounting for 45.5% of the total proteins. We identified 234 proteins in common between one-year-old and three-year-old grass carp after infection, and 62 unique proteins in the one-year-old carp and 64 unique proteins in the three-year-old carp.

Specific analysis of the differentially identified proteins between the one-year-old and three-year-old grass carp after infection showed that the proteins unique to three-year-old grass carp were mainly related to complement and blood coagulation, interleukin signaling, and antioxidant damage (Table 3). The rest of the proteins were mainly various enzymes involved in metabolism.

Table 3
Differential proteins related to antivirus in three-year-old grass carp after infection.

Catalog	Protein
complement and coagulation	complement component 3 complement component 8 adenylosuccinate synthase coagulation factor IX
antioxidant damage	glutathione peroxidase glutathione peroxidase 3 peroxiredoxin-6
interleukin signaling	interleukin-4 receptor interleukin-8

3.4. Expression patterns of immune-related genes after GCRV infection

The gene *c3* and the *il-8* were selected from unique proteins in the three-year-old carp to verify expression levels by RT-PCR in liver, spleen, and kidney tissue after GCRV infection. As the results showed, the expression levels of *il-8* in all tissue of three-year-old grass carp were higher than those in one-year-old (Fig. 4). And the expression levels of *c3* in liver and kidney of three-year-old grass carp were higher than those in one-year-old. These results indicated the accuracy of protein mass spectrometry.

Moreover, the antivirus gene *inf*-γ and *mx-1* were selected to invesitigated the immune response of grass carp after GCRV infection.

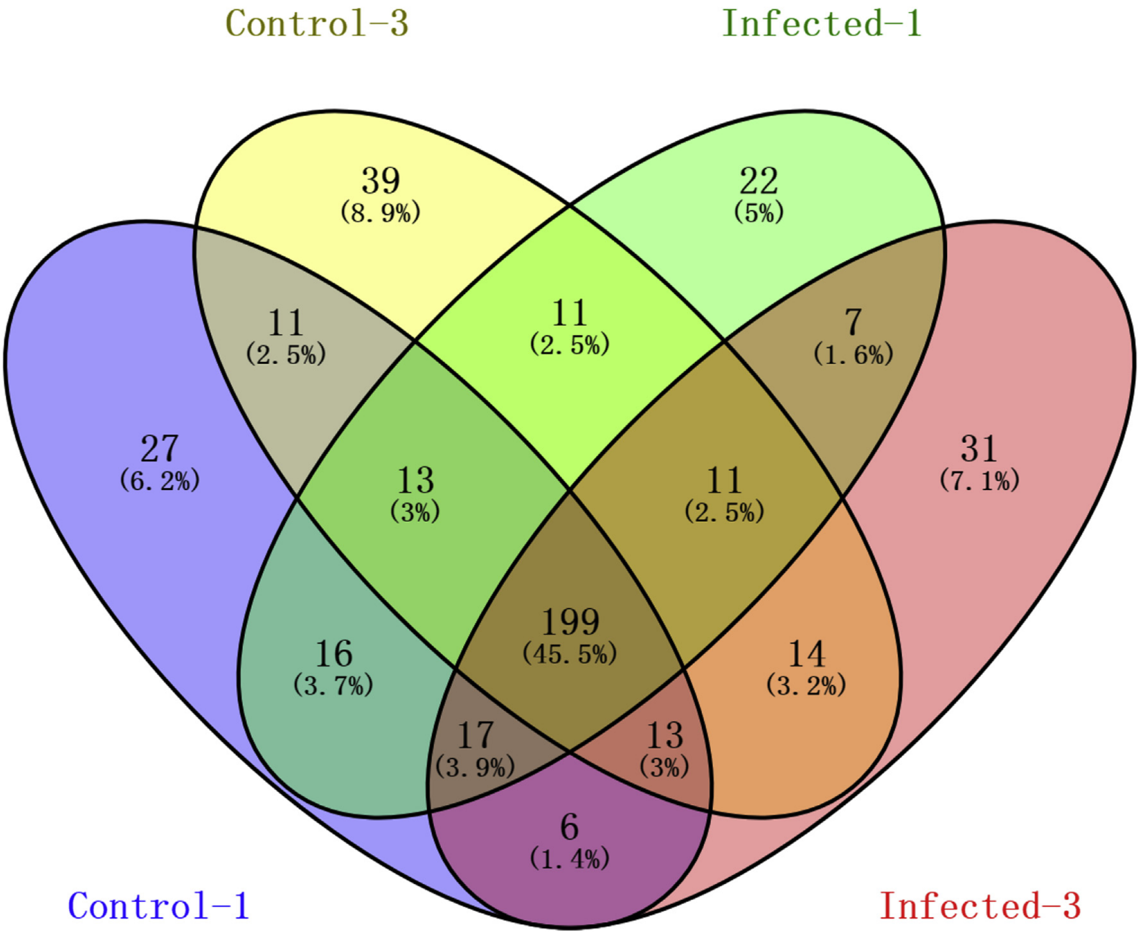

Fig. 3. Proteins identified in non-infected and infected grass carp. The Venn diagram results from a comparison of the identified blood proteins from the four groups (uninfected one-year-old grass carp, infected one-year-old grass carp, uninfected three-year-old grass carp, infected three-year-old grass carp).

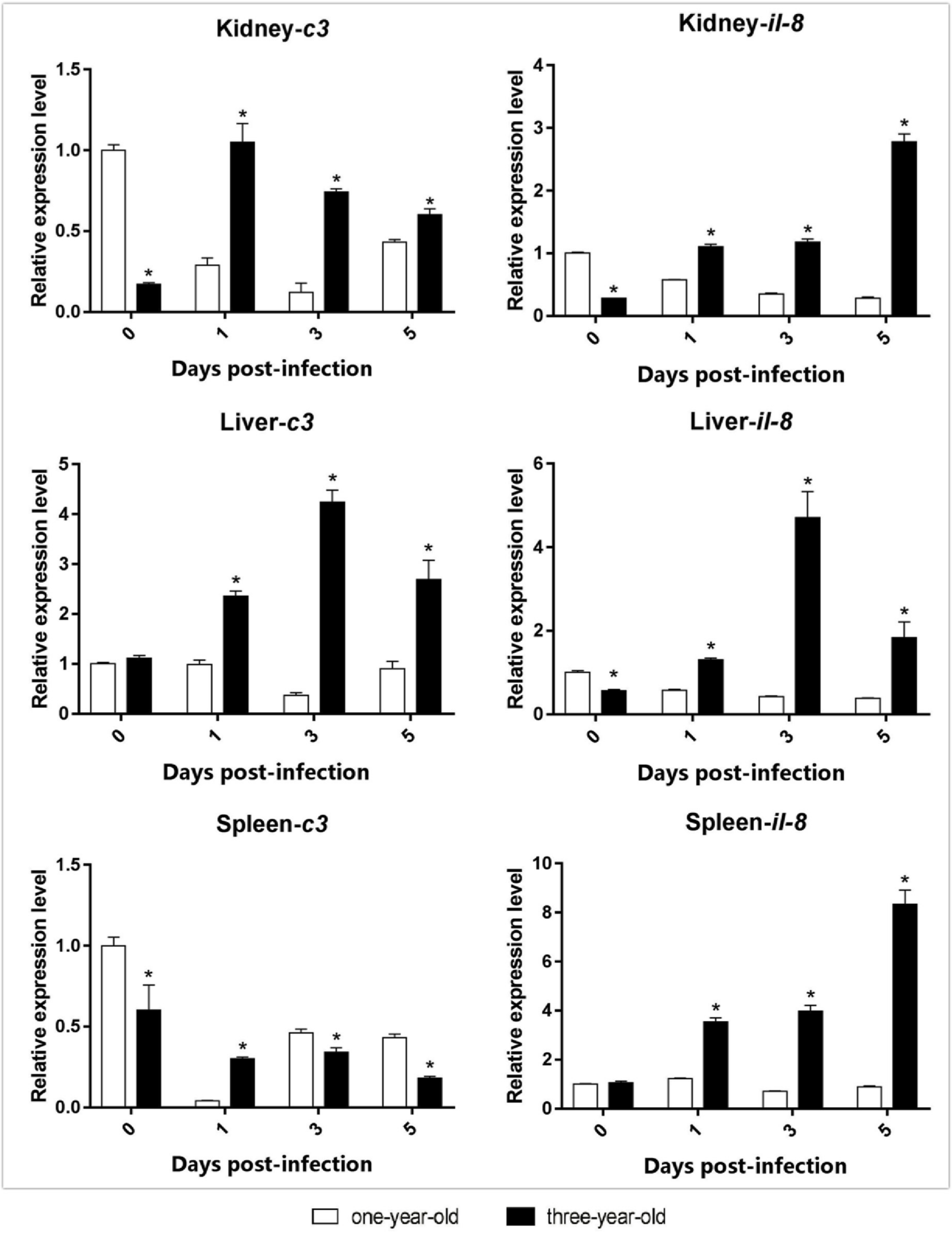

Fig. 4. qRT-PCR analysis of the expression levels of *c3* and *il-8*. The relative expression levels of the genes at 0 dpi in one-year-old grass carp was used as a reference for normalization. All data represent the mean ± standard deviation of three replicates (n = 3 for each biological duplicate). A significant difference ($P < 0.05$) in gene expression level between the two groups is indicated with an asterisk (*).

The expression level of *inf*-γ and *mx-1* were higher in liver, spleen, and kidney tissue of three-year-old grass carp than of one-year-old grass carp at 1 dpi (Fig. 5). This indicated that these genes are more responsive in three-year-old grass carp than one-year-old grass carp after infection. In summary, compared with one-year-old grass carp, the expression of *c3*, *il-8*, *inf*-γ and *mx-1* genes in three-year-old grass carp increased rapidly after GCRV infection.

3.5. Expression patterns of DNA methylation genes after GCRV infection

The expression levels of DNA methyltransferases *dnmt1* and *dnmt7* in liver, kidney, and spleen were examined to determine whether the

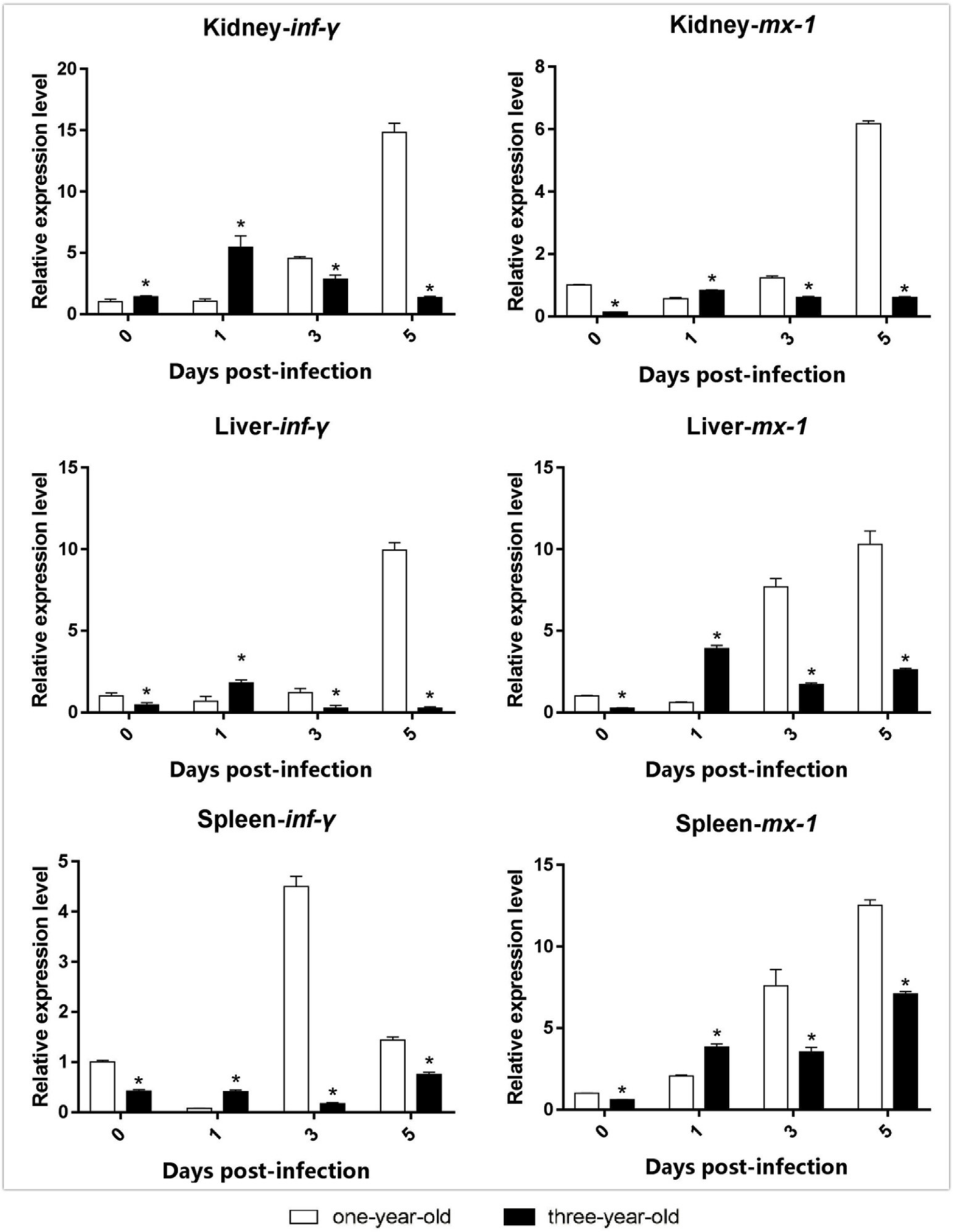

Fig. 5. qRT-PCR analysis of the expression levels of *inf*-γ and *mx-1*. The relative expression levels of the genes at 0 dpi in one-year-old grass carp was used as a reference for normalization. All data represent the mean ± standard deviation of three replicates (n = 3 for each biological duplicate). A significant difference ($P < 0.05$) in gene expression level between the two groups is indicated with an asterisk (*).

expression of the immune-related genes may be related to DNA methylation. The expression of *dnmt1* and *dnmt7* in liver and spleen tissue of three-year-old grass carp after infection was significantly lower than that of one-year-old grass carp (Fig. 6). On the other hand, the expression level of *dmnt1* and *dnmt7* in kidney tissue of three-year-old grass carp was higher than that of one-year-old carp in the first three days after infection, and was significantly lower than the one-year-old expression on 5 dpi.

The expression levels of the *tet3* and *gnmt* demethyltransferase genes were also examined. Expression levels of *tet3* and *gnmt* in liver and spleen after GCRV infection was significantly lower in three-year-old grass carp than in one-year-old grass carp (Fig. 7). The expression levels of *tet3* and *gnmt* in kidney were higher in three-year-old grass carp at 1 dpi than in one-year-old grass carp; expression levels were significantly lower in three-year-old grass carp that in one-year-old grass carp at 5 dpi.

4. Discussion

Reoviruses infection not only exhibits age restriction in grass carp but also in other species. Results of infection of reovirus in mice of different ages suggesting that maturation of type I interferon responses contributes to age-related deaths from reovirus infection [27]. Moreover, T cell responses in neonatal mice strongly favor the Th2 phenotype, which is less effective in clearing viral infections [28], and neonatal B cell responses develop more slowly and reach lower peak titers, resulting in antibodies with lower affinity than in adults [29]. Similarly to adaptive immune responses, innate immune responses also need to mature. For example, as age increases, the degree of microglia activation in the central nervous system and the production of pro-

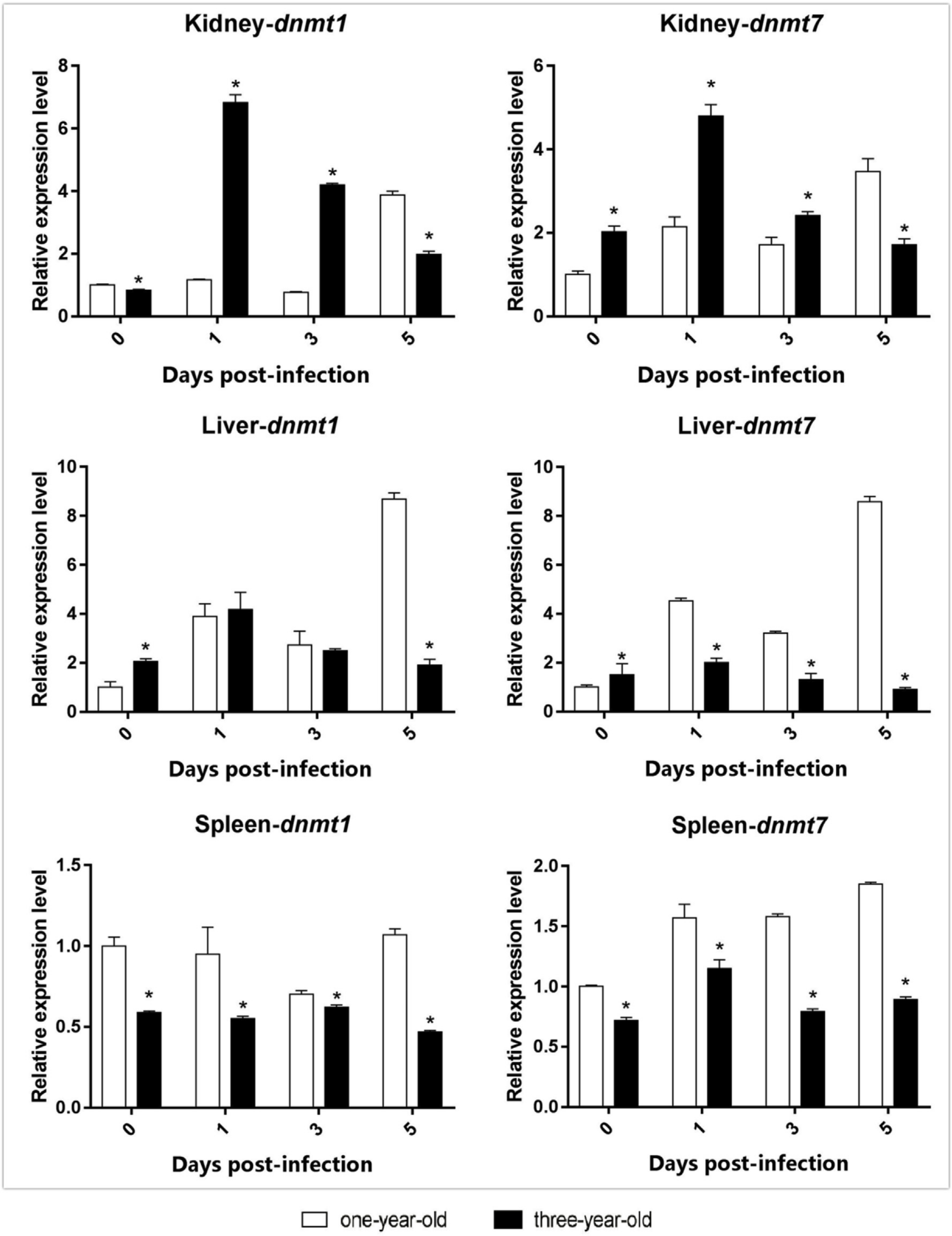

Fig. 6. The expression levels of *dnmt1* and *dnmt7*. The relative expression levels of the genes at 0 dpi in one-year-old grass carp was used as a reference for normalization. All data represent the mean ± standard deviation of three replicates. A significant difference ($P < 0.05$) in gene expression level between the two groups is indicated with an asterisk (*).

inflammatory cytokines is reduced [30], and expression of congenital immune sensor TLR3 is up-regulated in the intestine with increased age [12]. Natural antibodies are a well-known phenomenon in fish and have been shown to play an important role in their innate/acquired immune defence, and natural antibodies of rainbow trout and goldfish take part in both viral and bacterial defence [31,32]. Cod showed high natural antibody activity against haptenated proteins like TNP-BSA (bovine serum albumin), which increased with increasing age [33]. In addition, cell-surface expression of reovirus receptors may decrease with age, thus reducing reovirus binding to target cells [34,35]. These mechanisms may provide the basis for the age restriction seen in reovirus infection.

Differences in response to infection with GCRV in one-year-old and three-year-old grass carp confirmed the existence of GCRV age restriction in grass carp. One-year-old grass carp died on 9 dpi, while all three-year-old grass carp survived. Analysis of the relative copy number of

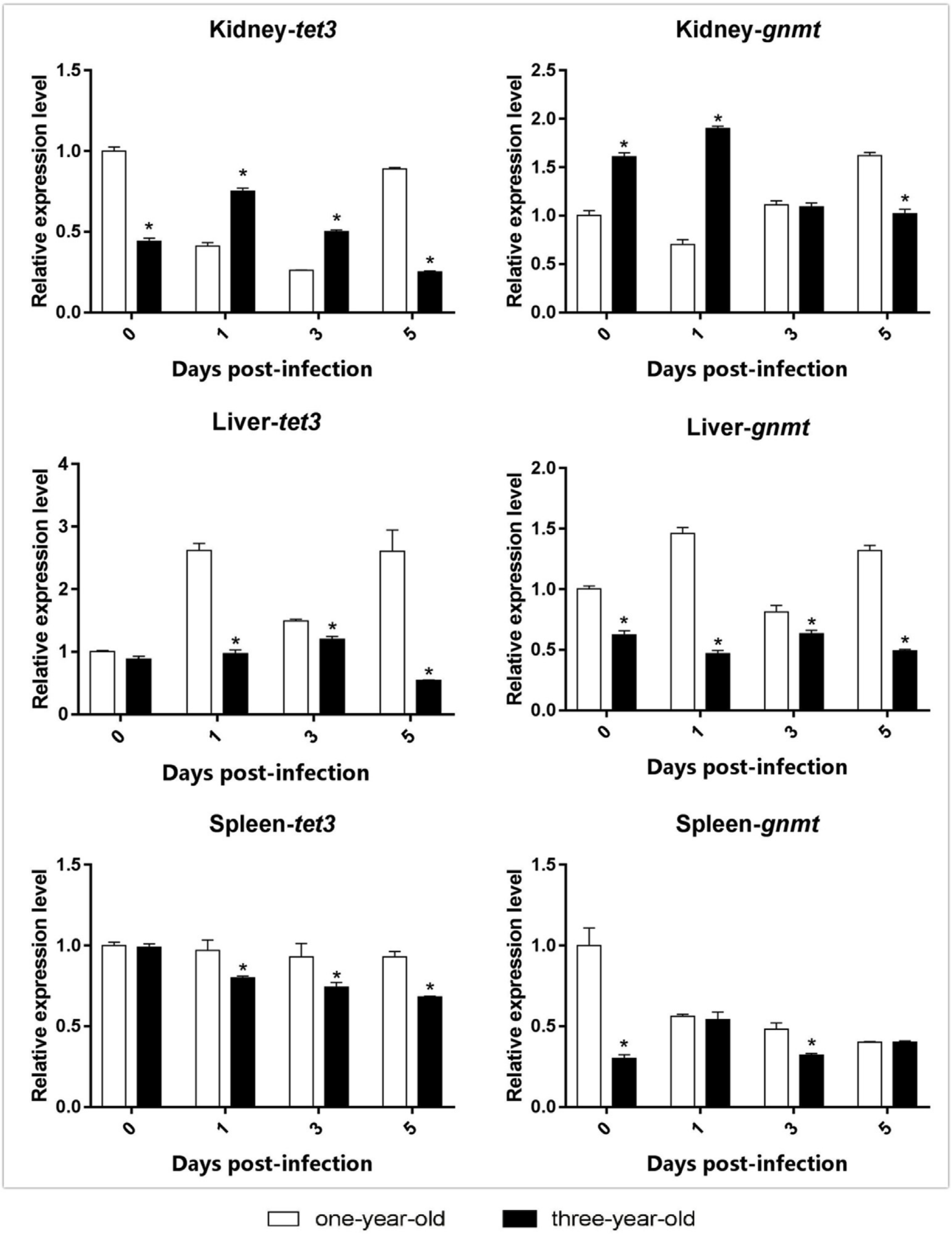

Fig. 7. qRT-PCR analysis of the expression levels of *tet3* and *gnmt* after GCRV infection. The relative expression levels of the genes at 0 dpi in one-year-old grass carp was used as a reference for normalization. All data represent the mean ± standard deviation of three replicates (n = 5 for each biological duplicate). A significant difference ($P < 0.05$) in gene expression level between the two groups is indicated with an asterisk (*).

GCRV showed that GCRV copy number in gill, intestine, liver, and spleen tissue in three-year-old grass carp was much lower than in one-year-old carp, and the virus was cleared in the first three days after infection. But the relative GCRV copy number in the kidney of three-year-old grass carp was higher than the one-year-old on 3 dpi, and the virus was not cleared until 5 dpi. As a critical organ in immune responses and blood circulation, the kidney may be the organ with the greatest virus accumulation. In fact, the symptoms caused by Hantavirus include kidney failure, hemorrhage, and shock [36]. And GCRV infection may trigger apoptosis in grass carp kidney cells [4]. Moreover, studies have found that the Hepatitis A virus may alter the immune surveillance of the host to provide a favorable kidney environment for the continued survival of the virus [37]. In summary, compared with the one-year-old grass carp, the three-year-old grass carp had lower GCRV copy number in most tissues, and quickly cleared the virus after infection.

Since the three-year-old grass carp can clear GCRV within three days after infection, we selected grass carp on 3 dpi for further research. Protein expression may be a more direct and accurate indication of response to GCRV infection than RNA expression. Therefore, we evaluated blood proteins from the one-year-old and three-year-old grass carp on 3 dpi by mass spectrometry. We identified differentially expressed proteins between the one-year-old and three-year-old grass carp; these proteins were enriched in complement and coagulation functions, anti-oxidative damage, and interleukin signaling. In previous studies, it was found that most of the genes involved in the complement and coagulation cascade pathway were significantly up-regulated after GCRV infection [38]. To explore whether similar expression patterns exist in the three tissues of liver, kidney, and spleen, we evaluated *c3* and *il-8* expressions level by qPCR. In addition, *inf*-γ and *mx-1* were selected to further understand the differences in the immune system between the one-year grass carp and the three-year-old grass carp after infection with GCRV. C3 plays a vital role in the activation of the complement system [39], and the complement system links the innate and adaptive immune responses through a variety of mechanisms, including enhanced humoral immunity, regulation of antibody effector mechanisms, and T cell functions [40]. IL-8 belongs to the CXC chemokine family [41], which induces chemotaxis of target cells, mainly neutrophils, and other granulocytes, causing them to migrate to sites of infection, and IL-8 will also induce phagocytosis [42]. INF-γ is the type II interferon member that has antiviral, immunomodulatory, and antitumor properties [43]. INF-γ is essential for innate and adaptive immunity against viral infections [44]. In mammals, there is sufficient evidence that antiviral activity is mediated by several IFN-inducible proteins, of which Mx is one of the most studied proteins [45]. Mx1 has also been shown to inhibit double-stranded RNA viruses in fish [46]. The expression levels of *c3* and *il-8* in the liver and kidney tissues of the three-year-old grass carp were higher than those in the one-year-old carp, while the expression of *inf*-γ and *mx-1* in the three tissues was higher in three-year-old carp than in one-year-old carp on 1 dpi. The similarity expression patterns between the protein data in the blood and the expression levels of the same genes in the liver, spleen and kidney further confirms the accuracy of the data. These results indicate that three-year-old grass carp may be able to rapidly clear the virus due to high expression of immune-related genes. And compared to *c3* and *il-8*, *inf*-γ and *mx1*-mediated antiviral defense mechanisms seems respond during the early stages of the GCRV infection. Moreover, studies have found that cytomegalovirus (CMV) appears to promote a robust immune response in infected aged mice compared to older mice that do not carry CMV [47]. Therefore, we suspected that during growth and maturity, increased age and exposure to more pathogens bolsters development of a more responsive immune system in three-year-old grass carp than in one-year-old grass carp, leading to rapid identification and elimination of GCRV infection.

The expression levels of enzymes involved in DNA methylation were examined to infer the global DNA methylation level after infection between the two groups. DNA methyltransferases (DNMTs) are essential enzymes involved in DNA methylation [48]. In contrast to DNMTs, TETs and GNMT are engaged in demethylation [49,50]. We found that the expression levels of enzymes that promote methylation, such as *dnmt1* and *dnmt7*, after GCRV infection in the liver and spleen of one-year-old grass carp were significantly higher than in the three-year-old carp. Therefore, we hypothesize that changes in the expression level of methyltransferases in grass carp after GCRV infection may be positively correlated with viral copy number. In the liver and spleen, the relative copy number of GCRV in one-year-old grass carp was significantly higher than in three-year-old carp. Higher GCRV copy number may be responsible for the increase in expression of DNA methyltransferases in one-year-old grass carp compared to three-year-old carp, and high DNA methylation levels may attenuate the expression of immune-related genes and other genes involved in antiviral response. In previous studies, it was found that the DNA methylation levels negatively correlated with gene expression levels [51]. It was also found that the methylation status of RIG-1 in grass carp was extremely highly correlated with resistance to GCRV, and may serve as a negative regulator of transcription of RIG-1 [24]. Notably, the expression levels of *dnmt1* and *dnmt7* in the kidney of three-year-old grass carp were higher than that of one-year-old grass carp, which may be related to higher viral copy number. At the same time-points, the expression levels of *tet3* and *gnmt* in three-year-old grass carp kidney tissue were higher than in one-year-old carp kidney. It is possible that the expression of immune-related genes in the kidneys of three-year-old grass carp is not affected by the synergistic action of DNA methylation enzymes. However, DNA methylation does not often cause side effects on viral hosts. In a study of HBV infection, the expression of host DNA methyltransferase gene (DNMT) was significantly up-regulated after viral infection, and at the same time, DNMT methylated viral DNA leads to decreased viral gene expression and reduced viral replication [52]. In a study of West Nile virus (WNV), it was found that the 2′-O methylation of the 5′ cap of viral RNA can mitigate the suppression of interferon responses against the virus, and differential methylation of cytoplasmic RNA may be used for pattern recognition and for limiting the proliferation of foreign viral RNA in host cells [53]. Therefore, the relationship between viruses, hosts, and DNA methylation needs further investigation. Overall, the three-year-old grass carp can survive after infection with GCRV, and the development of the immune system, especially the complement system, may play an important role, and may be related to levels of DNA methylation in response to viral copy number. In contrast, DNA methylation induced by GCRV infection may further inhibit the expression of antiviral-related genes, as evidenced by the increase in DNMT expression in one-year-old grass carp and their lack of ability to quickly clear viral infections.

In conclusion, our study investigated the age restriction of GCRV infection in grass carp; we focus in particular on the development of the immune system and epigenetic factors that may contribute to GCRV infection. We demonstrate that as age increases, the response of the immune system, especially the complement system, is more efficient and faster after GCRV infection, enabling the three-year-old grass carp to be more resistant to GCRV infection and more able to rapidly eliminate the viral infection. After infection with GCRV, the level of DNA methylation genes in the liver and spleen of the one-year-old grass carp was significantly higher than that of the three-year-old grass carp, which may lead to attenuation of expression of immune-related genes. These findings broaden our understanding of the age restriction of GCRV infection and may help to develop strategies to combat diseases caused by GCRV.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2018.11.074.

Abbreviations

GCRV	grass carp reovirus
TLR3	toll-like receptor 3
Dpi	days post-infection
C3	complement component 3
IL8	Interleukin 8
INF-γ	Interferon gamma
Mx1	Interferon-induced GTP-binding protein Mx1
DNMT	DNA methyltransferase
GNMT	glycine *N-methyltransferase*
TETs	ten-eleven translocation proteins

References

[1] Food and Agriculture Organization of the United Nations, Fishery and Aquaculture Statistics Yearbook, Food and Agriculture Organization of the United Nations, Rome, Italy, 2017.

[2] Y. Wang, Y. Lu, Y. Zhang, Z. Ning, Y. Li, Q. Zhao, H. Lu, R. Huang, X. Xia, Q. Feng, X. Liang, K. Liu, L. Zhang, et al., The draft genome of the grass carp (*Ctenopharyngodon idellus*) provides genomic insights into its evolution and vegetarian diet adaptation, Nat. Genet. 47 (2015) 625–631.

[3] H. Attoui, P.P. Mertens, J. Becnel, S. Belaganahalli, M. Bergoin, C.P. Brussaard, J.D. Chappell, M. Ciarlet, M. del Vas, T.S. Dermody, P.R. Dormitzer, R. Duncan, Q. Fang, et al., Family Reoviridae, in: A.M.Q. King, M.J. Adams, E.B. Carstens, E.J. Lefkowitz (Eds.), Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses, Elsevier, San Diego, CA, 2012, pp. 538–637.

[4] G. Chen, L. He, L. Luo, R. Huang, L. Liao, Y. Li, Z. Zhu, Y. Wang, Transcriptomics sequencing provides insights into understanding the mechanism of grass carp reovirus infection, Int. J. Mol. Sci. 19 (2018) 488.

[5] M. Dorson, C. Touchy, The influence of fish age and water temperature on mortalities of rainbow trout, Salmo gairdneri Richardson, caused by a European strain of infectious pancreatic necrosis virus, J. Fish. Dis. 4 (3) (1981) 213–221.

[6] Eveline J. Emmenegger, et al., Experimental infection of six North American fish species with the North Carolina strain of spring viremia of carp virus, Aquaculture 450 (2016) 273–282.

[7] M. Tardieu, M.L. Powers, H.L. Weiner, Age-dependent susceptibility to reovirus type 3 encephalitis: role of viral and host factors, Ann. Neurol. 13 (1983) 602–607.

[8] Markus Czub, et al., Age-dependent resistance to murine retrovirus-induced spongiform neurodegeneration results from central nervous system-specific restriction of virus replication, J. Virol. 65 (5) (1991) 2539–2544.

[9] Paul J. Cote, et al., Effects of age and viral determinants on chronicity as an outcome of experimental woodchuck hepatitis virus infection, Hepatology 31 (1) (2000) 190–200.

[10] M. Ramsay, D. Brown, Epidemiology of group A rotaviruses surveillance and burden of disease studies, Rotaviruses, Humana Press, 2000, pp. 217–238 2000.

[11] Wanda M. Wenman, et al., Rota virus infection in adults, N. Engl. J. Med. 301 (6) (1979) 303–306.

[12] Johanna Pott, et al., Age-dependent TLR3 expression of the intestinal epithelium contributes to rotavirus susceptibility, PLoS Pathog. 8 (5) (2012) e1002670.

[13] Lena Alexopoulou, et al., Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3, Nature 413 (2001) 6857 732.

[14] Taro Kawai, Shizuo Akira, Signaling to NF-κB by toll-like receptors, Trends Mol. Med. 13 (11) (2007) 460–469.

[15] Y. Rao, et al., Characterizations of two grass carp *Ctenopharyngodon idella* hmgb2 genes and potential roles in innate immunity, Dev. Comp. Immunol. 41 (2) (2013) 164–177.

[16] Y.Y. Pei, et al., Characterizations of four toll-like receptor 4s in grass carp *Ctenopharyngodon idellus* and their response to grass carp reovirus infection and lipopolysaccharide stimulation, J. Fish. Biol. 86 (3) (2015) 1098–1108.

[17] J. Su, et al., SNP detection of TLR8 gene, association study with susceptibility/resistance to GCRV and regulation on mRNA expression in grass carp, *Ctenopharyngodon idella*, Fish Shellfish Immunol. 43 (1) (2015) 1–12.

[18] Quanyuan Wan, et al., Genomic sequence comparison, promoter activity, SNP detection of RIG-I gene and association with resistance/susceptibility to grass carp reovirus in grass carp (*Ctenopharyngodon idella*), Dev. Comp. Immunol. 39 (4) (2013) 333–342.

[19] Chen Jin, et al., Transcriptome analysis of head kidney in grass carp and discovery of immune-related genes, BMC Vet. Res. 8 (1) (2012) 108.

[20] Yunfei Dang, et al., Transcriptome analysis of the innate immunity-related complement system in spleen tissue of *Ctenopharyngodon idella* infected with *Aeromonas hydrophila*, PloS One 11 (7) (2016) e0157413.

[21] B.H. Xu, et al., Characterization of grass carp spleen transcriptome during GCRV infection, Genet. Mol. Res. 15 (2016) 2.

[22] Quanyuan Wan, J. Su, Transcriptome analysis provides insights into the regulatory function of alternative splicing in antiviral immunity in grass carp (*Ctenopharyngodon idella*), Sci. Rep. 5 (2015) 12946.

[23] Mijuan Shi, et al., RNA-seq profiles from grass carp tissues after reovirus (GCRV) infection based on singular and modular enrichment analyses, Mol. Immunol. 61 (1) (2014) 44–53.

[24] X. Shang, et al., DNA methylation of CiRIG-I gene notably relates to the resistance against GCRV and negatively-regulates mRNA expression in grass carp, *Ctenopharyngodon idella*, Immunobiology 221 (1) (2016) 23–30.

[25] X. Shang, et al., The destiny of the resistance/susceptibility against GCRV is controlled by epigenetic mechanisms in CIK cells, Sci. Rep. 7 (1) (2017) 4551.

[26] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2 − ΔΔCt Method, Methods 25 (2001) 402–408.

[27] Allen G. WU, et al., Age-dependent susceptibility to reovirus encephalitis in mice is influenced by the maturation of the type-I interferon response, Pediatr. Res. 83 (5) (2018) 1057.

[28] Annie M. GARCIA, et al., T cell immunity in neonates, Immunol. Res. 22 (2–3) (2000) 177–190.

[29] M.A. Aranda, A. Fraile, F. Garcia-Arenal, Genetic variability and evolution of the satellite RNA of cucumber mosaic virus during natural epidemics, J. Virol. 67 (10) (1993) 5896–5901.

[30] Peter Ferrazzano, et al., Age-dependent microglial activation in immature brains after hypoxia-ischemia, CNS Neurol. Disord. - Drug Targets 12 (3) (2013) 338–349.

[31] R. Gonzalez, P. Matsiota, C. Torchy, et al., Natural anti-TNP antibodies from rainbow trout interfere with viral infection in vitro, Res. Immunol. 140 (7) (1989) 675–684.

[32] M.S. Sinyakov, M. Dror, H.M. Zhevelev, et al., Natural antibodies and their significance in active immunization and protection against a defined pathogen in fish, Vaccine 20 (31–32) (2002) 3668–3674.

[33] B. Magnadóttir, H. Jónsdóttir, S. Helgason, et al., Humoral immune parameters in Atlantic cod (Gadus morhua L.): II. The effects of size and gender under different environmental conditions, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 122 (2) (1999) 181–188.

[34] C. Tessitore, P.C. Brunjes, A comparative study of myelination in precocial and altricial murid rodents, Brain Res. 471 (1) (1988) 139–147.

[35] M. Tardieu, M.L. Powers, H.L. Weiner, Age-dependent susceptibility to reovirus type 3 encephalitis: role of viral and host factors, Ann. Neurol. 13 (1983) 602–607.

[36] Joachim J. BUGERT, et al., Hantavirus infection—haemorrhagic fever in the Balkans—potential nephrological hazards in the Kosovo war, Nephrol. Dial. Transplant. 14 (8) (1999) 1843–1844.

[37] Xumin OU, et al., Viral-host interaction in kidney reveals strategies to escape host immunity and persistently shed virus to the urine, Oncotarget 8 (5) (2017) 7336.

[38] Libo HE, et al., Differences in responses of grass carp to different types of grass carp reovirus(GCRV) and the mechanism of hemorrhage revealed by transcriptome sequencing, BMC Genomics 18 (2017) 452.

[39] Arvind SAHU, John D. LAMBRIS, Structure and biology of complement protein C3, a connecting link between innate and acquired immunity, Immunol. Rev. 180 (1) (2001) 35–48.

[40] Michael C. CARROLL, The complement system in regulation of adaptive immunity, Nat. Immunol. 5 (10) (2004) 981.

[41] W.S. Modi, M. Dean, H.N. Seuanez, N. Mukaida, K. Matsushima, S.J. O'Brien, Monocyte-derived neutrophil chemotactic factor (MDNCF/IL-8) resides in a gene cluster along with several other members of the platelet factor 4 gene superfamily, Hum. Genet. 84 (2) (1990) 185–187, https://doi.org/10.1007/BF00208938.

[42] Akihisa HARADA, et al., Essential involvement of interleukin-8 (IL-8) in acute inflammation, J. Leukoc. Biol. 56 (5) (1994) 559–564.

[43] Patrick W. GRAY, David V. GOEDDEL, Structure of the human immune interferon gene, Nature 298 (1982) 859, https://doi.org/10.1038/298859a0 5877.

[44] K. Schroder, P.J. Hertzog, T. Ravasi, D.A. Hume, Interferon-gamma: an overview of signals, mechanisms and functions, J. Leukoc. Biol. 75 (2) (2004) 163–189, https://doi.org/10.1189/jlb.0603252.

[45] S. Goodbourn, L. Didcock, R.E. Randall, Interferons: cell signalling, immune modulation, antiviral response and virus countermeasures, J. Gen. Virol. 81 (2000) 2341–2364.

[46] Rannveig Larsen, Torunn P. Røkenes, Børre Robertsen, Inhibition of infectious pancreatic necrosis virus replication by Atlantic salmon Mx1 protein, J. Virol. 78 (15) (2004) 7938–7944.

[47] Megan J. Smithey, Vanessa Venturi, Miles P. Davenport, et al., Lifelong CMV infection improves immune defense in old mice by broadening the mobilized TCR repertoire against third-party infection, Proc. Natl. Acad. Sci. Unit. States Am. (2018), https://doi.org/10.1073/pnas.1719451115.

[48] A. Hermann, H. Gowher, A. Jeltsch, Biochemistry and biology of mammalian DNA methyltransferases, Cell. Mol. Life Sci. 61 (2004) 2571–2587.

[49] Shinsuke ITO, et al., Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification, Nature 466 (7310) (2010) 1129.

[50] Y. Takata, Y. Huang, J. Komoto, T. Yamada, K. Konishi, H. Ogawa, T. Gomi, M. Fujioka, F. Takusagawa, Catalytic mechanism of glycine N-methyltransferase, Biochemistry 42 (2003) 8394–8402.

[51] L. Xiong, L. He, L. Luo, Y. Li, L. Liao, R. Huang, Z. Zhu, Y. Wang, Global and complement gene-specific DNA methylation in grass carp after grass carp reovirus (GCRV) infection, Int. J. Mol. Sci. 19 (2018) 1110.

[52] P. Vivekanandan, et al., Hepatitis B virus replication induces methylation of both host and viral DNA, J. Virol. 84 (9) (2010) 4321.

[53] S. Daffis, K.J. Szretter, J. Schriewer, J. Li, S. Youn, J. Errett, et al., 2′-o methylation of the viral mRNA cap evades host restriction by ifit family members, Nature 468 (7322) (2010) 452–456.