Study on the immune enhancers against *Micropterus salmoides* rhabdovirus infection

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ABSTRACT

Micropterus salmoides rhabdovirus (MSRV) is one of the most serious pathogens harming *M. salmoides* juvenile, which had brought huge economic losses to farming industry. Studies involving candidate genes to the clinical diseases, however, are limited. In this study, the viral target and clinical manifestation of MSRV on *M. salmoides* juvenile were analyzed, and the protective effects of a single immune enhancer and a compound immune enhancer were evaluated. The results showed that the brain, liver, intestine and muscle of *M. salmoides* showed obvious lesions after infection with MSRV. The relative expression levels of nucleoprotein (*N*) and matrix protein (*M*) genes showed a trend of increasing at first and then decreasing and reached the peak in each tissue at 36 h post-infection. The mortality rate of *M. salmoides* was over 90% after 7 days of MSRV infection. The immune enhancers containing free nucleotides and *Astragalus polysaccharide* added to the diet effectively inhibited the replication of *N* and *M* genes in *M. salmoides* and increased the survival rate by 25% to 28%. This study provided basic data and theoretical reference for the analysis of the pathological mechanism and prevention and treatment of MSRV.

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Key words: *Micropterus salmoides* rhabdovirus; pathological feature; nucleoprotein; matrix protein; immune enhancer.

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INTRODUCTION

Largemouth bass (*Micropterus salmoides*) is one of the important aquaculture species in China. The domestic production of largemouth bass occupied 99.8% of the total production in the world (Hussein *et al*., 2020). In 2021, the total breeding output of largemouth bass in China was about 702,000 tons, among which the largest production area was Guangdong in South China, where the total breeding output accounted for 52.5%, and the total breeding output of Zhejiang and Jiangsu in east China accounted for 23% (2022 China Fishery Statistical Yearbook). With the continuous expansion of breeding scale, the degradation of germplasm resources and high-density breeding had led to frequent diseases, which had been seriously affected the healthy development of largemouth bass industry. The pathogenic microorganisms causing largemouth bass diseases mainly included ngi, bacteria, parasites and viruses, among which viral diseases were the most serious. Viral diseases were highly infectious and destructive, with varying latency, rapid onset and high mortality (Lang and Qiya, 2019). The main pathogens of viral diseases in largemouth bass were iridovirus and rhabdovirus.

Rhabdovirus disease results from *Micropterus salmoides* rhabdovirus (MSRV) had caused high mortality rate of largemouth bass seedlings and led to huge economic losses to aquatic seed enterprises and aquaculturists. MSRV mainly harms the seedlings of largemouth bass, which is characterized by rapid transmission and high lethality. The virus infects seedlings by horizontal and vertical transmission using water environment as the vector (Yan, 2015). After MSRV infection, the sick fish mostly showed obvious phenomena such as spiral swimming, black body color, rotten body, rotten fins, loss of

appetite, roaming or outliers. In 1995, researchers isolated rhabdovirus from largemouth bass, identified the typical bullet-like structure containing five kinds of peptides, and found it was more than 95% similar to perch rhabdovirus isolated from perch *Perca fluviatilis* (Betts *et al.*, 2003). The outbreak of rhabdoviruses caused the death of nearly 20,000 juvenile largemouth bass (2.5-4.5 cm) with a mortality rate of about 40% in Zhongshan, Guangdong Province, China in 2011 (Ma *et al*., 2013). A large number of deaths of largemouth bass occurred in Foshan, Guangdong Province in 2014, with a fatality rate of about 90%, which the pathogen was confirmed to be MSRV (Lei *et al*., 2015). Fu *et al.* (2017) isolated the wild type of MSRV-SS from the fish body of infected largemouth bass and obtained the clone MSRV-SS-7 after purification. It was found that immunization with MRRV-SS and MSRV-SS-7 could prevent *Siniperca chuatsi* rhabdovirus (SCRV) infection in *S. chuatsi* (Zhang *et al.*, 2018). Lyu *et al*. (2019) isolated a new virus strain MSRV-YH01 in Yuhang, Zhejiang Province, with the mortality rate of about 90%.

MSRV belongs to the Rhabdoviridae family and the genus *Vesiculovirus*. It was encoded by five major structural proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and RNA polymerase (L). Although whole genome sequencing of MSRV had been completed, in-depth studies of the *N* or *M* gene are lacking. Nucleoprotein (N), the first protein expressed by rhabdovirus, is an important component of the nucleocapsid structure of rhabdovirus and plays an important role in the replication and transcription of genomic RNA. Up to now, no effective drug or vaccine had been developed against MSRV, and reports on the pathogenic mechanism of MSRV were less (Lu *et al*., 2023; Luo *et al*., 2024).

Fish have both non-specific and specific immune systems, and non-specific immunity is the basic immune mechanism at early stages. Enhancing the immune system was considered to be the most effective way to prevent fish diseases, and dietary immune enhancers had been continuously used to improve the health of fish (Kumar *et al*., 2022). Fish immune enhancers were divided into the following categories: animal and plant extracts, polysaccharides, nutritional elements, chemical compounds, hormones and other cytokines (Yutang, 2017). They could stimulate the body to produce immune responses, improve phagocytic activity, respiratory burst activity, complement activity, total protein and anti-protease activity, lysozyme activity and natural killer cell activity, enhance bactericidal effect, and prevent disease occurrence (Awad and Awaad, 2017; Sakai, 1999). Studies had found that dietary fiber (Lin *et al*., 2020), astragalus polysaccharide, chitooligosaccharides (Lin *et al*., 2017), starch (Lin *et al*., 2018), compound Chinese herbal medicine (*Scutellaria baicalensis*, Phelloberia, rhubarb, Daotaiye) (Bin *et al*., 2019), vitamin E (Li *et al*., 2018), vitamin C (Yusuf *et al*., 2020), antimicrobial peptides (Li *et al*., 2020) and *Bacillus subtilis* (Zhang *et al*., 2022) added to the diet of largemouth bass could enhance the specific or non-specific immunity of largemouth bass and promote the immune effect.

The aim of this study was to understand the pathological mechanism of MSRV and evaluate the protective effect of a single and a combined immune enhancer. This study could provide basic data and theoretical reference for further research on the pathogenic mechanism of MSRV, vaccine development, immune prevention and disease resistance breeding.

METHODS

Viruses and immune enhancers

MSRV was provided by Zhejiang Freshwater Fisheries Research Institute (China) and stored in our laboratory before use. Detailed strain information, references, genome information were shown in Lyu *et al*. (2019). The prepared virus suspension was centrifuged at 8000 rpm for 30 min at 4°C to remove the supernatant, filtered through a 0.45 μm filter membrane and stored at 4°C until use. Absolute fluorescence quantification was used to measure the viral load of the virus suspension. The component of single immune enhancer was free nucleotide. The compound immune enhancer was composed of 10~20% free nucleotide, 10~20% astragalus polysaccharide, 30~40% forsythus polysaccharide, 5~20% Daqing leaf polysaccharide and 5~20% vitamin C mixed in a certain proportion. This compound immune enhancer was under patent pending. Free nucleotide (>65%) was purchased from Nanjing Tongkai Biotechnology Co., LTD. Astragalus polysaccharide (>85%), forsythia polysaccharide (>85%), Daqing leaf polysaccharide (>85%), and vitamin C (>98%) purchased from Xi 'an Grass Plant Technology Co., LTD. The immune enhancer was first pre-mixed according to the formula proportion, mixed into the feed according to 5% proportion, and pressed into pellets.

Fish source and breeding management

Healthy largemouth bass juvenile (1.82±0.05 cm) were purchased from Huzhou Huwang Aquaculture Seed Industry Co., LTD. (Huzhou, China), and cultured in the laboratory of Huzhou University (Zhejiang, China). The fish were kept in 120 L tanks under natural light. The water temperature was maintained at about 28°C, the dissolved oxygen was more than 6 mg/L, and the pH was 7.5-8.5. The fish were fed at 8:00 and 17:00 every day, and the daily feeding amount was 4%-5% of body weight.

MSRV infection experiment

Largemouth bass were held for one week before the experiment, and feeding was suspended the day before the experiment. Largemouth bass juvenile were randomly divided into two groups set as the blank control group and the challenge group. Fish were immersed in virus suspension (2.62×10^4 copies/mL) for 30 min and then transferred to 120 L culture drums, while the control group was immersed in PBS solution. The concentration of the virus suspension is a semi-lethal concentration calculated by pre-experiment. The water temperature was maintained at 22 to 24°C, and appropriate feeding was allowed. The clinical manifestations and mortality of juveniles were observed and recorded during the infection experiment. Largemouth bass were euthanized at different time points after infection (0 h, 6 h, 12 h, 24 h, 36 h, 48 h, 72 h, 96 h, 120 h, 144 h and 168 h), and the brain, viscera, muscle and caudal fin tissues (n=15) were dissected and removed, frozen in liquid nitrogen, and then transferred to -80°C for storage. The dissection procedure was performed with as little pain to the fish as possible and in accordance with the regulations governing laboratory animals.

Histopathological observation

The brain, liver, intestine and muscle of largemouth bass with obvious symptoms of MSRV infection were collected and fixed in Bouin's fixative for 24 h, then embedded in paraffin and sectioned (4-6 μm). After H&E staining, the specimens were observed under a microscope (Olympus- CKX31, Tokyo, Japan) (100×).

Evaluation of the protective effect of immune enhancers

Healthy largemouth bass juveniles were randomly divided into four groups (750 fish per group): group A (blank control group), group B (challenge group), group C (single immune enhancer + challenge group) and group D (compound immune enhancer + challenge group). Groups A and B were fed with normal diet. Fish in C and D groups were fed with the mixture of immune enhancer (0.4% of body weight) for 5 d at 8: 00 and 17:00.

Feeding was suspended 24 h before artificial infection, and largemouth bass was immersed in virus suspension (2.62×10^4 copies/mL) for 30 min and then transferred to 120 L culture drums. The control group was immersed in PBS solution. During this period, the water temperature was maintained at 22 to 24°C, and appropriate feeding was allowed. The clinical manifestations of juveniles were observed during the experiment, and daily mortality was recorded. Fish were euthanized at different time points (0, 6, 12, 24, 36, 48, 72, 96, 120, 144 and 168 h) after infection, and brain, intestine, muscle and caudal fin (n=15) were dissected and removed, frozen in liquid nitrogen, and then transferred to -80°C for storage. The dissection procedure strictly followed the ethical protection norms of animals.

Transcript level analysis of *N* and *M* genes in different tissues

Tissue samples (brain, intestine, muscle and caudal fin) were collected at different time points after MSRV infection and the protective effect of immune enhancers was evaluated. Trizol reagent was used to extract RNA, and NanoDrop 2000 was used to determine the concentration. The RNA was reverse transcribed into cDNA with PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara Bio, Kusatsu, Japan) and stored in the -20°C refrigerator.

The *β-actin* was used as internal reference, and the specific primers (Tab. 1) were used to analyze the *N* and *M* genes by real-time PCR (SYBR Premix ExTaq, Takara Bio; Mx3005P, Stratagene, Agilent, Santa Clara, CA, USA), and each sample was analyzed three times. The 20 μL reaction system consisted of 10 μL SYBR Green Master Mix, 0.2 μL ROX, 1 μL qMSRV-F, 1 μL qMSRV-R, 1 μL cDNA, and 6.8 μL RNA-Free Water. The PCR reaction program was predenaturation at 95°C for 10 min. 40 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. The melting curve program was 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s. After the procedure was completed, the threshold cycle (Ct) value of each sample was taken. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Excel 2017 software was used for data processing, and the data was expressed as mean ± SD. SPSS 23.0 software was used for analysis. Significance was analyzed according to the Ducan comparison method in One-way ANOVA (*p<0.05, **p<0.01). Origin software was used for making figures.

RESULTS

Clinical manifestation of MSRV infection

Largemouth bass were immersed in virus suspension for infection, and monitored continuously for 14 d after MSRV infection to observe the clinical manifestations. Obvious symptoms began to appear 24 h after MSRV infection. A large number of fish died within 36 h to 48 h, with a fatality rate of about 50%

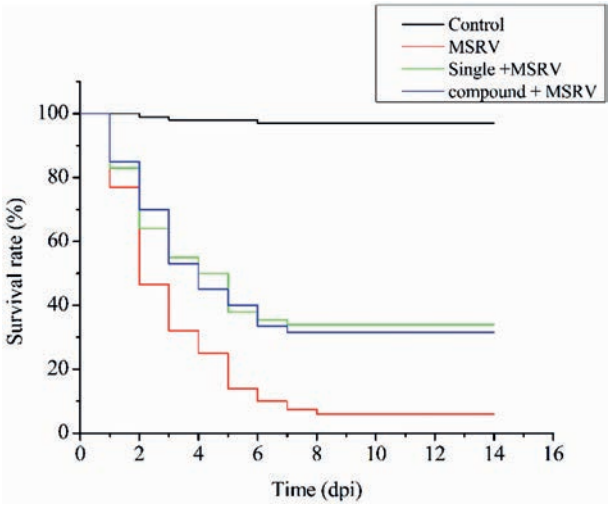

Fig. 1. Survival rate of *M. salmoides* after MSRV infection.

Tab. 1. qPCR primers used for amplification.

Primers	Primers sequence (5′→3′)	Temperature (°C)	Primers ID	Products
qMSRV-N	F: AGTGCTGGATTGGGTGTTCA R: CTGGATTCTTTGTGGCAGAGTAA	58.3 59.1	MK397811.2	155 bp
qMSRV-M	F: CCCCTGGACTGGGTTTGT R: TCGGACCTCTGCTTCTGCTA	57.3 58.4	MK397811.2	201 bp
qMS-β-actin	F: TACCCTGGAATCGCTGACC R: CATCGTACTCCTGCTTGCTGA	58.0 58.8	MH018565.1	175 bp

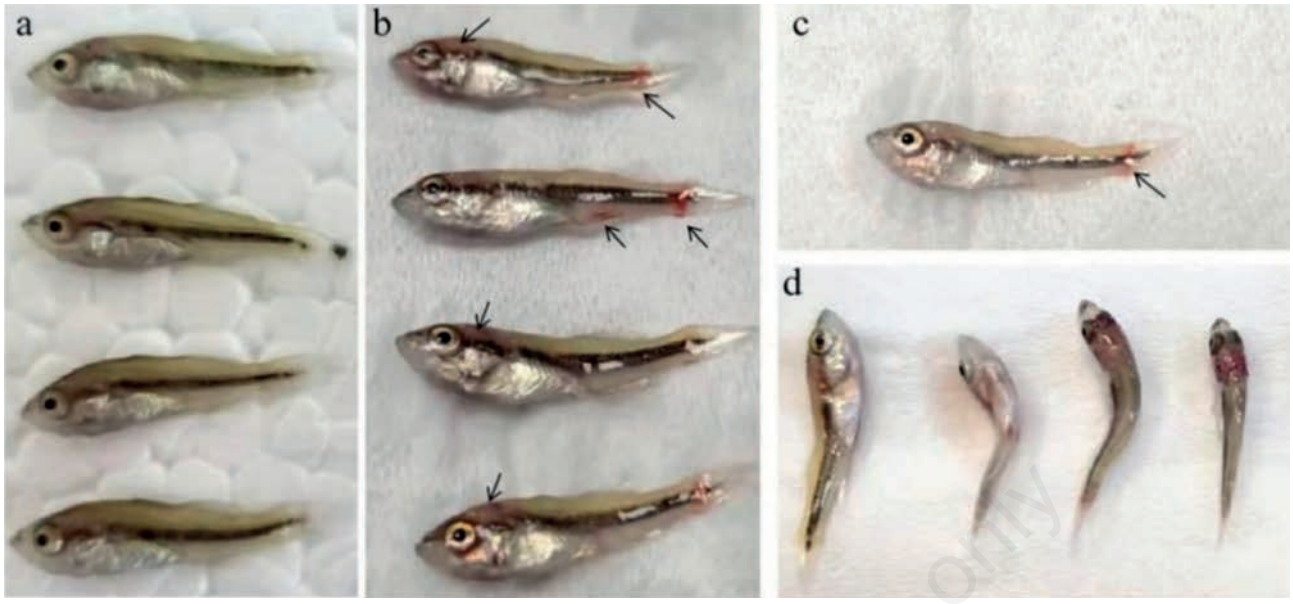

Fig. 2. Clinical signs of *M. salmoides* infected with MSRV. a) Healthy *M. salmoides*. b,c) Tissue hemorrhage. D) Crooked body.

and obvious symptoms. The mortality exceeded 90% by day 7 (Fig. 1). After MSRV infection, the diseased largemouth bass showed clinical manifestations such as black body color, spiral swimming, obvious bleeding symptoms in the head and tail tissues (Fig. 2 b,c), and bending of the fish body (Fig. 2d).

The histopathological analysis of brain tissue showed that the number of cells in granular layer decreased and the gap increased (Fig. 3 a,b). In the treated liver sections, edematous hepatocytes, loose cytoplasm, dilated sinusoids, and fat vacuoles were found (Fig. 3 c,d). The treated intestinal tissue structure was damaged, the cells were arranged disorderly, the intestinal villi were shed, some villous epithelial cells in the mucosal layer were necrotic, and the nuclei were fragmented and disappeared (Fig. 3 e,f). In the muscles of fish infected with the MSRV, it was found enlarged gaps, necrotic muscle fibers, hyperplastic fibrous tissue, significantly smaller peripheral muscle fibers, and a small number of infiltrating inflammatory cells (Fig. 3 g,h).

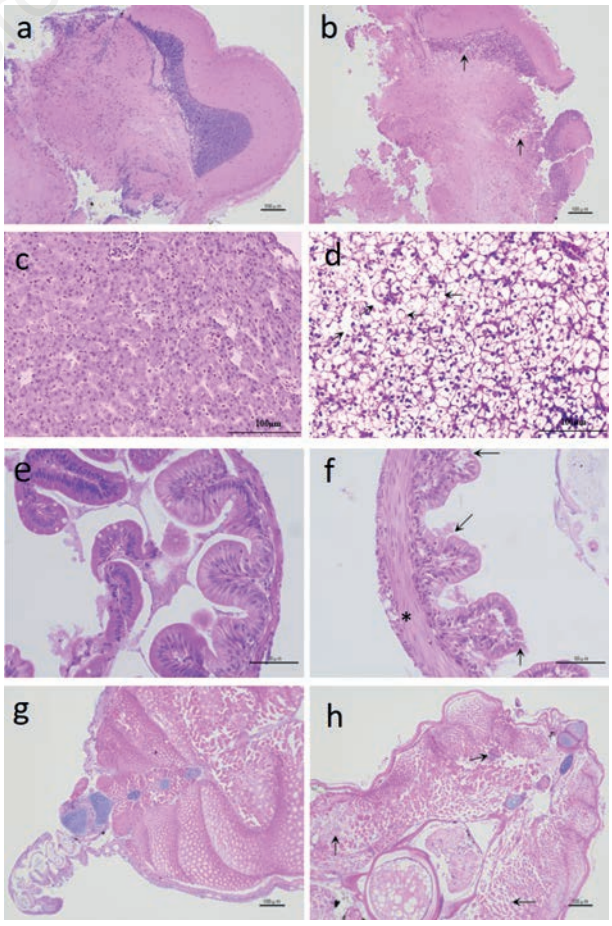

Fig. 3. Pathological tissue injury of *M. salmoides*. Brain: healthy tissue (a); number of granular cells decrease and the gap increases (b). Liver: healthy tissue (c); the vertical arrow indicates hepatocellular edema, tilted arrow indicates loose and disorganized intercellular space, and the horizontal arrow indicates cell vacuoles (d). Intestine: healthy tissue (e); vertical arrow refers to tissue structure damage and cell arrangement disorder (f); tilted arrow refers to intestinal villus shedding, horizontal arrow indicates epithelial cell necrosis and karyocyte fragmentation disappeared; the asterisk refers to muscular layer shallowing. Muscle: healthy tissue (g), vertical arrow refers to fibrous tissue hyperplasia (h); tilted arrow refers to inflammatory cell infiltration; horizontal arrow refers to tissue structure damage and loose disorder. Scale bars: c,g) 50 µm; a,b,d-f,h) 100 µm.

N and *M* gene expression

Real-time PCR was used to detect the expression levels of *N* and *M* in different tissues after MSRV infection (Fig. 4). *N* and *M* were expressed in brain, intestine, muscle and caudal fin. The relative expression of *N* and *M* increased first and then decreased, and the relative expression reached the peak at 36 h post-infection (p.i.). The relative expression of *N* was higher than that of *M* in brain and intestine, whereas the relative expression of *N* was lower than that of *M* in muscle and caudal fin.

After the infection of largemouth bass with MSRV, the spatial expression patterns of *N* transcribed at different time points were shown in Fig. 5A. The relative expression of *N* in each tissue increased first and then decreased. The abundance of *N* expression was low and almost undetectable at 6, 12 and 168 h p.i. During the period from 24 to 48 h p.i., the relative expression of *N* was the highest in intestine and the lowest in caudal fins. The highest relative expression of *N* was found in intestine at 36 h p.i. The highest relative expression of *N* was observed in brain during the period from 72 to 144 h p.i. After the infection of largemmouth bass with MSRV, the tissue spatial expression pattern of *M* transcription at different times was shown in Fig. 5B. The relative expression of *M* was almost undetectable at 6, 12 and 168 h p.i. The expression of *M* was relatively high at 24 to 48 h p.i. The relative expression of *M* in brain reached the peak at 36 h p.i., and the relative expression in caudal fin was the lowest. During the period from 72 to 144 h p.i., the relative expression of *M* in each tissue was not significantly different at other time periods except 96 and 144 h p.i., but it was obviously lower than that at 36 h p.i.

Evaluation of the protective effect of immune enhancers

After MSRV infection, the mortality rate of largemouth bass in each group was 93.8% at 8 d p.i. (Fig. 1). The mortality rate decreased to 66% and 68.5% after the addition of single and compound immune enhancers, respectively.

The anti-MSRV effects of different formulations of immune

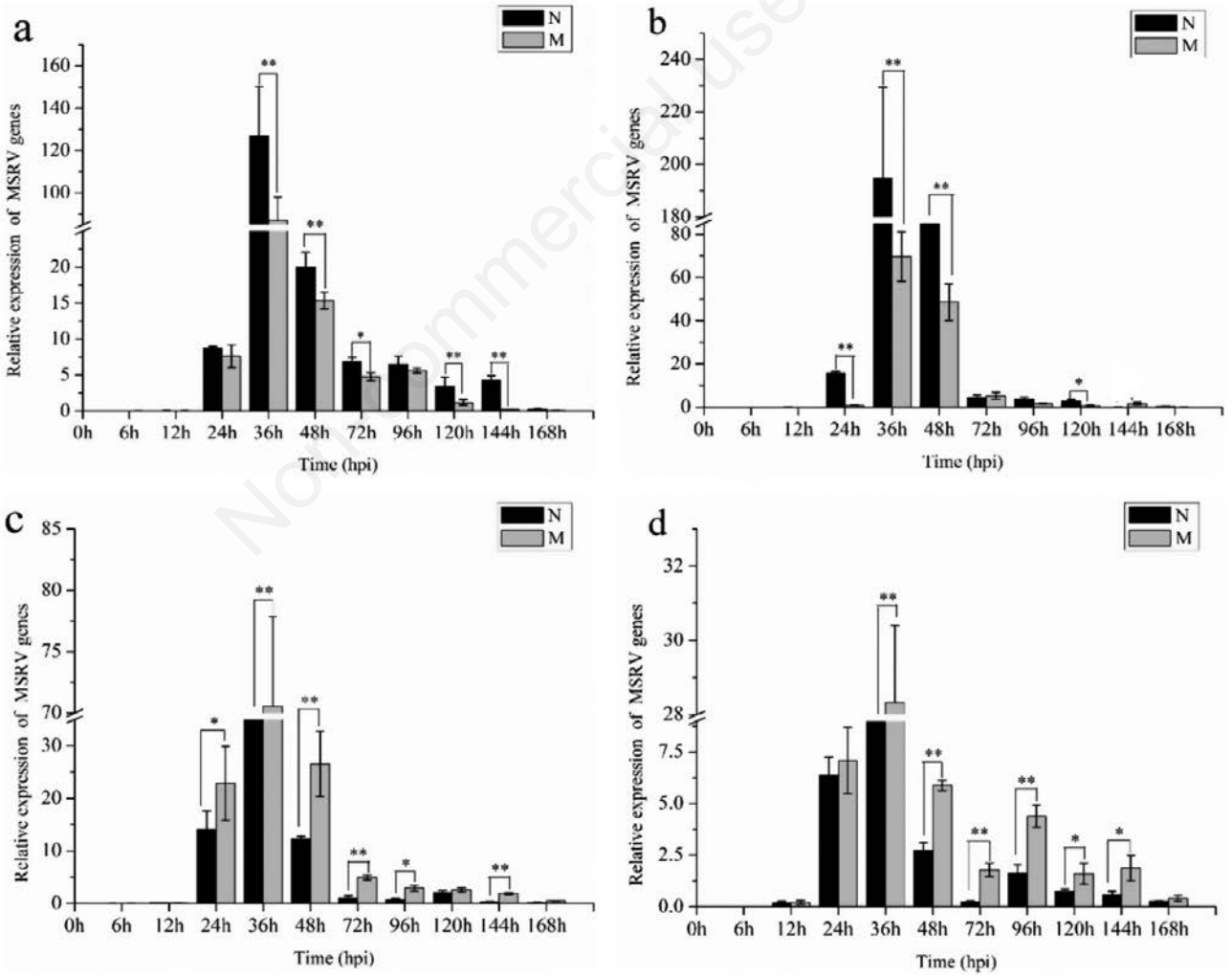

Fig. 4. Analysis of transcription expression of *N* and *M* genes in different tissues. a) Brain. b) Intestine. c) Muscle. d) Caudal fin. *p<0.05, **p<0.01.

enhancers were evaluated by real-time fluorescence quantitative PCR. The transcriptional expression of *N* was shown in Fig. 6. From 24 to 168 h after MSRV infection, the relative expression of *N* in brain and caudal fin tissues in group B was significantly higher than that in groups C and D ($p<0.05$). The relative expression of *N* in group B was significantly higher than that in groups C and D ($p<0.05$) in intestine except at 144 h p.i. However, the relative expression of *N* in muscle of group B was significantly higher than that of group C and group D at 24 to 48 and 120 h p.i. ($p<0.05$). At 72 and 96 h p.i., the relative expression of *N* in group B was not significantly different from that in group C, but was significantly higher than that in group D ($p<0.05$). At 144 and 168 h p.i., the relative expression of *N* in group B was not significantly different from that in groups C and D.

The transcriptional expression of *M* was shown in Fig. 7. In all tissues, the transcriptional expression of *M* in group B was significantly higher than that in groups C and D at 12 h p.i. ($p<0.05$) except in brain at 168 h p.i. There was no significant difference in the relative expression of *M* among group B, group C and group D ($p>0.05$) at 168 h p.i. The combined results showed that the single and compound immune enhancers used in this study had some inhibitory effects on the replication of *N* and *M* in largemouth bass.

DISCUSSION

Largemouth bass-MSRV infection

At present, the pathogenesis of MSRV had not been elucidated, and relevant studies were also lacking, which affected the prevention and control of this disease. Therefore, it was necessary to establish a suitable experimental animal model for the study of MSRV pathogenesis and immune prevention. The use of corresponding cell cultures and appropriate animal infection models was the classical method for virological research (Choi *et al*., 2021).

In this study, largemouth bass were immersed in MSRV suspension with a viral load of 2.62×10^4 copies/mL and maintained at 22°C to 24°C. At 24 h after MSRV infection, the largemouth bass began to show obvious symptoms and death. A large number of fish died within 36 to 48 h p.i., with a mortality rate of about 50%. The maximum mortality rate was over 90% on 7-8 d p.i. After MSRV infection, largemouth bass showed obvious symptoms, such as black body color, spiral swimming, tissue bleeding, and body bending, which were basically consistent with the experimental results of Ma *et al*. (2013) and Lyu *et al*. (2019). In order to further explore the pathogenicity of MSRV, histopathological analysis was performed. The results showed that there were obvious pathological injuries in the brain, liver, intestine and muscle of largemouth bass. For example, the number of cells in the granular layer of brain tissue decreased. Hepatocyte edema and fat vacuoles were observed. Some epithelial cells in the intestinal tissue were necrotic, and the nuclei were fragmented and disappeared. Some muscle fibers were necrotic and fibrous tissue was proliferated. In conclusion, MSRV was obviously pathogenic to largemouth bass, which could cause obvious pathological damage to fish and had a high mortality rate to largemouth bass seedlings.

Transcriptional expression of *N* and *M*

In this study, samples of tissue from the largemouth bass MSRV infection group at different periods were also examined. The results showed that *N* and *M* were expressed in the brain, intestine, muscle and caudal fin of largemouth bass after MSRV infection. The expression abundance of *N* and *M* genes were almost undetectable from 6 to 12 h p.i. The relative expression of genes increased significantly at 24 h p.i., and reached the peak at 36 h p.i. *N* and *M* expression began to decrease at 48 h p.i. and remained at low levels after 72 h p.i., which might be related to the activated immune response mechanism after fish rhabdovirus infection. IFN system was the first line of defense against viral infection in fish. IFN is an important antiviral factor in the early stage of fish, which has antiviral and immune regulation effects (Song *et al*., 2018). Gao and Chen (2018) showed that MSRV infection of epidermal cells of largemouth bass could significantly up-regulate the expression of genes such as *IFN-1*, *IFR-3* and *IFR-9*. Zebrafish had been used as a model organism to study fish rhabdoviruses, such as viral haemorrhagic septicemia virus (VHSV), spring viraemia of carp virus (SVCV) and snakehead rhabdovirus

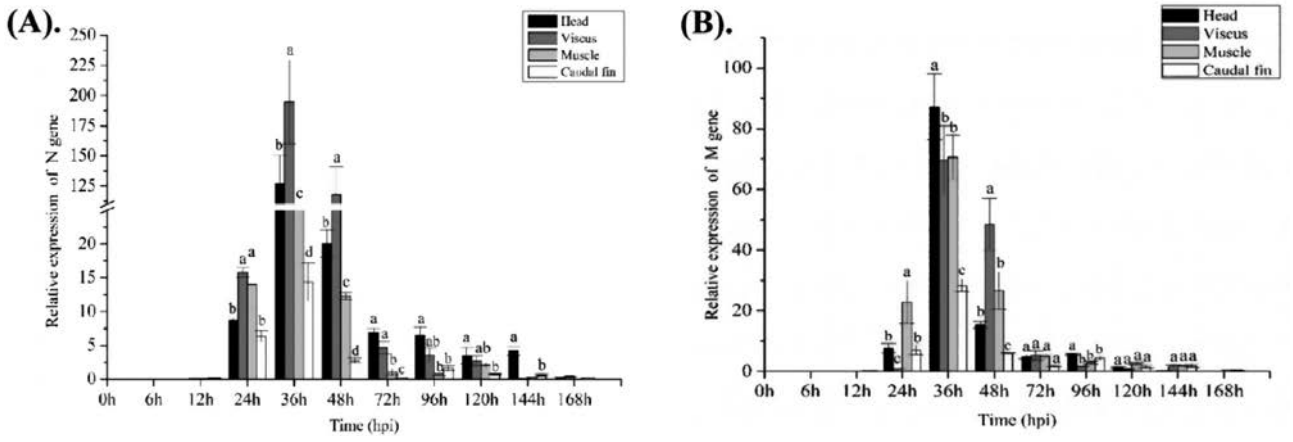

Fig. 5. The analysis of transcription and expression level of *N* (A) and *M* (B) gene in different tissues after infected with MSRV.

(SHRV) (Sanders *et al.*, 2003; Phelan *et al*., 2005; Novoa *et al*., 2006). Aggad *et al*. (2010) showed that the expression of *IFN-r1*, *IFN-r2* and *IFNΦ1* factors increased 48 h p.i. after SVCV infection in zebrafish, which was significantly higher than that in the control group. After 72 h of SVCV infection in zebrafish, the expression of *IFNΦ1* gene was up-regulated by 100 times, and the expression of *IFNΦ3*, *IFNΦ4* and other genes was up-regulated by 2-10 times. Over-expression of *IFN* could inhibit the replication of rotavirus (Levraud *et al*., 2007; López-Muñoz *et al*., 2009). *IFN* was also involved in the immune response of zebrafish infected with VHSV, and the results showed that the expression levels of *IFNΦ2*, *IFNΦ3* and other genes were significantly up-regulated to resist VHSV infection (Kavaliauskis *et al*., 2015, 2016). In addition, Phelan et al. infected zebrafish with SHRV, and found that the expression of *IFN* was maintained at the basal level during the period of 6 to 24 h p.i. after SHRV infection, the expression of *IFN* was 7.7 times higher than that of the control group at 48 hpi and 18 times higher at 72 h p.i. (Phelan *et al*., 2005). These results suggested that interferons were involved in the immune response to fish rhabdovirus infection.

In addition, qPCR was further used to analyze the transcriptional expression of *N* and *M* in different tissues of MSRV infection. It was found that the relative expression of *N* was higher than that of *M* in the head and visceral tissues. In muscle and caudal fin tissues, the relative expression of *N* was lower than that of *M*. Iverson and Rose (1981) inferred that the transcriptional expression level of *N* should be higher than that of *M* according to the characteristic that the transcriptional expression level of the genome decreases progressively, which was consistent with the experimental results of this study in head and visceral tissues. However, the expression of *N* in muscle and caudal fin tissue was lower than that of *M*, which did not conform to the transcriptional expression characteristics of genome. The specific reasons need to be further studied. The results showed that there were tissue differences in the transcriptional expression of *N* and *M* after MSRV infection.

Prevention and treatment methods of rhabdovirus in largemouth bass

Disease is a huge crisis in aquaculture. In China, more than 10% of farmed fish were lost to disease each year, with an annual loss of up to 15 billion RMB (Gui *et al*., 2018). MSRV was one

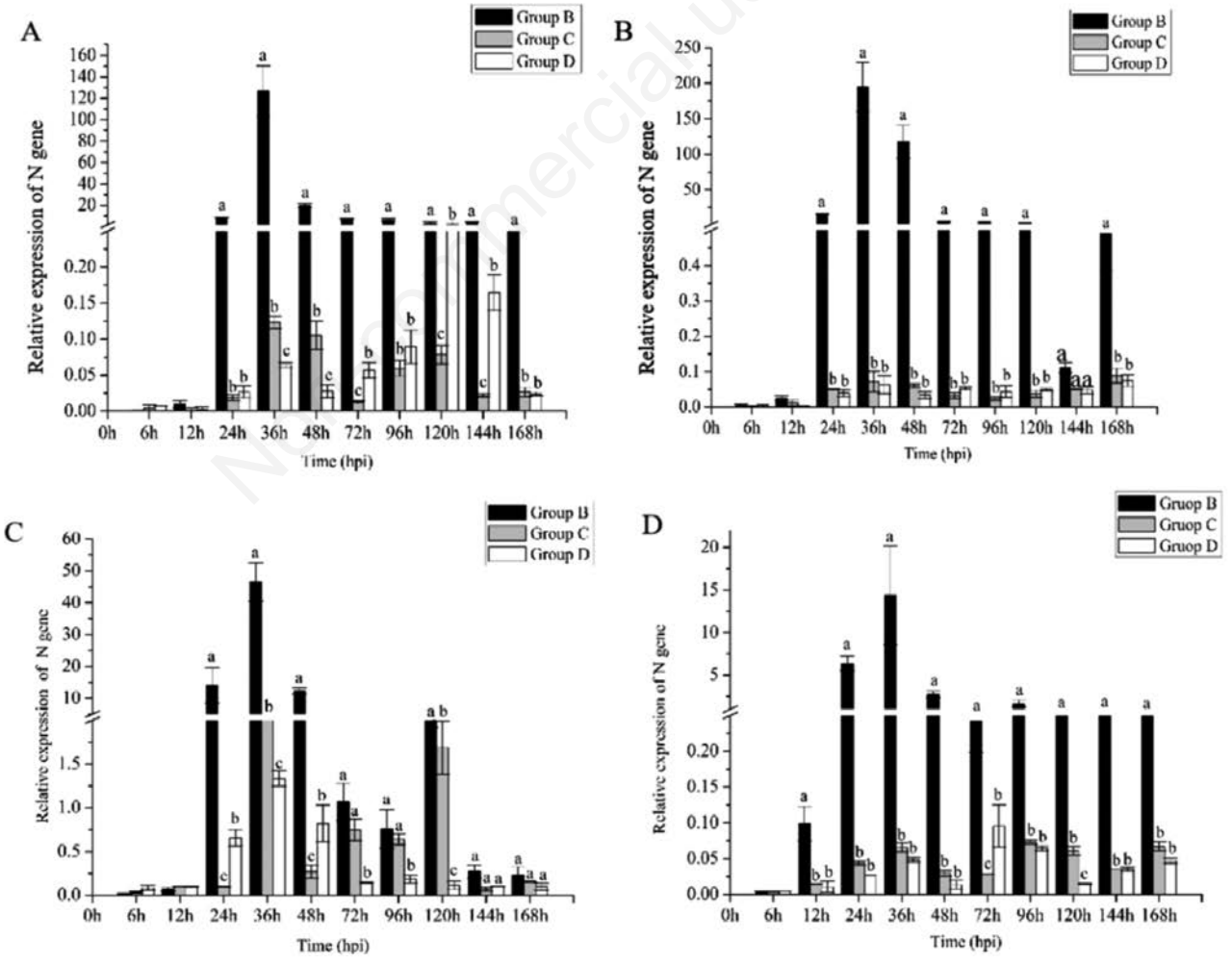

Fig. 6. Effect of immune enhancers on transcriptional expression of *N* gene. A) Brain. B) Intestine. C) Muscle. D) Caudal fin. $^{a,b,c}p<0.05$.

of the most serious pathogens that harmed the seedlings of largemouth bass and caused huge economic losses to farmers.

Vaccines were the effective means of disease prevention and control, and vaccines against MSRV in aquatic products had been studied. Guo *et al*. (2020) successfully constructed SWCNTs-G, a subunit vaccine of MSRV G protein, using SWCNT as a carrier. The levels of antibody, immune-related enzyme activity, complement C3 level and immune-related gene expression in the serum of largemouth bass increased with the increase of vaccine dose. The survival rate of the SWCNTs-G group was significantly higher than that of the control group, indicating that the G protein subunit vaccine could be used for MSRV prevention and treatment. Yang *et al*. (2022) also constructed an MSRV G protein subunit vaccine, different doses of vaccine groups (2.5, 5, 10 and 20 μg/fish) improved the survival rate of large mouth black bass by 6.1%-48.5%.

In addition, some drugs also had good inhibitory effects on MSRV. As antiviral drugs, the mechanism of action of chemically synthesized drugs was usually relatively clear. Studies showed that guanosine, arctigenin, vidarabine, ribavirin, 8-hydroxyquinoline and 4-8-2-bromoimidazole octyloxy-arctigenin (BOA) as antiviral drugs had highly effective anti-MSRV activity (Li *et al*., 2022; Shen *et al*., 2020; Yang *et al*., 2021). Hydrolysates of 3-bromo-4, 5-dihydroxybenzyl methyl ether, 3-bromo-4, 5-dihydroxybenzaldehyde, total casein and AS2-casein also had antiviral effects and could be used to prevent fish rhabdovirus infection (Kim *et al*., 2011; Rodríguez Saint-Jean *et al*., 2013).

It was an effective prevention and control method to prevent diseases by improving the host's own immunity. Studies had found that adding astragalus polysaccharide and chitooligosaccharides (Lin *et al*., 2017), compound Chinese herbal medicine (scutellaria, Phellodendri, rhubarb, Daqing leaf) (Bin *et al*., 2019), vitamin C (Yusuf *et al*., 2020), antimicrobial peptides (Li *et al*., 2020) and other immune enhancers to the diet could improve the specific and non-specific immunity of largemouth bass. Kang *et al.* (2012) found that *fructus forsythiae*, *Astragalus polysaccharide*, *Lonicerae flos* and *Paeoniae alba* and other plant extracts had significant inhibitory, blocking and neutralizing effects on MSRV

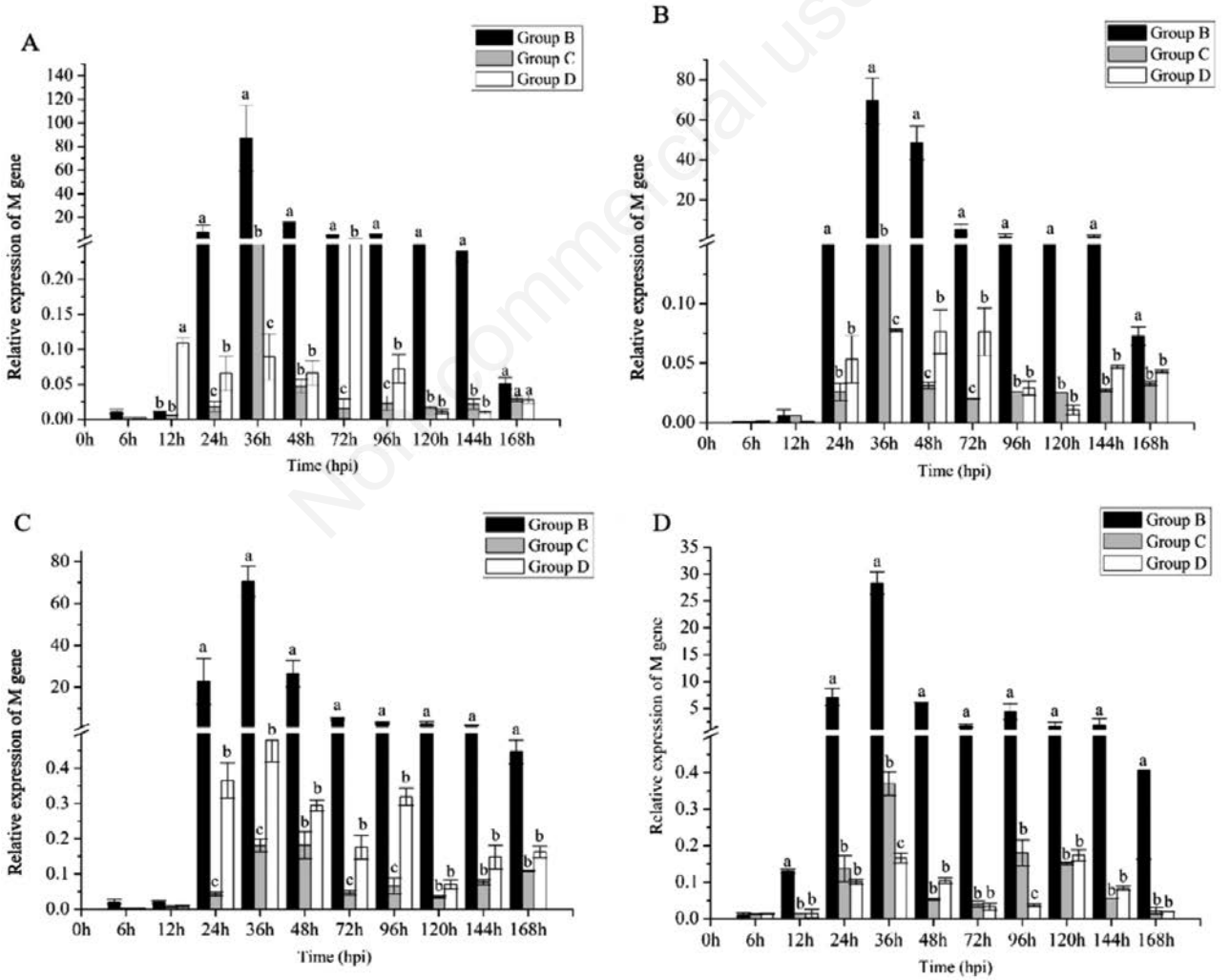

Fig. 7. Effect of immune enhancers on transcriptional expression of *M* gene. A) Brain. B) Intestine. C) Muscle. D) Caudal fin. [a,b,c]$p<0.05$.

through in vitro screening. Among them, *Astragalus polysaccharide* had the best anti-MSRV effect, which could inhibit the replication and proliferation of MSRV and up-regulated the expression level of lectin genes (Xiaoying *et al*., 2021). In this study, the immune enhancers (including free nucleotides, *Astragalus polysaccharide*, forsythiasis polysaccharide, macrosmus leaf polysaccharide and vitamin C) were added to the diet of largemouth bass. The results showed that the survival rate of largemouth bass increased by 25-28%, and the replication of *N* and *M* were effectively inhibited, which indicated that the selected immune enhancers had certain antiviral effects. The results could lay a foundation for further research on MSRV resistance.

CONCLUSIONS

In this study, the clinical manifestation and gene expression of *N* and *M* genes in largemouth bass were studied after MSRV infection and immune enhancers protection. MSRV caused obvious lesions in fish brain, liver, muscle and intestinal tissues. *N* and *M* genes were expressed in the brain, intestine, muscle and caudal fin of largemouth bass after MSRV infection, and the relative expression levels increased first and then decreased, reaching the peak at 36 h p.i. The relative expression of *N* was higher than that of *M* in brain and intestine, and lower than that of *M* in muscle and caudal fin tissues. The immune enhancers including free nucleotides and *Astragalus polysaccharides* could effectively inhibit the viral *N* and *M* gene expression of largemouth bass tissues and increase the survival rate of by 25-28%.

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