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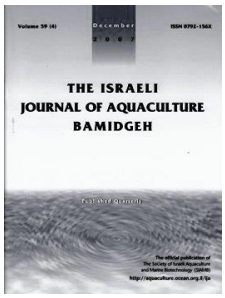

The Effect of Muscle-Regulatory Factor Genes and Satellite Cell Response to Recombinant Hsp70 Protein on *Megalobrama amblycephala* Skeletal Muscle

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Keywords: Hsp70; skeletal muscle; *Megalobrama amblycephala*; satellite cell.

Abstract

Hsp70 (70-kDa heat shock protein) plays a key regulatory role in muscle development. In order to evaluate whether recombinant Hsp70 protein (rMaHsp70) of *M. amblycephala* could affect muscle growth by inducing the expression of Myogenin (*MyoG*), Myogenic Differentiation Antigen (*MyoD*), Myogenic factor 5 (*Myf5*), myogenic regulatory factor 4 (*MRF4*) and myostatin (MSTN) in muscle, Hsp70 was injected intraperitoneally (IP) at two concentrations: 1.0 mg/mL and 3.4 mg/mL. The mRNA and protein levels of the transcription factor Pax-7 and the protein Hsp70 initially increased, then as concentration levels of injected recombinant *M. amblycephala* Hsp70 protein rMaHsp70 further increased, the protein Hsp70 decreased. In order to investigate the relationship between muscle hyperplasia, hypertrophy and satellite cell growth following rMaHsp70 administration, the number of satellite cells were analyzed and revealed that the numbers of satellite cells in low concentration group (1 mg/mL) was predominantly higher than in the other concentration groups in both red and white muscle. These results suggest that low concentrations of rMaHsp70 could decrease muscle-related gene expression and increase the number of satellite cells. Our research will be helpful in adapting Hsp70 as a feed additive to enhance *M. amblycephala* growth.

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Introduction

Fish skeletal muscle is predominantly composed of white muscle which is comprised of more than 70% myotomal muscle. This important and active metabolic tissue plays a key role in energy homeostasis. There are three different types of skeletal muscle, white, red and intermediate muscle, classified according to morphological, biochemical and physiological differences (Blasco et al., 1996). The two main types of muscles also differ in amount of fish body mass, with the red muscle representing only 5-10 % and white muscle representing 40-60% (Bone, 1978).

Muscle hyperplasia and hypertrophy are regulated by a series of positive and negative factors, such as myogenic regulatory factors including MyoD, Myf5, myogenin (MyoG), Mrf4 (Watabe, 2001), and myostatin (MSTN including MSTNa and MSTNb) (Thomas et al., 2000; Nishi et al., 2002; Chisada et al., 2011). MSTN belongs to the transforming growth factor (TGF)-beta superfamily and is a potent negative regulator of skeletal muscle development and growth (Thomas et al., 2000). It inhibits satellite cell proliferation and consequently activates myosatellite differentiation (Garikipati et al., 2012). Fish muscle hyperplasia and hypertrophy are involved in populations of myogenic precursor cells, also called satellite cells which are quiescent except in cases of injury or disease in mammals (Hawke and Garry, 2001).

Heat shock proteins 70, (Hsp70) are a family of proteins that restore protein homeostasis and contribute to cell survival (Liu et al., 2006; Miyabara et al., 2006). Hsp70 have also been associated with the muscle atrophy process (Dodd et al., 2009). During immobilization of skeletal muscles, Hsp70 expression can increase in response to the oxidative damage that occurs in atrophied muscles (Powers et al., 2005; Venojarvi et al., 2007). On the other hand, Hsp70 expression is depressed in atrophied muscles with prior long-term hindlimb unloading. This consequently impairs muscle mass recovery (Lawler et al., 2006). Studies demonstrating the role of Hsp in skeletal muscle atrophy have shown that whole body hyperthermia prior to, (Naito et al., 2000) and during (Selsby and Dodd, 2005) skeletal muscle disuse promotes high levels of Hsp70 and attenuates muscle wasting. The number of satellite cells during muscle atrophy was reduced along with decreased expression of genes that were regulated during the activation and proliferation of satellite cells (Mitchell and Pavlath, 2004).

Hsp70 has been considered a biological indicator of fish health (Webb and Gagnon, 2009). Under optimal conditions when Hsp70 protein is well expressed, indirect regulation of muscle development and growth of muscle satellite cells occurs. *Megalobrama amblycephala*, Cyprinidae, Cypriniformes, are popular as aquaculture species in China with an annual yield of 745,260 tons in 2007 (FBMA 2008). Interest in evaluating the influence of rMaHsp70-injection on skeletal muscle growth in *M. amblycephala* has increased.

Materials and Methods

Fish rearing conditions and experimental design. Sexually immature *M. amblycephala* were obtained from a breeding fishery center, in Ezhou, Hubei Province, China. Before initiation of the experiment, juvenile fish (body weight: 21.35 ± 2.60 g) were kept in two 500 L tanks, each with a circulating water system and controlled photoperiod (14 h/day and 10 h/night). Two weeks later, 270 fish were randomly distributed into three treatments with three replicates per treatment, 30 fish per experimental tank, a total of 90 fish per treatment. The treatments were: 1) control buffer injection; 2) low concentration group (0.1 mL of rMaHsp70 at 1 mg/ml); 3) high concentration group (0.1 mL of of rMaHsp70 at 3.4 mg/ml). All fish were fed continuously to apparent satiation with a commercial diet.During the experimental period tank water quality was maintained at 25^0 ± 1^0C, and dissolved oxygen > 6 mg/L.

Protein expression, purification, and identification. Recombinant Hsp70 protein of *M. amblycephala* (rMaHsp70) expression plasmid (produced in our lab) was transferred into *E. coli* BL21 cells (Chen et al. 2014). Protein expression was induced by incubating the cells overnight at 15°C in the presence of 1.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were suspended in lysis buffer (20 mM Tris-HCl 500 mM NaCl, 5 mM imidazole, pH=7.9) after centrifugation at 4000 × g for 5 min, and

disrupted by sonication and centrifuged for 30 min (4°C, 12.000 g). Histone-tagged (His-tagged) rMaHsp70 was purified by affinity purification using 6× His-tagged Purification Kit (Cowin Biotech, Beijing, China) in accordance with the manufacturer's instructions. Our recombinant Hsp70 was biologically active (Chen et al., 2014).

Sample collection. In order to investigate the effect of rMaHsp70 injection on muscle-regulatory gene expression patterns in *M. amblycephala*, fish were anesthetized using MS222 (0.1 g/L) (Sigma, Alcobendas, Spain). White muscle tissues were collected from all groups (n=8) and snap-frozen in liquid nitrogen 1 day post-injection.

Total RNA isolation and reverse transcription. Total RNA was isolated from different tissues using Trizol kit (Promega, Madison, USA). Isolated RNA quality and quantity (concentration) were determined by NANODROP 2000 spectrophotometer (ThermoScientific, Waltham, USA). First strand cDNA was synthesized using the PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Japan). Two micrograms of RNA and 0.5 μg of Olig (dT) $_{16}$ were reacted for 5 min at 70°C. After incubation for 2 min on ice, the mixture was reverse transcribed with 200 units of M-MLV reverse transcriptase, 5×buffer, 25 units RNasin and 0.8 mM dNTPs in a total volume of 25 μL and extended for an hour at 42°C.

Gene expression analysis. Temporal expression spectra of genes were analyzed by qRT-PCR (Bustin et al., 2009). Specific primer pairs for MRFs, MSTN and *β-Actin* were obtained (Zhu et al., 2014). Three reference genes, *β-Actin*, *EF1a* (elongation factor 1, alpha) and *18s RNA*, were selected based on expression stability. All primer sequences are described in Table 1. The qRT-PCR reactions were carried out in total 20 μL volume mixtures containing 50 ng cDNA, 0.3 μM of each primer and 10 μL SYBR Green qPCR Master Mix (Toyobo, Osaka, Japan). The three-step qRT-PCR amplification program consisted of an initial denaturation at 95°C for 30 s, followed by 40 cycles of amplification 5 s at 95°C, 20 s at specific annealing temperatures (Table 1), 20 s at 72°C, and final extension for 10 min at 72°C.

Table 1. Primers used for cloning and expression.

Gene	*Primer sequences (5'–3')*	*Tm (°C)*	*Application*
Pax-7-RT-F	GGTTTACTGCCACCAATAGC	60	Real-time PCR
Pax-7-RT-R	CTCTGCCCAATCACATCC		
Hsp70-RT-F	CTTTATCAGGGAGGGATGCCAGC	61	Real-time PCR
Hsp70-RT-R	CCCTGCAGCATTGAGTTCATAAGGT		
EF1α-RT-F	CGGACAATTTCTCTTTCGGCTG	57	Real-time PCR
EF1α-RT-R	ACCCACACCGTGCCCATCTA		
18s RNA-RT-F	CGGACAATTTCTCTTTCGGCTG	57	Real-time PCR
18s RNA-RT-R	ACCCACACCGTGCCCATCTA		

To select the reference genes with the most stable expression, the relative stability measure (*M*) of the reference genes was calculated by GeNorm (http://medgen.ugent.be/genorm/). The value M represents an average pairwise variation of a reference gene with all other reference genes and a lower M value corresponds to the higher expression stability (Tang et al., 2007).

Protein isolation and Western blot. Muscle was taken aseptically from rMaHsp70-stimulated *M. amblycephala* and used for total protein extraction by RIPA Lysis Kit (Dingguo Biotech, Beijing, China). The proteins were quantitated by BCA method (Smith et al. 1985), and then separated on 12% SDS-PAGE, and transferred to nitrocellulose membranes. The membrane was incubated for 2 h with a 1:100 dilution of Hsp70 polyclonal antibody (Boster, Wuhan, China). After extensive washing, the membrane was incubated with anti-rabbit peroxidase-labeled secondary antibody (1:2000) at room temperature for 1 h. Subsequently data imaging by chemiluminescence methodololgy showed the bands on photographic film.

Distribution of satellite cells. The distribution of satellite cells of *M. amblycephala* red and white muscle was estimated based on the immunofluorescence method at 1 d post-injection with different rMaHsp70 concentration (Zhu et al., 2014). The primary antibody, anti-Pax7 (ab34360, diluted 1:100, Santa Cruz) and anti-desmin (D8281, diluted 1: 20,

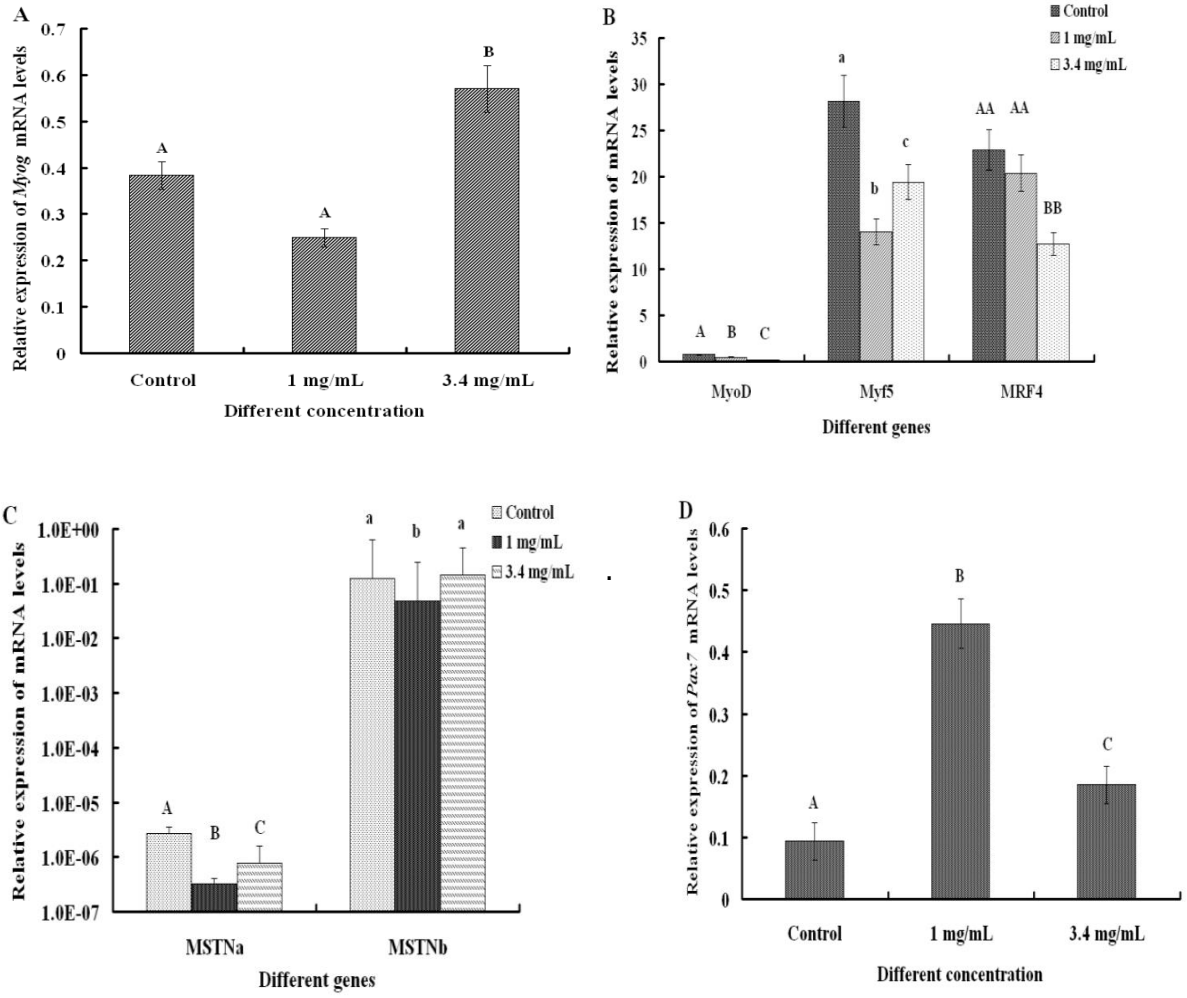
(Sigma, Aldrich) were used to label satellite cells and the intermediate filament, respectively. Fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG (Boster, China, diluted 1:50) and Cy3-labeled goat anti-rabbit IgG (Boster, China, diluted 1:100) as secondary antibodies were used to observe the Pax7 and desmin localization, respectively. Cell nuclei were stained with nuclear marker DAPI. Finally, the sections were observed using the fluorescence microscope (Zeiss, Germany), and 8 images were analyzed with Image-Pro Plus 6.0 software.

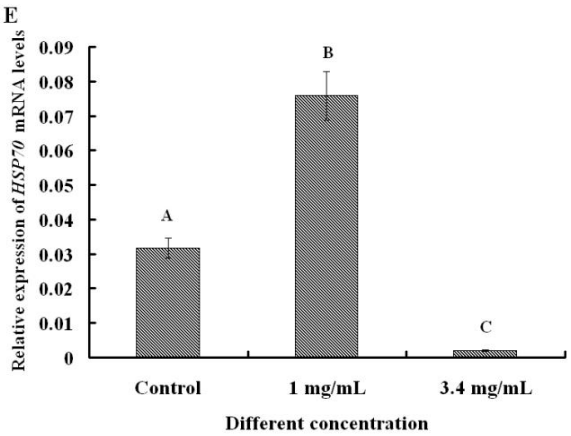
Statistical analysis. For statistical analysis, data from qRT-PCR were presented as the mean ± s.e.m. One-way ANOVA was used to examine the differential expression of MRFs and MSTN after injection. Differences were considered significant at $p < 0.05$ level.

Results

Effect of Hsp70 on muscle-regulatory genes expression in M. amblycephala white muscle. To evaluate the effects of Hsp70 on *M. amblycephala* white muscle, we used qRT-PCR to detect the muscle-related gene expression levels in fish. *β-Actin* was selected as the reference gene based on the ordinal *M* values of reference genes. As shown in Fig. 1, the mRNA levels of related genes changed dramatically 1 d post-injection in *M. amblycephala*. *MyoG* expression significantly increased ($p<0.05$) in the high concentration group (3.4 mg/mL) (Fig.1A), and noticeably decreased in the mRNA levels of *MyoD*, *Myf5* and *MRF4* (Fig.1B). Higher concentration of rMaHsp70-injection, *MSTNa* and *MSTNb* mRNA decreased initially, then increased, but *MSTNb* mRNA was dramatically higher than *MSTNa* (Fig.1C)

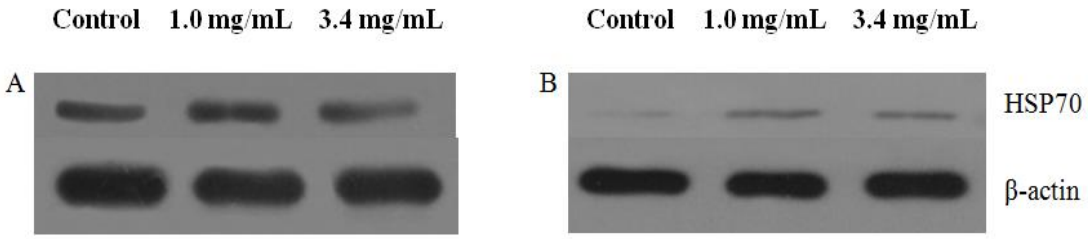
Figure 1. The effect of related-genes, *MyoG* (A), *MyoD*, *Myf5* and *MRF4* (B), *MSTN* (C), *Pax-7* (D), *Hsp70* (E), mRNA levels after injection of recombinant Hsp70 protein in muscle of *M. amblycephala.* Values with different letters show significant differences ($p<0.05$). Data are means ± s.e.m.





Effect of rMaHsp70 on Pax-7 and Hsp70 protein expression in M. amblycephala white muscle. To further characterize whether over-expression of Hsp70 and Pax-7, Hsp70 protein expression were linked, we quantified Hsp70 and Pax-7 protein expression by western blot in *M. amblycephala* white muscle. We found that when rMaHsp70 concentration increased, the protein levels of Pax-7 and Hsp70 initially increased and then decreased (Fig. 2).

Fig. 2. The effect of Pax-7 (A) and Hsp70 (B) protein levels after rMaHsp70 (Recombinant Hsp70 protein of *M.amblycephala*) injection in *M. amblycephala* muscle



Characterization of satellite cells. To further determine if a link existed between Hsp70-stimulated activation muscle and proliferation, differentiation of satellite cells, we quantified satellite cell numbers by immunostaining Pax-7 co-localized with desmin in *M. amblycephala* white muscle (Fig. 3). The frequency of satellite cells in the low concentration treatment (1 mg/mL) (54.6 % and 48.6 %) was predominantly higher than the higher concentration treatment in both of red and white muscle (Fig. 4), however no significant difference (p<0.05) was found between the control and high concentration treatment in both of red (38.6 % and 40.5 %) and white muscle. (34.6% and 32.1%).

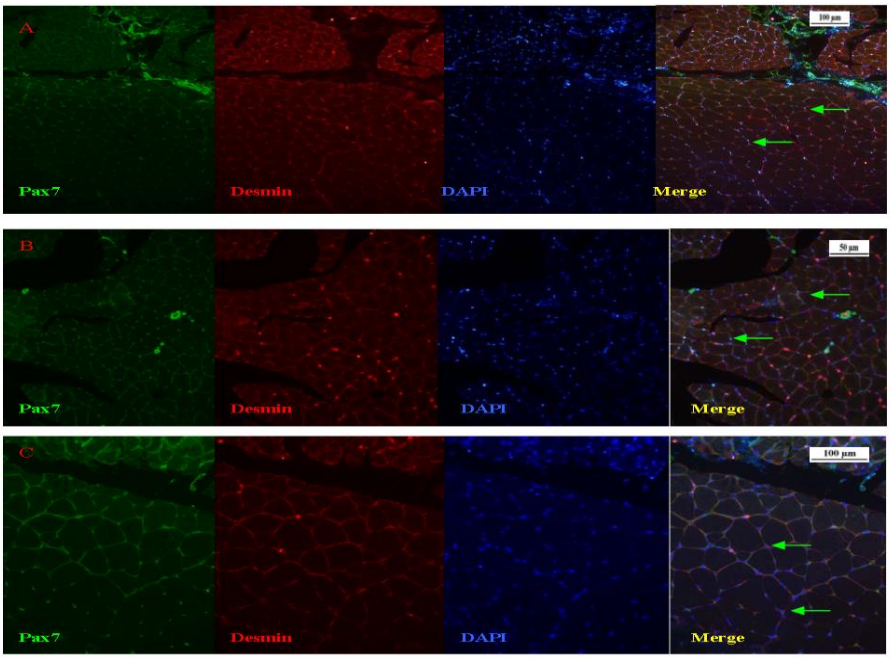


Fig.3 Affection of satellite cell distribution after injection of rMaHsp70 protein in red and white muscle of *M.amblycephala*. A. red and white muscle in the control group, Scale bars: 100 μm. B. red muscle in 1 mg/mL concentration group, Scale bars: 50 μm. C. white muscle in 3.4 mg/mL concentration group, Scale bars: 100 μm. Green arrows represent satellite cells.

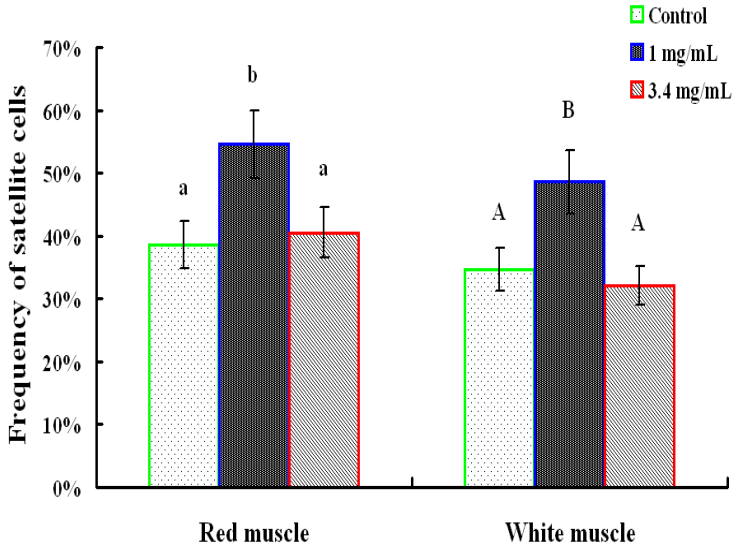


Fig. 4. Effect of satellite cell frequency after injection of rMaHsp70 protein in red and white muscle of *M. amblycephala.* Values with different letters show significant differences (p<0.05). Data are means ± s.e.m.

Discussion

Although it has been shown that Hsp70 is involved in the protection against muscle atrophy, the role of Hsp70 in muscle development of *M. amblycephala* is still unknown. The present study showed that the overexpression of inducible rMaHsp70 could decrease muscle-related gene expression, and increase the number of satellite cells.

MyoG expression may be related to intense differentiation and fusion of myoblasts to existant myofibers during hypertrophy, while higher *MyoD* and *Myf5* expression may be associated with intense myoblast proliferation (hyperplasia) (Johansen and Overturf, 2005). In our study, mRNA levels of *MyoD* and *Myf5* were significantly lower (p<0.05) in the low concentration treatment group (1 mg/mL) than the control group, nevertheless no difference in the expression of *MyoG* between two groups was observed. Our results might also indicate enhanced expression of *MSTNa* and *MSTNb* in response to possible increased myotube differentiation and hypertrophy, possibly attenuating the effect of *MyoD* and *Myf5* in low concentration groups (Johansen and Overturf, 2005; Zhu et al., 2014).

Pax7 is a marker gene of satellite cells, and Hsp70 plays a key regulatory role in muscle development (Gros et al., 2005; Relaix et al., 2005). In the present study, the result of Pax-7 and Hsp70 mRNA levels and protein expression were similar, both showing higher expression levels in the low rMaHsp70 concentration group than in the control group. This suggests that the increased expression levels of Hsp70 and Pax-7 could be induced by rMaHsp70, yet over-expression of Hsp70 retarded its own expression.

Satellite cells in red and white muscle initially increased and then decreased. This is consistent with the Pax-7 mRNA and protein levels obtained with the enhancement of rMaHsp70 concentration (Relaix et al., 2005). This indicates that Hsp70 injections at low concentrations could stimulate the quantity of muscle satellite cells in both of red and white muscle. Different mechanisms related to the proliferation and differentiation of satellite cells might exist in red and white muscle of fish (Hawke and Garry, 2001; Zhu et al., 2014). This could be associated with greater blood supply and capillary proliferation in the red muscle than in the white muscle which resulted in different density of satellite cells in the two types of muscle fiber (Hawke and Garry, 2001).

These results suggest that low concentrations of rMaHsp70 could reduce muscle hyperplasia and hypertrophy, and activate satellite cells to repair the skeletal muscle. Our study demonstrated that the stimulation of rMaHsp70 was beneficial to muscle development. Moreover, the results will be helpful for adapting Hsp70 as feed additive to enhance *M. amblycephala* growth. Further research is necessary to study the applicable aspects of the present research.

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