Morphological analysis and muscle-associated gene expression during different muscle growth phases of *Megalobrama amblycephala*

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ABSTRACT. Skeletal muscle growth is regulated by both positive and negative factors, such as myogenic regulatory factors (MRFs) and myostatin (MSTN), and involves both hyperplasia and hypertrophy. In the present study, morphological changes during muscle development in *Megalobrama amblycephala* were characterized and gene expression levels were measured by quantitative real-time polymerase chain reaction (qRT-PCR) analysis in juvenile [60, 90, 120, and 180 days post-hatching (dph)] and adult fish. Our results show that during muscle development, the frequency of muscle fibers with a diameter <20 μm dramatically decreased in both red and white muscles, with a concomitant increase in the frequency of >30 μm fibers in red muscle and >50 μm fibers in

white muscle. At 90-120 dph, the ratio of hyperplastic to hypertrophic areas in red and white muscles increased, but later decreased at 120-180 dph. The effect of hypertrophy was significantly larger than hyperplasia during these phases. qRT-PCR indicated *MRF* and *MSTN* (*MSTNa* and *MSTNb*) genes had similar expression patterns that peaked at 120 dph, with the exception of *MSTNa*. This new information on the molecular regulation of muscle growth and rapid growth phases will be of value to the cultivation of *M. amblycephala*.

Key words: *Megalobrama amblycephala*; Muscle fiber; Gene expression; Morphological analysis; Hyperplasia and hypertrophy

INTRODUCTION

Skeletal muscle in fish contributes 40-60% of the body mass and is specifically adapted to the mechanical requirements of aquatic life (Bone, 1978). In most species, the myotomal organization of the axial musculature (epaxial and hypaxial muscles) is stratified into three layers: the superficial red muscle, more deeply located myotomal muscle (white muscle) that contributes >70% of muscle mass, and an intermediate (pink muscle) layer between these. The three layers vary among species with regard to extension and histochemical properties (Mascarello et al., 1995).

Post-embryonic skeletal muscle development involves muscle hyperplasia (fiber number increase) and hypertrophy (fiber volume increase) (Junghyo et al., 2009). Hyperplastic growth generally occurs during larval stages and completes the formation of the main muscle layers. During this process, new fibers are generated along a distinct germinal layer and named “stratified” hyperplasia. “Mosaic” hyperplasia is defined as the dissemination of new fibers across the whole myotome. Muscle fiber hypertrophy continues throughout the post-embryonic life until the fibers reach a functional maximum diameter in the range 100-300 μm for white fibers, but rather smaller for red fibers (Sanger, 1993). In *Atractoscion nobilis*, *Eleginops maclovinus*, and *Patagonotothen tessellata* both hyperplasia and hypertrophy play major roles in muscle growth throughout the entire life span. Recruitment of new fibers during muscle growth ceases when the fish attain about 36.5-49% of their final size; muscle growth then mainly depends on hypertrophy (Fernandez et al., 2000; Rowleson and Veggetti, 2001).

Muscle hyperplasia and hypertrophy are regulated by the myogenic regulatory factors (MRFs), MyoD, Myf5, myogenin (MyoG), and Mrf4 (Watabe, 2001) and MSTN (including MSTNa and MSTNb) (Thomas et al., 2000). MyoD and Myf5 are primary MRFs and are responsible for proliferation and differentiation of myoblasts (Sabourin and Rudnicki, 2000). MyoG and MRF4 are considered secondary MRFs and control muscle differentiation at later stages through regulation of myoblast fusion and consequent formation of myotubes (Rudnicki and Jaenish, 1995). MSTN belongs to the transforming growth factor (TGF)- β superfamily and is a potent negative regulator of skeletal muscle development and growth (Thomas et al., 2000; Matsakas et al., 2010; McGivney et al., 2012; Tripathi et al., 2013).

Megalobrama amblycephala, commonly known as Wuchang bream, is an endemic species in China that is widely appreciated for its delicate flavor and is currently an important aquaculture species. Total production of bream increased from 541,115 tonnes in 2001 to

1,000,000 tonnes in 2009 (CAFS, 2001; Song et al., 2011). In the present study, we investigated the morphological characteristics of red and white muscle in *M. amblycephala* at different growth phases. Additionally, the levels of expression of selected genes associated with muscle development were also measured.

MATERIAL AND METHODS

Experimental fish

Development-related changes in the expression of *MRFs* and *MSTN* were investigated using juvenile [60, 90, 120, and 180 days post-hatching (dph), N = 8 at each stage] and adult (N = 8) *M. amblycephala* obtained from a breeding farm in Wuhan, China. The fish were euthanized using tricaine methanesulfonate [MS-222 (0.1 g/L), Sigma, Alcobendas, Spain]. Body weights, body heights, and total lengths were determined at each growth stage (Table 1). Muscle was also collected, immediately frozen in liquid nitrogen, and then stored at -80°C until use.

Table 1. Body weight, body height, and total length in 60, 90, 120, 180 dph and adult *Megalobrama amblycephala*.

Growth stage*	Body weight (g)	Body height (cm)	Total length (cm)
60 dph	0.89 ± 0.12 ^a	1.19 ± 0.15 ^a	4.67 ± 0.31 ^a
90 dph	3.01 ± 0.98 ^b	1.95 ± 0.22 ^b	6.74 ± 0.61 ^b
120 dph	5.98 ± 1.21 ^c	2.77 ± 0.30 ^c	8.18 ± 0.68 ^c
180 dph	8.31 ± 1.23 ^d	3.10 ± 0.24 ^d	9.17 ± 0.39 ^d
Adult	360.6 ± 30.5 ^e	11.05 ± 1.82 ^e	29.20 ± 3.4 ^e

*N = 8 at each sampling interval. Values with the different letters are statistically significant (P < 0.05). Data are reported as means ± SEM.

Immunohistochemistry studies

Muscle morphology was characterized using 4 µm transverse sections of tissue; the sections were stained immunohistochemically (Braun et al., 2011; Neusser et al., 2010; Zhu et al., 2014) using MYH1/2/4/6 (F59, sc-32732, diluted 1:200, Santa Cruz) as the primary antibody and mouse IgG horseradish peroxidase-conjugated (BA1050, diluted 1:200, BOSTR) as the secondary antibody.

Muscle fibers from red and white muscle at different growth stages were classified by diameter (d, µm): class 10, d ≤ 10; class 20, d = 10-20; class 30, d = 20-30; class 40, d = 30-40; etc. (de Assis et al., 2004). Muscle fiber frequency was calculated as the number of fibers from each diameter class relative to the total number of fibers measured.

The contributions of hypertrophy and hyperplasia to white muscle growth were assessed by grouping fibers into three diameter classes: class 1, d ≤ 20; class 2, d = 20-50; class 3, d > 50 (Valente et al., 1999). The new hyperplasia muscle fibers were defined by ordering of smaller muscle fiber diameter. The relative increased areas were constituted by new hyperplasia muscle areas and old hypertrophy muscle areas. The relative contributions of hyperplasia and hypertrophy to the increase in transverse section areas was estimated as follows (Valente et al., 1999):

$$C = \Delta N_t \bar{a} (\mu\text{m}^2) + N_t \Delta \bar{a} (\mu\text{m}^2)$$

where, Δ is the calculated difference in fiber areas between two sampling times (t and $t+1$), and N_t and $\bar{a}$ are the total number of fibers and fiber area at t , respectively. “C” was both the relative contribution of “hyperplasia” and of “hypertrophy”.

Total RNA isolation and reverse transcription PCR

Total RNA was extracted from frozen white muscle samples of juvenile and adult fish using Trizol kit (Promega, Madison, WI, USA). The quality and concentration of the isolated RNA were measured using a NANODROP 2000 spectrophotometer (ThermoScientific, Waltham, MA, USA). First strand cDNA was synthesized using a PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Japan) following the manufacturer protocol.

Gene expression analysis

Changes in gene expression during development were examined using *MyoG*, *MyoD*, *Myf5*, *MSTNa*, and *MSTNb* and analyzed by quantitative reverse transcription (qRT)-PCR (Zhu et al., 2014). qRT-PCR was performed with a Rotor-Gene 6500 real-time PCR Thermo-cycler (QIAGEN, Dusseldorf, Germany) using 20- μ L reaction mixtures containing 50 ng cDNA, 0.3 μ M of each primer pair and 10 μ L SYBR Green qPCR Master Mix (Toyobo, Osaka, Japan). The thermal cycling protocol was as follows: 2 min pre-denaturation at 95°C; 40 cycles of 95°C for 15 s; the appropriate T_m for 15 s; 72°C for 30 s; and a final extension at 72°C for 10 min. *β -actin* was used as the internal reference gene, and the relative quantification of the target and reference genes was evaluated with the standard curve method.

Statistical analysis was performed by one-way ANOVA (analysis of variance), and the Duncan test was used for the multiple comparisons. The qRT-PCR data are reported as means $\pm$ SEM from triplicated samples. $P < 0.05$ was considered to be statistically significant.

RESULTS

Morphological and morphometric analysis

Analysis of stained muscle sections showed that white muscle comprised most of the muscle mass at all growth stages. Muscle mass increased continually throughout the developmental stages examined here. The muscle tissue consisted of round or polygonal muscle fibers separated by a fine septum of connective tissue, termed the endomysium. Thicker septa of connective tissue separated muscle fibers into fascicles. Red muscle fibers were confined to a narrow superficial strip along the lateral line with a wedge-like insertion in the region of the horizontal septum, just below the skin. Mosaic hyperplastic growth in white muscle was evident as shown the increase in fiber numbers and in the appearance of many small fibers (Figure 1).

Nuclei were almost exclusively subsarcolemmal (SS) in the small fibers of adult fish, whereas intermyofibrillar (IM) nuclei were present in the larger fibers of red and white muscle (Figure 1E and G); however, the number of IM nuclei was lower than of SS nuclei. Only SS nuclei were observed in juvenile fish (Figure 1A-D).

The range of muscle fiber diameters in red and white muscle tissues was greater in

adult than juvenile fish (Figure 2). The largest mean diameter for red muscle fibers was 26.66 μm (range 8.07-49.47 μm ; Figure 2E) and for white muscle fibers was 47.56 μm (range 10.11-108.56 μm ; Figure 2J) and was observed in adult fish. Moreover, the variation in red muscle fiber diameter was smaller among different stages (Figure 2A-E). In order to estimate the contribution of hyperplastic muscle growth to red muscle development we assigned the fibers to three further diameter classes: class 1, $d \leq 20$; class 2, $d = 20\text{-}30$; and class 3, $d > 30$ to estimate the contributions of hyperplastic muscle growth in red muscle.

Figure 1. Transverse sections of lateral muscle tissue from juvenile fish at 60 days post-hatching (dph) (A), 90 dph (B), 120 dph (C), and 180 dph (D), and from adult fish (E-G). The sections were stained immunohistochemically with a primary antibody (F59) against myosin. E. Section showing red muscle from an adult fish at the level of the epaxial muscle. The area within the square is shown enlarged in the panel to the right. F. Section showing white muscle from an adult fish at the level of the epaxial muscle. Small fibers are apparent within the white muscle, indicating mosaic hyperplasia (red arrowheads). G. Transverse section of white muscle of an adult fish. W, white muscle; R, red muscle; blue arrowheads indicate intermyofibrillar nuclei; green arrowheads indicate subsarcolemmal nuclei. Muscle fibers are separated by endomysium (e). Perimysium (p) separates muscle fibers into fascicles. Scale bars (A-F) = 50 μm ; scale bar (G) = 20 μm .

Figure 2. The proportion (%) of fibers in red (A-D) and white muscle (E-H) in each fiber diameter class from 60 (A, F), 90 (B, G), 120 (C, H), and 180 (D, I) days post-hatching (dph) and from adult fish (E, J); N = 8 at each sampling interval.

In general, the frequency of fibers of $<20\ \mu\text{m}$ diameter gradually decreased in red and white muscle as the fish aged. In red muscle, fibers with diameter $<20\ \mu\text{m}$ comprised 87.6, 83.9, 80.8, 76.5, and 14.04%, respectively, of the total of all size classes in 60, 90, 120, 180 dph, and adult fish (Figure 3A); the comparative figures in white muscle were 22.9, 21.2, 27.9, 21.2, and 5.1%. Fibers of $>50\ \mu\text{m}$ diameter in white muscle comprised 11.6, 12.5, 10.8, 19.9, and 50.6%, respectively, in 60, 90, 120, and 180 dph, and in adult fish (Figure 3B).

Figure 3. Proportion of fibers of different diameters in red (A) and white muscle (B) at 60, 90, 120, and 180 dph and in adult fish (N = 8 at each sampling interval). Statistically significant differences ($P < 0.05$) among developmental stages for the same diameter class are indicated using capital letters; significant differences among classes at the same developmental stage are indicated by lower script letters. Values with the same letters are not statistically significant. The data are reported as means $\pm$ SEM.

Skeletal muscle growth in *M. amblycephala*, including both hyperplasia and hypertrophy at 60, 90, 120, and 180 dph, occurred in a time-dependent manner for total fiber numbers in red (690 to 1406) and white muscle (5151 to 14,321), and for total fiber area in red (386,683.38 to 1,811,664.8 μm^2) and white muscle (3,632,231.4 to 14,318,086.1 μm^2). At 90 to 120 dph, the relative sizes of hyperplastic and hypertrophic areas in red muscle increased; subsequently, however, they decreased at 120 to 180 dph. The relative sizes of hyperplastic and hypertrophic areas in white muscle showed the same changes as in red muscle (Table 2).

The rates of hyperplasia (9.03-8.95%) and hypertrophy (90.97-91.05%) did not vary significantly during the 90 to 180 dph period in red muscle (Figure 4A). In white muscle, however, the rate of hyperplastic growth increased from 3.63 to 30.71% during this period, while hypertrophic growth fell from 96.33 to 69.29% (Figure 4B).

Expression of *Myog*, *MyoD*, *Myf5*, *MSTNa*, and *MSTNb*

To investigate the relationship between muscle morphological characteristics and the patterns of expression of muscle-related genes in *M. amblycephala*, we performed a qRT-PCR analysis of the changes in expression of selected genes during development. We found that the changes in *MyoG*, *MyoD*, and *Myf5* expression followed a pattern in which mRNA levels

Table 2. Fiber numbers and areas of red and white (left epaxial) muscle in *Megalobrama amblycephala* at different growth stages.

Growth stage (dph)	Total fiber numbers		Total fiber area (μm^2)		Hyperplastic area ^a (μm^2)		Hypertrophic area ^b (μm^2)	
	Red muscle	White muscle	Red muscle	White muscle	Red muscle	White muscle	Red muscle	White muscle
60	690 $\pm$ 89 ^a	5151 $\pm$ 565 ^a	386683.38 $\pm$ 12593.3 ^a	3632231.4 $\pm$ 226532.1 ^a	-	-	-	-
90	948 $\pm$ 106 ^b	6377 $\pm$ 648 ^b	740284.7 $\pm$ 35263.5 ^b	7325711.5 $\pm$ 563214.5 ^b	31940.8 $\pm$ 1683.9 ^a	135418.9 $\pm$ 8759.6 ^a	321660.6 $\pm$ 11297.5 ^a	3558061.2 $\pm$ 396578.5 ^a
120	1130 $\pm$ 153 ^c	9899 $\pm$ 1025 ^c	1365504.1 $\pm$ 55896.5 ^c	11637402.7 $\pm$ 856354.5 ^c	44504.1 $\pm$ 19853.7 ^b	923871.1 $\pm$ 45963.2 ^b	580715.3 $\pm$ 25684.5 ^b	3387820.1 $\pm$ 356895.1 ^a
180	1406 $\pm$ 168 ^d	14321 $\pm$ 1563 ^d	1811664.8 $\pm$ 79634.7 ^d	14318086.1 $\pm$ 986573.3 ^d	39934.8 $\pm$ 12867.1 ^b	823246.4 $\pm$ 35864.9 ^b	406226.0 $\pm$ 38648.9 ^c	1867437.7 $\pm$ 113586.1 ^b

Values with different letters are significantly different. Data are reported as means $\pm$ SEM. $P < 0.05$. ^aRelative hyperplastic area which was estimated as: $A = \Delta N_t / \bar{a}$; ^bRelative hypertrophic area which was estimated as: $B = N_t \Delta \bar{a}$, where Δ was calculated between two sampling times (t and $t + 1$) and N_t and $\bar{a}$ refer to the mean total number of fibers and fiber area at t .

gradually increased from 60 to 120 dph, and then decreased thereafter (Figure 5A and B). The highest levels of expression were seen in *MyoG*, followed by *Myf5* and *MyoD*. The pattern of expression of *MSTNb* was similar to that of *MyoG*, *MyoD*, and *Myf5*; however, *MSTNa* mRNA levels only increased until 90 dph and then subsequently gradually declined with a slight increase in adult fish (Figure 5C). The level of *MSTNb* expression was higher than that of *MSTNa* during the stages studied of muscle development.

Figure 4. Relative contribution of hyperplastic and hypertrophic growth to red (A) and white muscle (left epaxial) (B) development in juvenile and adult fish.

Figure 5. Patterns of *MyoG* (A), *MyoD* and *Myf5* (B), and *MSTNa* and *MSTNb* (C) expression at 60, 90, 120, and 180 dph and in adult fish. The data are reported as means $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$.

DISCUSSION

Morphological analysis

The analysis of muscle morphology in *M. amblycephala* at different stages of development stages identified two main tissue layers at all analyzed times: superficial red muscle, located below the dermis, formed by smaller fibers; and, more deeply located white muscle with larger fibers, which constituted approximately 90% of the total muscle mass. These morphological characteristics are consistent with those reported for other fish species whose white muscle mass is of economic importance (Fernandez et al., 2000; Dal Pai-Silva et al., 2003a,b; Aguiar et al., 2005; de Almeida et al., 2008, 2010). Moreover, mosaic hyperplasia was ubiquitous at detected stages, as previously reported in other species (Rowlerson and Veggetti, 2001; de Almeida et al., 2008).

The present study demonstrated that white muscle fibers of *M. amblycephala* underwent extreme hypertrophic growth during development until they reached a threshold size. At this point, the SS nuclei were apparently unable to sufficiently serve the entire myonuclear domain, leading to the appearance of IM nuclei. The characteristics of the *M. amblycephala* myonucleus were similar to those reported previously for *Centropristis striata* (Priester et al., 2011) and in crustaceans (Hardy et al., 2010; Bruusgaard et al., 2012), in which the distribution of nuclei changed with muscle development. Mosaic hyperplasia in *C. striata* followed the proliferation of IM nuclei, supporting the notion that diffusion constraints might trigger the formation of new fibers (Priester et al., 2011). However, no morphological evidence was found in the present study to support the interpretation that new small diameter fibers are formed following the proliferation of IM nuclei in red or white muscle of adult fish.

In some teleost species, both hyperplasia and hypertrophy play major roles in muscle growth throughout the entire life span (Rowlerson and Veggetti, 2001). Here, we found that red muscle showed no significant differences in the frequency of fibers with <20 μm diameter during the development of juvenile fish, although there was a large difference between juvenile and adult fish. Our results indicated that hyperplastic growth was more prevalent in juvenile than adult fish. The highest and lowest frequencies of fiber of <20 μm diameter occurred in the white muscle of 120 dph and adult fish, respectively. The presence of fibers of <20 μm diameter indicated active hyperplastic growth, which clearly contributed to *M. amblycephala* skeletal muscle growth at 120 dph (Valente et al., 1999; Rowlerson and Veggetti, 2001; de Almeida et al., 2010). Our study showed that muscle fiber recruitment in adults was lower than during juvenile development (60-180 dph). The lower frequency of fibers of >50 μm diameter at 120 dph indicated that muscle fiber hypertrophy was less prominent at this stage (de Almeida et al., 2008, 2010); the highest rate of fibers of >50 μm diameter occurred at 180 dph and in adults suggesting that muscle fiber hypertrophy was occurring (Valente et al., 1999; Rowlerson and Veggetti, 2001; de Almeida et al., 2010).

The total areas occupied by fibers in red and white muscle increased from 386,683.38 to 1,811,664.8 μm^2 and from 3,632,231.4 to 14,318,086.1 μm^2 between 60 dph and adulthood, respectively. Compared to red muscle, white muscle tissue had significantly larger areas with fibers suggesting that white muscle growth was the principal contributor to overall muscle growth. In *Pagellus bogaraveo*, hyperplasia is mainly responsible for the increase in white muscle areas at 70 to 100 dph (Silva et al., 2009); by contrast, in *M. amblycephala*, hypertrophy

was the main contributor during the analogous growth phases, while red muscle also increased as a result of hypertrophy (Silva et al., 2009; Chang et al., 2012).

The highest relative increase in hyperplastic areas occurred at 120 dph, whereas the highest relative increase in fiber number was at 180 dph in red and white muscle. A previous study also reported that the increase in fiber number did not correspond well with the increase in areas of fibers during fish growth (Paul and Rosenthal, 2002). In addition, our study also showed that hypertrophy was a significantly more important factor in muscle growth than hyperplasia; however, the rate of hyperplastic growth gradually increased, while that of hypertrophic growth gradually decreased during 90 to 180 dph in white muscle. This result suggests that hypertrophy was the main contributor to muscle growth but that hyperplasia strengthened dramatically during this period.

***MyoG*, *MyoD*, *Myf5*, and *MSTN* mRNA expression**

During skeletal muscle growth, *MyoD* and *Myf5* directly control proliferation of undifferentiated myoblasts, whereas *MyoG* regulates the differentiation and fusion of myoblasts to form myofibers (Rudnicki and Jaenisch, 1995; Watabe, 1999; de Almeida et al., 2010). These proliferating cells provide the essential nuclei for new muscle fiber formation (hyperplasia) and hypertrophy (Koumans and Akster, 1995). The high level of *MyoG* expression at 120 dph found here might be related to intense differentiation and fusion of myoblasts to existing myofibers during hypertrophy. The high levels of *MyoD* and *Myf5* expression at this stage might be associated with intense myoblast proliferation and, thus, to hyperplastic growth in skeletal muscle (Johansen and Overturf, 2005). Our morphometrical analysis also showed the highest relative increases in hyperplastic and hypertrophic areas in red and white muscle at 120 dph. Moreover, we observed higher mRNA levels for *MyoG* than for *MyoD* and *Myf5*. We suggest that *MyoG* regulates hypertrophic muscle growth at this developmental stage. In contrast to *M. amblycephala*, the highest level of *MyoG* expression in *Piaractus mesopotamicus* occurred at 180 dph (de Almeida et al., 2010).

MSTN (including MSTNa and MSTNb) is a member of the TGF- β superfamily and normally acts to restrain muscle growth to prevent overgrowth (Thomas et al., 2000). Transgenic zebrafish with suppression of *MSTNb* expression exhibited an approximately 10% increase in myofiber number compared to non-transgenic lines (Xu et al., 2003). Zebrafish embryos injected with an *MSTNb* morpholino showed faster growth than controls (Amali et al., 2004). However, these effects are negligible in comparison to the approximately 200% increase in muscle mass in mice lacking *MSTN* (McPherron et al., 1997). These studies demonstrated that MSTN may exhibit differences in function between mammals and fish.

Fish *MSTNa* and *MSTNb* have evolved under strong purifying selection, leading to a well-conserved MSTN structure. The coding sequences in fish for *MSTNa* orthologs are more divergent than those among *MSTNb* orthologs (Gabillard et al., 2013). *MSTNb* expression is also higher than *MSTNa* in most tissues and during early development stages in zebrafish (Helterline et al., 2007). In *Oncorhynchus mykiss*, *MSTNa* orthologs appear to be limited primarily to the brain to regulate neurogenesis (Garikipati et al., 2007). In the present study, expression of *MSTNb* was higher than that of *MSTNa* during the stages studied. *MSTNa* and *MSTNb* mRNA levels peaked at 90 and 120 dph, respectively, suggesting a possible restriction of muscle growth at 120 dph. Interestingly, we also found that *MyoG*, *MyoD*, and *Myf5*

expression increased at 120 dph, indicating that expression of the *MSTNa* and *MSTNb* may have risen in response to hyperplasia and hypertrophy to attenuate the effects of *MyoG*, *MyoD*, and *Myf5*.

The results reported in the present study are preliminary and require further extensive study. However, the new information presented here will be helpful in understanding the morphology and molecular control of skeletal muscle growth in the economically important species *M. amblycephala*, especially the developmental stage at 120 dph is of particular interest for further study.

Conflicts of interest

The authors declare no conflict of interest.

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