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Comparative Analysis of Muscle Development in Zebrafish with Different Intermuscular-Bones Patterns

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Authors' Contribution
YK and XS conceived the study. GT carried out the genetic screen of mutant. YJ and YK wrote the manuscript. JY, ZS and DC carried the histological analysis. JY, XZ and WL carried out the qRT-PCR analysis.

Key words
Zebrafish, Intermuscular-bones, Muscle development, Muscle-specific genes, Muscle fiber

ABSTRACT

In this study, we used an intermuscular-bones (imbs) partial deletion zebrafish (*Danio rerio*) mutant to analyze the impact of imbs deficiency on adjacent muscle development and growth by examining expression of muscle-specific genes and muscle structure. Five muscle-specific genes including *myod*, *myog*, *myf5*, *mef2ca* and *sox6* were selected to test and verify the expression differences in embryonic development stages (3 hpf, 6 hpf, 12 hpf, 24 hpf, and 72 hpf) and post-embryonic stages (15 dpf, 30 dpf, 45 dpf, 60 dpf and 75 dpf). Compared to the wild-type (WT) siblings, the mutants showed no significant differences in the 5 gene expressions. Among different development stages, the expression levels and patterns of the 5 genes in the mutants were similar to that of WT zebrafish in both embryonic and post-embryonic development stages. Furthermore, the results of histological analyses of the muscle fiber showed that there were no significant differences in muscle fiber density between imbs mutant and WT zebrafish, and no significant differences between anterior and posterior part to the dorsal fin at the same developmental stages. In WT and mutants, the density of muscle fiber declined gradually over time. In conclusion, the lack of intermuscular-bones has no influence on adjacent muscle development.

INTRODUCTION

Intermuscular-bones (imbs) are small bones in muscles on the both sides of the vertebra, which are ossified from myoseptum (Patterson *et al*., 1995; Meng, 1987), which are popular in freshwater fish species (Lv *et al*., 2007), such as common carp and the four other major Chinese carps (*Mylopharyngodon piceus*, *Ctenopharyngodon idellus*, *Hypophthalmichthys molitrix*, *Hypophthalmichthys nobilis*). These bony structures are inconvenience for producing flesh products, as well as reducing the flesh quality because of the difficulty to remove. At present, most of the studies on imbs focus on using them as features for classification (Rom *et al*., 1975; Johmn *et al*., 2001), distribution (Dong *et al*., 2006; Li *et al*., 1987, 2013; Bing, 1962; Gao, 1984) and morphological development (Ke *et al*., 2008; Karsenty *et al*., 2002: Lv *et al*., 2012). Molecular and genetic analyses of the imbs' development are still rare (Wan *et al*., 2016; Nie *et al*., 2017; Liu *et al*., 2017), especially on their specific function as a tissue.

Studies on the histology and calcification of imbs in fishes with different swimming modes (e.g. zebrafish, and Japanese eel, *Anguilla japonica*) have shown that imbs act as structural support to the muscles and transmission of strength (Yao *et al*., 2015). However, fish with the same swimming mode as zebrafish, crucian carp (*Carassius cuvieri*), blunt snout bream (*Megalobrama amblycephala*), bighead carp (*Aristichthys nobilis*) and silver carp (*Hypophthalmichthys molitrix*), have significant differences in number and form. Crucian carp has less imbs than the other three fishes and might rely more on muscle than imbs to swim compared to blunt snout bream, bighead carp and silver carp (Dong *et al*., 2006; Li *et al*., 2013; Li *et al*., 2017). Studies of zebrafish have also shown that strength transmission rely mainly on muscle fiber and not imbs (Sun, 2008). Therefore, we speculate that imbs might not be necessary for fish swimming. However, there is no study to verify the developmental relationship between muscle fiber and imbs. As suggested by lack of imbs in teleost species such as catfish (*Silurus asotus* Linnaeus) and tilapia (*Oreochromis niloticus*), inhibition of imbs formation probably will not impact the survival. It is unconfirmed that imbs' deficiency would be harmful to muscle attachment.

Zebrafish muscle contains two kinds of muscle fiber: one is slow twitch muscle fibers which are needed

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for slow-swimming; the other is fast twitch muscle fiber required for fast swimming (Stickney *et al*., 2000). Both types of muscles develop immediately from progenitor cells of mesoblast at early stage of embryonic development. Myogenic regulatory factor (MRF) family genes include *myod*, *myf5* and *myog*, which regulate and control the specification and differentiation of muscle cells (Pownall *et al*., 2002). Transcription factors, *myod* and *myf5*, are markers of muscle precursor cells (Coutelle *et al*., 2001; Weinberg *et al*., 1996), both of them are expressed in developing somites, and are essential for initiating the skeletal muscle program in an embryo. Gene *myf5* initiates skeletal muscle development at gastrula stage, whereas *myog* expresses later than *myf5* and functions in both myoblast and skeletal muscles. Gene *myod* plays an important role in differentiation and maturation of muscle fibers (Watabe, 1999; Tan *et al*., 2002; Iban *et al*., 2012: Daniel *et al*., 2008). Gene *mef2* is an important regulator in skeletal muscle differentiation (Ticho *et al*., 1996: Olson, 1992). In all known skeletal muscle development processes, *myf5/myod* expression is followed by upregulation of *myog* and *mef2* family factors; the latter's enhancing expression of differentiation genes (Yun and Wold, 1996). Gene *sox6* is a key transcriptional regulator of fast-twitch muscle fiber differentiation in the zebrafish and ectopic over-expression of *sox6* is sufficient to downregulate slow-twitch specific gene expression in zebrafish embryos (Harriet *et al*., 2015). These 5 genes are muscle-specific expression genes and could be used to indicate the development of muscle.

With the advantage of forward and reverse genetic technologies, it becomes feasible to study the relationship between imbs and muscles during early development. Through genetic screens, we obtained a mutant with partial loss of imbs, which is homozygous and fertile (Fig. 1). Using this imbs mutant, we analyzed the impact of abnormality or loss of imbs to the development of muscles in two aspects, the expression of 5 muscle-specific genes, *myod*, *myf5*, *myog*, *mef2ca* and *sox6*, and the histological differences of muscle fibers.

MATERIALS AND METHODS

Lineage of experimental zebrafish

In the previous studies, we screened an imbs partial deletion mutant lineage and established homozygous lines which were demonstrated through classical Mendelian inheritant experiments that the mutants were homozygous recessive. To eliminate the impact resulted from different genetic backgrounds, we established the experimental lineages using a strategy illustrated in Figure 2 and described as follows. First, we crossed the mutant and wildtype zebrafish to construct F2 generation families. We used bone staining method to check the phenotypes of F2 families and found that the mutational phenotypes occupied about 25% individuals which are consistent with Mendel's law; it confirmed the results in the previous studies that the imbs mutants were homozygous recessive. Among F2 families, healthy individuals were inter-crossed to establish F4 families, after characterizing phenotypes of F4 families by bone-staining, 23 homozygous imbs mutant families and 13 homozygous wildtype families were chosen to carry out the studies.

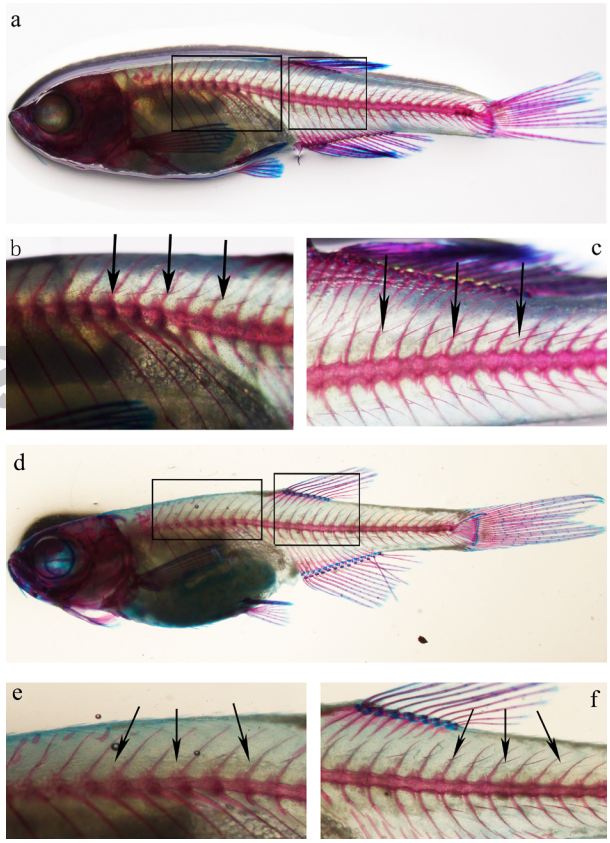

Fig. 1. Whole-body bone-staining images of a WT fish (45dpf, body length= 17.77 mm) and of an imbs deletion mutant zebrafish (47dpf, body length= 19.28mm). a) Whole body bone staining of a WT zebrafish, b) an area anterior to the dorsal fin of the WT zebrafish, and c) an area posterior to the dorsal fin of the WT zebrafish (Arrowheads point at imbs). d) whole body bone staining of a mutant zebrafish with partial loss of imbs, e) an area anterior to the dorsal fin of the mutant zebrafish, and f) an area posterior to the dorsal fin of the same mutant zebrafish. In image e, imbs are completely missing in the area anterior to the dorsal fin of the mutant fish. In image f, imbs are still present but appear shorter compared to ones in the WT zebrafish as shown in b and c, respectively. Arrowhead indicated imbs.

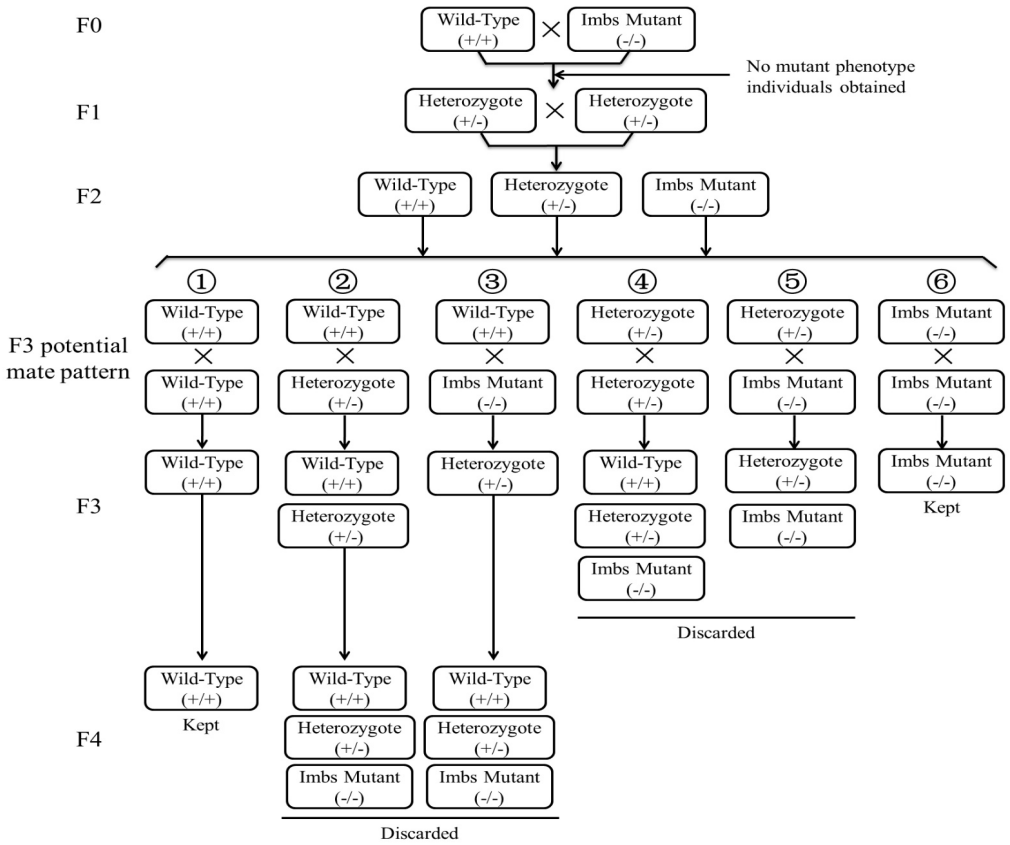

Fig. 2. Screening of experimental zebrafish lineages. + and – represented wild-type allele and mutant allele, respectively; +/+ represented homozygous wild-type genotype; -/- represented homozygous mutant genotype; +/- represented heterozygous genotype, only individuals with homozygous mutant genotype (-/-) expressed the mutant phenotype, i.e. imb partial deletion. For avoiding the influence of the mutant allele to traits of individuals with heterozygous genotypes, only families with homozygous genotypes (+/+ or -/-) were chosen for this study.

Fish culture and breeding

Families of wild-type and mutant were chosen as female-male ratio 1:1 to conduct the breeding experiments. After spawn, eggs in each family were counted and hatched in one culture dish at 28℃. The dead eggs or abnormal larvae were counted and clean up each day. The breeding experiments were replicated four times in four weeks using the same parents. The fertilized rate, hatched rate and deformed rate were represented with the mean value of the four replicates.

Growth measure

After 7dpf, larvae of WT and imbs mutant were divided randomly into three parallel groups, each containing 30 individuals. Fish in each group were cultured in polystyrene plastic tanks (27.5×23.5×19 cm) containing 8L water at water temperature 28℃ and fed with fairy shrimp purchased from Tianjin Fengnian Aquaculture LTD. Growth of individuals in different imbs phenotype group was measured at 5 development stages (15 dpf, 30 dpf, 45 dpf, 60 dpf and 75 dpf). For biometric analysis, fish were anesthetized with tricaine (0.1mg/mL), weighed, and measured in lateral decubitus for standard length measurements.

Gene expression

Gene expressions were tested in 5 embryonic development stages (3 hpf, blastula; 6 hpf, midgastrula; 12 hpf, segmentation period; 24 hpf, pharyngula; and 72 hpf, hatching period) and 5 post-embryonic development stages (15 dpf, 30 dpf, 45 dpf, 60 dpf and 75 dpf). In embryonic development, we sampled wild-type and mutant fertilized eggs or larvae, each sample in the same stage were triplicate; each sample contained 30 fertilized eggs in 3 hpf, 6 hpf, 12 hpf and 24 hpf, and 10 larvae in 72 hpf. In post-embryonic development, we dissected muscles of anterior and posterior to dorsal fin separately in 5 stages. After anesthetization, fish was placed on ice for

muscle sample collection. The samples were collected in triplicate for each period.Total RNA was extracted from each sample using Trizol reagent (Invitrogen, CA, USA), and cDNA was synthesized using High Capacity cDNA Reverse Transcription kits (Roache, CA, USA).

Five muscle-specific genes including *myod*, *myog*, *myf5*, *mef2ca* and *sox6* were chosen to assess their expression by qRT-PCR. The primers for amplification were designed using Primer3 program and are listed in Table I. Quantitative real time PCRs were performed using Luna Universal qPCR Master Mix M3003 kits (NEB, MA, USA) with10 μL reaction volume which contained 5 μL 2×qPCR mix, 0.25 μL 10 μM forward and reverse primers, 1μL 50ng/μL cDNA template, 3.5 μL nuclease-free water. The amplification program was set up as follows: 95℃ pre-denaturation 60 s, followed by 40 cycles each of 95℃ denaturation 15 s, 60℃ extension 30 s. Expression of target genes was normalized against reference gene *gapdh*.

Table I. Muscle-specific gene primers for qRT-PCR.

Gene bank	Gene	Sequence
NM_001328013.1	myod	F: 5'TCCGAGGACATGAGCCAGAT3'
		R: 5'GACGCCGTTTTGCCTGAATA3'
NM_131006.1	myog	F: 5'AGAGACCTCAGGTTGGATTGC3'
		R: 5'TCCTCTAGTGATCAGGGCTCT3'
NM_131576.1	myf5	F: 5'GCGTCAAAGTTGTAGCTATTCCC3'
		R: 5'TACTACAGCCTGCCGATGGA3'
NM_131301.2	mef-2ca	F: 5'CTCTTTCCGTCTGTGCCTCT3'
		R: 5'CCGAGGAAGAGAAAGCACCA3'
NM_001123009.1	sox6	F: 5'TCGTGTGGAAAAATGGGGATCA3'
		R: 5'ATCGCCAGACAACAGCAGCA3'
NM_001115114.1	gapdh	F: 5'ACCCGTGCTGCTTTCTTGAC3'
		R: 5'GACCAGTTTGCCGCCTTCT3'

Muscle histology

Muscles of anterior and posterior to dorsal fin at 5 stages, 15 dpf, 30 dpf, 45 dpf, 60 dpf and 75 dpf were used for histological analysis. Muscle tissues were fixed in Bouin's solution for 4h and preserved in 70% ethanol. Histological samples were prepared using classic methods (Li, 2009). The samples were cut into 6 μm sections, stained with hematoxylin/eosin, and analyzed for density and diameter of muscle fibers.

A standard circle was used for counting the number of muscle fibers and calculating muscle fibers' density. Samples at 15 and 30 dpf were measured using a standard circle of radius 30.0 μm (area 2827.4 μm^2) considering the small size of the sample. For samples at 45, 60 and 75 dpf a standard circle of radius 50.0 μm (area 7853.9 μm^2) was used in the measurement. Density of muscle fibers was calculated using the formula as follows:

$$\text{Density} = \frac{(\text{number of muscle fiber within standard circle})}{\text{area of standard circle}}$$

Statistical analyses

Differences of growth, gene expression, muscle fiber density and diameter among 5 growth stages were used one-way ANOVA to analyze the differences among stages, and differences between mutant and WT were analyzed with two-way ANOVA followed by Bonferroni posttest. Relative gene expression was calculated using the $2^{-\triangle\triangle CT}$ method (Kenneth *et al*., 2001).

RESULTS

Embryonic development

Fertilization rate, hatching rate and deforming rate of wild-type zebrafish were 0.908±0.095, 0.902±0.072 and 0.024±0.011, respectively. Rate of fertilization, hatch and deformity of mutant individuals were 0.927±0.051, 0.933±0.061 and 0.021±0.018, respectively. The analysis revealed that there were no significant differences between wild-type and mutant zebrafish in embryonic development (Fig. 3).

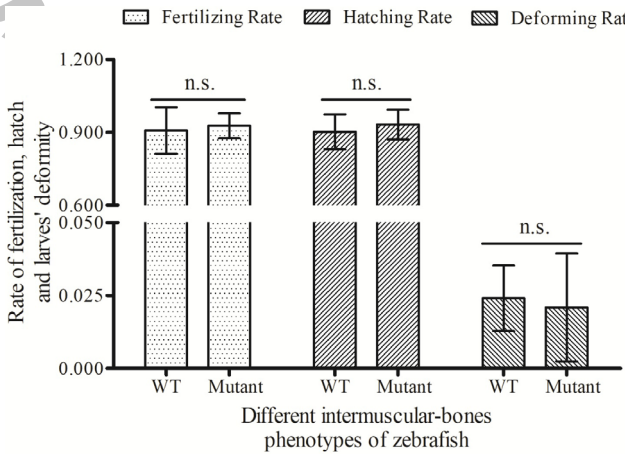

Fig. 3. Comparisons on fertilized rate, hatched rate and deformed rate between wildtype zebrafish and lmbs deficient mutants. Fertility rate, hatched rate and deformed rate were presented as mean ± SD. n.s., no significance (P<0.001).

Growth

The weight and body length of the experimental fish increased dramatically along with their growth. The two-way ANOVA analysis showed that there was no significant difference between WT and mutants in body weight and body length at of 5 developmental stages measured. The survival of the animals was 100% during the growth experiment (Fig. 4).

Gene expression in embryonic development

Expression of 5 muscle-specific genes in 5 embryonic developmental stages increased at the beginning, and then was reduced later on. The expression of *myf5* and *myod* reached a peak at 12hpf, and the other 4 genes reached the peak at 24 hpf. Furthermore, *mef2ca*, *myog* and *sox6*, had a low expression level in blastula and gastrula period, and reached a peak in segmentation and pharyngula period. Comparison between wild-type and mutant samples showed no significant differences of the 5 gene expressions in the same developmental stages (Fig. 5).

Gene expression in post-embryonic development

In general, there are no significant differences in the expression of 5 muscle-specific genes between imbs mutant and WT in the same anatomical locations of individual at the same developmental stages. The expression level of each gene in anterior and posterior to the dorsal fin did not seem much different. Moreover, the expression level of each gene at the same anatomical location decreased gradually as development proceeded (Fig. 6).

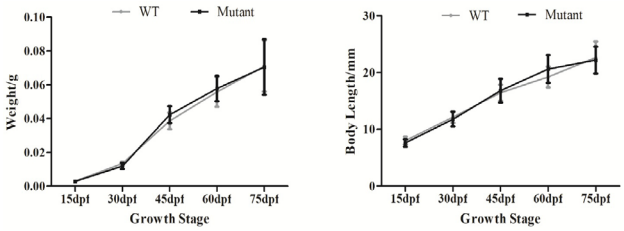

Fig. 4. Growth curves of WT and imbs mutants at different development stages. Data are expressed as mean ± SD of body weight and length.

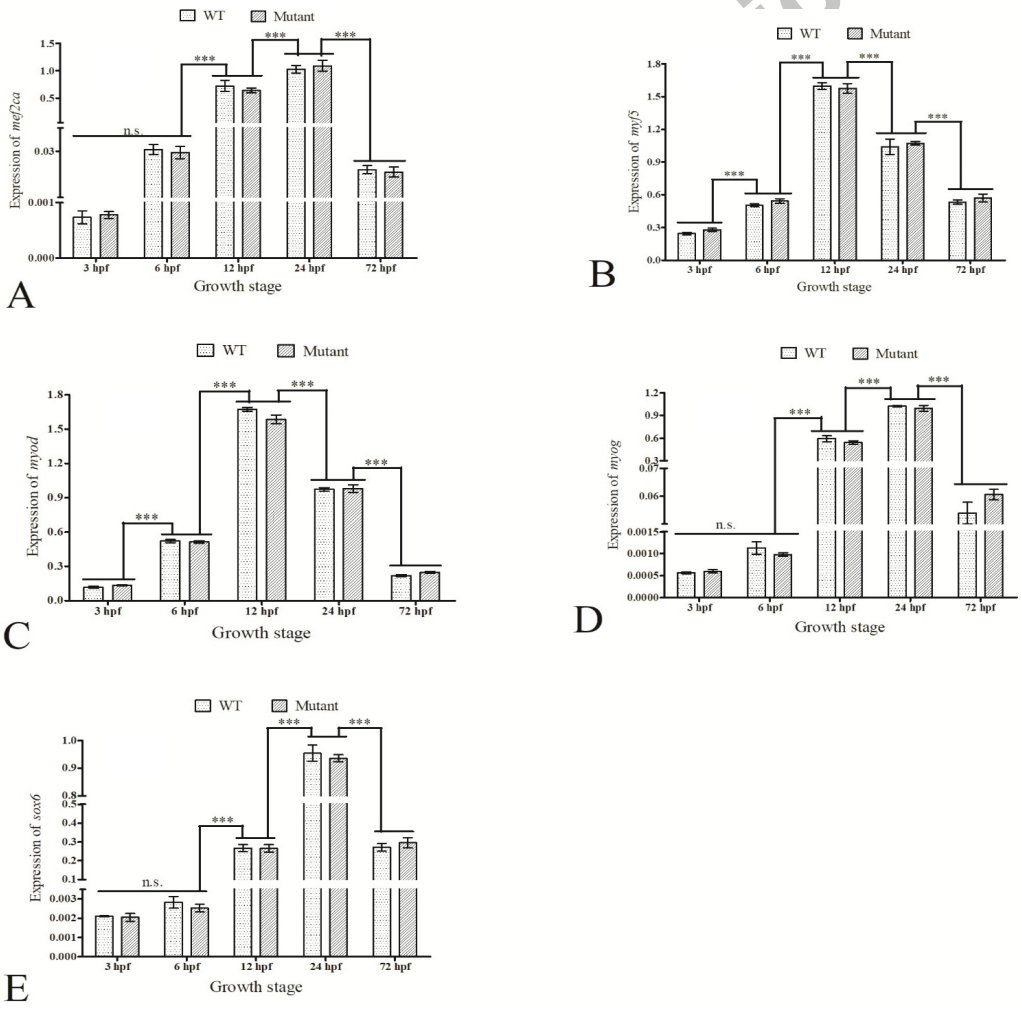

Fig. 5. Expression of five muscle-specific genes in five embryonic development stages. The gene expression was normalized by expression of reference gene *gapdh* and represented using $2^{-\triangle\triangle CT}$. Data were expressed as mean ± SD of (n=3). ***, $P<0.001$; n.s. = no significance.

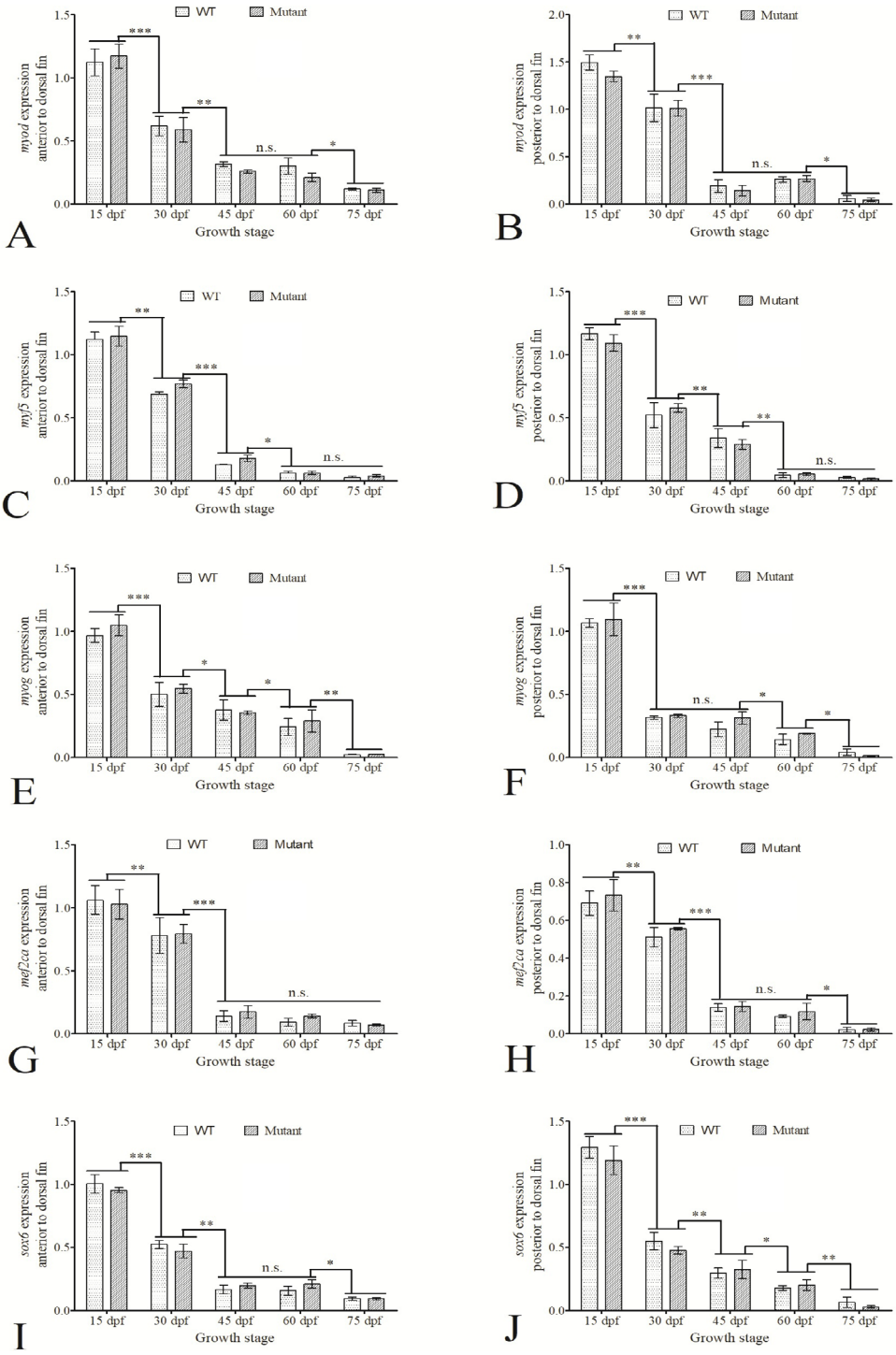

Fig. 6. Expression of five muscle-specific genes in the anterior and posterior muscles to the dorsal fin in both WT and imbs mutant at five developmental stages. A, C, E, G and I represented the expression of *myod*, *myf5*, *myog*, *mef2ca* and *sox6* in the anterior muscles of the dorsal fin at the five stages (15 dpf, 30 dpf, 45 dpf, 60 dpf and 75 dpf), respectively. B, D, F, H and J showed the expression of the five muscle-specific genes in the posterior muscles of the dorsal fin at five different stages. The gene expression was normalized to the expression level of the reference gene *gapdh*. Data are expressed as mean ± SD of $2^{-\triangle\triangle CT}$ (n=3). *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; n.s., no significance.

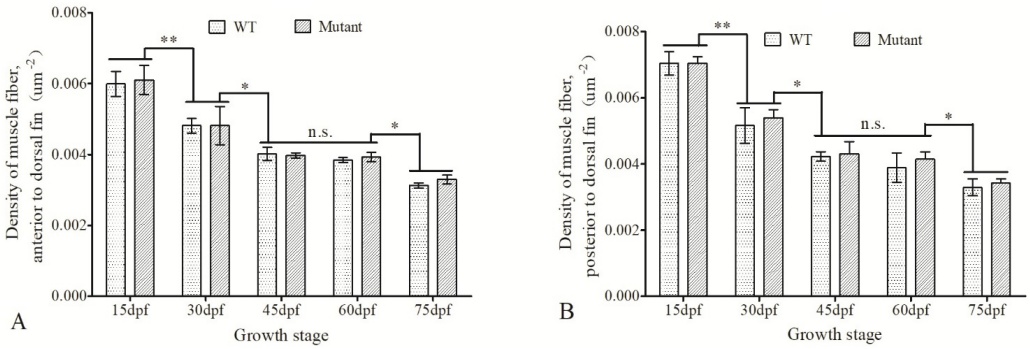

Fig. 7. Density of muscle fibers in the anterior and posterior muscle to the dorsal fin in WT and imbs mutant zebrafish at different developmental stages. The density of the anterior muscles to (i) and posterior muscles(ii) to the dorsal fin in WT and imbs mutant fish were measured at 15 dpf, 30 dpf, 45 dpf, 60 dpf and 75 dpf, data were expressed as mean ± SD of density of muscle fiber. *, $P<0.05$; **, $P<0.01$; n.s., no significance.

Muscle histology

The muscle histological analyses showed that there was no significant difference in the density of muscle fibers between imbs mutant and WT in the same anatomical locations at the same growth stages. The number of muscle fibers in a unit area at the anterior area to dorsal fin was less than the posterior one. Besides, the density of muscle fibers at the same location decreased gradually along with the growth of individuals.

The diameters of muscle fibers showed the same trend as the density of muscle fibers. There were also no significant differences between WT and imbs mutants in the same anatomical locations at the same developmental stages (Fig. 7, Table II).

Table II. Diameter of muscle fibers at different locations of the zebrafish.

Growth stage	Group	Diameter of anterior dorsal fin (μm)	Diameter of posterior dorsal fin (μm)
15 dpf	WT	12.13 ± 1.76^{a}	11.47 ± 0.90^{a}
	Mutant	13.07 ± 1.90^{a}	11.93 ± 0.35^{a}
30 dpf	WT	15.87 ± 0.58^{b}	12.70 ± 0.75^{b}
	Mutant	16.33 ± 0.91^{b}	13.27 ± 0.60^{b}
45 dpf	WT	17.13 ± 0.91^{c}	15.03 ± 0.45^{c}
	Mutant	17.03 ± 0.57^{c}	14.90 ± 0.79^{c}
60 dpf	WT	17.83 ± 1.45^{d}	16.27 ± 0.81^{d}
	Mutant	18.33 ± 1.79^{d}	16.10 ± 0.53^{d}
75 dpf	WT	19.40 ± 1.18^{e}	17.37 ± 1.12^{e}
	Mutant	19.27 ± 1.38^{e}	17.07 ± 1.03^{e}

Data on different body location were analyzed separately. Diameter of muscle fibers are expressed as mean ± SD. Significant differences ($P<0.05$) occurred between different development stages represented by different letters (a, b, c, d and e), while there were no significant difference between imbs mutants and WT individuals represented by the same letter.

DISCUSSION

Development of somites and muscles in fish is similar to that of amphibians, birds and mammals (Kimmel *et al*., 1995). Precursors of adult slow- and fast-twitch muscle fibers already emerge in early embryo development. By the end of segmentation period, fast-twitch muscle fibers move into deeper areas of myotomes, and slow-twitch muscle fibers form a monolayer on the surface of myotome. Slow- and fast-twitch muscle fibers further differentiate to become red muscle and white muscle, respectively in the end (Sun, 2008). MRFs (*myod*, *myf5*, *myog*) and *mef2ca* play a great role in differentiation and maturation of skeletal muscle cells. Therefore, *myod* and *myf5*, expressed in developing somites, are essential for initiating the skeletal muscle program in the embryo (Coutelle *et al*., 2001; Weinberg *et al*., 1996), whereas *myod/myf5* expression is followed by an up-regulation of *myog* and *mef2ca* family factors (Yun and Wold, 1996). As for *sox6*, studies on zebrafish and mice show that it displays an increase in slow-specific gene expression and a concomitant decrease in the expression of fast-twitch specific genes suggesting that *sox6* normally functions to promote the fast-twitch differentiation and repress slow-specific gene expression in fetal muscle fibers (Hagiwara *et al*., 2007; An *et al*., 2011; Quiat *et al*., 2011; Von Hofsten *et al*., 2008: Harriet *et al*., 2015). Gene expression indicated that all five genes were increased in first and decreased the last during five periods of embryonic development. The expression of *myf5* and *myod* reached a peak in 12hpf (segmentation period), and maintained a high level in 24 hpf (pharyngula period). Besides, gene *mef2ca*, *myog* and *sox6* have a low expression level in blastula and gastrula period, and reached a peak in segmentation and pharyngula period. The results agreed well with the previous literature. Moreover,

most studies focus on the expression of MRFs during embryonic development period. Few studies discussed about the expression in juvenile and adult fish. In our study, we explore the expression of 5 muscle-specific genes in post-embryonic development. The results showed that the expression of 5 genes decreased along with the growth of zebrafish. As the growth of fish and the maturation of muscle fibers, the expression of genes functioning in development of muscle declined slowly which might result in increase in the density and diameter of muscle fiber slowly in the adult zebrafish individuals.

As for the difference of muscle development between anterior to posterior to dorsal fin, this study showed that the expression level of five muscle-specific genes was close to each other which indicated that the development pattern of muscle in anterior to dorsal fin was similar to that of muscle of posterior to dorsal fin. But the density of muscle fibers of posterior to dorsal fin was a little larger than anterior ones, and the diameter of posterior ones was smaller than anterior ones, which might be because of the tail part of fish which required more muscle fibers to provide strength to fish movement compared to front part. The development pattern of muscle is still influenced by its functional requirement.

With regard to the influence of imbs elimination to the development of muscle fiber, our study showed that no negative affect occurred on muscle development from three aspects. In gene expression aspect, there are no significant differences between imbs mutant and WT at the same part of zebrafish during the same growth stage. In muscle histology aspects, there is no significant difference in the muscle fibers' density between imbs mutant and WT of the same part and growth stage. In the aspect of embryonic development and post-embryonic development growth, there was no significant difference between WT and mutants in the rate of fertility, hatching and deformities, body weight and body length. Therefore, we speculated that the elimination of imbs did not affect muscle-specific genes' expression and may not have detrimental influence on muscle's development.

Researchers began to explore the value of eliminating imbs in fish as early as the 1960s. Available studies show that significant difference occurs among fishes of different species and ploidy. Some researches have shown that the existence of imbs is closely related to the evolution of fish. The number of imbs became larger as the teleost appeared. Along with the evolution of fish, the number of imbs decreased gradually; even disappeared completely in some fishes (Patterson *et al*., 1995; Ma *et al*., 2012). Moreover, studies show that because of small body and swim bladder, zebrafish have no need of strong bones but powerful muscles to support body movement. Therefore, primary somites of zebrafish are myotomes, not sclerotomes (Sun, 2008). Some researchers also speculate that imbs might be kind of rudimentary tissues (Lv *et al*., 2007). Therefore, imbs were considered that they only have limited supplementary role in supporting muscles and transmitting strengthen, and the elimination of imbs might not have negative effect on body shape, tissue structure and life activity. However, there are no sufficient number of examples to draw a definitive conclusion that there are no disadvantages after the deletion of imbs in fish with imbs. Through genetic screen methods, we could establish the imbs deletion model to investigate the role of imbs. In this study, we demonstrated that the lack of imbs didn't impact the muscle development and growth of zebrafish, while it is still unknown whether imbs' deletion would have an influence on reproductive performance, avoiding predators, predation and other living activities.

CONCLUSION

In conclusion, intermuscular-bone is a rudimentary organ according to speciation evolution study. Our results indicated that the deletion of Imbs had no influence on the embryonic development and growth and there was no significant difference in muscle structure and development no matter in embryonic or post-embryonic stages. However, it is still unconfirmed whether the deficiency of imbs would be pernicious to fish which already have imbs, and more studies should be carried out to access the impact of the deficiency of Imbs.

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Statement of conflict of interest

The authors declare there is no conflict of interest.

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