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Journal	Anatomia, histologia, embryologia, 36(2): 103-106
URL	<a href="http://hdl.handle.net/10130/443">http://hdl.handle.net/10130/443</a>
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Changes in the muscle fiber properties of the mouse temporal muscle  
after weaning

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With 1 table and 4 figures.

Short title: Muscle Fiber Properties

**Key Words:** myosin heavy chain, MHC2a, MHC2b, mdx, LightCycler<sup>2</sup>

## **Summary**

To clarify changes in the muscle fiber properties of the temporal muscle related to the start of masticatory movement, we immunohistochemically investigated myosin heavy chain (MyHC) isoform protein expression using pre-weaning and post-weaning mice. In addition, we examined the expression of a gene coding for those MHC proteins. Immediately after weaning, isoforms with fast and potent contractility were frequent. This suggests that the temporal muscle plays an important role in a marked functional change in the oral cavity from lactation to mastication, contributing to oral function in cooperation with other masticatory muscles.

## **Introduction**

Many studies into the expression of MyHC isoforms in the process of muscular growth and development have investigated the limb muscles. However, recently, several studies have reported changes in the muscle fiber properties of the head and neck muscles including the masticatory muscle in the process of growth and development (Abe et al., 2002; Doi et al., 2003; Usami et al., 2003). Gojyo et al. focused on weaning in the process of development, and investigated changes in the muscle fiber properties of the mouse masticatory muscle before and after weaning at the protein level. In addition, Shida et al. quantitatively examined changes in the muscle fiber properties of the mouse masticatory muscle at the gene level. These studies suggest that a functional change in the oral cavity after weaning (from lactation to mastication) markedly influences the muscle fiber

properties of the mouse masticatory muscle. Thereafter, another study similarly reported changes in the muscle fiber properties of the mouse tongue muscle after weaning, as demonstrated for the masticatory muscle (Maejima et al., 2005). Thus, the role of the head and neck muscles responsible for ingestion function in the developmental stage has been clarified. The mouse temporal muscle may play an important role in masticatory movement. However, no study has examined changes in the muscle fiber properties of the mouse temporal muscle in the developmental stage.

The muscle-contracting protein, myosin, comprises more than 50% of the total amount of protein in myogenic fibers, and is vital to contractile muscular movement. In particular, myosin heavy chains (MyHC), which constitute the highest proportion of the molecular structure of myosin, most accurately reflect muscular function (Pette and Sartori, 1990). It has become clear in recent years that there are different isoforms of MHC, such as MHC-fast (MHC- 2a, 2b and 2d) and MHC-slow (MHC-1). It has also been reported that among MHC-fast isoforms, the contraction speed of MHC-2b is the most rapid whilst that of MHC-2a is slower. It has also been suggested that composition ratios of these isoforms determine muscle contraction properties (Table 1) (Brueckner et al, 1996; Hori et al., 1998; Schiaffino and Reggiani, 1996).

In this study, to clarify changes in the muscle fiber properties of the temporal muscle related to the start of masticatory movement, we immunohistochemically investigated MHC isoforms using pre-weaning and post-weaning mice. In addition, we examined the expression of a gene coding

MHC protein at the transcription level.

## **Materials and Methods**

### **Animals and treatment**

As the mean interval from birth until weaning in mice is about 3 weeks (Gojyo et al., 2002), we used 2-week-old, 4-week-old, and 9-week-old ICR mice (immediately before weaning, immediately after weaning, and adulthood, respectively). After 3 weeks of age, these mice were separated from their parent mice, and solid food was given. We employed 5 mice per age group for immunohistochemical investigation, and 5 mice per age group for the investigation of mRNA expression (total: 30 mice). Based on the guidelines for laboratory animals established in Tokyo Dental University, these mice were killed by deep anesthesia with pentobarbital sodium. Immediately after the temporal muscle was extirpated (Fig. 1), specimens were snap-frozen in liquid nitrogen, and stored in isopentan at -80°C until this experiment.

### **Immunostaining**

Using a cryostat, we prepared serial frozen sections 8- $\mu$ m thick transversely crossing the longitudinal axis of muscle fibers. Immunostaining was performed as described below. As primary antibodies, we used mouse monoclonal antibodies (Eason et al., 2000; Schiaffino et al., 1996) extracted from hybridoma cells, SC-71 (anti-MyHC-2a) and BF-F3 (anti-MyHC-2b)(American Type Tissue Culture); hybridoma cells were cultured in DMEM (10%FBS) at 37°C for 72 hours

under a condition of 5%CO<sub>2</sub>, and centrifuged to isolate the supernatant as an antibody. As secondary antibodies, we employed an FITC-labeled goat anti-mouse IgG antibody against SC-71, and an RITC-labeled goat anti-mouse IgM antibody against BF-F3 (Novocastora Laboratories, Newcastle, UK) for detection. For observation and photographing, a cofocal laser microscope (MRC-1024/2P; Nippon Bio-Rad Lab., Tokyo, Japan) was used. FITC-labeled muscle fibers were regarded as positive for MHC-2a, and RITC-labeled muscle fibers were regarded as positive for MHC-2b. The contrasts of the images were managed by computer to make the morphology of all cells clear. The stained and unstained cells were observed based on these images.

#### **RNA extraction and the analysis of mRNA expression using a Light Cycler<sup>®</sup>**

We quantified MHC-2a and MHC-2b mRNAs in each site at each age using a Light Cycler<sup>™</sup> (Roche Diagnostics, Mannheim, Germany), which facilitates the measurement of RNA levels. Total RNA in each site at each age was extracted using a Quick Prep Micro mRNA Purification Kit (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK), and cDNAs were prepared with a Ready-To-Go kit (Amersham Pharmacia Biotech UK Ltd.). After the optimal conditions for all primers were established, this experiment was conducted according to the standard protocol of the Light Cycler<sup>™</sup>. As a hot start PCR reaction buffer for the Light Cycler<sup>™</sup>, we used adjusted LC Fast Start DNA Master SYBR Green I (Roche). We prepared the dilution series. Then, it was mixed with 5  $\mu$ l of each diluted PCR product so that the final reactive capacity reaction volume was 20  $\mu$ l.

Concerning PCR mixtures for detection of transcripts in experimental samples, 14.2  $\mu$ l of sterile water was mixed with 1.6  $\mu$ l of  $MgCl_2$  (25 mM) and 2  $\mu$ l of LC Fast Start DNA Master SYBR Green I. In addition, the solution was mixed with 0.6  $\mu$ l of a forward primer (10 pmole/ $\mu$ l) and 0.6  $\mu$ l of a reverse primer (10 pmole/ $\mu$ l), which were prepared using an Oligo 5 Primer Design (Biogene, Ltd.). Then, it was mixed with 5  $\mu$ l of each diluted PCR product so that the final reactive capacity was 20  $\mu$ l. We employed MyHC-2b and MyHC-2a primers; the two primers were designed by extracting specific sites from the full sequences of MyHC-2b and MyHC-2a DNAs. The base sequences of MyHC-2b and MyHC-2a were as follows: MyHC-2b (Forward: 5'-ACAGACTAAAGTGAAAGCC-3', Reverse: 5'-CTCTCAACAGAAAGATGGAT-3'; Accession: XM $\mu$ 126119), and MyHC-2a (Forward: 5'-CGATGATCTTGCCAGTAATG-3', Reverse: 5'-TGATAACTGAGATACCAGCG-3'; Accession: NM $\mu$ 144961).

Concerning PCR mixtures, 14.2  $\mu$ l of sterile water was mixed with 1.6  $\mu$ l of  $MgCl_2$  (25 mM) and 2  $\mu$ l of LC Fast Start DNA Master SYBR Green I. In addition, the solution was mixed with 0.6  $\mu$ l of a forward primer (10 pmole/ $\mu$ l) and 0.6  $\mu$ l of a reverse primer (10 pmole/ $\mu$ l). Then, it was mixed with 1  $\mu$ l of the target DNA so that the final reactive capacity was 20  $\mu$ l. The two PCR mixtures (20  $\mu$ l)(MyHC-2b and MyHC-2a) were added to the capillary glass tube. Cycle conditioning was performed at 95°C for 10 minutes, 95°C for 10 seconds, 62°C for 10 seconds, and 72°C for 7 seconds. A total of 50 cycles were performed. Gene amplification was performed according to a melting program (70°C, 15

seconds). In the transitional phase from 70°C to 95°C, fluorescence was serially monitored at a rate of 0.1°C per second. Concerning the fluorescence channel, we employed F1 (530 nm), and the gains indicated 89.9°C for MyHC-2b and 88.2°C for MyHC-2a. We calculated mRNA expression by dividing the levels of MHC isoforms, which were calculated as described above, by the level of GAPDH, which was used as a House Keeping Gene. The base sequence of GAPDH was as follows: Forward: 5'-TGAACGGGAAGCTCACTGG-3', Reverse: 5'-TCCACCACCCTGTTGCTGTA-3'; Accession: NM<sub>μ</sub>008084.

Cycling conditions were one cycle of denaturation at 95°C for 10 minutes followed by 50 cycles of an amplification sequence that consisted of denaturation for 10 seconds at 95°C, annealing for 10 seconds at 62°C and extension for 7 seconds at 72°C.

## **Statistical analysis**

Student's *t*-test was used for statistical analysis in this study.

## **Results**

### **Immunostaining**

The immunohistochemical images are shown in Fig. 2. In the 2-week-old mice, a large number of cells were positive for the MyHC-2a antibody. Furthermore, there were no MyHC-2b antibody-positive cells. In the 4-week-old mice, the number of MyHC-2b antibody-positive cells was similar to that of MyHC-2a antibody-positive cells. The MyHC-2b antibody-positive cells were slightly larger.



In the 9-week-old mice, the number of MyHC-2a antibody-positive cells was decreased, and the number of MyHC-2b antibody-positive cells was markedly increased. There were no age-related changes in the size of MyHC-2a antibody-positive cells. However, the size of MyHC-2b antibody-positive cells at 9 weeks of age was 2 times larger than that at 4 weeks of age.

### **Analysis of mRNA expression using a Light Cycler (Figs. 3 and 4)**

MHC-2a mRNAs were frequent in the 2-week-old mice. In the 4-week-old mice, MHC-2a mRNA expression was about one-third of the value. In the 2-week-old mice, only a few MHC-2b mRNAs were detected; however, MHC-2b mRNA expression was increased in the 4-week-old mice, and was further increased in the 9-week-old mice.

### **Discussion**

Concerning the isoforms of myosin, a fast muscle type muscle-contracting protein, MHC-2b contributes to the fastest contractions, and MHC-2a contributes to the slowest contractions (Bottinelli et al., 1991; Sakiyama et al., 2005). Muscular function can be compared based on the ratios of the two isoforms. Several studies compared the two isoforms using mouse masticatory muscle and tongue specimens in the developmental stage, and reported a marked change in the muscle fiber properties after weaning. These studies suggest that, in the oral region responsible for ingestion, the composition of muscle isoforms changes in response to various functional changes represented by weaning. No study has

investigated the mouse temporal muscle, which was examined in this study. Our data resembled those previously reported for the mouse masticatory muscle. According to previous studies, the MyHC-2a level in the superficial masticatory muscle remained high after weaning, that is, at 4 weeks of age. However, in the temporal muscle, the MyHC-2b level was increased to the MyHC-2a level at 4 weeks of age.

Many studies have reported the functional role of MyHC-2b. A study using the musculus extensor digitorum longus (EDL) of adult rats, which is classified in the fast muscle category, as is the mouse masticatory muscle, and considered to require potent contractility, showed that EDL consisted of MHC-2b alone. In addition, experimental EDL hypofunction related to denervation resulted in the expression of MHC-2a, decreasing the expression of MHC-2b (Erzen et al., 1999). This suggests that EDL requiring potent contractility finally acquires the MHC-2b isoform, and that the hypofunction-related absence of the necessity of potent contractility leads to the expression of MHC-2a. In addition, the contractility of MHC-2a may be less potent than that of MHC-2b. Based on the study results, various studies have accepted the functional roles of MyHC-2b and MyHC-2a without objection. These findings suggest that the mouse temporal muscle exhibits potent contractility in the early phase after weaning, followed by the superficial masticatory muscle.

Previously, some studies involving humans compared the muscle fiber properties of the temporal muscle, and indicated that the temporal muscle consisted of slow muscle type isoforms and fast muscle type isoforms.

According to a study (Eriksson and Thornell, 1983), the ratio of fast muscle type isoforms in the temporal muscle was higher than that in the masticatory muscle. The role of the temporal muscle in mastication function differs between mice and humans; however, in both mice and humans, the temporal muscle exhibited more potent contractility during masticatory movement compared to the masticatory muscle, contributing to mastication.

This study suggests that a change in oral function, that is, weaning, influences the properties of the temporal muscle at a timing different from that for other masticatory muscles, and that the temporal muscle acquires muscle fiber properties to play a functional role in the oral cavity. For complex masticatory movement, various muscles with different roles may comprehensively function.

## **Acknowledgements**

This study was supported by grants-in-aid for scientific research (14704046: S.A.) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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### **Further reading**

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## Figure legends

### Fig. 1. Observation specimen

The course of the mouse temporal muscle is from the posterior superior direction to the inferior anterior direction against the bite plane. This muscle may pull the mandible in the posterior superior direction.

### Fig. 2. Immunohistochemical staining

Green cells were positive for the MyHC-2a antibody, and red cells were positive for the MyHC-2b antibody (Bar=100  $\mu$ m) (Shida et al., 2005). In the 2-week-old mice, there were no MyHC-2b antibody-positive cells. However, these cells were frequent in the 4-week-old mice. At 9 weeks of age, the greater portion consisted of MyHC-2b fibers. Furthermore, there were no marked changes in the muscle fiber size for MyHC-2a. However, the muscle fiber size for MyHC-2b was serially increased.

### Fig. 3. Expression of MHC-2a mRNA (Light Cycler) (Shida et al., 2005)

MHC-2a mRNAs were frequent in the 2-week-old mice. In the 4-week-old mice, MHC-2a mRNA expression was about one-third of the value.

### Fig. 4. Expression of MHC-2b mRNA (Light Cycler) (Shida et al., 2005)

In the 2-week-old mice, only a few MHC-2b mRNAs were detected; however, MHC-2b mRNA expression was increased in the 4-week-old mice, and was further increased in the 9-week-old mice.

Table 1 Myosin heavy chain isoforms identified in skeletal muscle

Designation	Nomenclature	Distribution
Embryonic	MHC <sub>emb</sub>	Myobubes, intrafusal fibers, regenerating fibres
Neonatal	MHC <sub>neo</sub>	Neonatal muscles, masseter, intrafusal fibres
Fast-twitch	MHC-2b	} Fast-type isoforms in masseter of mice > contraction speed: 2b > 2d > 2a
Fast-twitch	MHC-2d	
Fast-twitch	MHC-2a	
Fast-twitch	MHC <sub>com</sub>	Super-fast fibers in extraocular muscles
Fast-twitch	MHC-2m	Super-fast fibers in muscles derived from the first branchial arch
Slowtwitch	MHC-1	Type 1 fibers

Source: Brueckner et al. (1996)

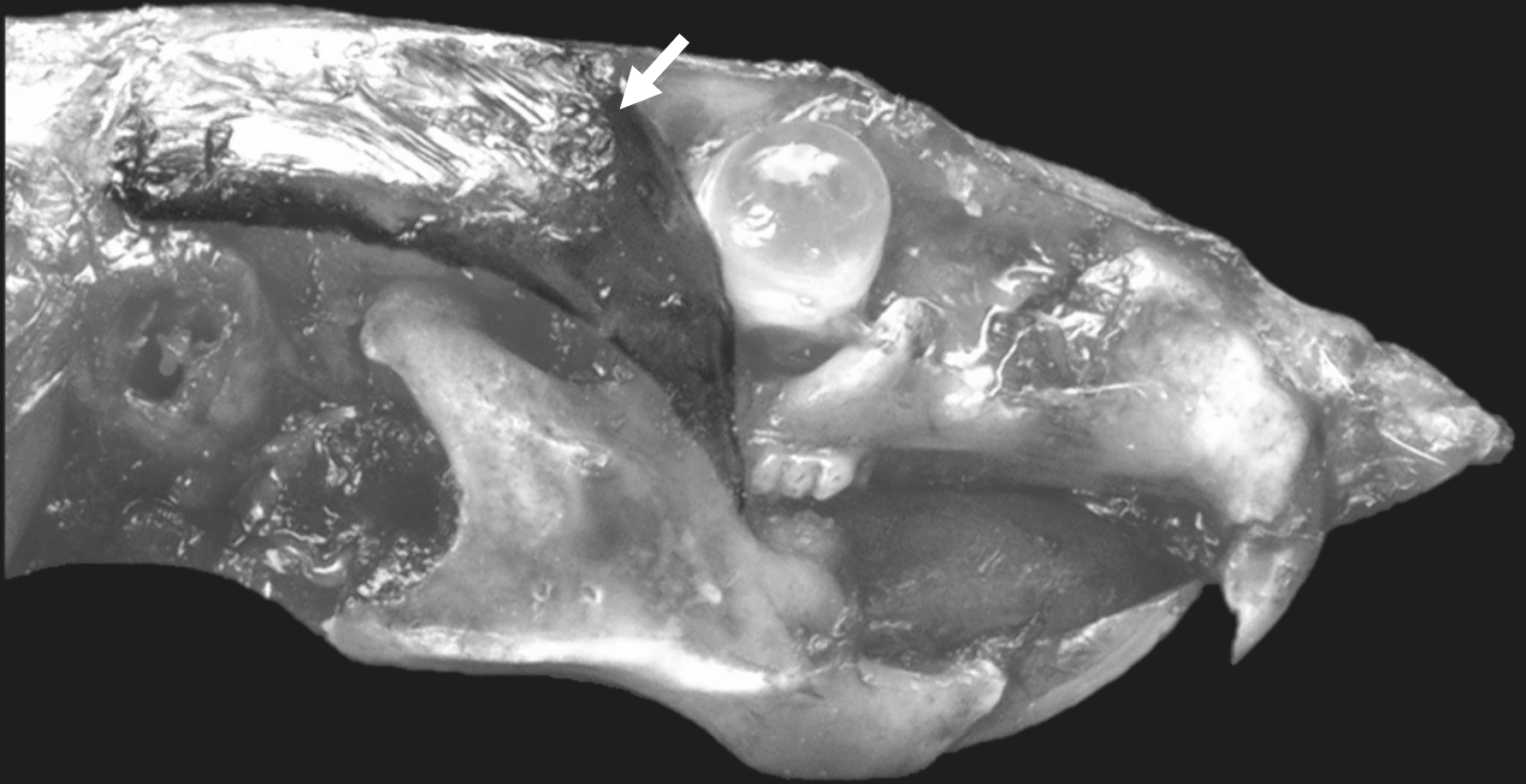
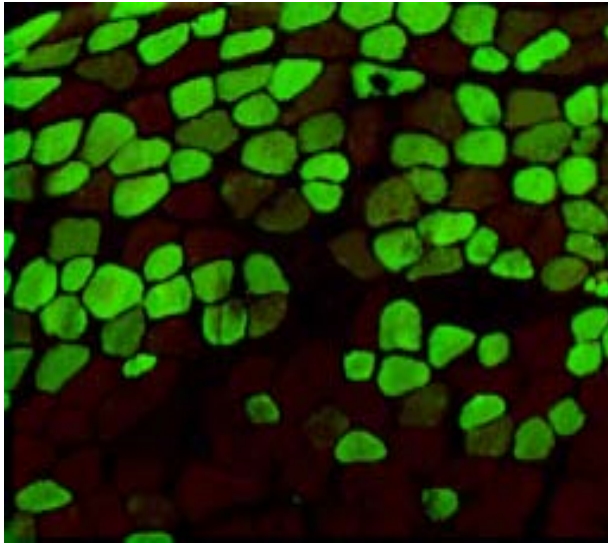


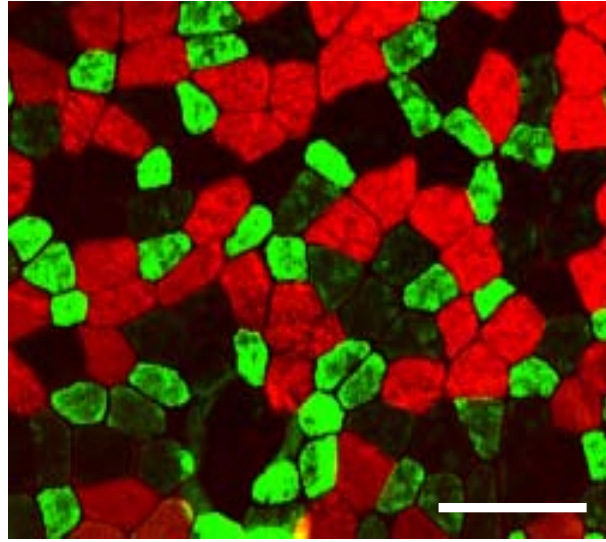
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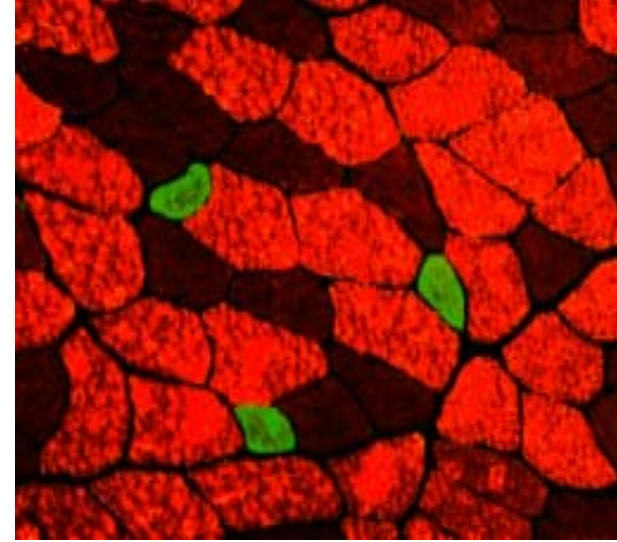




**2weeks**



**4weeks**



**9weeks**

Fig. 2. Immunohistochemical staining

Green cells were positive for the MyHC-2a antibody, and red cells were positive for the MyHC-2b antibody (Bar=100  $\mu\text{m}$ ). In the 2-week-old mice, there were no MyHC-2b antibody-positive cells. However, these cells were frequent in the 4-week-old mice. At 9 weeks of age, the greater portion consisted of MyHC-2b fibers. Furthermore, there were no marked changes in the muscle fiber size for MyHC-2a. However, the muscle fiber size for MyHC-2b was serially increased.

**\*p<0.05**

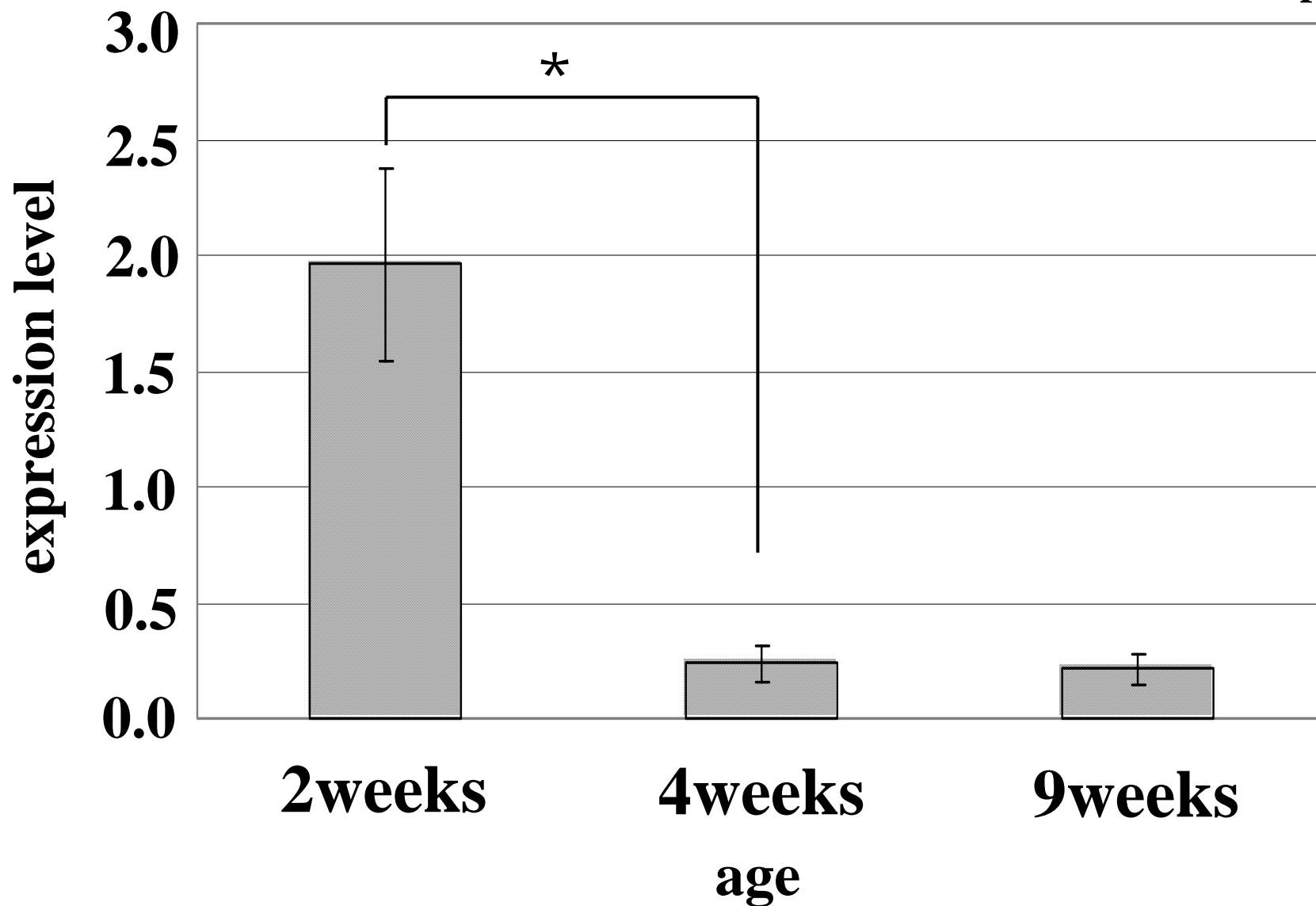


Fig. 3. Expression of MyHC-2a mRNA (Light Cyclor)  
MyHC – 2a/GAPDH

**\*p<0.05**

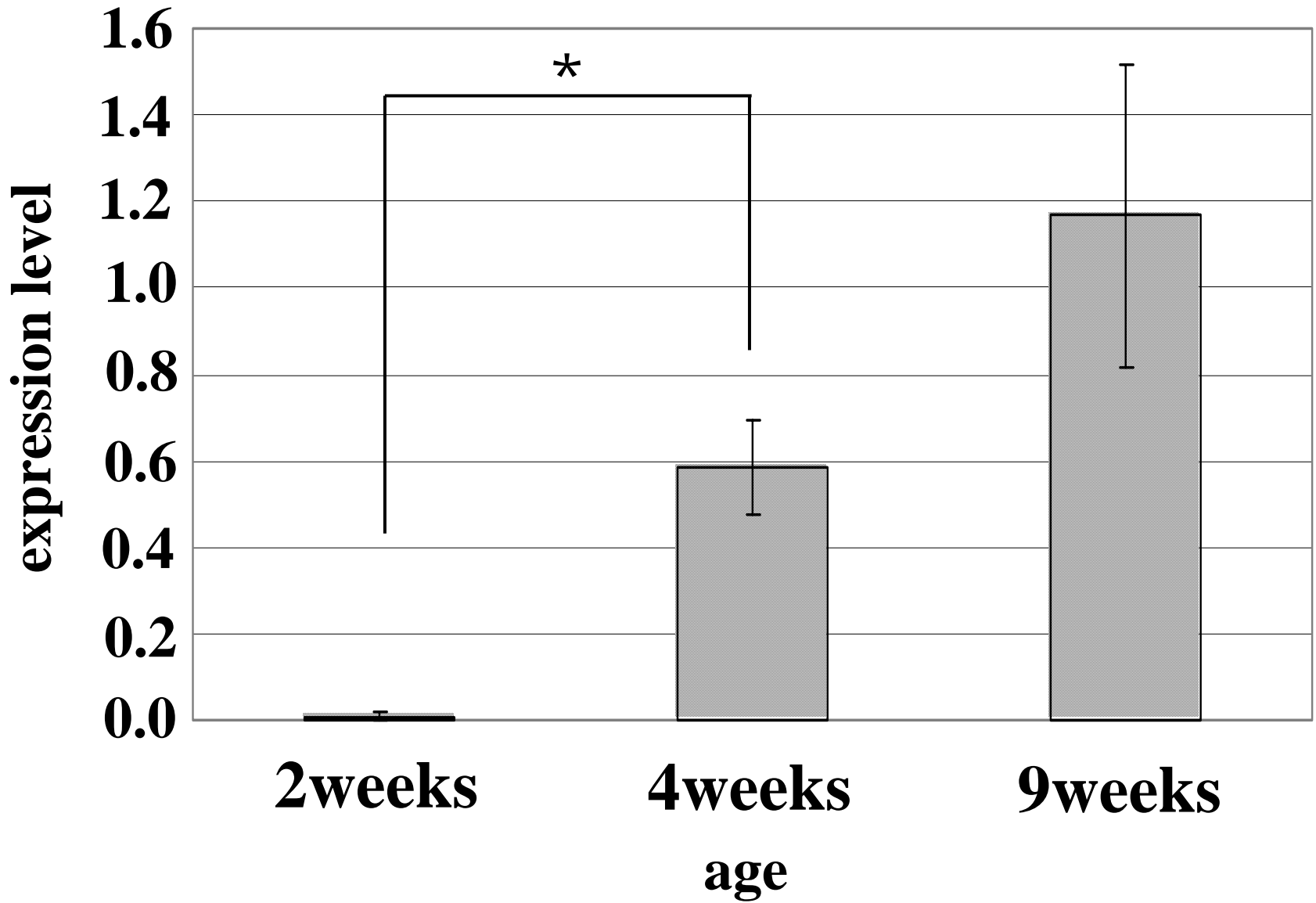


Fig. 4. Expression of MyHC-2b mRNA (Light Cycler)  
MyHC – 2b/GAPDH