Development of the Lateral Muscle in the Japanese Sardine *Sardinops melanostictus**

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Histochemical characteristics of lateral muscle fiber types in the Japanese sardine *Sardinops melanostictus* were confirmed and their developmental process investigated. In 102 and 106 mm young specimens in standard length (SL), four muscle fiber types, i.e., red, pink, white, and tonic-like fibers were recognized. Pink fibers mainly occurred in the anterior trunk region. Two types of tonic-like fibers were recognized around the red triangular wedge along the horizontal septum. Newly-hatched larvae (free embryos) had a single layer of superficial fibers containing a few myofibrils and inner white fibers. Histochemical reactions for NADHD and lipid in the superficial fibers gradually increased and became strong enough to be classified as red fibers by the first-feeding stage. Beyond about 20 mm SL, stratification of the red fibers, differentiation of the tonic-like fibers, and mosaic appearance of the white fibers occurred. By 30 mm SL, pink fibers and two types of tonic-like fibers were differentiated. The structure of the lateral muscle in a 37 mm SL juvenile was complete and did not differ from those of young specimens, 102 and 106 mm SL.

Key words: Japanese sardine, *Sardinops melanostictus*, lateral muscle, red fiber, white fiber, pink fiber, tonic-like fiber, larval development

The Japanese sardine *Sardinops melanostictus* is one of the most important commercial fish in Japan for human food consumption, animal feeds, and fertilizer. Many studies about the resource of this species have been done,¹⁾ but its biological knowledge is not sufficient, especially about developmental biology.^{2,3)}

In spite of much research on the lateral muscle structure of adult fish, there is little literature on the developmental processes of lateral muscle.^{4–9)} In the Clupeidae, Batty¹⁰⁾ and Fukuda¹¹⁾ reported that swimming style or speed change with growth and development of lateral muscle in herrings *Clupea harengus* and *C. pallasii*, respectively. Recently, it was examined that temperature influences on muscle differentiation and the relative timing of organogenesis¹²⁾ and developmental plasticity of muscle phenotype¹³⁾ in *C. harengus* larvae. In most fish, the lateral muscle consists of two major zones, red and white muscles. The red muscle relies on an aerobic form of metabolism while the white muscle operates principally by anaerobic glycolysis.¹⁴⁾ The former is functional in sustained swimming and the latter during in bursts of high activity. Besides, the pink muscle and very small diameter fibers (sdf) have been reported in some teleosts.^{8,9)} The early development of the lateral muscle may influence the ability to feed and survival of larvae. This report clarifies the types of lateral muscle fibers and their differentiation and development in *S. melanostictus*.

Materials and Methods

Specimens

Specimens examined were laboratory-reared, using wild

eggs collected by plankton net, from newly-hatched (3.65 mm in notochord length (NL)) to 16.2 mm NL larvae which required for 29 days. Further, specimens smaller than 37 mm in standard length (SL) were collected from the field by a scoop net using a fish gathering lamp. Larger specimens (85–178 mm SL) were obtained from a commercial catch by a seine net.

Histology

Larvae and juveniles were fixed in Bouin's solution and embedded in paraffin. Sections were cut at 6–10 μ m and stained by alcian blue-hematoxylin-eosin or azan stain.

Histochemistry

Larvae and juveniles were frozen immediately in isopentane cooled with dry ice, either as a whole body or the head removed, depending on body size. For 85–106 mm SL specimens, before freezing, the muscular blocks near the horizontal septum were dissected out from three parts of the body, anterior trunk region, central region, and posterior caudal region. These samples were preserved with dry ice in the jar until examination. Frozen transverse sections were cut at 8–10 μ m, mounted on glass coverslips, and dried in a desiccator at –20°C for one night. Histochemical reactions were examined by the following methods:

(1) Lipid. Sections were stained with a saturated solution of Sudan Black B in 70% ethyl alcohol for 5–30 min at room temperature and mounted in glycerin-gelatin.

(2) Succinate dehydrogenase (SDH, E. C. 1. 3. 99. 1). Sections were stained by the method of Barka and Anderson.¹⁵⁾ Sections were incubated in a stain solution for three

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hours at room temperature and mounted in glycerin-gelatin.

(3) NADH dehydrogenase (NADHD, E. C. 1. 6. 99. 3). Sections were stained by the method of Barka and Ander-

son.¹⁵⁾ Sections were incubated in a stain solution for 15-60 min at room temperature and mounted in glycerin-gelatin.

Fig. 1. Cross sections of fresh lateral muscle in adult Japanese sardine, 178 mm in standard length (SL).

A: anterior trunk region. B: central region. C: posterior caudal region. P, pink muscle; R, red muscle; W, white muscle. Vertical direction. Scale bar indicates 10 mm.

Fig. 2. NADHD activities of cross sections of lateral muscle in 102 (A and B) and 106 mm SL (C and D) Japanese sardine.

A: central region. B: magnified section of the red triangular wedge in A. Note two types of tonic-like fibers (arrows). C: anterior trunk region. D: posterior caudal region. P, pink muscle; R, red muscle; T, tonic-like fibers; W, white muscle. Horizontal (A, B, C) and vertical (D) directions. Scale bars indicate 500 μ m.

Results

Fiber Types

Figure 1A, B, and C show the cross sections of three regions of the lateral muscle (anterior, central, and posterior) of a fresh adult sardine, 178 mm SL. Red and white muscles were observed in all the regions, but only the anterior region had distinct pink muscle.

Red muscle contains lipid and abundant large mitochondria, while white muscle has little lipid and few smaller mitochondria.¹⁴ So, histochemical staining of lipid or oxidative enzyme activity (NADHD, SDH, etc.) in mitochondria can use in discrimination between red and white mus-

cles. Pink muscle shows intermediate reaction for lipid, NADHD, and SDH.⁸⁾ Figure 2A shows the NADHD activity of the central region of a 102 mm SL young specimen. Two main zones, red and white muscles, occupied most of lateral muscle, and between these types there were a few pink muscle fibers. Figure 2B is a magnified section of the red triangular wedge in Fig. 2A. There were very weakly reacted fibers for NADHD (arrow) similar to tonic-like fibers in the roach.¹⁶⁾ Some fibers, however, showed a relatively strong reaction for NADHD at the apex of the red muscle "triangle" (arrow). Away from the horizontal septum, red and white muscles adjoined through a boundary of a thin fibrous tissue. Figure 2C indicates the anterior region of a 106 mm SL specimen, the pink muscle forming

Fig. 3. Initial development of superficial red and white fibers in free embryonic (prelarval) and larval Japanese sardines.

A: histological section of a 3.7 hour-old larva. B: NADHD activity of a 3.7 hour-old larva. C: NADHD activity of a 33 hour-old larva. D: NADHD activity of a 58 hour-old larva. E: NADHD activity of a 82 hour-old larva (at the first-feeding stage). F: NADHD activity of a 14.40 mm in notochord length (NL) larva. NC, notochord; SF, superficial fibers; SR, superficial red fibers; W, white fibers. Horizontal direction. Scale bars indicate 50 μ m.

Fig. 4. Development of lateral muscle in larvae and juveniles of the Japanese sardine.

A: NADHD activity of a 20.4 mm SL larva. Arrows show the original superficial red fibers at both sides of the horizontal septum. B: NADHD activity of anterior trunk region of a 27.7 mm SL larva. C: azan stain of central region of a 29.25 mm SL larva. Note two types of tonic-like fibers (arrows). D: NADHD activity of anterior trunk region of a 37.0 mm SL juvenile. P, pink muscle; R, red muscle; SR, superficial red fibers; SSR, second layer of superficial red fibers; T, tonic-like fibers; W, white muscle. Horizontal direction. Scale bars indicate 50 μ m.

a distinct layer between red and white muscles. Figure 2D shows the posterior caudal region of 106 mm SL specimen where only red and white muscles were recognized.

Development of Muscle Fibers

In 3.7 hour-old larvae after hatching (3.65 mm NL, free embryos), there were several layers of the developing white fibers containing myofibrils. A single layer of superficial fibers containing a few myofibrils was observed (Fig. 3A). Histochemical reactions could not distinguish the superficial fibers from the inner white fibers at this stage (Fig. 3B). In 33 hour-old larvae, NADHD activity in the superficial fibers became clearer than in the inner white fibers (Fig. 3C). In 58 hour-old larvae, the reaction in the superficial fibers was distinct (Fig. 3D), and in 82 hour-old larvae (5.6 mm NL) at the first-feeding stage, the superficial fibers showed a very unambiguous reaction for NADHD sufficient to be termed as the superficial red fibers (Fig. 3E). The reaction for lipid was similar to NADHD, although SDH activity was very weak in *S. melanostictus*.

The increase in the number of muscle fibers after the first-feeding stage occurred only in the dorsal and ventral direction. Beyond about 13.5 mm NL, newly formed small white fibers were recognized laterally outward, so the larval body width began to increase.

The structure of the lateral muscle such as a superficial single layer of red fibers surrounding the inner white

fibers, remained until 16–17 mm NL (Fig. 3F). Beyond this size, a superficial single layer of red fibers began to bend to the inner region along the horizontal septum. In a 20.4 mm SL larva, a second layer of the superficial red fibers was formed. The original superficial red fibers at both sides of the horizontal septum consisted mostly of myofibrils (Fig. 4A, arrows). These fibers seemed to develop to tonic-like fibers by later division into small fibers. Small white fibers began to be formed among the large existing white fibers, so that the white muscle showed a mosaic appearance after about 20 mm SL.

Figure 4B shows the anterior region of the lateral muscle of a 27.7 mm SL larva in which the pink muscle fibers were formed. In the central region of a 29.25 mm SL larva, there were two types of tonic-like fibers distinguished from the azan stain (Fig. 4C, arrows). In a 37.0 mm SL juvenile, the anterior trunk region consisted of red, white, pink, and tonic-like fibers as similar to young specimens, 102 and 106 mm SL (Fig. 4D).

Discussion

Fiber Types

In *Pagrus major*,⁸⁾ the pink muscle develops from the anterior trunk region through to the caudal region. In *S. melanostictus*, however, the pink muscle exists mainly in the anterior trunk region. Johnston *et al.*¹⁷⁾ suggested the

pink muscle is associated with intermediate swimming speeds between sustained and burst swimming in the carp. It seems that intermediate swimming speeds may be less important for *S. melanostictus*. Higgins¹⁸⁾ suggested that the pink fibers are most evident in younger fish and become less distinct with growth in the Atlantic salmon *Salmo salar*. In *S. melanostictus*, however, an adult specimen had the pink muscle in the anterior trunk region (Fig. 1A).

One type of the tonic-like fibers with a very weak reaction for NADHD closely resembled the very small diameter fibers (sdf).¹⁶⁾ In *P. major*⁸⁾ and other there were some teleosts,^{19–22)} these fibers usually exist between the red and pink muscles. However, in *S. melanostictus*, the tonic-like fibers concentrated in the red triangular wedge along the horizontal septum, and the other type having relatively strong reaction for NADHD was observed. Gillerman²³⁾ found in *Scomber scombrus* a muscle fiber group at the apex of the red muscle triangle and thought it intermediate (pink) muscle from histochemical reactions. The other type of the tonic-like fibers in *S. melanostictus*, however, had small diameter and reacted more strongly for NADHD than pink fibers. The contractile system in tonic muscle fibers reflects their capacity for generating a sustained force involved in maintaining tonicity of relaxed twitch-muscle system.¹⁶⁾ The tonic-like fibers in *S. melanostictus* seem to be connected to the adjacent bases of the cartilage chevrons (intermuscular cartilages) which exist between anterior 33–34 myotomes.³⁾

Development of Muscle Fibers

The superficial cells in newly-hatched *P. major* larvae possess no myofibrils.⁸⁾ In *S. melanostictus*, however, a few myofibrils are contained in the superficial fibers, although the difference can not be detected from histochemical characters between the superficial fibers and the inner white fibers. At the first-feeding stage, the superficial single layer of red fibers which react strongly for NADHD and lipid is formed. These fibers function for initial, sustained, and slow swimming. Larvae at this stage do an undulation type of swimming, similar to eel, and obtained food organisms by S-stroke movement. The former swimming motion is thought to be conducted by the superficial red fibers and the latter movement by the inner white fibers. True sustained swimming can occur only after the stratification of the red fibers and is related to the filter feeding of fine organisms by *S. melanostictus*.

Distinct changes in the lateral muscle development occurred at the first-feeding stage and between about 20 and 30 mm SL. At the first-feeding stage, larvae have aerobic metabolism as red fibers, mentioned above. Beyond about 20 mm SL, clear stratification of red fibers around the horizontal septum, differentiation of the tonic-like fibers, and appearance of small white fibers among large fibers occur. By about 30 mm SL, the two types of the tonic-like fibers are recognized and the pink fibers differentiate. Matsuoka³⁾ osteologically defined the ontogenetic intervals in *S. melanostictus* which are composed of four periods and eight phases (see, Fig. 17). Distinct changes of the lateral muscle development almost occur parallel to the osteological development. For example, at the first-feeding stage, the cartilaginous-osteological elements develop in the jaw, suspensorium, hyoid arch, lower branchial arches, neu-

rocranium, and pectoral fin supports. At about 20 mm SL, ossification of various skeletal elements occurs, e.g., the neurocranium and vertebral column. Metamorphosis (rapid morphological changes from larva to juvenile) based on the osteological development lasts from 20 to 34 mm SL. At 34 mm SL, most basic osteological elements are established and completion of the structure occurs at 60–70 mm SL. In the lateral muscle development, all structures appear by about 30 mm SL, earlier than the osteological development. In this respect, fiber growth such as number, diameter, and area seems to perform an important role in the lateral muscle development. Beyond about 30 mm SL, true sustained swimming by the red muscle, intermediate speed swimming by the pink muscle, and burst swimming by the white muscle begin to be properly used for feeding, avoidance, and another behavior with respective fiber growth. Moreover, the maneuverability in swimming seems to increase from this size, with the aid of fin rays which are formed by 28.4 mm SL.

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