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ORIGINAL ARTICLE

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The development of muscle fiber type identity in zebrafish cranial muscles

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Abstract Cranial skeletal muscles underlie breathing, eating, and eye movements. In most animals, at least two types of muscle fibers underlie these critical functions: fast and slow muscle fibers. We describe here the anatomical distribution of slow and fast twitch muscle in the zebrafish (*Danio rerio*) head in the adult and at an early larval stage just after feeding has commenced. We found that all but one of the cranial muscles examined contain both slow and fast muscle fibers, but the relative proportion of slow muscle in each varies considerably. As in the trunk, slow muscle fibers are found only in an anatomically restricted zone of each muscle, usually on the periphery. The relative proportion of slow and fast muscle in each cranial muscle changes markedly with development, with a pronounced decrease in the proportion of slow muscle with ontogeny. We discuss our results in relation to the functional roles of each muscle in larval and adult life and compare findings among a variety of vertebrates.

Keywords Slow muscle · Feeding · Pharyngeal arches · Myosin · Fish

Introduction

Differences in feeding and swimming performance are often due to architectural differences in musculoskeletal design and basic cytoarchitectural differences within individual muscles. For example, the relative proportion of fast and slow trunk muscle fibers correlates well with swimming behavior in a variety of fishes (Mosse and Hudson 1977). Fiber type composition has a significant effect on animal performance (Mosse and Hudson 1977; Ono and Kaufman 1983), and because it is hereditary in rats (Suwa et al. 1996, 1998), it is likely hereditary in other vertebrates. Thus, evolutionary changes in muscle fiber type may play a key role in the evolution of different locomotory styles.

Ontogenetic changes in the number and distribution of slow and fast twitch fibers also occur as animals mature from larvae into adults. For example, in many teleosts slow fibers in the early larval trunk form a monolayer on the surface of the fast fibers, whereas slow fibers in the adult form a comparatively small region at the position of the horizontal myoseptum (Stoiber et al. 1999). These ontogenetic changes in anatomy may be necessary to provide optimal swimming movements in fish of different sizes, which use different swimming modes. Ontogenetic changes in feeding behavior are at least as important as changes in swimming behavior (Liem 1991; Osse 1990). However, although the biomechanics and functional morphology associated with feeding in teleost fishes are well understood (Ferry-Graham and Lauder 2001; Westneat 1990, 2003), much remains unknown about the muscle fiber type composition of muscles involved in feeding. Understanding the developmental changes in the size and fiber type composition of cranial muscles may suggest mechanisms for these behavioral changes. The zebrafish has emerged as a powerful system for studying vertebrate development because of the combination of genetics and embryology that it affords. These advantages have led to rapid advances in understanding fiber type patterning in trunk muscles (Currie and Ingham 2001; Stickney et al. 2000). Comparatively little research has been done on fiber type patterning in cranial muscles.

The musculoskeletal system of the zebrafish head, as in other vertebrates, is assembled from muscle precursors derived from head and trunk mesoderm and from cartilage precursors derived primarily from the cranial neural crest (Noden 1983; Schilling and Kimmel 1994,

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1997). During early cranial development, three streams of neural crest cells migrate into the pharynx and together with cranial paraxial mesoderm and overlying ectoderm form seven pharyngeal arches, which will give rise to the musculoskeletal elements of the jaws and gills. The first two arches give rise to musculoskeletal elements associated with movements of the jaws, while the next five arches, known as the branchial arches, are associated with support and movement of the gills. Neural crest cells within the first arch give rise to the palatoquadrate and Meckel's cartilage (mandible). Muscles associated with the first arch include the adductor mandibulae, the intermandibularis muscles, the levator arcus palatini, and the dilator operculi. Neural crest cells within the second arch give rise to the hyosymplectic as well as the different elements of the hyoid (basihyal, interhyal, and ceratohyal). Muscles associated with this arch include the interhyoideus, hyohyoideus, adductor hyomandibulae, adductor operculi, and levator operculi. Finally, neural crest cells within the last stream give rise to the cartilaginous elements associated with the gills. Muscles associated with these arches include the transversus ventralis and branchial levators. In addition to these muscles derived from cranial mesoderm, a long muscle derived from trunk paraxial mesoderm, the sternohyoideus, underlies the transversus ventralis (Schilling and Kimmel 1997).

We describe the distribution and relative proportion of fast and slow muscle in larval and adult zebrafish, with particular emphasis on muscles previously determined to be important in feeding (Hernandez 2000; Hernandez et al. 2002). We found during growth a marked decrease in the proportion of slow muscle within any given cranial muscle. We discuss the functional, evolutionary, and developmental implications of our data.

Materials and methods

Wild-type zebrafish from the Oregon AB line maintained at Wesleyan University since 1997 were used for this analysis. Larvae used were 6 days postfertilization and had a mean total length (TL) of 3.6 mm. Adults used had a mean standard length (SL) of 26 mm. The "Principles of Animal Care" of the National Institutes of Health (USA) were followed.

For immunohistochemistry on sections, larvae were fixed in Carnoy's solution (60% ethyl alcohol, 30% chloroform, 10% glacial acetic acid) overnight at room temperature. Adults were fixed for several days in Carnoy's at 4°C. Fish were dehydrated through an ethanol series to 95% ethanol and then rehydrated into phosphate buffered saline (PBS) before being embedded in 1% agar before sectioning. Agar blocks were placed in a 30% sucrose solution overnight and then frozen in a Leica cryostat. Sections (12 μm) were mounted on Fisher Superfrost slides. Slides were placed on a 40°C hot plate for 2 h after sectioning and then stored at −20°C.

Sections were rehydrated using PBT (0.1% Tween in PBS). Nonspecific binding was blocked using blocking solution (PBT with 1% bovine serum albumin and 5% normal goat serum). After several minutes, blocking solution was removed, and primary antibodies diluted in blocking solution were applied at a concentration of 5 μg/ml. Primary antibodies used were S58, which stains slow muscle fibers, and MF20, which stains all muscle fibers. Fibers that stained with MF20 but not S58 were assumed to be fast muscle fibers. After 45 min, antibody was removed. Two quick rinses followed by three longer washes with PBT were then done. Blocking solution was then applied for several minutes as before. Secondary antibodies (Jackson ImmunoResearch) diluted in blocking solution were then applied for 30 min. Sections were washed as before with PBT. Hoechst stain (0.3 ng/ml in PBT) was then applied to serve as a nuclear counterstain. After several minutes Hoechst was washed away with several washes of PBT. Slides were coverslipped in 50% glycerol and stored at −20°C when not being viewed. For whole mount immunohistochemistry, fish were fixed in Dent's fixative overnight at 4°C, then bleached in Dent's bleach (Dent et al. 1989). After bleaching they were rehydrated into PBT. Larvae and adults were treated with a 10 μg/ml solution of Proteinase K for a length of time dependent on their size (5 min for 3.7 mm, 15 min for 11 mm). Proteinase K was removed with washes of PBT. Specimens were then treated with blocking solution. Zebrafish were incubated overnight in MF20 (Bader et al. 1982) or S58 (Miller et al. 1985) in blocking solution at room temperature while rocking. All antibody solution was washed out with PBT. Specimens were treated with blocking solution, secondary antibody was applied, and zebrafish were incubated overnight at 4°C. Specimens were carefully washed with PBT. For adults and larvae where a horseradish peroxidase conjugated secondary antibody was used, DAB color reaction solution (0.05% DAB and 0.004% hydrogen peroxide in PBT) was applied and monitored closely. Once fully reacted, DAB was washed out with PBT. After thorough washing, specimens were postfixed in 4% paraformaldehyde. After fixing, specimens were washed in PBT and placed in 50% and then 80% glycerol in PBT to clear.

To expose branchial musculature in adults, opercula were surgically removed with microdissection scissors and the lateral musculature of the head dissected away with sharpened tungsten needles.

For myosin ATPase (mATPase) histochemistry, 13-mm (SL) juvenile zebrafish were anesthetized with MS-222 (Tricaine) and flash-frozen in isopentane cooled with liquid nitrogen. Twelve-micrometer frozen sections were cut with a cryostat and stored at −20°C until use. Slides were processed without prior fixation using the methods described in Johnston et al. (1974). mATPase histochemistry was performed both on sections preincubated at alkaline pH (5 min at pH 10.4) and sections without preincubation.

Results

Cranial muscles: overview and fiber types

We have primarily investigated the following muscles: intermandibularis posterior, interhyoideus, adductor mandibulae, sternohyoideus, transversus ventralis, the most anterior ventral hypaxial musculature (inserting on the pectoral girdle), and the most anterior epaxial muscles (attaching to the posterior edge of the neurocranium). In addition, we present some preliminary results on the muscles of the hyomandibula and opercula of larvae, as well as the branchial levators in both larvae and adults. During the adult stage, several of the lateral muscles associated with the operculum and hyomandibula hypertrophy, making identification of individual muscles in whole mount preparation difficult. Therefore, we show a juvenile in Fig. 1 to clearly illustrate the pattern of cranial musculature and to provide orientation for subsequent images.

The intermandibularis posterior makes up the anterior portion of the protractor hyoideus, while the interhyoideus makes up the posterior portion. The adductor mandibulae originates on the lateral face of the hyomandibula and inserts on the lower jaw. The sternohyoideus originates on the cranial face of the cleithrum and inserts on the hyoid. The transversus ventralis muscles, which control motions of the gill bars, originate from the midventral line of the hypohyals and insert on the ceratobranchials (Winterbottom 1974).

The abductors and adductors of the hyomandibula and opercula include the dilator operculi, adductor operculi, adductor hyomandibulae, levator hyomandibulae, levator arcus palatini, and levator operculi. Adductors originate from the base of the neurocranium and insert on either the medial face of the hyomandibula or opercula. Abductors originate from the dorsolateral side of the neurocranium and insert on the lateral aspects of the hyomandibula or opercula. Branchial levators (the levatores externi; also called dorsal pharyngeal wall muscles in Schilling and Kimmel [1997]) originate from the neurocranium and insert on the branchial cartilages (Winterbottom 1974).

Determination of muscle fiber type identity has been done both by immunocytochemistry and enzyme histochemistry. In zebrafish, both of these approaches identify at least four different fiber types in the adult trunk myotomes: fast, intermediate, red muscle rim, and slow (van Raamsdonk et al. 1980). We have used myosin

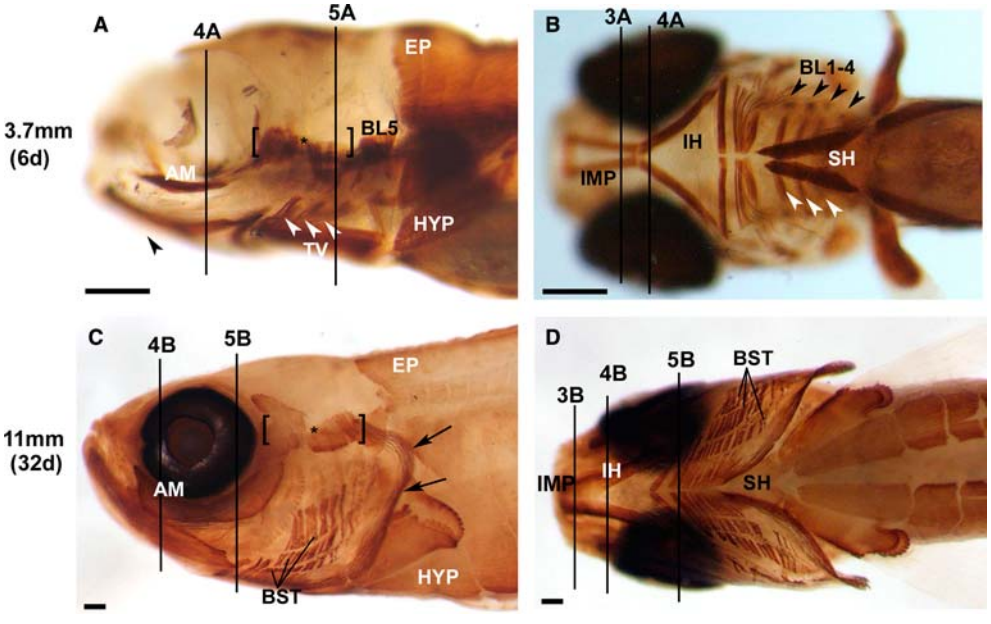


Fig. 1 Cranial musculature in a 6-day-old larva (**a**, **b**; 3.7 mm TL) and a 32-day-old juvenile (**c**, **d**; 11 mm TL), labeled in whole mount with MF20 antibody. **a** Whole mount preparation of lateral cranial muscles in a 6-day-old larva showing the adductor mandibulae (*AM*), dorsal muscles (*asterisk*) of the head, transversus ventralis (*TV*), hypaxial (*HYP*), 5th branchial levator (*BL5*), and epaxial (*EP*). *White arrowheads* in **a** and **b** show individual muscles of the transversus ventralis, ventral muscles of the branchial arches. **b** Ventral view of the larva showing the intermandibularis posterior (*IMP*) and interhyoideus (*IH*), which together make up the protractor hyoideus, and the sternohyoideus (*SH*). *Black arrowheads* in **b** point to branchial levator (*BL*) muscles, which originate on the neurocranium and insert on the cartilaginous elements of arches 3–6. **c** Lateral view of cranial muscles in a juvenile, representative of the postlarval condition, showing the adductor mandibulae (*AM*), dorsal muscles of the head (*asterisk*), muscles associated with the branchiostegal rays (*BST*), HYP muscles, and EP. *Black arrows* show staining of muscles associated with the opercular valve, the musculus hyohyoideus marginalis (Anker 1978). **d** Ventral view in a juvenile showing the IMP, IH, and SH. Whole mount immunohistochemistry using MF20 with an HRP secondary was employed. *Labeled lines* represent histological sections taken through larvae and adults and shown in subsequent figures to characterize fiber type distribution in a number of cranial muscles. All *scale bars* represent 100 μm. *Asterisk* indicates dorsal muscles of the head, which include the levator arcus palatini, levator operculi, adductor arcus palatini, dilator operculi, and adductor operculi

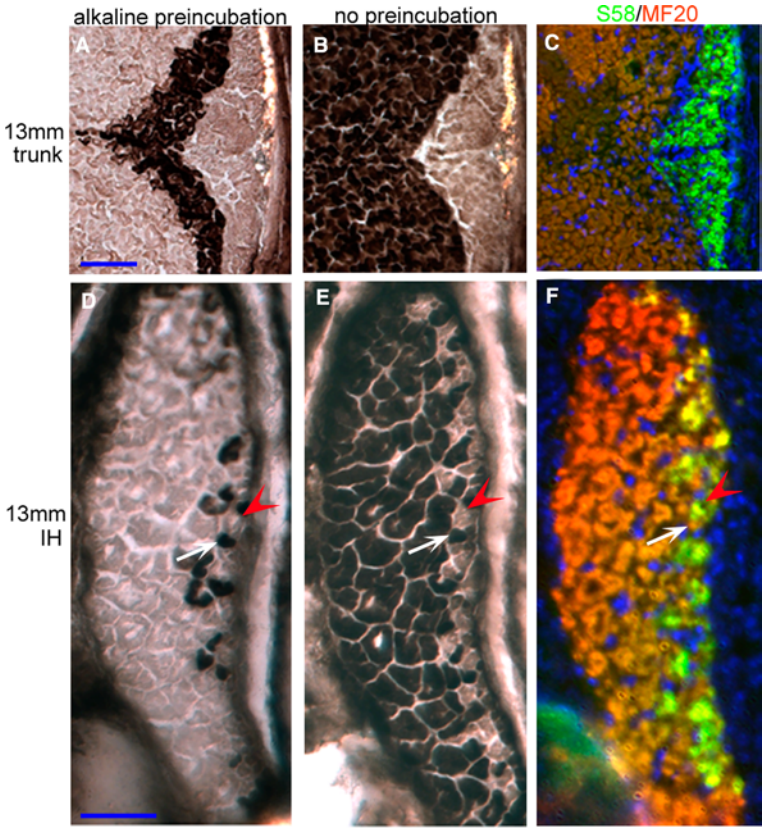


Fig. 2 Enzyme histochemistry and immunohistochemistry in a juvenile (13 mm SL). **a**–**b** Adjacent sections through the trunk, labeled for mATPase activity, following either alkaline (**a**) or no pre-incubation (**b**) of section. **c** Neighboring section to those shown in **a** and **b**, labeled for S58 (*green*) and MF20 (*orange*); counterstaining with Hoechst shows nuclei (*blue*). **d**–**e** Adjacent sections through the interhyoideus (IH), labeled for ATPase activity, following either alkaline (**d**) or no (**e**) pre-incubation of section. **f** Neighboring section to those shown in **d** and **e**, labeled for S58 (*yellow*) and MF20 (*orange*); counterstaining with Hoechst shows nuclei (*blue*). *Arrowheads* in **d**–**f** denote an individual slow fiber with low ATPase activity and S58 labeling. *Arrows* in **d**–**f** point to an intermediate fiber with high ATPase activity that is S58 negative. All *scale bars* represent 50 μm

ATPase (mATPase) histochemistry and immunocytochemistry to determine the fiber types present in cranial muscles (Fig. 2). Slow muscle fibers in the trunk are labeled with both the S58 and MF20 antibodies and have low levels of mATPase activity following alkaline pre-incubation or no preincubation (Fig. 2a–c). Fast muscle fibers in the trunk are labeled with the MF20 but not the S58 antibody and have low levels of mATPase activity following alkaline pre-incubation and high levels of mATPase with no preincubation (Fig. 2a–c). The same perfect correspondence exists in all cranial muscles we have examined (shown for the interhyoideus in Fig. 2d–f). These results show that S58 stains slow fibers within both the axial and cranial musculature. Moreover, the correspondence between staining patterns for both mATPase staining and staining with S58 validates the less laborious antibody staining method for characterizing the development and distribution of slow muscle fibers. Following preincubation at pH 10.4, high mATPase activity remains exclusively in a clear layer of intermediate fibers in both the trunk and interhyoideus

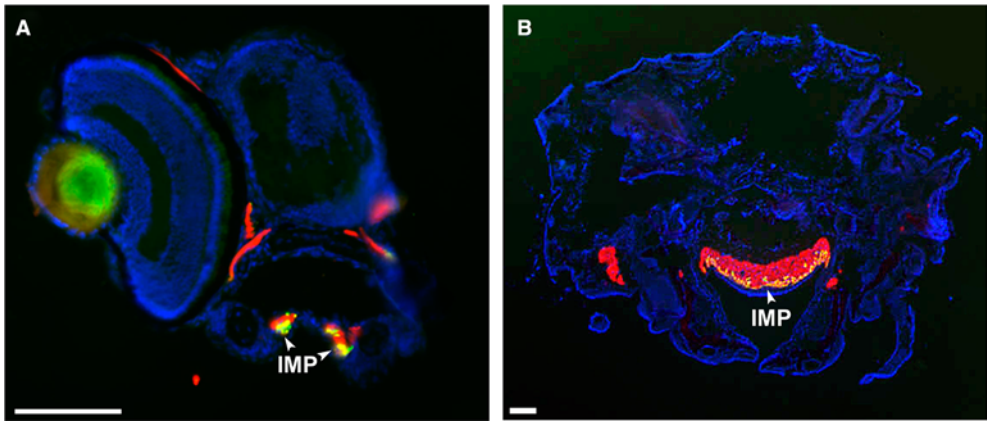


Fig. 3 Section through the intermandibularis posterior (IMP) of a larval (**a**) and adult (**b**) zebrafish. An equal proportion of slow (*green*) and fast (*red*) muscle makes up the IMP in larvae (**a**), whereas there is a much greater proportion of fast muscle within the adult (**b**). Slow muscle is stained with S58 and an FITC secondary, while all muscle is stained with MF20 and an Alexa 546 secondary. Counterstaining with Hoechst shows nuclei (*blue*). All *scale bars* represent 100 μm

(Fig. 2a, d). Thus, cranial muscles have at least three types of muscle fiber types. The intermediate muscle fibers are masked within the MF20-positive, S58-negative population, as they are in the trunk. Therefore, when referring to fast muscle throughout this paper, we include the intermediate fibers. It is worth noting that while the pattern of mATPase staining seen in zebrafish is inconsistent with the pattern of inactivation in mammalian muscle, it is consistent with the pattern of staining seen in another cyprinid, the crucian carp, using the same methods (Johnston et al. 1974).

Muscles associated with pharyngeal arches 1 and 2

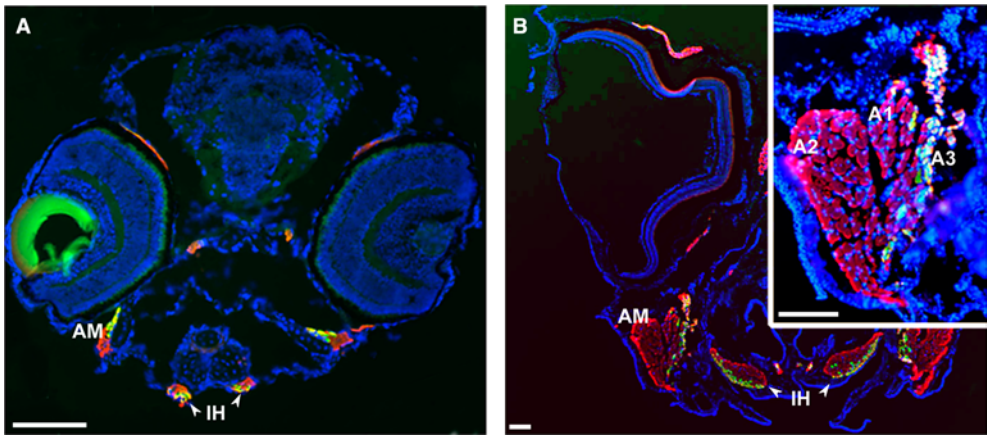
The intermandibularis posterior (IMP) and interhyoideus fuse during development to make the protractor hyoideus, which originates on the hyoid and inserts on the mandible (Winterbottom 1974). In a 3.7-mm (6d) larva the bilaterally symmetrical muscle straps of the intermandibularis posterior and interhyoideus form an hourglass (Fig. 1b), while in an 11-mm juvenile (32d) the two anterior components of the intermandibularis posterior have joined to form one strap (Figs. 1d, 3b). In larvae, fast and slow muscle contributes equally to the anterior portion of the protractor hyoideus (IMP, Fig. 3a). Fast fibers make up a much larger portion of this muscle in the adults compared with larvae (Fig. 3b).

Fig. 4 Sections through the interhyoideus (IH) and the adductor mandibulae (AM) in larval (**a**) and adult (**b**) zebrafish showing distribution of fast and slow fibers. Whereas larvae show roughly equal numbers of slow (*green*) and fast (*red*) fibers in both the IH and AM, adults have a much greater proportion of fast muscle. *Inset* Close-up of the IH and AM in the adult zebrafish showing a much greater proportion of fast muscle (*red*) than slow muscle (*light blue*). Moreover, fast fibers show a greater diameter than slow fibers. While the larval AM is composed of only one muscle mass, in the adult zebrafish the AM has broken up into three branches characteristic of most teleosts: A1, A2, and A3. Note that while A3 consists primarily of slow muscle (*light green*), A2 is made up entirely of fast muscle (*red*). A1 has both slow and fast fibers. Slow muscle fibers were stained with S58 and an FITC secondary, while all muscle fibers were stained with MF20 and an Alexa 546 secondary. Counterstaining with Hoechst was used to visualize all nuclei. All *scale bars* represent 100 μm

This pattern is also seen in the posterior portion of the protractor hyoideus, the interhyoideus. While larvae show a more equal distribution of slow and fast fibers (Fig. 4a), juveniles and adults have much more fast muscle in the interhyoideus (Figs. 2d–e, 4a, b). Moreover, as in trunk muscles, most fast fibers have a greater diameter than slow fibers do (Figs. 2d–e, 4b). Slow and fast fibers show a zoned distribution in which there is very little overlap between slow and fast fibers.

The architecture and fiber type composition of the adductor mandibulae differ significantly between these two ontogenetic stages. The larval adductor mandibulae is composed of an equally-sized ventral fast fiber zone and a dorsal slow fiber zone (Fig. 4a). In contrast, a relatively small proportion of the adult adductor mandibulae is composed of slow fibers (Fig. 4b). The larval adductor mandibulae is relatively small and consists of only one muscle mass (Fig. 4a), whereas the adult adductor mandibulae is quite large and has become subdivided into several different branches (Fig. 4b). This is a characteristic subdivision that is found in teleosts (Anker 1978; Winterbottom 1974). Three of these branches are the adductor mandibulae A1, A2, and A3. Another common branch, the Aω, was not examined in this study. A2 is the most substantial part of this complex, making up the biggest lateral portion of the adductor mandibulae. A3 is the most medial branch of this muscle. A1 is found between these two muscle branches. A2 is composed primarily of fast fibers, while A3 is composed almost exclusively of slow fibers. A1 contains predominantly fast fibers but does contain a few slow fibers (Fig. 4b, inset). Slow and fast fibers of the adductor mandibulae show a zoned distribution in both larvae and adults.

Dorsal muscles associated with the hyomandibula and opercula (dilator operculi, adductor operculi, adductor hyomandibulae, levator arcus palatini, levator hyomandibulae, and levator operculi) are used for expansion and contraction of the buccal and opercular cavity. In larvae, slow fibers make up most of these muscles (Figs. 5a, 6b).



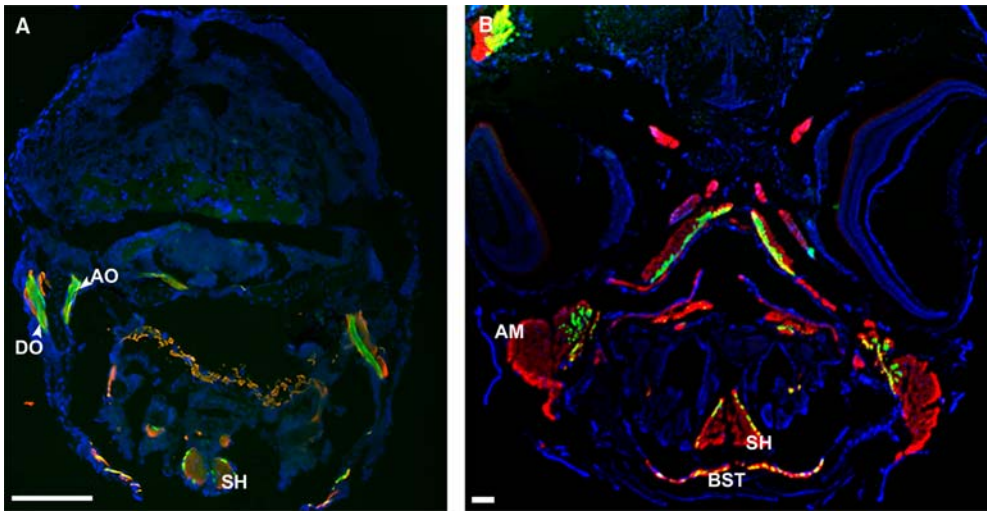


Fig. 5 Sections through the sternohyoideus (SH) of a larva (**a**) and an adult (**b**). **a** The section through the larva is taken at a more caudal position than that in the adult to get a larger cross-section of the SH. This section also shows the larval dilator operculi (*DO*) and the adductor operculi (*AO*), which are both composed primarily of slow (*green*) fibers, and the adult branchiostegal ray muscles (*BST*), which are also composed primarily of slow fibers. Both larvae (**a**) and adults (**b**) have very few slow fibers in the SH. The shape of the SH is markedly different: The larval SH (**a**) is perfectly rounded, whereas the adult SH (**b**) is triangular. *Arrowheads* in **a** show the preponderance of slow fibers within the dilator operculi and the adductor operculi. Slow muscle fibers were stained with S58 and an FITC secondary, while all muscle fibers were stained with MF20 and an Alexa 546 secondary. Counterstaining with Hoechst was used to visualize nuclei (*blue*). All *scale bars* represent 100 μm

Muscles associated with pharyngeal arches 3–7

The transversus ventralis muscles are responsible for respiratory movements in most fishes (Winterbottom 1974) and insert on the ventrolateral surfaces of the ceratobranchial cartilages of the pharyngeal skeleton. They are made up primarily of slow fibers during the larval stage (Fig. 6a, b), but as in many of the cranial muscles examined, there is an ontogenetic decrease in the proportion of slow muscle making up the transversus ventralis in the adult (Fig. 6c). Moreover, as in all other cranial muscles investigated, these muscle types show a marked zoned distribution in both the adult and larval stage. However, contrary to the situation seen in several of the muscles associated with arches 1 and 2, the diameter of individual slow muscle fibers in the adult transversus ventralis appears equal to that of fast muscle fibers (Fig. 6c).

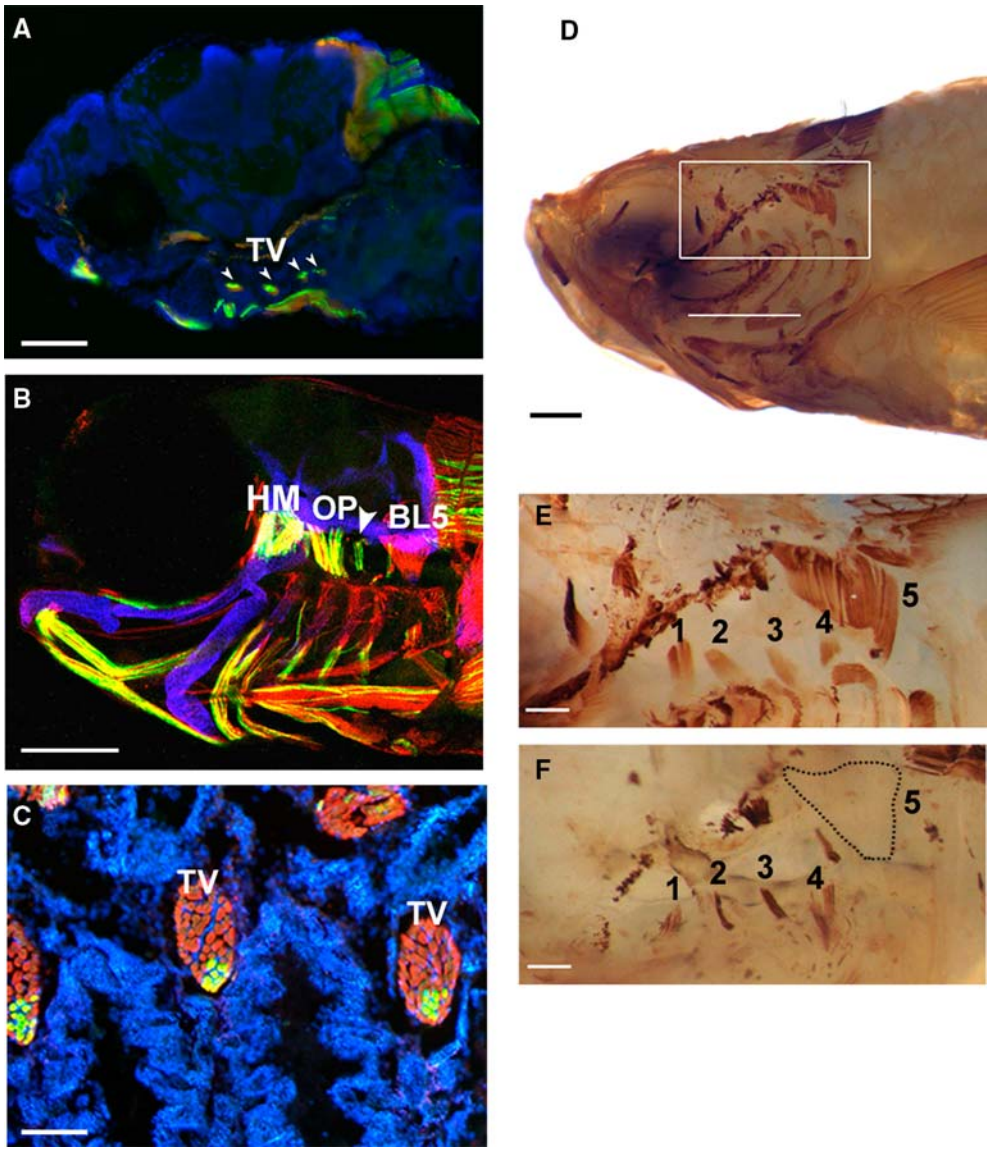
Branchial levators (dorsal pharyngeal wall muscles) originate from the base of the neurocranium and insert on the epibranchial cartilages. They play a role in the expansion of the buccal chamber in the adult (Winterbottom 1974). The four most anterior branchial levators are relatively small in both adult and larval zebrafish (Fig. 6b, e), with slow fibers forming a relatively small portion in the adult (Fig. 6f). The most posterior branchial levator plays a key role in feeding in adult cyprinids (Sibbing 1982). It is not only significantly larger than the other branchial levators in both larval and adult zebrafish (Fig. 6b, e), but it is also, in both adult and larval zebrafish, composed entirely of fast fibers (Fig. 6b, e, f).

Muscles derived from somitic mesoderm

Several muscles involved in head and jaw movements, including the epaxial, hypaxial, and sternohyoideus, are likely derived from somitic mesoderm (Huang et al. 1999; Schilling and Kimmel 1997; Winterbottom 1974).

▶

Fig. 6 Muscles associated with the branchial arches in both larvae (**a**, **b**) and adults (**c**–**f**). **a** Parasagittal section through a 6-day-old larva showing a significant portion of individual transversus ventralis (TV) muscles (*arrowheads*) predominantly made up of slow muscle fibers. **b** Hyomandibular (*HM*), opercular (*OP*), and 5th branchial levator (BL5) muscles in larval zebrafish. Whole mount immunostained larva showing a preponderance of slow muscle fibers in both OP and HM. Alternatively, in larvae the BL5 contains only fast muscle fibers. The serially homologous branchial levator 3 (*arrowhead*) is composed predominantly of slow fibers. **c** Cross-section through the TV muscles of the adult (section taken through *white line* in **d**) showing a preponderance of fast muscle and a small zoned distribution of slow fibers. **d** MF20 staining of the branchial muscles in the adult zebrafish showing all muscle fibers. Operculum and lateral muscles have been surgically removed to reveal the branchial musculature. **e** Close-up of the branchial levators showing all muscle fibers. Note the size of the BL5 compared with that of more anterior, serially homologous branchial levators (*numbered 1–4*). **f** Close-up of the branchial levators showing the position of slow fibers (S58). Note that although much larger in size, the BL5 contained no slow fibers. For **a**, **b**, and **c**, slow muscle fibers were stained with S58 and an FITC secondary, while all muscle fibers were stained with MF20 and an Alexa 546 secondary. Counterstaining with Hoechst was used to visualize nuclei (*blue*). For **b**, slow muscle fibers were stained with S58 and an FITC secondary, all muscle fibers were stained with MF20 and an Alexa 546 secondary, and cartilage was stained with II-II6B3 and a Cy5 secondary. For **d** and **e**, all muscle fibers were stained with MF20 and an HRP secondary. For **f**, slow muscle fibers were stained with S58 and an HRP secondary. *Scale bars* in **a**–**c** = 100 μm, in **d** = 1 mm, and in **e** and **f** = 200 μm



These muscles are composed primarily of fast muscle fibers. In both larval and adult stages, the anterior epaxial and hypaxial musculature are each composed primarily of fast fibers (Fig. 7), deep to a superficial zone of slow fibers, typical of most cranial muscles (Fig. 7a, c). Anterior epaxial muscle, which elevates the cranium in the larvae (Liem 1991), is composed of a thick layer of fast fibers surrounded by a small number of slow fibers (Fig. 7a). Anterior epaxial musculature in the adult also consists primarily of fast fibers, but the pattern of distribution is slightly different in the adult. Whereas slow fibers are found solely along the periphery in larval zebrafish, in adults the slow fibers are located dorsomedially within one discrete area (Fig. 7b). Anterior hypaxial muscle has been proposed to pull back the cleithrum and thus allow the sternohyoideus to more effectively depress the hyoid (Hernandez et al. 2002; Osse et al. 1997). In the larva, the anterior hypaxial muscle is composed of both slow and fast fibers (Fig. 7a). Anterior hypaxial muscle in the adult is composed primarily of fast fibers, and the slow fibers are found deeper in the myotome (Fig. 7c).

The sternohyoideus is responsible for depression of the hyoid during feeding (Hernandez 2000; Hernandez et al. 2002). The sternohyoideus consists predominantly of fast fibers in both larvae and adults (Fig. 5a, b), with little change in their relative proportion during ontogeny. However, the shape of the sternohyoideus changes dramatically. Whereas the larval sternohyoideus is completely rounded in cross-section, the anterior portion of the sternohyoideus in the adult is triangular in cross-section. Both larvae and adults have slow fibers only in the periphery of this muscle (Fig. 5).

Discussion

We have characterized the fiber type composition of the major muscles involved in feeding and breathing in zebrafish. With one exception, all of these muscles contain

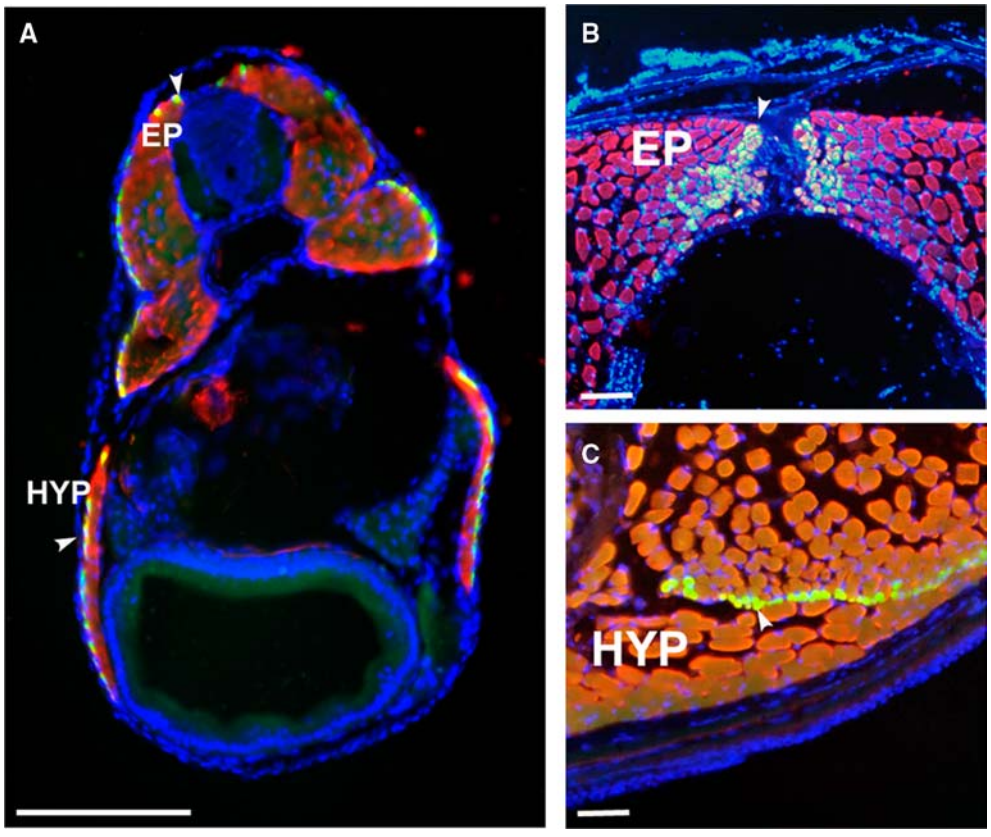


Fig. 7 Anterior epaxial and hypaxial muscles of larval and adult zebrafish. **a** Immunostained larval specimen showing anterior epaxial (EP) and hypaxial (HYP) muscles. Both these larval muscles show a pattern very similar to that seen in trunk muscles of larval fish in which slow fibers (*green*, *arrowhead*) surround a core of fast muscle (*red*). **b** Slow muscle fibers (*light green*, *arrow*) within the anterior EP muscles in the adult show a different pattern, with slow muscles making up a discrete zoned area within this muscle mass. Such medial growth of slow muscle fibers also occurs in the trunk. **c** Slow fibers (*green*, *arrowhead*) within the HYP muscle retain the distribution of slow fibers seen in larvae. This portion of the HYP musculature is found deep to additional hypaxial musculature in the adult (*arrowhead*). Slow muscle fibers were stained with S58 and an FITC secondary, while all muscle fibers were stained with MF20 and an Alexa 546 secondary. Counterstaining with Hoechst was used to visualize nuclei (*blue*). All *scale bars* represent 100 μm

both slow and fast muscle fibers. During larval and juvenile growth, the proportion of slow muscle decreases, suggesting the preferential addition of fast and/or intermediate muscle fibers. Only the most posterior of the branchial levators contain no slow muscle. Finally, contrary to amniote cranial muscles (Marcucio and Noden 1999), none of the zebrafish cranial muscles we examined showed a mosaic distribution of slow and fast fibers in either the larva or the adult.

Although there is a marked increase in the size of all cranial muscles going from the juvenile to the adult stage, the distribution pattern of slow and fast fibers remains nearly identical in these two stages. Distribution patterns for the interhyoideus, intermandibularis, and adductor mandibulae are essentially identical to those seen in adult zebrafish (Fig. 2; Table 1; data not shown). Such consistent distribution patterns between juveniles and adults suggests that after metamorphosis muscle fiber type distribution is largely determined and that subtle changes in muscle proportion are due primarily to increases in mass that accompany growth. Data on muscle fiber type distribution from both juvenile and adult zebrafish suggest that there is preferential growth of areas composed predominantly of fast muscle fibers within cranial muscles. Such growth gives rise to a preponderance of fast muscle fibers in the postlarval condition.

Ontogenetic changes associated with feeding

Ontogenetic changes in feeding kinematics have been examined in zebrafish (Hernandez 2000; Hernandez et al. 2002). Briefly, larvae use a very rapid and pronounced hyoid depression and cranial elevation to create suction and bring food into the mouth. While postlarval stages also use suction generated by hyoid depression, the better-developed muscles of the hyomandibula and operculum allow for suction to be generated via lateral abduction of the suspensoria and opercula. Moreover, while larvae are able to depress the hyoid at nearly the same velocity as adults, the larval hyoid is elevated and brought back to its resting position quite slowly, differing from the process in adults, in which hyoid elevation is quite rapid. Finally, jaw-closing velocity is much faster in adults than in larvae (Hernandez 2000).

Table 1 Summary of fiber type development (*ND* not determined)

Region and muscle	Larval		Juvenile		Adult	
	Slow	Fast	Slow	Fast	Slow	Fast
Mandibular arch						
Intermandibularis posterior (IMP)	+	+	+	+ +	+	+ + +
Adductor mandibulae (AM)	+	+	+	+ +	+	+ +
Dilator operculi (DO)	+	+	ND		ND	
Hyoid arch						
Interhyoideus (IH)	+	+	+	+ +	+	+ + +
Adductor operculi (AO)	+	–	ND		ND	
Branchial arches						
Transversus ventralis (TV)	+	+	ND		+	+ +
Branchial levators 1–4 (BL1-4)	+	+ +	ND		+	+
Branchial levator 5 (BL5)	–	+ +	ND		–	+ +
Branchiostegal (BST)	ND		ND		+	+
Somite-derived						
Sternohyoideus (SH)	+	+ +	ND		+	+ + +
Anterior hypaxial (HYP)	+	+ +	ND		+	+ + +
Anterior epaxial (EP)	+	+ +	ND		+	+ + +

Although part of the difference in feeding mode is likely due to differences in Reynolds number regime among different ontogenetic stages (Hernandez 2000), the distribution and proportion of slow and fast muscle also correspond to these differences in feeding. The intermandibularis posterior and interhyoideus are both made up of a nearly equal proportion of fast and slow muscle in larvae, whereas the adult has a much lower proportion of slow muscle (Fig. 3). The significantly slower velocity at which the hyoid is elevated in larvae (Hernandez 2000) may be due at least in part to this preponderance of slow fibers.

There are striking differences between larvae and adults in morphology of the adductor mandibulae. Jaw-closing velocity is significantly faster in adult than larval zebrafish (Hernandez 2000). This is likely due at least in part to the relatively small number of fast fibers within the larval adductor mandibulae (AM). In the adult this muscle has hypertrophied greatly and contains many fast fibers (Fig. 4b). Moreover, while the larval AM is composed of a single muscle mass with equal contributions from slow and fast fibers, adult zebrafish have three discrete branches of the AM: A1, A2, and A3 (Fig. 4b, inset). While A3 plays a role primarily in respiratory movements, A2 and A1 are used during feeding (Osse 1969). The preponderance of slow fibers in A3 corresponds to the type of slow rhythmic contractions associated with respiratory movements (Barends 1979; Liem 1985; Osse 1969), while the more powerful contractions associated with the compressive phase of suction feeding events (Lauder 1985) would likely necessitate a greater proportion of fast muscle.

In adult zebrafish, both dorsal and ventral branchial muscles are made up primarily of fast fibers (Fig. 6c, e, f). This is in contrast to most larval branchial muscles (transversus ventralis muscles, as well as dorsal branchial levators), which have a significant proportion of slow muscle (Fig. 6a, b). One notable exception is the branchial levator associated with branchial arch 5 (dorsal pharyngeal body wall muscle 5). This muscle is specialized for feeding in cyprinids (Sibbing 1982) and contains no slow fibers in either zebrafish larvae or adults (Fig. 6b). It is also significantly larger than the other branchial levators (see arrowhead in Fig. 6b for comparison) at both ontogenetic stages. Selection for a larger adult muscle may result in the development of a larger muscle in the larva, even if that muscle is not likely to play a role in the larva.

Correlations between fiber type composition and function suggest that it may be possible to use fiber type proportion as a predictor of function. Fisheries researchers are deeply interested in what factors determine successful feeding in larval fishes. As functional data are difficult to gather for larvae, it may be possible to glean information about feeding behavior from patterns of fiber type composition.

Interspecific comparisons of cranial muscle composition

Correlations between fiber type distribution and function have been reported in a variety of teleosts examined. Ono and Kaufman (1983), investigating muscle fiber type in pharyngeal musculature of teleosts, found a significant correlation between fiber type distribution and functional role. Comparing centrarchids and cichlids, they found that those which used pharyngeal teeth for heavy crushing differed significantly in fiber type from those fish that used pharyngeal dentition primarily for processing prey. Akster and Osse (1978), investigating the relationship between fiber type and feeding in the perch, also found that muscles used in respiration had a preponderance of slow fibers whereas those used in feeding tended to have a greater proportion of fast muscle. They attributed the preponderance of fast fibers in the sternohyoideus to the rapid hyoid depression that effects suction, a finding similar to ours.

Scapolo et al. (1989) found that the more medial portions of the AM complex in the carp are composed primarily of slow fibers, a pattern shared with the A3 branch of the adductor mandibulae within adult zebrafish. As in the carp, the A2 branch of the adductor mandibulae in adult zebrafish is composed exclusively of fast muscles, while the A1 branch has a combination of both fast and slow fibers. A similar pattern is found in perch (Barends 1979). These distribution patterns differ from those seen in the air-breathing fish, *Channa punctatus*, in which the AM is composed entirely of fast fibers (Ojha and Datta Munshi 1975). It is not clear whether this is true for all branches of the AM; however, it is possible that the high preponderance of fast muscle is related to air-breathing, a habit in which the use of the AM in aerial respiration may have selected for a unique distribution of muscle fiber types.

As in zebrafish, other teleost species also showed a mixed population of fibers within the opercular muscles of the head. Ojha and Datta Munshi (1975) found, in *Channa punctatus*, a combination of slow and fast muscles making up the adductor operculi, dilator operculi, and levator operculi, with fast fibers making up most of the muscle. Given that these muscles are responsible for both explosive lateral abduction during suction feeding (Lauder 1985) and slower, rhythmic motions during breathing (Liem 1985), a mixed population of slow and fast fibers within these cranial muscles is probably common in adult teleosts.

The increased size of the branchial levator of the 5th branchial arch (dorsal pharyngeal wall muscle 5) appears to be a synapomorphy of the Cyprinidae since the equivalent muscle in the carp *Cyprinus carpio* makes up 33% (by wet weight) of the pharyngeal musculature. In the carp these muscles are responsible for moving the pharyngeal bone against the chewing pad located at the base of the skull (Sibbing 1982). It is most likely that this muscle performs the same function in zebrafish, as these species are closely related. Understanding the molecular genetic mechanisms that regulate fiber type development and size of pharyngeal arch muscles may shed considerable light on the possible evolutionary changes that led to this synapomorphy.

Distribution of slow and fast fibers within individual muscles

Both adult and larval zebrafish show a zoned distribution of slow and fast muscle fibers in all cranial muscles examined, as has consistently been found in other anamniotes (Engel and Irwin 1967; Lannergren and Smith 1966; Lassar et al. 1989; Watanabe et al. 1980). In contrast, amniote cranial muscles, like their trunk and limb counterparts, typically have a more mosaic distribution of fiber types (Cobos et al. 2001; Marcucio and Noden 1999; but see also Throckmorton and Saubert 1982). For example, while the rat geniohyoideus is composed predominantly of fast fibers (Cobos et al. 2001), a trait shared by the homologous intermandibularis posterior of the zebrafish, the pattern of fiber type distribution is quite different. Slow fibers in the rat geniohyoideus are not regionalized but rather mixed in among fast fibers. Such a mosaic distribution characterizes mammalian muscles.

Moreover, a pattern in which a superficial layer of slow fibers surrounds a deeper layer of fast fibers is typical for most cranial muscles examined in zebrafish. Ono and Kaufman (1983), investigating fiber type in two families of freshwater fish, centrarchids and cichlids, also found a zoned distribution of slow and fast fibers for several of the branchial muscles examined. However, they identified a distinct fiber type pattern in which a mosaic population of fibers was found adjacent to a homogenous area of only one fiber type and suggested that this pattern might be an epigenetic response to processing tough foods. In their study, muscle fibers within the 5th branchial levator in both centrarchids and cichlids showed this unique zoned distribution in which a much greater population of fast fibers surround a small number of slow and intermediate muscle fibers. Given that this muscle is essential for chewing motions within the pharyngeal jaws of cyprinids (Sibbing 1982), such a unique pattern of fiber type distribution is interesting and merits further attention in zebrafish.

Evolutionary developmental implications

The great diversity of feeding mechanisms that characterize teleost fishes necessitated two essential evolutionary changes within the structure of pharyngeal arches (Mallatt 1996). First, arches had to undergo morphological specialization such that the functional anatomy of individual arches differed from that of other arches within the same organism. Second, modifications of individual arches had to occur in different vertebrate lineages such that the functional anatomy of a single arch could vary significantly among species. The 5th branchial levator (dorsal pharyngeal wall muscle 5) illustrates both of these modifications: It is larger than the other branchial levators, and it is composed solely of fast muscle fibers. Moreover, these differences appear to be specific to cyprinids. Thus, the pharyngeal skeleton of the zebrafish provides an ideal model in which to study developmental mechanisms leading to the evolution of the vertebrate head.

Some of the changes in muscle may be driven by changes in the size or shape of the cartilaginous elements associated with it. However, changes in fiber type composition and distribution are not easily explained by changes in the cartilage. We suggest that these types of changes must be a direct result of changes in the developmental events that pattern the muscle itself. Changes in the adult fiber type pattern may also be influenced by nervous activity (Wigmore and Evans 2002). These hypotheses can be directly tested through examining fiber type patterning in the large collection of mutants

disrupting cartilage and neural patterning (Fetcho and Liu 1998; Schilling and Kimmel 1997). Identification of mutations that make relatively small changes in morphology may identify genes whose alteration plays a role in evolution (Parichy and Johnson 2001). Examination of the role of muscle patterning genes in related species with altered cranial muscle patterning may suggest hypotheses for how evolutionary changes in form and function have taken place. Because feeding behavior is critical in determining ecological niche, an understanding of the evolution of the developmental mechanisms underlying changes in muscle form and function is likely to greatly enhance our understanding of rapid and small scale evolutionary changes.

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