

Fast Drum Strokes: Novel and Convergent Features of Sonic Muscle Ultrastructure, Innervation, and Motor Neuron Organization in the Pyramid Butterflyfish (*Hemitaurichthys polylepis*)

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ABSTRACT Sound production that is mediated by intrinsic or extrinsic swim bladder musculature has evolved multiple times in teleost fishes. Sonic muscles must contract rapidly and synchronously to compress the gas-filled bladder with sufficient velocity to produce sound. Muscle modifications that may promote rapid contraction include small fiber diameter, elaborate sarcoplasmic reticulum (SR), triads at the A–I boundary, and cores of sarcoplasm. The diversity of innervation patterns indicate that sonic muscles have independently evolved from different trunk muscle precursors. The analysis of sonic motor pathways in distantly related fishes is required to determine the relationships between sonic muscle evolution and function in acoustic signaling. We examined the ultrastructure of sonic and adjacent hypaxial muscle fibers and the distribution of sonic motor neurons in the coral reef Pyramid Butterflyfish (Chaetodontidae: *Hemitaurichthys polylepis*) that produces sound by contraction of extrinsic sonic muscles near the anterior swim bladder. Relative to adjacent hypaxial fibers, sonic muscle fibers were sparsely arranged among the endomysium, smaller in cross-section, had longer sarcomeres, a more elaborate SR, wider t-tubules, and more radially arranged myofibrils. Both sonic and non-sonic muscle fibers possessed triads at the Z-line, lacked sarcoplasmic cores, and had mitochondria among the myofibrils and concentrated within the peripheral sarcoplasm. Sonic muscles of this derived euteleost possess features convergent with other distant vocal taxa (other euteleosts and non-euteleosts): small fiber diameter, a well-developed SR, and radial myofibrils. In contrast with some sonic fishes, however, Pyramid Butterflyfish sonic muscles lack sarcoplasmic cores and A–I triads. Retrograde nerve label experiments show that sonic muscle is innervated by central and ventrolateral motor neurons associated with spinal nerves 1–3. This restricted distribution of sonic motor neurons in the spinal cord differs from many euteleosts and likely reflects the embryological origin of sonic muscles from hypaxial trunk precursors rather than occipital somites. *J. Morphol.* 274:377–394, 2013. © 2012 Wiley Periodicals, Inc.

KEY WORDS: sonic muscle; hypaxial muscle; Z-line; t-tubules; sonic motor neurons; spinal nerves

INTRODUCTION

Sound production organs have arisen repeatedly and independently in the course of teleost fish

evolution (Parmentier and Diogo, 2006; Mok et al., 2011). Unlike the relatively conserved pattern of vocal organ evolution in various tetrapod lineages (Bradbury and Vehrencamp, 1998), teleosts have evolved a variety of mechanisms for sound production (Bass and Ladich, 2008). Many disparate fish lineages have co-opted the swim bladder hydrostatic organ as a sound radiator. Sound is produced through the vibration of this gas filled bladder by swim bladder muscles that have independently evolved from trunk musculature in a wide-array of teleosts. For example, apomorphic intrinsic swim bladder muscles found in some fishes (toadfishes and midshipmen, Batrachoididae; sea robins and gurnards, Triglidae; Walleye Pollock, *Theragra chalcogramma*, Gadidae; and John Dory, *Zeus faber*, Zeidae) insert and originate entirely on the swim bladder, and elongate after contraction because of the tension produced by pressure inside the swim bladder (Tavolga, 1971; Fine et al., 2001; Connaughton, 2004; Onuki and Somiya, 2004, 2006). In contrast, other species (soldier and squirrelfishes, Holocentridae; grunTERS

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and tigerperches, Terapontidae; and croakers, Sciaenidae) have extrinsic swim bladder muscles that originate on skeletal features on the head, body, or both and insert on the swim bladder surface or adjacent tendons and bones. Extrinsic muscles elongate after contraction because of elasticity of the swim bladder, connective tissue, or bones and internal or external pressure on the swim bladder. Thus, the independent evolution of sonic musculature in fishes has yielded two primary strategies for sonic muscle attachment to invoke this rapid sound production.

Sound production mechanisms that involve the swim bladder must produce rapid synchronous contractions to vibrate the swim bladder with sufficient velocity to generate sound. In most species examined, the muscle contraction rate determines the fundamental frequency of sounds. Species that produce tonal sounds like toadfishes and sea robins produce rapid, continuous contractions. In fact, the sonic muscle of the Oyster Toadfish (*Opsanus tau*) produces the fastest known contraction rate of any vertebrate muscle (Rome, 2006). Several features of sonic muscles are hypothesized to facilitate the high contraction rates. These include a reduced fiber diameter, more developed sarcoplasmic reticulum (SR), and cores of sarcoplasm surrounded by a contractile cylinder in which mitochondria are present adjacent to myofibrils in the sarcolemma and in the core (Fine et al., 1993). This morphology maximizes calcium ion exchange at the site of contraction and maintains adenosine triphosphate and phosphocreatine near the myofibrils at both the center and periphery of the contraction cylinder (Fine et al., 1993). As these fibers grow, fragmentation of the cores maintains the small cylinder size necessary for efficient contraction (Fine et al., 1993). Lastly, several soniferous fishes have swim bladder muscles with t-tubules present at the A-I boundary (which results in two triads per sarcomere) rather than the Z-line (Parmentier and Diogo, 2006). In addition, the sonic muscles of type I (most soniferous) male phenotype of the Plainfin Midshipman (*Porichthys notatus*) have widened Z-discs (Bass and Marchaterre, 1989). However, not all of these features are present in the limited set of taxa examined. The wide variability of muscle properties among distantly related sonic fish groups likely reflects the different embryonic origins and the independent evolution of apomorphic muscle characteristics involved in the fast contractions associated with sound emission.

The independent evolution of sonic musculature from occipital and anterior trunk precursors has yielded several convergent patterns of innervation. Sonic muscles are innervated by occipital nerves in several distantly related sonic fish lineages with examples that include extrinsic muscles that originate on the head and entirely

intrinsic muscles with no origin in the occipital region (Onuki and Somiya, 2007). A less common pattern of spinally innervated sonic musculature is seen in piranhas (Characidae), Walleye Pollock (Gadidae), John Dory (Zeidae), and croakers (Sciaenidae) (reviewed in Onuki and Somiya, 2007). A mixed pattern of innervation, with a combination of occipital and spinal nerves is documented for two pimelodid catfishes (*Pimelodus blochii* and *P. pictus*; Ladich and Fine, 1994). Furthermore, the neuroanatomical location of the occipital and spinal nerve motor neurons that innervate sonic muscles varies within and among taxa. Most known sonic motor pathways involve discrete motor nuclei present within the caudal medulla and rostral spinal cord. These nuclei consist of motor neurons located medial to the ventral motor column or adjacent to the central canal/fourth ventricle (Ladich and Bass, 2005). The sonic muscles of piranhas, however, are unusual because they are innervated by motor neurons that are located solely within the spinal cord and exit via multiple spinal nerve roots (Ladich and Bass, 2005; Onuki et al., 2006). This pattern may be more widespread among teleosts with spinal nerve innervated sonic muscles, but such pathways are not yet described across diverse percomorph taxa.

The prevalence and degree of evolutionary convergence for these morphological features remain unresolved among teleost taxa. Social sound production was described recently for several genera within the butterflyfish family Chaetodontidae (Tricas et al., 2006; Boyle and Tricas, 2010, 2011; Parmentier et al., 2011a). This family includes approximately 122 species that are conspicuous and highly social members of coral reefs (Nelson, 2006). The Pyramid Butterflyfish (*Hemitaurichthys polylepis*) produces very rapid pulse train sounds with extrinsic swim bladder muscles that also cause a rapid buckling of the tissues lateral to the anterior swim bladder (Boyle and Tricas, 2010). This species lacks the anteriorly directed swim bladder horns and laterophysic connection, which are apomorphies of the genus *Chaetodon*. These features are hypothesized to increase hearing sensitivity and to transduce sound pressure to the normally flow-sensitive lateral line, respectively (Webb, 1998; Webb et al., 2006, 2012). In this study, we examine the sonic muscle ultrastructure found in a percomorph species with extrinsic swim bladder muscles, and compare the innervation patterns of their motor neurons to other soniferous fishes. We test for differences between the ultrastructure of sonic muscle and adjacent white trunk muscle, determine sonic motor pathways and compare these convergent features with those of other sonic teleosts. These results demonstrate that Pyramid Butterflyfish sonic muscles possess smaller fiber diameters and a well developed SR relative to

hypaxial muscle fibers, but unlike several sonic fish species, possess typical Z-line type triads, and lack cores of sarcoplasm. Pyramid Butterflyfish are members of the most speciose teleost subdivision, the Euteleostei (Nelson, 2006), a taxon which includes many families with swim bladder muscles. Unlike other euteleosts studied thus far, Pyramid Butterflyfish sonic muscles are innervated entirely by spinal nerves and motor neuron somata that are not clustered in a discrete motor nucleus, a pattern convergent with the non-euteleost piranhas (subdivision Otocephala or cohort Otomorpha sensu Wiley and Johnson, 2010).

MATERIALS AND METHODS

Pyramid Butterflyfish, *Hemitaurichthys polylepis*, (Bleeker, 1857) used in this study were obtained from commercial suppliers in the Hawaiian Islands. Housing of live animals and experimental protocols complied with the University of Hawaii at Manoa IACUC protocol. Research was conducted during fall 2009 through summer 2010.

Gross Anatomy

Gross dissections and examinations were conducted on fish that were cleared and stained ($n = 1$), first for bone with alizarin red-s (Taylor, 1967) and then later for nerves with Sudan Black B (Song and Parenti, 1995), as well as on formalin-fixed fish ($n = 4$).

Muscle Histology

Transmission electron microscopy. Two samples of muscle, one from sonic musculature in a region with very loosely packed muscle fibers described previously (Boyle and Tricas, 2010) and one from hypaxial trunk musculature were compared from each of four fish (two males, two females) 90–94 mm standard length (SL). Sonic muscle tissue was removed in the region over the swim bladder, between the fourth and fifth vertebra (Fig. 1) in an area where sonic buckling is maximal. Trunk muscle tissue was removed in an area immediately ventral to the sonic buckling area, between the ribs of vertebra 4 and 5, and at a dorso-ventral level approximately even with the dorsal margin of the pectoral fin insertion (Fig. 1).

For muscle extraction, fish were euthanized with an overdose of tricaine methanesulfonate (MS-222) and a small piece ($\leq 1 \text{ cm}^3$) of muscle was placed immediately in fixative (2% paraformaldehyde/2% glutaraldehyde in 0.1 M cacodylate/0.35 M sucrose). Muscle tissue was further dissected while immersed in fixative into a smaller piece (approximately $1.5 \times 4 \times 4 \text{ mm}$, with the long axis parallel to fibers) and kept overnight in fresh fixative. Tissue was washed in 0.1 M cacodylate/0.35 M sucrose, postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h, dehydrated through a graded series of ethanol, cleared with propylene oxide, and embedded in LX112 resin (Ladd Research Industries). Ultrathin sections were stained with uranyl acetate, observed and imaged with a Zeiss LEO 912 energy filtering transmission electron microscope.

Light microscopy. Sonic and adjacent hypaxial muscle fibers from one fish (female, 88 mm SL) on the non-reacted side of the sonic motor study were removed after transcardial perfusion (see below), postfixed and stored in 4% paraformaldehyde overnight, rinsed in several changes of 70% ethanol until embedded in paraffin and sectioned at $10 \mu\text{m}$ and stained with hematoxylin and eosin. Light micrographs were observed and photographed at $400\times$ on a Zeiss Axioskop microscope.

Sonic Motor Neurons

Retrograde biocytin labeling. Fish were anesthetized with 100 mg/L MS-222. A small 1–1.5 cm incision was then made dorso-ventrally across sonic muscle fibers that run rostrocaudally over the rib of vertebra 4 (Fig. 1). An additional cut with small surgical scissors was used to sever muscle fibers anteriorly near the supra-cleithrum, at a location where sonic muscle fibers are organized in a medial/lateral fashion. We then placed crystals of biocytin (Sigma or Anaspec) onto the severed ends of these sonic muscle fibers and associated nerve rami with a small insect pin. Because of the large area of muscle fibers and contiguity with connective tissue of adjacent trunk musculature, we were unable to cut and label all sonic nerve rami within each individual fish. Thus, biocytin labeled motor neurons likely represent only a portion of the total population of motor neurons that innervate the sonic musculature. Further, it is possible that this labeling technique labeled axons of proprioceptors. Muscle spindle proprioceptors are not well-studied in teleost axial muscles, but free nerve endings may be involved in proprioception (Syme, 2005). In addition, this labeling technique does not distinguish between sensory and motor axons. A previous study (which assumed that small diameter axons are sensory in nature), reported mixed populations of motor and sensory axons that which innervated the ventral rami of the spinal cord of two teleosts (Egginton and Johnston, 1986). To our knowledge, this assumption remains to be confirmed and the somata location of these presumptive sensory neurons was not determined in the previous study. Thus, without detailed electrophysiological experiments, the innervation pattern of sonic muscle sensory axons cannot be inferred. Incisions were sealed with a small piece of parafilm adhered to the fish with Vetbond (3M). Animals were then returned to an aquarium for recovery and fed daily.

After survival periods of approximately four days, fish were deeply anesthetized in MS-222 and perfused transcardially with 0.9% heparinized saline followed by 4% paraformaldehyde/1% glutaraldehyde in 0.1 M phosphate buffer (PB). The brain and rostral spinal cord were removed and postfixed as above for 1 h. Neural tissues were stored at 4°C in 0.1 M PB and cryoprotected in a 30% sucrose solution overnight prior to sectioning. Brains and spinal cords were then embedded in Histoprep (Fisher Scientific). Sections were cut on a cryostat at a thickness $40 \mu\text{m}$ in transverse (five fish, three males 80–94 mm SL, and two females 88–94 mm SL) and horizontal planes (three females 90–92 mm SL) and were collected in 12 well plates filled with 0.1 M PB. All fish were sectioned and stained within one week of perfusion.

Free floating sections were quenched in 1% hydrogen peroxide in PB, incubated in 0.4% Triton-X in phosphate-buffered saline, then incubated for 3 h in avidin-biotin-horseradish peroxidase complex (ABC Elite kit, Vector Laboratories), rinsed in PB for 15 min, reacted with diaminobenzidine chromogen substrate kit with nickel intensification (Vector Laboratories) for 5–6 min, and rinsed in distilled water for 10 min. Sections were collected on chrom-alum slides, dried overnight, and counterstained with 0.5% cresyl violet or 0.1% methyl green, dehydrated in an ethanol series, cleared in toluene, and coverslipped with Cytoseal 60 mounting media (Richard Allen Scientific, Thermo Scientific). Sections were photographed both before and after counterstaining.

Reference brain. A single *H. polylepis* was deeply anesthetized in MS-222, perfused transcardially with 0.9% heparinized saline, followed by alcohol-formalin-acetic acid (AFA) solution. The brain and rostral spinal cord were removed and postfixed in AFA overnight, dehydrated in an ethanol series, and embedded in paraffin. The brain and spinal cord were serially sectioned in the transverse plane at $10 \mu\text{m}$ and mounted on slides. Sections were deparaffinized and stained with 0.5% cresyl violet, dehydrated in an ethanol series, cleared in toluene, and coverslipped with Cytoseal 60.

Data Analyses

Length and area estimates from digitized images were made with ImageJ software (v. 1.44a) straight line and outline tools, respectively.

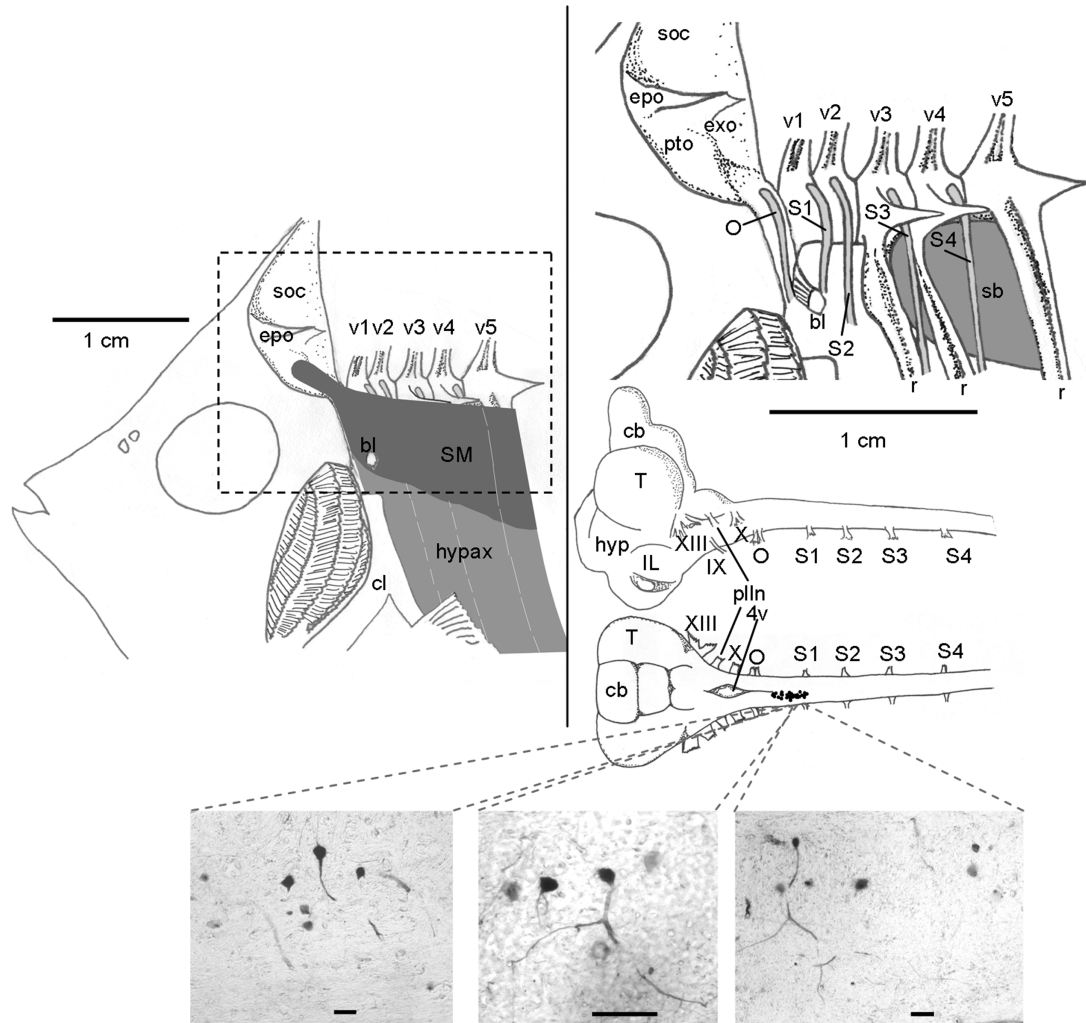


Fig. 1. Neuromuscular organization of the sound production mechanism in the Pyramid Butterflyfish (*Hemitaurichthys polylepis*). Diagrammatic representation of the head and trunk (upper left) shows sonic muscle location and normal hypaxial trunk muscles. The supraclathrum, posttemporal, and operculum are removed and the pectoral fin rays reduced. Inset (top right) shows medial position of occipital and spinal nerves after sonic muscle fibers are removed. Right middle, diagram of lateral (upper pane) and dorsal (lower pane) views of brain and spinal cord in relative position as fish above (telencephalon not shown). Dots on dorsal spinal cord indicate positions for A-type labeled motor neuron somata in horizontal sections after application of biocytin to sonic muscle fibers in three fish. Bottom, three images of horizontal sections of the rostral spinal cord with biocytin labeled A-type cell bodies and fibers, whose dense branching patterns are more apparent in transverse sections. Scale bars on bottom panel are 100 μm . 4v = 4th ventricle, bl = Baudelot's ligament, cb = cerebellum, cl = cleithrum, epo = epioccipital, exo = exoccipital, hypax = hypaxial muscle, IL = inferior lobe of the hypothalamus, IX = glossopharyngeal nerve, hyp = hypothalamus, pto = pterotic, O = occipital nerve, r = rib, S1-4 = spinal nerves 1-4, sb = swim bladder, SM = sonic muscle, soc = supraoccipital, T = tectum, v1-5 = vertebrae 1-5, X = vagal nerve, and XIII = eighth nerve.

Sarcomere lengths. Sarcomere lengths were estimated from measurements of transmission electron micrograph (TEM) digital micrographs. Because these fish were deeply anesthetized, but not curarized prior to sacrifice (see above), the estimated values may be lower than actual sarcomere lengths. However, this measurement should provide a low-end estimate and a valid comparison between adjacent sonic and non-sonic muscles which were prepared identically and simultaneously. Several measurements were taken from each micrograph (1-9, total of 182) and values were averaged for each micrograph (a total of 33 micrographs, 17 sonic and 16 non-sonic, $n = 4$ fish).

Transverse tubule diameter. Initial observations (see Results) of the TEM micrographs indicated that the triads of sonic muscle were unusual in the diameter of their transverse tubule membranes (t-tubules). To examine this further, the

space between the membranes of the transverse tubules of triads was measured with 33 micrographs (17 sonic and 16 non-sonic, $n = 4$ fish). A total of 318 measurements were made (2-18 per micrograph) and values were averaged for each micrograph.

Muscle fiber diameters. Muscle fiber diameters were measured from cross sections of 67 fibers chosen haphazardly from paraffin embedded sonic and control hypaxial tissue ($n = 1$ fish, see above). Diameters were calculated using area measurements using the formula for a circle, diameter = $2(\text{area}/\pi)^{1/2}$. Median observations are presented because sonic muscle fiber area and diameter was not normally distributed.

Somata size. Somata cross-sectional area (μm^2) of biocytin labeled neurons were estimated from digital micrographs. Somata were divided into two classes, A-type and B-type, based

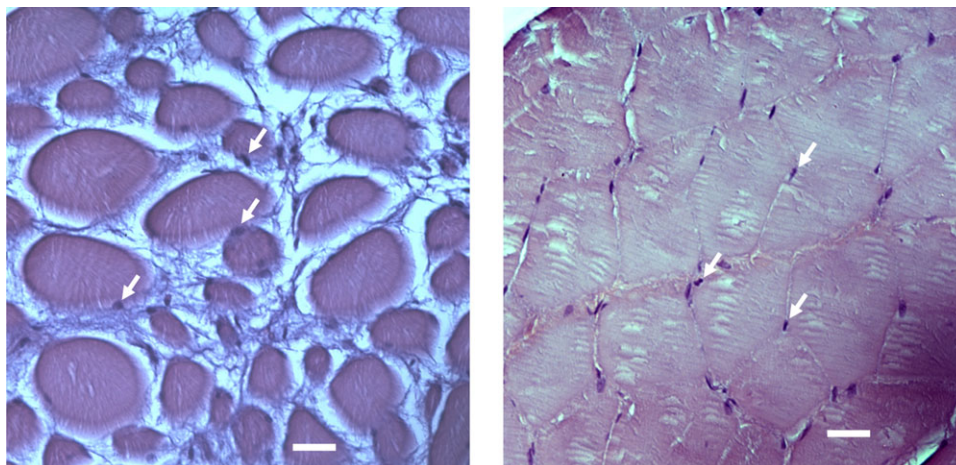


Fig. 2. Adjacent sonic and non-sonic muscle fibers in the trunk of the Pyramid Butterflyfish (*Hemitaurichthys polylepis*). Cross section of sonic (left) and hypaxial (right) muscle fibers stained with hematoxylin and eosin. Scale bars = 20 μm . Arrows point to nuclei. Note that the rounded shape and smaller fiber diameter of sonic fibers and polygonal shape of regular hypaxial fibers. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

on a combination of their morphology, location, and pattern of processes.

Statistical analyses. Replicate measurements were averaged for each individual fish. In all cases, normality tests did not indicate skewed distributions ($P > 0.05$), so data were not transformed. Paired t -tests with a type I alpha level of 0.05 were used to test for differences in sarcomere length and t-tubule diameter between sonic and non-sonic muscle and for differences in soma size between A- and B-type somata. A Bonferroni adjusted alpha level of 0.025 was used for the two tests conducted with TEM micrographs.

Sexes. No obvious qualitative differences between sexes were observed for any histological feature associated with the sonic apparatus. Small within-sex sample sizes, however, precluded statistical tests for sex differences.

RESULTS

Pyramid Butterflyfish possess an unusual loose arrangement sonic muscle fibers, described previously by gross dissection (Boyle and Tricas, 2010), that are distributed sparsely within the endomygium. Muscle fibers originate on the pterotic, epioccipital, and exoccipital of the neurocranium, run posteroventrally, and have insertions on the anterolateral faces of the ribs of vertebra 3, 4, and 5 (Fig. 1). Additionally, some fibers attach to the connective tissue surrounding the swim bladder and to the medial face of the supracleithrum. Baudelet's ligament passes through this musculature (Fig. 1). A large intercostal space exists over the anterior swim bladder, where the sonic musculature is widest (Fig. 1). This location corresponds to the area of swim bladder which buckles during pulse sound emission (Boyle and Tricas, 2010). The rib of vertebra 5 has a large anterolateral face for muscle insertion and lies just anterior to a large aponeurosis that runs the length of the lower half of the body. Ventral to the sonic musculature, more rigid hypaxial muscle fibers occur. These hypaxial

muscle fibers are darker in appearance in preserved specimens than are those from the sonic musculature.

Muscle Histology

Sonic muscle fibers are cylindrical in cross section, highly variable in size, and surrounded by large extracellular space (Fig. 2). Adjacent hypaxial trunk muscle fibers, by contrast, are polygonal in cross section, show less variation in size and are packed tightly with little extracellular space (Fig. 2). Fibers were small in cross section in sonic ($n = 39$; area median and quartiles 1083, 400, and 1785 μm^2 , estimated diameter median and quartiles 37, 23, and 48 μm) and large for non-sonic ventral hypaxial muscle ($n = 28$; area median and quartiles 3256, 2225, and 3842 μm^2 , estimated diameter 67, 53, and 70 μm). Cores of sarcoplasm were not observed in paraffin sections with light microscopy.

Muscle ultrastructure. In sonic muscle fibers, we observed both radial architecture (Fig. 3A), in which long, spoke-like myofibrils were flanked by linear arrangements of sarcotubules and non-radial architecture (Fig. 3B, C). However, no cores of sarcoplasm within the contractile cylinder were observed. Sonic muscle mitochondria were located within sarcoplasm along the periphery (Fig. 3A) and between myofibrils (Fig. 3B, C). Ventral hypaxial musculature had a non-radial arrangement, with myofibrils of variable cross-section morphologies, and mitochondria located between myofibrils and along the peripheral sarcoplasm (Fig. 3D, E). Mitochondria were not densely concentrated in either type of muscle fiber, but were more abundant along the peripheral sarcoplasm of the sonic muscle fiber.

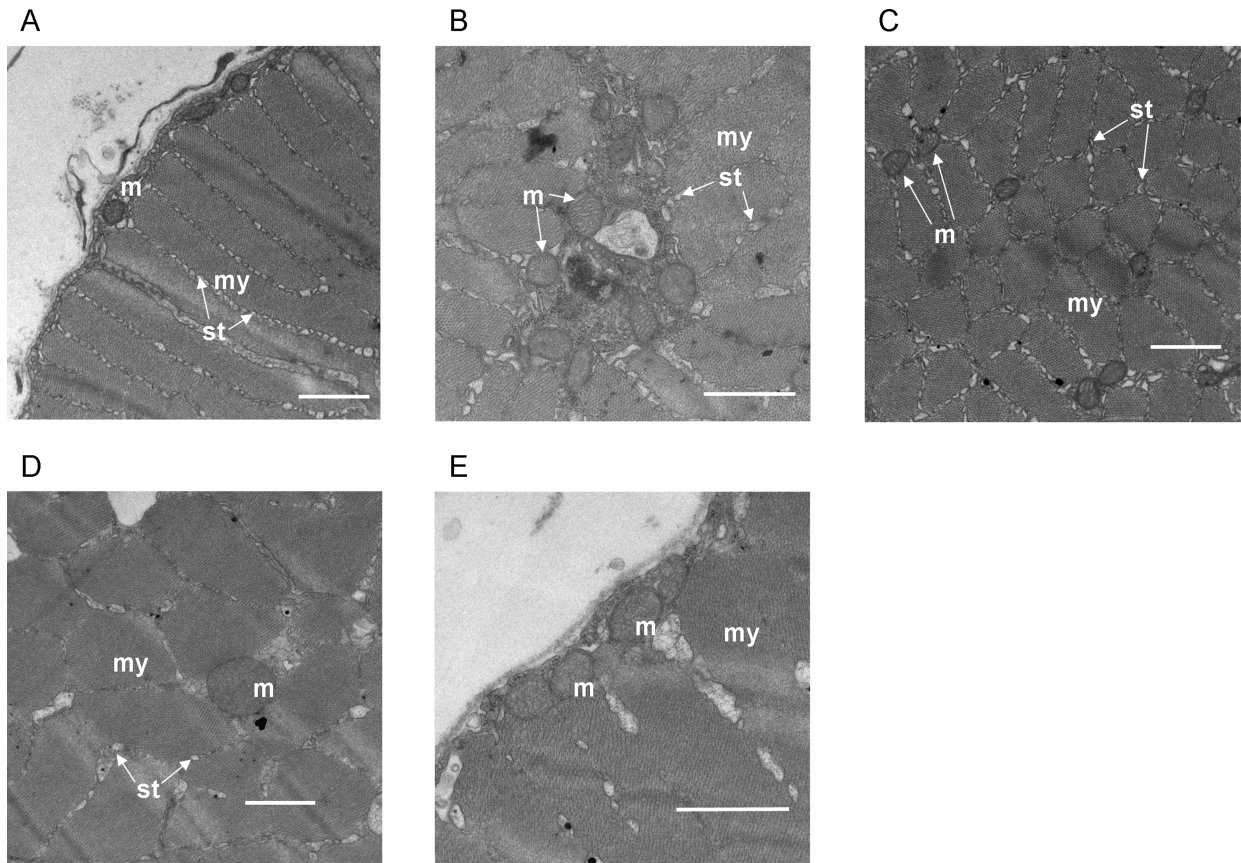


Fig. 3. Transmission electron micrograph cross sections of sonic muscle and white hypaxial trunk muscle of the Pyramid Butterflyfish (*Hemitaurichthys polylepis*). (A) Cross section of sonic muscle fiber shows spoke-like, radially arranged myofibrils (my). Unlike toadfish and weakfish sonic muscle, however, no cores of sarcoplasm were identified in the center of the muscle fiber. (B) Small concentration of mitochondria (m) and sarcoplasm in the center of the contractile cylinder of sonic muscle. (C) Non-ribbon-like myofibrils with interspersed mitochondria in sonic muscle. (D) Cross section of white trunk muscle fiber shows irregular, tightly packed myofibrils with mitochondria along the fiber periphery. (E) Cross section of white trunk muscle fiber showing rounded, tightly packed myofibrils. Scale bars = 1 μ m. st = sarcotubules of the SR.

Longitudinal sections of sonic muscle fibers (Fig. 4A–C) and ventral hypaxial muscle fibers (Fig. 4D–F) revealed all triads were located along the Z-line. Terminal cisternae of sonic muscle fibers tended to be flask-shaped, and enlarged relative to ventral hypaxial musculature (Fig. 4). Electron dense glycogen granules were visible in terminal cisternae of both sonic (Fig. 4C) and non-sonic muscles (Fig. 4F). Sonic muscle fibers had a more complex SR with numerous invaginations of the membrane and a large surface area (Fig. 4B) than did ventral hypaxial muscle fibers (Fig. 4E). Sonic muscle t-tubules were greater in diameter than hypaxial t-tubules (Fig. 4, paired *t*-test, d.f. = 3, $P = 0.003$, mean t-tubule diameter \pm s.e., $0.075 \pm 0.008 \mu\text{m}$ for sonic muscles and $0.051 \pm 0.005 \mu\text{m}$ for hypaxial musculature).

Sarcomere lengths estimated from micrographs indicated variability between individuals (mean \pm s.e., $1.75 \pm 0.043 \mu\text{m}$ for sonic muscle and $1.70 \pm 0.031 \mu\text{m}$ for hypaxial fibers). No statistical difference between sonic and non-sonic muscle sarcomere

length was found (paired *t*-test, d.f. = 3, $P = 0.07$), though the average length of the sonic sarcomere was longer in each fish, the sample size ($n = 4$) and observed variability in magnitude between sarcomere length differences of each muscle type (s.d. = $0.035 \mu\text{m}$) resulted in a low statistical power (0.31).

Sonic Muscle Innervation and Motor Neurons

Retrograde labeling of sonic muscle fibers filled motor neuron somata in the rostral spinal cord but not the occipital nerves. Sonic muscle fibers are innervated by spinal nerves (S1–S3) and horizontal sections indicated that sonic motor neurons have a broad rostrocaudal distribution (Fig. 1). No contralateral labeling of fibers or cell bodies was observed in any section.

Sonic motor somata were not clustered into a discrete spinal nucleus. Instead labeled cells were loosely distributed along a 400–1640 μm length of

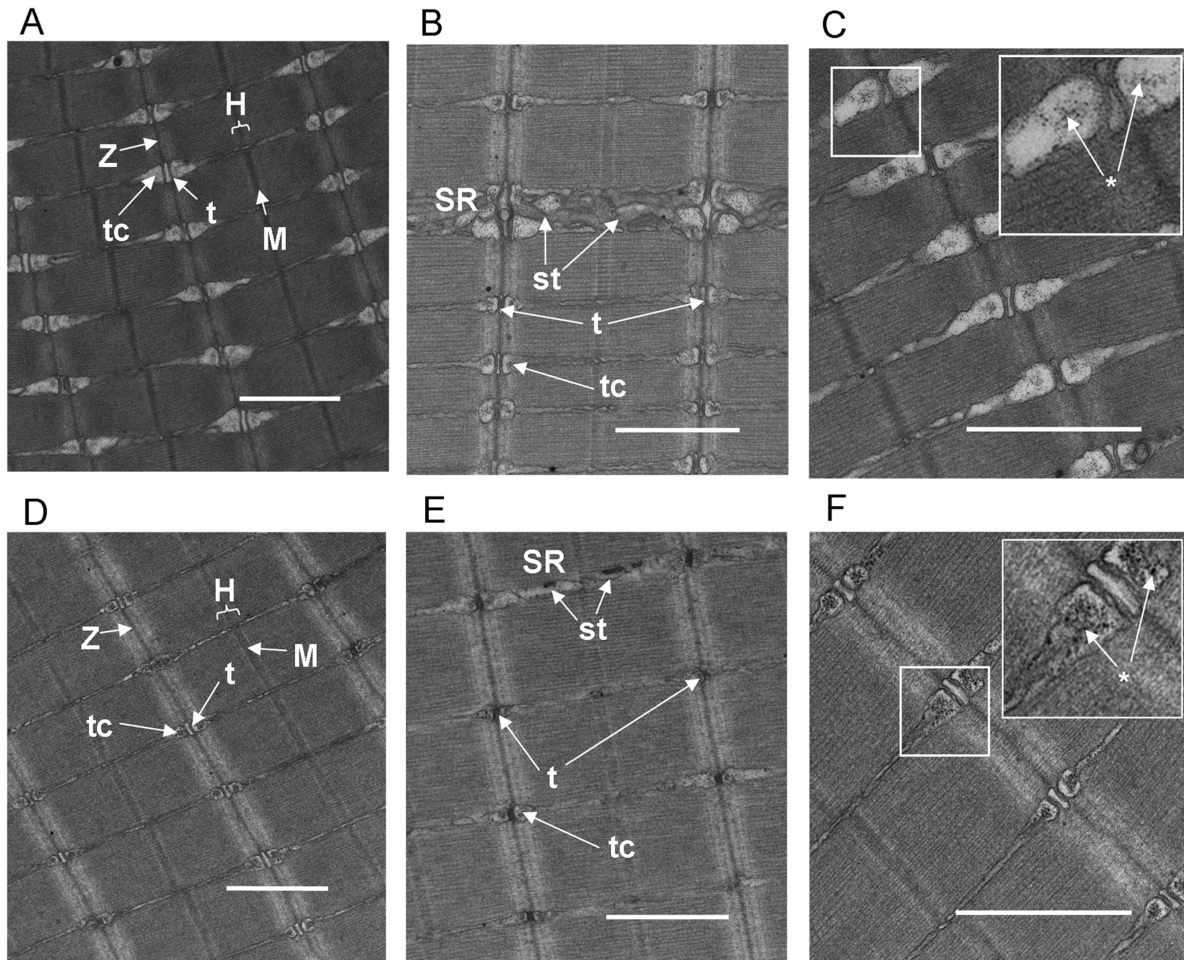


Fig. 4. Longitudinal TEM micrographs of the sonic (A, B, and C) and white trunk (D, E, and F) hypaxial muscle fibers in the Pyramid Butterflyfish (*Hemitaenichthys polylepis*). Note position of t-system triads at the Z-line (Z) in muscle fibers of both regions. Relative to white trunk hypaxial fibers, sonic muscle fibers have transverse tubules (t) with larger diameters and enlarged flaring of the terminal cisternae (tc) of the sarcoplasmic reticulum (SR). In panel B, note that the well developed SR of the sonic muscle fibers. Note the presence of numerous electron dense particles in the terminal cisternae (*, inset) in both fiber types (C and F). Scale bars = 1 μm , inset in panel C and F $0.5 \times 0.5 \mu\text{m}$. H = h-zone, M = M-line, and st = sarcotubules of the SR.

spinal segments 1–2 (Figs. 5 and 6) in which two cell morphologies were identified. A-type motor neuron somata were ovoid to discoid in shape and often showed multipolar processes. They were located in a medial and dorsal position, ventrolateral to the central canal, and dorsolateral to the Mauthner axon and medial longitudinal fasciculus (MLF; Fig. 5A–C). A-type cells were sparse in transverse sections (1–3 co-labeled cells, median 1 per/section). Processes of these cells bifurcated and extended laterally and to a lesser extent ventrally and dorsally (Fig. 5A–C), whereas a larger single process coursed ventrally towards the Mauthner axon (Fig. 5C). A-type cells often showed bifurcated processes. Labeled cells were not distinct in cytoarchitecture, size, or location from adjacent non-labeled cells.

In contrast, B-type neurons were positioned ventral and lateral to A-type neurons, and lateral to

the MLF and ventral fasciculus (FV; Figs. 5C, D and 6A–F). Cell bodies were fusiform in shape and showed a broad but sparse distribution along the spinal cord (1–5, median 2 per section). Adjacent unlabeled neurons had a similar cytoarchitecture, size, and location. Furthermore, B-type cells typically showed only one or two processes that extended dorsolaterally (Fig. 6A, B) and in several sections had faintly stained axonal projections to ventral spinal roots (Fig. 6C). Somata of A-type neurons were greater in area than B-type neurons (paired *t*-test, d.f. = 2, $P = 0.029$, mean soma area \pm s.e., $553 \pm 38 \mu\text{m}^2$ for A-type and 305 ± 15 for B-type, Fig. 7).

DISCUSSION

This study tested the hypothesis that the extrinsic sonic muscle and motor neurons of the Pyramid

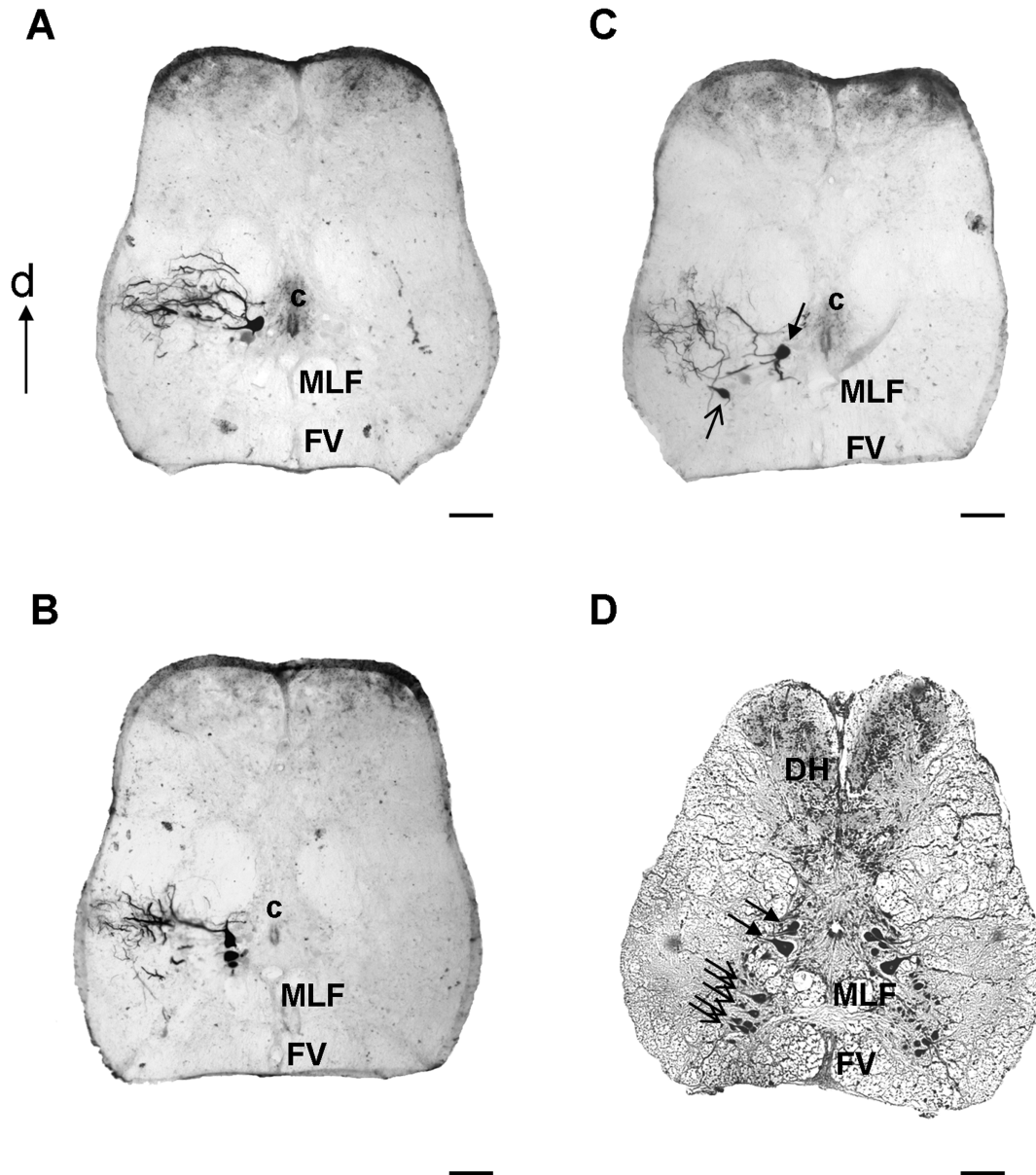


Fig. 5. Photomicrographs of transverse sections show sonic motor neuron organization in the spinal cord of the Pyramid Butterflyfish (*Hemitaurichthys polylepis*). (A–C) Counterstained sections with sonic motor neurons labeled with retrograde application of biocytin in the sonic muscles. (A, B) Cell bodies of A-type motor neurons are located medially near the central canal and show dense, highly branched, laterally oriented processes, and sometimes (C), a single process that projects ventrally to the Maunther axon. (C) Fewer sections contained both A-type (solid arrowhead) and B-type (open arrowhead) labeled cell bodies. (D) For comparison, a cresyl-violet stained paraffin section from a similar location within the spinal cord shows numerous motor neuron somata that are of similar size and location to labeled B-type (closed arrowhead) and A-type (open arrowhead) cell bodies. These motor neurons may innervate non-labeled portions of sonic musculature or non-sonic muscle fibers. Scale bars = 100 μm . c = central canal, d = dorsal, DH = dorsal horn, FV = ventral fasciculus, and MLF = medial longitudinal fasciculus.

Butterflyfish show morphological specializations that promote fast muscle contractions during sound production. Specialized features of the butterflyfish sonic muscles and motor neurons share similarities with some, but not all sonic fish species examined so far. Our data provide evidence that compared to adjacent non-sonic trunk muscle, sonic muscles possess morphological differences that include reduced fiber size and density, elabo-

ration of the SR, larger diameter t-tubules, and radially arranged myofibrils bordered by an extensive sarcotubule network. Most of the fish species examined in previous studies have occipital innervated sonic muscles which result in clustered nuclei in the caudal medulla and rostral spinal cord. In Pyramid Butterflyfish, however, sonic muscle was innervated by spinal nerves S1–S3 and sonic motor somata were found to have a

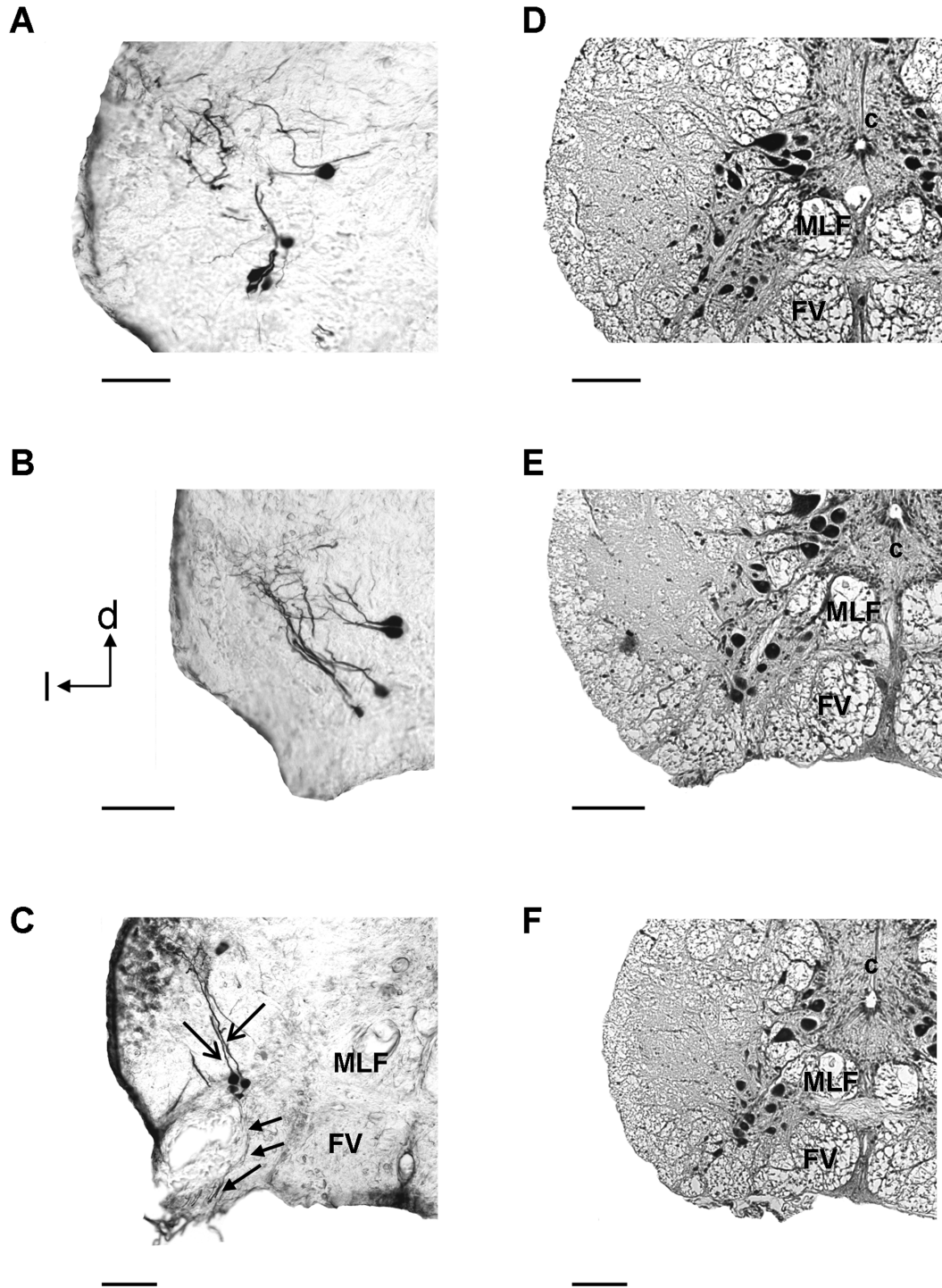


Fig. 6. Photomicrographs of transverse sections of the rostral spinal cord that emphasize B-type motor neurons in the Pyramid Butterflyfish (*Hemitaurichthys polylepis*). (A–C) Sonic motor neurons labeled with biocytin. Note the extensive dorsal processes present in A and the prominent extension of dorso-lateral processes in B and C. Arrows with closed heads in C point to axons that exit a ventro-lateral spinal nerve and arrows with open heads point to dorso-lateral oriented dendrites. (D–F) Cresyl-violet stained paraffin sections from a similar location within the spinal cord are shown for comparison. Note numerous B-type motor neurons that may include innervation of sonic or non-sonic muscle fibers. Centrally-located A-type fibers are also visible in D and F. Scale bars = 100 μ m. c = central canal, d = dorsal, FV = ventral fasciculus, and MLF = medial longitudinal fasciculus.

broad, sparse distribution entirely within the spinal cord, an arrangement convergent with the distantly related, non-euteleost piranhas. However,

unlike piranhas, Pyramid Butterflyfish sonic motor neurons consisted of two cellular morphologies. A-type cells were larger, had bifurcated processes

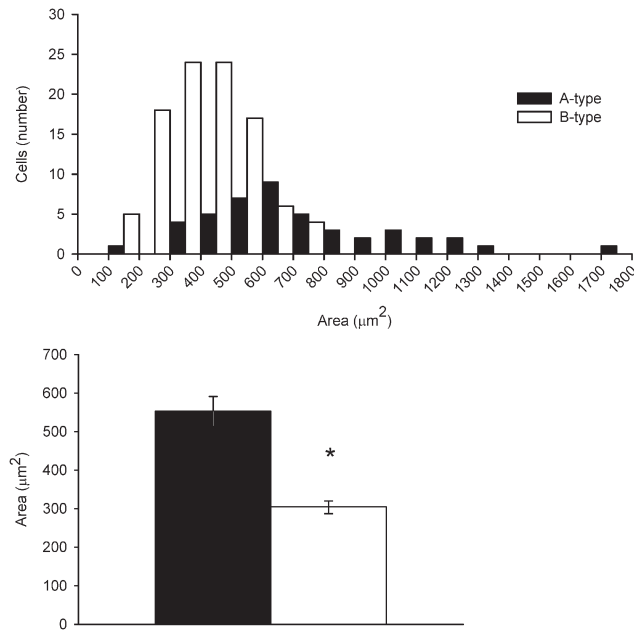


Fig. 7. Sonic motor neuron cell sizes in the Pyramid Butterflyfish (*Hemitaenichthys polylepis*). Histogram (A) of cell area and comparison of cell size between A-type (black bars) and B-type (white bars) cell bodies of three individuals and bar graph (B) of mean cell area after back-transformation of log₁₀ transformed data. (*Denotes cell size difference, $P < 0.05$, paired t -test, error bars indicate s.e.).

and were located slightly ventrolateral to the central canal. B-type somata were smaller and located near the ventrolateral margin of the spinal cord. Neither motor neuron cell type was morphologically distinct from adjacent motor neurons. These findings underscore the importance of independent evolutionary events that have shaped the diversity of fish sound production mechanisms.

Sonic Musculature

Pyramid Butterflyfish possess apomorphic extrinsic muscle fibers that originate on the otic region of the neurocranium and the medial supraclathrum of the pectoral girdle and insert on the first three ribs along with an aponeurosis of the hypaxial musculature (Boyle and Tricas, 2010). The location of this musculature and indirect association with the swim bladder bears similarity with a variety of distantly related sound producing fishes. The holocentrid squirrelfishes and soldierfishes, like the Pyramid Butterflyfish, possess extrinsic sonic muscles associated with the rostral end of the swim bladder (Winn and Marshall, 1963; Salmon, 1967; Carlson and Bass, 2000; Parmentier et al., 2011). Holocentrids possess muscles that originate on the ventral skull, run under the dorsal pectoral girdle and insert on the first or first and second (v3 and v4) ribs and ribbon-like

fascia anterior of the third rib (Winn and Marshall, 1963; Parmentier et al., 2011). Similarly, the Pinecone fish (*Monocentrus japonica*; Monocentridae) has extrinsic swim bladder muscles that originate on the back of the skull, but insert on the anterior swim bladder (Onuki and Somiya, 2007). Similar muscles were described in scorpaeniform fishes such as the Southern Pigfish (Congiopodidae: *Congiopodus leucopaecilus*; Packard, 1960), and the Marbled Rockfish (Scorpaenidae: *Sebasticus marmoratus*), which has extrinsic muscles that originate on the neurocranium and insert on the anterior swim bladder (Miyagawa and Takemura, 1984; Suzuki et al., 2003). The perciform Tigerperch (Terapontidae: *Terapon jarbua*) has sonic muscles that originate on the caudoventral neurocranium and supraclathrum and insert on the anterior swim bladder (Eichelberg, 1976; Onuki and Somiya, 2007). Sonic muscles originate on the pterotic of the neurocranium and insert on the anterior swim bladder of the perciform Silver Sweeper (Pempheridae: *Pempheris schwenkii*; Takayama et al., 2003) and the closely related Pearl Perch (Glaucosomatidae: *Glaucosoma buergeri*), which possesses an antagonistic smooth muscle that is thus far unique among known sonic fish mechanisms (Mok et al., 2011). Doradid catfishes possess an extrinsic drumming muscle that originates in the occipital region of the neurocranium and inserts on the transverse process of the 4th vertebra, which is attached to the swim bladder (Ladich, 2001). Paracanthopterygian cusk-eels (Ophidiidae) also possess sonic muscles that originate on the caudal end of the neurocranium, course medial to the dorsal pectoral girdle, and insert on bony elements (epineurals and an ossification of the anterior swim bladder) in close association with the swim bladder (Parmentier et al., 2006, 2010; Fine et al., 2007). Though extrinsic sonic muscles of many distantly related fishes have similar origins, insertions, and actions, a variety of apomorphic histological features of the muscles and their motor neurons demonstrate the independent origins of these sound producing organs.

A trend towards smaller muscle fiber diameters is apparent among many sonic fish species. Muscle fiber diameters of Pyramid Butterflyfish are similar to fiber diameters of other sound producing fish species (Batrachoididae, Carapidae, Dactylopteridae, Triglidae, Terapontidae, and Sciaenidae; reviewed in Parmentier and Diogo, 2006). White trunk muscle fiber diameters from Pyramid Butterflyfish were larger than adjacent sonic muscles, similar to observations of other sonic fish taxa (Parmentier and Diogo, 2006). However, the fiber diameters of these white trunk muscle fibers (27–35 μm) were at the low end of the more variable range of observations from other studies (Parmentier and Diogo, 2006). Smaller sonic muscle

fiber diameters are proposed to be an adaptation for more efficient transfer of metabolites, oxygen, and calcium ions because of the increased surface to volume ratio (Eichelberg, 1976; Parmentier and Diogo, 2006). Sonic muscle fibers of male Weakfish enlarge during the spawning season (though to a size substantially smaller than adjacent hypaxial fibers, see Ono and Poss, 1982) when sounds are produced but a central core of sarcoplasm also enlarges and fragments (Connaughton et al., 1997). This fragmented sarcoplasm core is thought to mitigate the loss of surface to volume ratio of the myofibrils and provides space for proliferation of mitochondria within hypertrophied fibers (Fine et al., 1993; Connaughton et al., 1997). Cores of sarcoplasm were not observed in Pyramid Butterflyfish, and it is not known if features of the sonic musculature differ among seasons or sex. Field observations in the spring and summer indicated that males produce sounds for courtship and agonistic competition among males, but both females and males produce putative distress or alert calls in the laboratory at all times of the year (Boyle and Tricas, 2010). Additional studies are required to determine if changes in sonic muscle fiber diameter and concentration of sarcoplasm occur seasonally, ontogenetically, or between sexes as reported for several other fishes (Bass and Marchaterre, 1989; Connaughton et al., 1997; Loesser and Fine, 1997).

The radial arrangement of many myofibrils in Pyramid Butterflyfish sonic muscle fibers is consistent with the hypothesis of increased transport efficiency of calcium ions and metabolic substrates. Radially arranged, ribbon-like myofibrils are hypothesized to be adaptations for rapid contraction that minimize the distance between SR and myofibrils to maximize calcium exchange from the SR to contractile proteins (Fine et al., 1993). This morphology is reported from a variety of fish sonic muscles that include pimelodid and doradid catfishes (Ladich, 2001), Oyster Toadfish (Fawcett and Revel, 1961; Loesser and Fine, 1997), Plainfin Midshipman (Bass and Marchaterre, 1989), soldierfishes (Parmentier et al., 2011b), Weakfish (Ono and Poss, 1982), Tigerperch (Eichelberg, 1976), and Pearl Perch (Mok et al., 2011). Pyramid Butterflyfish sonic muscle, like the sound producing muscles of these other fishes, possesses a well developed SR. This SR is visible as a dense network of sarcotubules between myofibrils that may provide rapid exchange of calcium ions with the myoplasm for crossbridge formation and detachment (Feher et al., 1998; Rome, 2006). The SR of Pyramid Butterflyfish sonic muscle appears more developed and convoluted than trunk muscle fibers, but notably less voluminous than the complex SR observed in Oyster Toadfish sonic muscle (Appelt et al., 1991). This difference likely reflects the higher contraction rates and longer sustained

calls (routine calls c.a. 200 Hz for 200–400 ms) of the Oyster Toadfish (Fine et al., 2001). The significance (if any) of the variation in sonic muscle myofibrils (radial vs. non-radial arrangements) in the Pyramid butterflyfish remains to be determined, but may reflect the level of modification required to produce sounds of short duration relative to the long calls and fast contractions of species like the Oyster Toadfish.

In addition to modifications of the SR, the t-system of certain fish sonic muscles vary in location and morphology. Similar to fish axial muscles, the t-tubules of sonic muscles in some species are located at the Z-line (Johnston, 1981). Pyramid Butterflyfish sonic muscles possessed Z-line type triads in both trunk and sonic muscle. Several other sound producing fishes have Z-line type muscle fibers. These include piranha (Eichelberg, 1977), pimelodid and doradid catfishes (Ladich, 2001), and Weakfish (Ono and Poss, 1982). However, the t-system in sonic muscles of the Oyster Toadfish (Fawcett and Revel, 1961), the Plainfin Midshipman, Northern Searobin (*Prionotus carolinus*; Bass and Marchaterre, 1989), and Tigerperch (Eichelberg, 1976) are found at the A–I boundary similar to mammalian muscles. The sonic muscles of Pearl Fish (*Carapus acus*) are unusual in that triads are found at both the Z-line and A–I boundary (Parmentier et al., 2003). In Marbled Rockfish, triads are located at the A–I boundary in the middle portion of sonic muscle fibers, at the Z-line at the end points (neurocranium and swim bladder), and at both the Z-line and A–I boundary at an area adjacent to the middle of the fiber (Suzuki et al., 2003). Thus, the position of triads at the A–I boundary in sonic muscle fibers has evolved through convergence and multiple times.

The location of triads at the A–I boundary is hypothesized to be adaptive for fast contractions. This triad morphology results in two t-tubules per sarcomere rather than one (Ladich and Fine, 2006) and may also reduce the distance of calcium ion diffusion to the site of contraction and thus increase the speed of depolarization (Parmentier et al., 2003). Eichelberg (1977) proposed that the short sarcomeres in Z-line type sonic muscles of piranha (Serrasalminae; 1.2 μm vs. 1.4 μm in trunk muscle) may allow for similar conduction velocities to those found in muscles with longer sarcomeres and triads at the A–I boundary. This hypothesis gains some support from the observations that some of the fishes with A–I located triads tend to have long sarcomeres: Toadfish (2.0–2.22 μm ; Fawcett and Revel, 1961; Loesser and Fine, 1997), Plainfin Midshipman (2.0–3.4 μm ; Bass and Marchaterre, 1989), Marbled Rockfish (2.2–2.3 μm ; Suzuki et al., 2003), and Tigerperch (2.2 μm ; Eichelberg, 1976). Pyramid Butterflyfish sonic muscles measured in this study had smaller sarcomere lengths (1.74 μm), similar to weakfish

(1.5 μm ; Ono and Poss, 1982), but were longer than those of the piranha (Eichelberg, 1977). However, this association is not observed universally as two sound producing catfishes (*Platydoras costatus* and *Pimelodus pictus*) have Z-line type triads despite long sarcomeres (2.2–2.3 μm ; Ladich, 2001). Further, the relationship between sonic and non-sonic muscle sarcomere length varies among taxa. Sarcomeres within Pyramid Butterflyfish sonic muscles were of similar size to adjacent trunk muscle fibers. Tigerfish have longer sonic muscle sarcomeres than white trunk muscle fiber sarcomeres (Eichelberg, 1976), but piranha have shorter sonic muscle fiber sarcomeres than trunk muscle (Eichelberg, 1977). Thus, variability in triad location in sonic muscles of sound producing fishes may be related to sarcomere length.

The triads of Pyramid Butterflyfish sonic muscle were unusual in morphology compared to other sonic fishes. The terminal cisternae often were enlarged and flask shaped in sonic muscle but not in trunk muscle. Additionally, the diameters of sonic muscle t-tubules were greater than their counterparts in trunk muscles. The functional relevance for the observed differences in muscle cytoarchitectures remains to be tested. T-tubules are involved in transmission of the action potential from the surface of the muscle fiber to deep inside the muscle cell, where release of calcium ions from the SR is initiated (Feher et al., 1998; Rome, 2006). An enlarged t-tubule volume increases the volume-to-surface ratio of the lumen and could function to increase the speed of action potential propagation along the t-tubule network (Launikonis and Stephenson, 2002). An additional, nonmutually exclusive hypothesis, is that t-tubule swelling may be a plastic, reversible response caused by fast accumulation of intracellular lactate (a byproduct of muscle contraction) with a local release at the t-tubule that accumulates water (Usher-Smith et al., 2007). The enlarged terminal cisternae, which are the site of calcium ion release, may enhance the transmission of the action potential from the t-tubule to the SR, deliver and resequester calcium ions to and from the myoplasm more efficiently, or both.

Sonic Motor Neurons

Patterns among teleosts. Phylogenetic and functional patterns of sonic motor pathways have received attention in a number of fish studies (Bass and Baker, 1991; Ladich and Bass, 1998; Carlson and Bass, 2000; Ladich and Bass, 2005; Onuki and Somiya, 2007). The variety of distantly related teleost lineages with highly soniferous members that employ muscle-driven swim bladder mechanisms has led to interest in sonic motor neural circuits and the pattern of muscle innervation. Although there is evidence of possible deep

homology for some aspects of vocal organ development among fish and tetrapods (Bass et al., 2008), the diversity of musculo-skeletal features involved in sound generation indicates many independent evolutionary events have occurred among taxa. Further, sound producing mechanisms are known currently from a diverse array, yet significant minority of fishes. Thus, teleost-wide sound production homologies would require many independent evolutionary losses, which would seem unlikely (Parmentier and Diogo, 2006).

Previous authors examined the patterns of sonic motor innervation within a phylogenetic framework (Ladich and Bass, 1998; Carlson and Bass, 2000; Ladich and Bass, 2005; Onuki and Somiya, 2007). This analysis has yielded several patterns of conserved innervation and motor neuron position that indicate possible homology across sonic fish taxa. Recent revision of the phylogenetic hypothesis of teleostean relationships (Wiley and Johnson, 2010) casts doubt on the homology of some of the previously recognized patterns (Table 1). Sonic motor pathways described in fishes thus far involve spinal nerves, occipital nerves or both, with one notable exception of vagal innervation (Table 1). Within the Ostariophysi alone, there are examples of occipital, spinal, and combined occipital spinal innervation of sonic organs and a diverse pattern of motor neuron distribution (Table 1). Most of the euteleost fishes (excluding Pyramid Butterflyfish) examined for sonic motor pathways possess occipital nerve innervated sonic muscles with sonic motor nuclei located in the caudal medulla and rostral spinal cord, in ventral motor columns, lateral to the MLF (Table 1). The well-studied exception of batrachoidids have occipital nerve innervated apomorphic intrinsic sonic muscles with sonic motor nuclei that extend from the caudal medulla to the rostral spinal cord, but are present in a centralized location, ventral to the central canal (Fine et al., 1982; Bass, 1985; Bass and Baker, 1991; Fine and Mosca, 1995). Occipital sonic organ innervation and ventrolateral motor neuron distribution was hypothesized as a conserved feature among Cottidae, Triglidae, and Scorpaenidae (Ladich and Bass, 1998; Yoshimoto et al., 1999; Ladich and Bass, 2005); however, recent phylogenetic hypotheses have disputed the inclusion of cottoid fishes within the Scorpaeniformes (Wiley and Johnson, 2010). Currently, occipital nerve sonic organ innervation is most prevalent among euteleosts studies that have used neuroanatomical tracing techniques. These taxa, however, represent only a small fraction of the diversity of euteleosts, for which new examples of sound production continue to be discovered. Further several of the soniferous euteleosts which are known to have spinal innervation of sonic muscles have not yet been examined for their motor neuron distribution. Thus, a high-degree of homoplasy

TABLE 1. Sonic motor pathways and patterns among teleost fishes

	Sonic organ	Innervation	Rostrocaudal extent	Position in transverse plane
Cohort Osteoglossomorpha				
Order Osteoglossiformes				
Family Mormyridae				
<i>Breniomyrus</i> sp. ¹	Ex SM	Vagal	Vmn. in Med.	dors. cent.
Cohort Otomorpha				
Subcohort Ostariophysi				
Section Otophysi				
Superorder Characiphysae				
Order Characiformes				
Family Characidae				
<i>Pygocentrus nattereri</i> ^{2,3}	Ex SM	Spinal Only	rSC only	cent.
<i>Serrasalmus rhombeus</i> ²	Ex SM	Spinal Only	rSC only	cent.
Order Siluriformes				
Family Ariidae				
<i>Arius felis</i> ⁴	Ex SM	Occ.	cMed &rSC	vent. lat.
<i>Bagre marinus</i> ⁴	Ex SM	Occ.	cMed &rSC	vent. lat.
Family Doradidae				
<i>Platydoras costatus</i> ⁴	Ex SM	Occ.	cMed &rSC	cent.
Family Mochokidae				
<i>Synodontis nigriventris</i> ⁵	Ex SM	Occ., Sp 1	cMed &rSC	cent.
<i>Synodontis nigromaculatus</i> ⁵	Ex SM	Occ., Sp 1	cMed &rSC	cent.
Family Pimelodidae				
<i>Pimelodus blochii</i> ⁴	Ex SM	Occ., Sp 1,2	cMed &rSC	vent. lat. & cent.
Cohort Euteleostei				
Subcohort Neoteleostei				
Section Ctenosquamata				
Subsection Acanthomorphata				
Division Berycacea				
Order Beryciformes				
Family Holocentridae				
<i>Holocentrus rufus</i> ⁶	Ex SM	Occ.	cMed &rSC	vent. lat.
<i>Neoniphon sammara</i> ⁶	Ex SM	Occ.	cMed &rSC	vent. lat.
<i>Sargocentron seychellense</i> ⁶	Ex SM	Occ.	cMed &rSC	vent. lat.
<i>Sargocentron xantherythrum</i> ⁶	Ex SM	Occ.	cMed &rSC	vent. lat.
Family Monocentridae				
<i>Monocentris japonica</i> ⁷	ExSM	Occ.	cMed	vent. lat.
Division Percormorphacea <i>incertae sedis</i>				
Order Anabantiformes	Pect. Strid.	Occ.	cMed &rSC	vent. lat.
Family Osphronemidae				
<i>Trichopsis vittatus</i> ⁸				
Order Batrachoidiformes	In SM	Occ.	cMed &rSC	cent.
Family Batrachoididae	In SM	Occ.	cMed &rSC	cent.
<i>Opsanus tau</i> ^{9,10}				
<i>Porichthys notatus</i> ^{1,11}				
Order Cottiformes	Pect. Vib.	Occ.	cMed &rSC	vent. lat.
Family Cottidae	Pect. Vib.	Occ.	cMed &rSC	vent. lat.
<i>Leptocottus armatus</i> ⁵				
<i>Myoxocephalus scorpius</i> ¹¹				
Order "Perciformes"	Ex SM	Spinal Only	rSC only	vent. lat. & cent.
Family Chaetodontidae				
<i>Hemitaenichthys polylepis</i> ^{PS}				
Order Scorpaeniformes	Ex SM	Occ.	cMed &rSC	vent. lat.
Family Scorpaenidae				
<i>Sebasticus marmoratus</i> ¹²	In SM	Occ.	cMed &rSC	vent. lat.
Family Triglidae				
<i>Prionotus carolinus</i> ^{1,13}				

cMed, caudal medulla; cent., central; dors., dorsal; ExSM, extrinsic swim bladder, muscles; In SM, intrinsic swim bladder muscles; Pect. Strid., pectoral fin ray-tendon stridulation; Pect. Vib., pectoral girdle vibration; Occ, occipital; lat., lateral; rSC, rostral spinal cord; Sp 1, spinal nerve 1; Sp 1,2, spinal nerves 1 and 2; vent., ventral; Vmn. in Med., ventral medial nucleus in medulla.

Rostrocaudal extent describes the rostro-caudal distribution of sonic motoneurons relative to the brain and spinal cord. Right describes the location of sonic motoneurons in the transverse plane: cent., centralized, ventral to central canal; dors. cent., centralized but dorsal to central canal; vent. lat., ventral and lateral to medial longitudinal fasciculus; and vent. lat. & cent., nuclei present ventral and lateral to central canal.¹Bass (1985), ²Ladich and Bass (1998), ³Onuki et al. (2006), ⁴Ladich and Bass (1998), ⁵Ladich and Bass (1996), ⁶Carlson and Bass (2000), ⁷Onuki et al. (2010), ⁸Ladich and Fine (1992), ⁹Fine et al. (1982), ¹⁰Fine and Mosca (1989), ¹¹Bass and Baker (1991), ¹²Yoshimoto et al. (1999), ¹³Finger and Kalil (1985), ^{PS}Present study. Teleost classification based on Wiley and Johnson (2010). Quotes around Perciformes indicate putative designation not supported yet by synapomorphies (Wiley and Johnson, 2010).

among sonic organ evolution may be present among all major sonic teleost clades. There is a clear need for more studies on sonic motor neuron innervation in additional euteleost fish taxa to determine phylogenetic patterns of conservation and convergence related to the neural circuitry of acoustic signaling.

The location of sonic motor neurons of the Pyramid Butterflyfish is contained entirely within the spinal cord, not in a distinct nucleus, and in ventrolateral and central locations that differ from that reported for many other sonic fishes (Table 1). This pattern is similar to that described for the very distantly related and non-euteleost piranha. Piranha sonic muscle has spinal nerve innervation with sonic motor neurons located entirely within the spinal cord and adjacent to motor neurons that appear to innervate non-sonic areas (Ladich and Bass, 2005; Onuki et al., 2006). However, unlike piranhas, small motor neurons (B-type) located along a ventral motor column were also labeled in Pyramid Butterflyfish. Sonic motor neurons located entirely in the spinal cord are likely more widespread among acanthomorph fishes, as a variety of taxa with spinal nerve innervation of sonic muscle are not yet examined with nerve tracing methods (Onuki and Somiya, 2007). These taxa include the gadiform Walleye Pollock (Gadidae: *Theragra chalcogramma*; Onuki and Somiya, 2006), the zeiform John Dory (Zeidae: *Zeus faber*; Onuki and Somiya, 2004), and the highly soniferous perciform sciaenid fishes (Ono and Poss, 1982; Vance et al., 2002). The diversity of sonic motor neuron patterns among distantly related teleosts indicates multiple independent evolutionary modifications of features associated with the occipital region of the central nervous system and anterior spinal nerves.

Somata sizes. Overall, the sonic motor neurons of Pyramid Butterflyfish were similar in size to other sonic fishes (Ladich and Bass, 1998; Carlson and Bass, 2000; Onuki et al., 2006). Two discrete labeled somata types were observed in this study. These cells are similar in morphology and neuroanatomical location to primary and secondary motor neurons of other studies (Fetcho, 1986; Westerfield et al., 1986). However, our neuroanatomical tracing did not yield B-type motor neurons with somata or axons immediately lateral to the Mauthner axon as is seen in secondary motor neurons. Further, although A-type motor neurons send a process towards the Mauthner axon, these presumed axons do not appear to course in close proximity to the entire medial boundary of the Mauthner axon as is reported for primary motor neurons (Fetcho, 1986; Westerfield et al., 1986). Red muscle fibers in the Goldfish *Carassius auratus* are innervated by secondary motor neurons, whereas white muscle fibers are mainly innervated by primary motor neurons with some secondary motor neurons also (Fetcho, 1986). The fiber type

of Pyramid Butterflyfish sonic muscles is yet to be confirmed with histochemical analyses, but the nonburst like pattern of electromyograms during sonic contractions (Boyle and Tricas, 2011) indicates they are likely fast-twitch fibers associated with rapid contractions, similar to fibers of other sound producing fishes (Fine and Pennypacker, 1988; Chen et al., 1998). The A-type sonic motor neurons were larger than many reported so far, with the notable exception of the trunk motor neurons in the Goldfish *Carassius auratus* (Fetcho, 1986). Further, these cells were larger than epaxial and pectoral fin musculature motor neurons of Croaking Gourami (Ladich and Fine, 1992), comparable to the sonic motor neurons of Plainfin Midshipman (Bass and Baker, 1991) and Pinecone-fish (Onuki et al., 2010), and were smaller on average, but overlap in range with sonic motor neurons from Oyster Toadfish (Fine and Mosca, 1995) and Marbled Rockfish (Yoshimoto et al., 1999). In addition, the A-type motor neurons, though smaller on average, broadly overlap the size range of pectoral fin abductor superficialis of the osteoglossiform *Pantodon buchholzi* that are used in a rapid escape response (Starosciak et al., 2008). Large motor neuron somata are hypothesized to be an adaptation for innervation of muscles with high contraction rates (Bass, 1985) and may provide a metabolic advantage for increased call production (Fine and Mosca, 1995). Further, to facilitate synchronous contractions, the sonic muscle fibers of several fish species are innervated by multiple motor endplates (Ono and Poss, 1982; Hirsch et al., 1998; Kobayashi et al., 2004) and in Oyster Toadfish the neuromuscular junction (NMJ) size increases with body size (Hirsch et al., 1998). Thus, large motor neurons may be necessary for neurotransmitter requirements of neurons that innervate multiple large NMJs, though the innervation patterns at the level of the muscle fiber in Pyramid Butterflyfish are not known. Thus, the larger A-type motor neurons of Pyramid Butterflyfish, may be necessary to meet the demands of repeated and fast contractions.

This study found distinctive small (B-type) and large (A-type) motor neurons based on location and cytoarchitecture. B-type neurons resemble the ventral motor column neurons present in a variety of sonic taxa (Table 1), whereas A-type neurons labeled in this study resemble epaxial, non-sonic motor neurons labeled as control muscle in Croaking Gourami (Ladich and Fine, 1992) and Threespot Squirrelfish *Sargocentron cornutum* (Carlson and Bass, 2000). Labeled A-type neurons in this study clearly did not come from epaxial myotomes, as all biocytin application took place well below the midlateral horizontal septum. The homology of sonic muscle in Pyramid Butterflyfish is not known. The sonic musculature lies below the lateral septum and inserts along an aponeurosis of

hypaxial myotomes, however, anatomical investigations across an ontogenetic series (e.g., Tracy, 1959, 1961; Hill et al., 1987) are necessary to determine if this muscle is derived from the hypaxialis. Intrinsic sonic muscles of Oyster Toadfish migrate embryologically from occipital somites to a more caudal location around the swim bladder (Tracy, 1959, 1961). Though some sonic muscle fibers originate on the occipital region of the skull (Fig. 1), the pattern of origin within Pyramid Butterflyfish is consistent with the obliquus superioris hypaxial musculature of many teleosts (Winterbottom, 1974).

Our analysis is consistent with the presence of two motor neuron types. In this study, the A-type motor neurons were observed in all fish labeled, whereas B-type motor neurons were more numerous, but labeled in only three of five fish. In other studies, biocytin was hypothesized to label premotor neurons via transneuronal transport (Ladich and Bass, 1996; Carlson and Bass, 2000). A study on Marbled Rockfish sonic motor neurons, however, did not find transneuronal transport of biocytin (Yoshimoto et al., 1999; Ladich and Bass, 2005). The B-type neurons of this study do not appear to be premotor neurons, as their size overlaps many sonic and non-sonic motor neurons found in other fishes, they appear to have axons that exit from ventral nerve roots, and they are similar in morphology to other ventral column motor neurons (e.g., the sonic motor neurons of squirrelfishes; Carlson and Bass, 2000). Thus, multiple neuron types may innervate different areas of sonic muscle fibers present in Pyramid Butterflyfish.

Aspects of fiber connections. No evidence of contralateral dendritic connections from sonic motor neurons was observed for the Pyramid Butterflyfish even though sound production in this species involves highly synchronous bilateral motor activation in sonic musculature (Boyle and Tricas, 2010). Highly synchronized muscle activation is evidenced by electromyography (EMG) experiments that show waveforms that resemble two (doublet) or one (singlet) muscle action potentials indicative of synchronous activity within musculature. In addition, the EMGs between right and left muscles are highly synchronized (Boyle and Tricas, 2010). Synchronous contraction of muscles around the swim bladder may be important for sound production in a variety of fish taxa (Ladich and Fine, 2006) and several species show connections between contralateral motor neurons to synchronize motor neuron firing. These include the batrachoidids (Bass and Baker, 1990), the Marbled Rockfish (Yoshimoto et al., 1999), but not piranhas (Ladich and Bass, 2005; Onuki et al., 2006) or Short-spined Sculpin (*Myoxocephalus scorpius*; Bass and Baker, 1991). In addition, electrical synapses couple the activity of sonic motor

neurons in batrachoidids (Pappas and Bennett, 1966; Bass and Baker, 1991). Bilateral motor neuron connections also are not found in Northern Seabob, a species that produces antiphase bilateral contractions which are hypothesized to involve a network of reciprocating pacemaker neurons (Bass and Baker, 1991; Connaughton, 2004). In squirrelfishes, contralateral projections were interpreted as premotor neurons that were labeled transneuronally (Carlson and Bass, 2000). Thus, premotor neurons may be important for the production of synchronized bilateral firing of sound production muscles in fish such as the Pyramid Butterflyfish that do not appear to have direct contralateral connections between motor neurons.

Comparison with Other Butterflyfishes

Sound production is known from multiple genera within the butterflyfish family Chaetodontidae (Tricas et al., 2006; Boyle and Tricas, 2010, 2011; Parmentier et al., 2011). Experiments with Forcepsfish (*Forcipiger flavissimus*), a species in the genus sister to a clade that comprises *Hemitaurichthys*, *Heniochus*, and *Johnrandallia* (Fessler and Westneat, 2007; Bellwood et al., 2009), demonstrate a dramatically different kinematic pattern associated with sound emission (Boyle and Tricas, 2011). Sound production in Forcepsfish involves synchronous muscle activity in the anterior epaxial musculature behind the supraoccipital of the cranium, the sternohyoideus, and the adductor mandibulae that occurs at the initial onset of sound emission and results in a rapid elevation of the cranium without protrusion of the oral jaws, which are presumably kept shut by activity of the adductor mandibulae (Boyle and Tricas, 2011). Unlike sonic muscle activity in Pyramid Butterflyfish (Boyle and Tricas, 2010) and Pennant Bannerfish (*Heniochus chrysostomus*; Parmentier et al., 2011), muscle activity in Forcepsfish sound production events involves burst activity within each muscle group. Electromyograms of Forcepsfish indicate the occurrence of many unsynchronized muscle action potentials during activity and no synchronous activity of anterior hypaxial musculature was observed with sound emission (Boyle and Tricas, 2011). Thus, sonic motor kinematics in Forcepsfish likely involves diverse motor nuclei with muscles innervated by many different nerves that may include occipital and spinal nerves for epaxial myotomes (Westerfield et al., 1986; Thys, 1997; Carlson and Bass, 2000), occipital nerve rami for sternohyoideus (Parenti and Song, 1996; Boyle and Tricas, 2011), and trigeminal for adductor mandibulae (Winterbottom, 1974; Nakae and Sasaki, 2004). Thus, the evolutionary origin of sound production in the chaetodontid clade is still very much clouded as the similarity of the sound production pattern and mechanism of the Pyramid

Butterflyfish and Pennant Bannerfish is juxtaposed with the divergent action pattern of Forcepsfish. It is possible that trunk muscle derived sound production may be a feature that evolved in parallel in *Hemitaurichthys* and *Heniochus*, a synapomorphy for the *Hemitaurichthys* + *Heniochus* + *Johnrardallia* clade, or be a conserved among deeper chaetodontid clades and lost within the genus *Forcipiger*. In addition, further study on the sound production mechanism(s) and innervation patterns in the more distantly related, speciose genus *Chaetodon* may shed light on the evolution and adaptive significance of the laterophysic connection.

CONCLUSIONS

Results of this study show specialization of sonic muscle fiber size, structure of SR, and transverse tubules, all of which may influence call production in the Pyramid Butterflyfish. Other features associated with sonic muscles in distantly related sound producing species, such as t-tubules at the A–I boundary and cores of sarcoplasm were not detected. This study found sonic motor neurons entirely in the spinal cord in a pattern similar to piranhas. Future studies should address ontogenetic and sex differences related to sonic muscle morphology and physiology in this and other species to assess divergence of sound production in butterflyfishes and higher phylogenetic levels.

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