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The ontogeny of muscle structure and locomotory function in the long-finned squid

**Doryteuthis pealeii**

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**SUMMARY**

Understanding the extent to which changes in muscle form and function underlie ontogenetic changes in locomotory behaviors and performance is important in understanding the evolution of musculoskeletal systems and also the ecology of different life stages. We explored ontogenetic changes in the structure, myosin heavy chain (MHC) expression and contractile properties of the circular muscles that provide power for jet locomotion in the long-finned squid *Doryteuthis pealeii*. The circular muscle fibers of newly hatched paralarvae had different sizes, shapes, thick filament lengths, thin:thick filament ratio, myofilament organization and sarcoplasmic reticulum (SR) distribution than those of adults. Viewed in cross section, most circular muscle cells were roughly triangular or ovoid in shape with a core of mitochondria; however, numerous muscle cells with crescent or other unusual cross-sectional shapes and muscle cells with unequal distributions of mitochondria were present in the paralarvae. The frequency of these muscle cells relative to ‘normal’ circular muscle cells ranged from 1:6 to 1:10 among the 19 paralarvae we surveyed. The thick filaments of the two types of circular fibers, superficial mitochondria-rich (SMR) and central mitochondria-poor (CMP), differed slightly in length among paralarvae with thick filament lengths of 0.83±0.15 μm and 0.71±0.1 μm for the SMR and CMP fibers, respectively (P < 0.05; ANOVA). During ontogeny the thick filament lengths of both the CMP and SMR fibers increased significantly to 1.78±0.27 μm and 3.12±0.56 μm, respectively, in adults (P < 0.0001 for both comparisons; ANOVA with Tukey’s highly significant difference post hoc tests). When sectioned parallel to their long axes, the SMR and CMP fibers of both paralarvae and adults exhibited the myofilament arrangements typical of obliquely striated muscle cells but the angle of obliquity of the dense bodies was 22.8±2.4 deg. and 4.6±0.87 deg. for paralarvae and adults, respectively. There were also differences in the distribution of the anastomosing network of SR. In paralarvae, the outer and central zones of SR were well developed but the intramyoplasmic zone was greatly reduced in some cells or was scattered non-uniformly across the myoplasm. Whereas in adults the intramyoplasmic SR region was composed primarily of flattened tubules, it was composed primarily of rounded vesicles or tubules when present in the paralarvae. The ontogenetic differences in circular muscle structure were correlated with significant differences in their contractile properties. In brief tetanus at 20°C, the mean unloaded shortening velocity of the paralarval circular muscle preparations was 9.1 L₀ s⁻¹ (where L₀ was the preparation length that generated the peak isometric stress), nearly twice that measured in other studies for the CMP fibers of adults. The mean peak isometric stress was 119±15 mN mm⁻² physiological cross section, nearly half that measured for the CMP fibers of adults. Reverse transcriptase-polymerase chain reaction analysis of paralarval and adult mantle samples revealed very similar expression patterns of the two known isoforms of squid MHC. The ontogenetic differences in the structure and physiology of the circular muscles may result in more rapid mantle movements during locomotion. This prediction is consistent with jet pulse durations observed in other studies, with shorter jet pulses providing hydrodynamic advantages for paralarvae.

Key words: cephalopod, jet locomotion, mechanics, obliquely striated muscle, hydrodynamics, ontogeny, muscle ultrastructure.

**INTRODUCTION**

Muscle fibers can be modified to produce a broad continuum of contractile properties. These modifications, in conjunction with changes in other parts of the motor system, have resulted in muscles that can generate force economically (Twarog, 1967), produce relatively high force over an impressive range of lengths (Hoyle et al., 1965; Lanzavecchia, 1977; Herrel et al., 2002; Woods et al., 2008), shorten with great speed (Rome et al., 1996; Schaeffer et al., 1996; Elemans et al., 2004), act as powerful motors (Lutz and Rome, 1994; Marsh and Olson, 1994), as brakes or shock absorbers (Full et al., 1998), as struts (Robert et al., 1997; Biewener et al., 1998) or as springs (Tu and Dickinson, 1994). A recognized, but understudied, phenomenon is that muscle fibers can be modified extensively during ontogeny (e.g. Greer-Walker, 1970; Goldspink and Ward, 1979; Anapol and Herring, 1989; Gilly et al., 1991; Kier, 1996; Thompson and Kier, 2006; Etnier et al., 2008). The ultimate causes of ontogenetic muscle modifications are not always apparent from a functional or ecological perspective but they may represent developmental constraints, selection for different levels of performance during ontogeny or both. Understanding the extent to which changes in muscle form and function underlie ontogenetic changes in locomotory behaviors and performance is important to understanding the evolution of musculoskeletal systems and also the ecology of different life stages.

We explored the ontogeny of muscle structure, the expression of isoforms of myosin heavy chain (MHC) and the function of the
mantle in squid, with respect to how changes in these parameters affect jetting behavior and performance. Unlike vertebrates and some arthropods, in which striated muscle fibers may be altered during ontogeny via expression of different isoforms of MHC (Bárány, 1967; Goldspink, 1968; Goldspink, 1983; Gauthier et al., 1978; Bandman, 1985; Gondret et al., 1996), changes in the ratio of the alkali light chains (Sweeney et al., 1988) or changes in calcium sensitivity via expression of isoforms of troponinT (Fitzhugh and Marden, 1997), squids appear to modify muscle mechanical properties by altering the lengths of the thick filaments. For example, the thick filaments of the obliquely striated transverse muscles of the arms of the oval squid Sepioteuthis lessoniana increase in length during ontogeny, from 2.2 μm in hatchlings to 6.4 μm in adults, while the thick filament lengths of the cross-striated transverse muscles of the tentacles decrease from 2.4 μm to 1.2 μm during the same interval (Kier, 1996). The ontogenetic change in thick filament length of the tentacle muscle is clearly related to changing function (see Kier, 1982) of the tentacles but the functional basis for the arm muscle change is less clear (Kier, 1996).

Ontogeny of circular muscle structure and function

Production of jet thrust in squids requires the integration of internal (i.e. the components of the motor system, including connective tissues) and external (i.e. jet dynamics) factors. In jetting, contraction of the circular muscles of the mantle pressurizes the mantle cavity and drives water out of the mantle cavity via the funnel (Young, 1938). Mantle contraction rate, the amplitude of mantle cavity pressure, the mass flux of the jet (i.e. the mass of water ejected from the funnel per unit time) and jet velocity are determined largely by the contractile properties of the circular muscles of the mantle, although the muscles of the funnel can adjust the aperture and, therefore, also affect jet velocity. Thus, the contractile properties of the circular muscles directly affect the hydrodynamics of the jet wake, jet thrust and jet propulsive efficiency. Both the rate of mantle contraction and the mass flux of the escape jet, however, change in some squid during growth (Thompson and Kier, 2001a; Thompson and Kier, 2002), and evidence suggests that the contractile properties of the circular muscles may change during ontogeny (Thompson and Kier, 2006).

Two important changes in the circular muscles of the mantle occur during the ontogeny of squids. The first is the relative abundance of the two types of circular muscle fibers. Many squid species possess two types of circular muscle cells: centrally located, mitochondria-poor (CMP) fibers and superficially located, mitochondria-rich (SMR) fibers (Bone et al., 1981; Mommsen et al., 1981) (terminology from Preuss et al. (Preuss et al., 1997)). Both fiber types are obliquely striated, have a core of mitochondria, a single nucleus and are electrically coupled to adjacent circular muscle fibers, presumably by gap junctions (Marceau, 1905; Young, 1938; Hanson and Lowy, 1957; Kawaguti and Ikemoto, 1957; Millman, 1967; Ward and Wainwright, 1972; Moon and Hulbert, 1975; Bone et al., 1995; Milligan et al., 1997). The SMR circular muscle fibers are hypothesized to provide power for ventilation of the mantle cavity and prolonged, slow-speed jetting (Bone et al., 1981; Mommsen et al., 1981; Bartol, 2001). Conversely, the CMP circular muscle fibers are hypothesized to provide power for high velocity jets, including escape jets (Bone et al., 1981; Mommsen et al., 1981; Gosline et al., 1983; Bartol, 2001). The ratio of SMR: CMP fibers decreases from about 1:1 to 1:10 in the mantle of Sepioteuthis lessoniana and Doryteuthis pealeii (formerly Loligo opalescens) during growth from a tiny paralarva to a large adult (Preuss et al., 1997; Thompson and Kier, 2001b). The second important change is that the thick filaments of both the SMR and CMP circular muscles increase 1.5-fold in length, at least during the ontogeny of Sepioteuthis lessoniana (Thompson and Kier, 2006).

The ontogenetic increase in thick filament length may affect the contractile properties of the circular muscles. The shortening velocity of striated muscles depends on the lengths of the thick filaments and sarcomeres, the load on the muscle and the rate of cross-bridge cycling (e.g. Josephson, 1975). Thick filament length is inversely proportional to unloaded shortening velocity and is directly proportional to peak isometric force (e.g. Josephson, 1975). Assuming all else about the fibers is equal, we hypothesize that the circular muscles of paralarval squids produce lower peak isometric stresses and higher unloaded shortening velocities than the circular muscles of adult squids. To better understand how the structure and function of the circular muscles that provide power for jet locomotion change throughout ontogeny in squids, we examined (1) muscle properties using mechanical tests, (2) muscle morphometrics using histological techniques, and (3) the expression of isoforms of MHC using reverse transcription-polymerase chain reaction in paralarvae and, wherever data were lacking from previous studies, adult life stages of the long-finned squid Doryteuthis pealeii.

MATERIALS AND METHODS

Animals

We performed our experiments on the paralarvae and sexually mature adults of the long-finned squid Sepioteuthis (formerly Loligo) pealeii (formerly Loligo pealei) Lesueur. Egg fingers were collected from the waters near Woods Hole, MA, and Walpole, ME, USA between 1 June and 30 September 2006 and 2007. They were housed in a flow-through seawater system with temperature and salinity ranging from 14 to 18°C and 28 to 32 p.p.t. (parts per thousand), respectively. Copepods, small mysid shrimp and larval brine shrimp were available as prey but feeding by the paralarvae was observed only rarely. Healthy paralarvae (i.e. squid that had normal coloration, that did not rest on the bottom of the tank and that were able to hold their vertical position in the water column via vigorous jetting) were used in experiments within 12 h of hatching. The mean ± s.d. dorsal mantle length (DML) of the paralarvae was 1.6±0.1 mm.

We caught male and female D. pealeii at night from lighted piers in South Bristol and Walpole, ME, USA, in July 2007. We trapped all of the squid with a 4.2 m-diameter cast net and then transported them immediately to the lab in 241 buckets. Squid were housed in a 1 m × 2 m × 0.5 m tank provided with flow-through seawater at 14–18°C and 28–32 p.p.t. salinity. The animals were fed small fish (Clupea spp.) daily and were used within four days of capture. We used only squid that were healthy, had no visible damage to the skin or mantle and swam vigorously. The adults ranged in size from 150 to 167 mm DML.

Muscle mechanical testing

Paralarvae were anesthetized in cold seawater (3°C) (O’Dor and Shadwick, 1989; Bower et al., 1999), transferred to a drop of ice-cold modified squid saline solution containing (in mmol l−1): NaCl (450), MgCl2·6H2O (10), Hepes (10), EGTA (10), pH adjusted to 7.8 with 2 mol l−1 NaOH (Milligan et al., 1997), and then killed by impaling the brain with a straight pin. The posterior tip (i.e. about 0.5 mm) of the mantle was sliced off. The mantle was then separated from the rest of the body, slit along the dorsal side and unrolled to form an approximately rectangular piece of tissue from what was once a hollow cylinder. The pen was removed and then each slit edge of the mantle was glued with Vetbond (3M, St Paul, MN, USA).
to a T-shaped foil clip (Milligan et al., 1997). The clips were attached so that the long axes of the circular muscle fibers were parallel to the long axis of the preparation. The preparation was then transferred to a temperature-controlled bath. The bath was filled with standard squid saline containing (in mmol l\(^{-1}\)): NaCl (470), KCl (10), CaCl\(_2\) (10), MgCl\(_2\) ·6H\(_2\)O (50), glucose (20), Hepes (10), pH adjusted to 7.8 with 2 mol l\(^{-1}\) NaOH (Milligan et al., 1997).

The muscle preparations were attached at one end to an ASI 400A force transducer (Aurora Scientific Inc., Aurora, ON, Canada) and at the other end to an ASI 322 high-speed length controller (Aurora Scientific Inc.). The length–force relationship of each tissue preparation was determined using supramaximal brief tetanic (2 ms pulses, 50 Hz, 200 ms) and twitch stimulations. Stimuli were provided via platinum foil electrodes that spanned the entire length and width of the preparation. The maximum unloaded shortening velocity (\(V_{\text{max}}\)) was determined at 0.9\(L_0\) (where \(L_0\) was the length of the preparation at which isometric tension was highest) using slack tests (Edman, 1979). We chose 0.9\(L_0\) because passive tension was close to zero for all preparations at this length. We performed regular isometric control stimulations to monitor the health of the preparation. If the force produced during isometric contraction at \(L_0\) decreased by more than 10%, we terminated the experiment and discarded all data collected subsequent to the previous control stimulation. We analyzed data from the muscle preparations of 19 paralarvae.

The muscles of the mantle are arranged primarily in two orientations: circumferentially (the circular muscles) and radially (the radial muscles) (Marceau, 1905; Williams, 1909; Young, 1938). Contraction of the circular muscles drives water out of the mantle cavity via the funnel while contraction of the radial muscles helps to refill the mantle cavity with water at the end of the power stroke (Young, 1938). As mentioned earlier, two types of circular muscle have been identified in loliginid and ommastrephid squid: CMP and SMR fibers (Bone et al., 1981; Mommersen et al., 1981) [terminology from Preuss et al. (Preuss et al., 1997)]. As in a previous study of the contractile properties of the CMP and SMR circular muscle fibers of adult D. pealeii (Thompson et al., 2008), we attempted to use a vibratome to cut sheets of circular muscles from the mantles of paralarvae. The paralarval mantles were thin (about 0.06 mm thick) and were much less stiff than the adult mantles, perhaps because they have significantly fewer intramuscular connective tissue fibers than the adults (see Thompson and Kier, 2001b). The result was that the vibratome blade tended to deform the paralarval mantles rather than slice them, and we had no success in isolating the SMR or CMP circular muscle fibers. The paralarval mantle preparations, therefore, contained intact SMR and CMP circular muscle fibers as well as radial fibers. We discuss the limitations of the paralarval intact preparations in the Discussion.

**Morphometrics**

Following the mechanical tests, each preparation was pinned at \(L_0\) in a Sylgard dish. Most of the preparations were fixed for 6–8 h (3% glutaraldehyde, 0.065 mol l\(^{-1}\) phosphate buffer, 0.5% tannic acid and 6% sucrose) and then postfixed for 45 min at 4°C in a 1:1 solution of 2% osmium tetroxide and 2% potassium ferrocyanide in 0.13 mol l\(^{-1}\) cacodylate buffer (Kier, 1985). The tissue was rinsed in chilled 0.13 mol l\(^{-1}\) cacodylate buffer for 15 min, dehydrated in a graded series of acetones and embedded in epoxy resin (Embed 812, Electron Microscopy Sciences, Hatboro, PA, USA). A few of the preparations were fixed in a modified Karnovsky fixative (2% paraformaldehyde, 3% glutaraldehyde, 0.065 mol l\(^{-1}\) phosphate buffer and 2% sucrose), rinsed in phosphate buffer, dehydrated in ethanol and then stained in 1% phosphotungstic acid in absolute ethanol prior to embedding in LR White (Electron Microscopy Sciences, Hatfield, PA, USA). As is typical in squid muscle, dense bodies did not stain well in the 3% glutaraldehyde fixative (Kawaguti and Ikemoto, 1957; Ward and Wainwright, 1972; Moon and Hulbert, 1975; Bone et al., 1981; Kier, 1985) but the preservation of the myofilaments, sarcoplasmic reticulum (SR), plasma membrane and mitochondria was excellent. The tissues fixed in the modified Karnovsky and stained in phosphotungstic acid showed excellent preservation and staining of the dense bodies and thick filaments but the preservation and staining of the mitochondria, plasma membrane and SR were poor. For all tissues, transverse sections of the preparations were cut, stained and examined with brightfield and electron microscopy to measure the physiological cross section (pcs) of the circular muscle fibers (for details, see Thompson et al., 2008) and to explore the ultrastructure of the cells.

We prepared the mantle tissue of three adult D. pealeii for histology in order to examine circular muscle cell size, shape and structure in detail, and also to confirm the thick filament lengths reported by Thompson et al. (Thompson et al., 2008). We anesthetized the animals in a 1:1 solution of 7.5%MgCl\(_2\) ·6H\(_2\)O:seawater (Messenger et al., 1985), decapitated them and then removed small blocks of mantle tissue from the ventral midline approximately ½ DML from the anterior edge of the mantle. The tissue blocks spanned the thickness of the mantle wall (i.e. they included radial muscle fibers plus both types of circular fibers). They were fixed, post-fixed and then embedded in epoxy resin as described above.

The protocol we followed for measuring thick filament lengths is described in detail elsewhere (Thompson and Kier, 2006; Thompson et al., 2008). Briefly, embedded tissue blocks were sectioned in a plane perpendicular to the longitudinal axis of the mantle (i.e. parallel to the long axes of the circular muscles) using a diamond knife. Thick sections (0.5–1 mm) were cut initially and stained in an aqueous solution of 0.1% Methylen Blue and 0.1% Azure II. Sections were then examined using brightfield microscopy to determine if the long axes of the circular muscle fibers were parallel to the knife edge. Once alignment was achieved, thin sections (silver interference color) were cut, mounted on grids and stained with 2% aqueous uranyl acetate (Bozzola and Russell, 1992) and 0.4% lead citrate (Venable and Coggeshall, 1965). Thin sections were examined with either a Zeiss EM-902 (Oberkochen, Baden-Wurttemberg, Germany) or JEOL JEM-100SX (Tokyo, Japan) transmission electron microscope and photographed.

The electron micrograph negatives were scanned at 4800 dpi (dots per inch), and thick filament lengths measured using ImageJ software (Abramoff et al., 2004). We measured thick filaments from at least five CMP and SMR muscle fibers per preparation, and a total of about 1200 thick filaments from paralarvae and 700 from the three adults. The mean thick filament length per animal was used for the statistical comparisons. Although great care was taken to align the long axes of the circular muscle fibers with the section plane, the thick filament lengths we report here may nevertheless be slight underestimates.

**RNA purification and RT-PCR**

We used reverse transcription-polymerase chain reaction (RT-PCR) to determine if different isoforms of MHC are expressed in the mantle during ontogeny. We anesthetized two adult D. pealeii (155 and 167 mm DML) in cold water, decapitated them, quickly excised small blocks of tissue and then placed the blocks into RNAlater™ (Ambion, Inc., Austin, TX, USA). The tissues included the central
zone of the mantle (containing CMP and radial muscle fibers but no SMR fibers), portions of the mantle containing all muscle fiber types and the funnel retractor muscle. We also placed 25 whole paralarval mantles (including the fins, skin and small fragments of the ctenidia) in RNAlater™.

The tissues in RNAlater™ were stored at 2°C for several weeks then frozen in liquid nitrogen and pulverized using a chilled mortar and pestle. We then isolated total RNA using a kit (RNAqueous-4PCR kit, Ambion, Inc.) and performed RT-PCR. We used primer sequences designed by Kier and Schachat (Kier and Schachat, 2008) for the RT-PCR. The forward primer was 5'-AGCTTGCGCTGAAAAGATAA-3'; the reverse primer was 5'-CAGCACCGGCAATTTTACCTT-3'. The primers bracketed the putative alternative RNA splice site identified by Matulef et al. (Matulef et al., 1998) in the funnel retractor muscle of D. pealeii. Expression of MHC isoform A or isoform B (sensu Matulef et al., 1998) was determined by the lengths of cDNA products, with the isoform A CDNA being 15 bp longer than that of isoform B. Following 40 cycles of PCR amplification, the products were diluted to 20 μg ml⁻¹ and then resolved by gel electrophoresis using a 4% high-resolution agarose gel in 1× Tris/Borate/EDTA buffer.

**Statistics**

For comparison of morphological or muscle mechanics data between two sample populations, we used one-way analysis of variance (ANOVA). For comparisons among multiple groups, we used one-way ANOVA with Tukey’s highly significant difference (HSD) post tests. Where indicated in the text, we compared thick filament length and muscle mechanics data from paralarvae collected in this study with similar data from adults reported by Thompson et al. (Thompson et al., 2008) and used here with their permission.

**RESULTS**

**Mantle musculature**

The mantle musculature of paralarval and adult D. pealeii differed notably in several ways. First, when circular muscle cells of paralarvae were cut transverse to their long axes, their shapes were variable (Fig. 1A,B). Although most muscle cells were roughly triangular or ovoid in cross section with a core of mitochondria (typical of squid mantle muscles), numerous muscle cells with crescent or other unusual cross-sectional shapes, and muscle cells with unequal distributions of mitochondria, were present. The frequency of these fibers relative to ‘normal’ circular muscle cells ranged from 1:6 to 1:10 among the 19 paralarvae we surveyed. We did not find crescent-shaped circular fibers or fibers with unusual cross-sectional shapes in the circular muscles of adult squid (Fig. 1C,D).

Second, the circular fibers of paralarvae were smaller than those of adults (Table 1). Among paralarvae, the CMP circular muscle fibers had significantly smaller total cross sectional areas than the SMR fibers (P<0.05), and this difference was also seen in the adults (P<0.0001). Both the SMR and CMP fibers of paralarvae had significantly smaller cross sectional areas than their counterparts in the adults (P<0.0001 for both comparisons).

Third, the general organization of the myofilaments in the cross sections of the circular muscle fibers was different between paralarvae and adults. When sectioned transverse to their long axes, circular muscle cells of paralarvae exhibited a centrally located mitochondrion and two thick filaments, whereas adult muscle cells usually contained two to three mitochondria and were regularly crescentic. This difference was reflected in the number of mitochondria in paralarvae (mean ± s.d. 1.7 ± 0.5) compared to adults (3.0 ± 0.8).

**Table 1. Circular muscle metrics**

<table>
<thead>
<tr>
<th></th>
<th>Paralarval SMR</th>
<th>Paralarval CMP</th>
<th>Adult SMR</th>
<th>Adult CMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cross sectional area (μm²)</td>
<td>25.7±9.1</td>
<td>14.2±5.4</td>
<td>53.2±12.2</td>
<td>37.5±9.1</td>
</tr>
<tr>
<td>Thick filament diameter (nm)</td>
<td>24.2±3</td>
<td>19.8±3</td>
<td>28±3.1</td>
<td>22.7±2.6</td>
</tr>
<tr>
<td>Thin:thick filament ratio</td>
<td>8.5±2.1</td>
<td>7.3±2.4</td>
<td>8.3±1.7</td>
<td>5.1±1.1</td>
</tr>
</tbody>
</table>

Comparison of total cross sectional area, thick filament diameter and thin:thick filament ratios between the superficial mitochondria-rich (SMR) and central mitochondria-poor (CMP) circular muscle fibers of paralarval and adult Doryteuthis pealeii. The means ± s.d. are listed. The data are from 19 paralarvae and three adults. The thick filament diameter and the thin:thick filament ratio data were obtained from 10 cells in each animal. We measured at least 65 thick filaments in each cell. The cross sectional area data were obtained from the same individuals.
the SMR and CMP muscle fibers of adults showed the banding pattern typical of obliquely striated muscle cells (Figs 1, 2). Briefly, the bands result from alternation of lightly stained regions containing only thin filaments, SR and irregularly spaced dense bodies with more darkly stained regions containing overlapping thick and thin filaments. In the paralarvae, this pattern was not apparent in most of the circular fibers (Fig. 1, Fig. 2A,B). Cross sections of most paralarval circular fibers were composed primarily of regions with both thick and thin filaments, with a few isolated regions composed of either thin filaments or thick filaments only (Fig. 2B). When sectioned parallel to their long axes, the SMR and CMP fibers of both paralarvae and adults exhibited the myofilament arrangements typical of obliquely striated muscle cells but the angle of obliquity of the dense bodies was much greater (i.e. closer to cross striation) in paralarvae than in adults (Figs 3, 4). The angle of obliquity is sensitive to the plane of section (see Rosenbluth, 1965), and thus the mean angle of obliquity must be reported with great caution. Nevertheless, among the hundreds of paralarval and adult muscle cells observed, the means ± s.d. of the 25 lowest angles of obliquity (for SMR and CMP fibers combined) visible in the micrographs were 22.8±2.4 deg. and 4.6±0.8 deg. for paralarvae and adults, respectively. In addition to the difference in angle of obliquity, the dense bodies in some of the paralarval circular fibers did not follow straight trajectories (Fig. 3C). This resulted in adjacent thick filaments being out of the register typically seen in obliquely striated cells. Indeed, some thick filaments within a few circular fibers followed trajectories that were almost perpendicular to the majority of the thick filaments (Fig. 3C).

Fourth, there was a difference in the distribution of SR between paralarvae and adults. In adults, the SR of the circular fibers was well-organized into three zones: a peripheral zone associated with the sarcolemma, a second, central zone surrounding the core of mitochondria, and a third, intramyoplasmic region of flattened tubules located in the plane of the dense bodies that appeared to connect the outer and central zones of SR (Fig. 2). In paralarvae, the outer and central zones of SR were well developed but the intramyoplasmic zone was greatly reduced in some cells or was scattered non-uniformly across the myoplasm (Fig.2A,B). Whereas in adults the intramyoplasmic SR region was composed primarily of flattened tubules that occasionally swelled into a vesicle, it was composed primarily of large vesicles in the paralarvae, although flattened tubules were present in parts of a small number of paralarval fibers.

Fifth, among paralarvae the thick filaments of the SMR and CMP circular muscle fibers differed slightly in length (P=0.05, Figs5, 6). During ontogeny, the lengths of the thick filaments of the SMR and CMP fibers increased significantly (P<0.0001 for both comparisons), with the SMR fibers increasing 3.7-fold in length and the CMP fibers 2.5-fold (data for adults from Thompson et al., 2008).

Finally, the thick filament diameter and thin:thick filament ratio changed during ontogeny. Among paralarvae, the CMP thick filaments were significantly (P=0.009) smaller in diameter than the SMR thick filaments. A similar difference was found among adults (P=0.0001) (Table 1). The CMP and SMR thick filaments of paralarvae were significantly smaller in diameter than their counterparts in adults (P=0.037 and P=0.0014 for SMR and CMP fibers, respectively) (Table 1). The thin:thick filament ratio was significantly lower in the CMP fibers of adults compared with the SMR fibers of adults (P=0.05) and with both the CMP and SMR fibers of paralarvae (P<0.05). The thin:thick filament ratios of the SMR and CMP fibers of paralarvae did not differ significantly from each other (P>0.4) (Table 1).
Paralarval circular muscle contractile properties

All of the slack tests resulted in tight linear relationships between step length and force recovery time, with the $R^2$ values for the least-squares linear regressions of all preparations exceeding 0.98 (not shown). For brief tetanus (2ms pulse width, 50Hz, 200ms duration) at 20°C, the mean maximum unloaded shortening velocity ($V_{max}$) of the paralarval preparations was 9.1 $L_0$ s$^{-1}$, where $L_0$ was the preparation length that generated the peak isometric stress (Fig. 7). $V_{max}$ ranged from 4.3 to 14.8 $L_0$ s$^{-1}$.

In the mantle preparations of the paralarvae, the maximum isometric stress in brief tetanus ($P_0$) was 119±15 mN mm$^{-2}$ pcs with a range of 85–160 mN mm$^{-2}$ pcs.

The temporal aspects of brief tetani are summarized in Table 2.

**MHC mRNA expression**

Both isoforms of MHC were expressed in the mantles of adults and paralarvae. Isoform ‘A’ (204 bp) composed at least 90% of the RT-PCR product in all animals, based on relative staining intensity of the bands in the agarose gel (Fig. 8).

DISCUSSION

The fine structure of the circular muscles that provide power for jet locomotion changed in several ways during ontogeny in *D. pealeii*. These include a difference in myofilament organization, the shapes and distribution of SR and the lengths of the thick filaments. All observed changes may have implications for contractile properties of the circular muscles.

Myofilament organization

When cut transverse to their long axes, the CMP and SMR circular muscle fibers of adults showed the alternating band pattern typical of obliquely striated muscle cells (e.g. Kawaguti and Ikemoto, 1957; Rosenbluth, 1965; Rosenbluth, 1968; Ward and Wainwright, 1972; Moon and Hulbert, 1975; Lanzavecchia, 1977) but it was absent from virtually all of the CMP and SMR circular fibers of the paralarvae (Figs 1, 2). The pattern visible from the transverse plane of an obliquely striated cell results from the staggered arrangement of the myofilament lattice proteins (see Rosenbluth, 1965). Thus, a change in the banding pattern suggests a difference in the arrangement of the myofilaments.
suggested that oblique striation constrains shortening velocity implications of oblique striation are not yet clear. Rosenbluth on muscle performance is uncertain because the functional (Fig. 4). Examining sections that are slightly off-axis the fiber will primarily contain A-bands (i.e. overlapping thick and thin filaments; Fig. 4). This, in combination with short thick filaments, result in the cross sectional views of the paralarvae exhibiting far fewer A-bands than are actually visible in photomicrographs. The paralarval schematic illustrates the different appearance of cross sections of the fiber when shortened (section plane indicated by the dashed line) or stretched (at far right side of fiber). The greater the angle of obliquity, the greater the proportion of A-bands exhibited in a given cross section of the fiber. The angle of obliquity (22.8 deg. and 4.6 deg. in paralarval and adult circular fibers, respectively) is overestimated in each schematic. Thin filaments were not included in the adult schematic. Db, dense body; Mt, mitochondrial core; Tf, thick filaments.

We propose that the difference in the arrangement of myofilaments between paralarvae and adults results from a greater angle of obliquity of the dense bodies in the circular muscle fibers of paralarvae (Fig. 3). The angle of obliquity increases from 6–12 deg. at rest to 14–18 deg. during contraction in Loligo sp. (Hanson and Lowy, 1957; Rosenbluth, 1965; Millman, 1967) and can change with section plane (Rosenbluth, 1965); thus, making comparisons of angle of obliquity between animals is difficult. Nevertheless, the paralarval fibers consistently exhibited substantially higher angles of obliquity even when sections from highly stretched (prior to fixation) paralarval preparations were compared with highly contracted (during fixation) adult preparations. Higher angles of obliquity make it likely that sections transverse to the long axis of the fiber will primarily contain A-bands (i.e. overlapping thick and thin filaments; Fig. 4). Examining sections that are slightly off-axis or sectioning cells that contracted during the fixation process decreases the probability of seeing the banding typical of adults (Fig. 4).

The effect of this change in the organization of the dense bodies on muscle performance is uncertain because the functional implications of oblique striation are not yet clear. Rosenbluth suggested that oblique striation constrains shortening velocity because the friction associated with shearing of the thick filaments may act as a brake (Rosenbluth, 1968). The higher angle of obliquity in paralarval fibers should reduce this friction and result in higher $V_{\text{max}}$ relative to otherwise identical fibers that have a lower angle of obliquity.

The difference in the arrangement of myofilaments between paralarvae and adults we found is consistent with the photomicrographs of the circular muscle fibers in paralarval and juvenile D. (formerly Loligo opalescens) in Preuss et al. (Preuss et al., 1997), who also noted a difference in the organization of the myofilaments between the SMR fibers and CMP fibers of paralarvae. Preuss and colleagues reported that the myofilaments of paralarvae

![Fig. 4. Schematics to illustrate hypothesized differences in the angle of obliquity (θ) of the dense bodies in the circular muscle fibers of a paralarva (top) and adult (bottom). Dense bodies are indicated as solid black circles, thick filaments as hollow black lines and thin filaments as solid red lines. Both schematics greatly under-represent the numbers of thick and thin filaments. This, in combination with short thick filaments, result in the cross sectional views of the paralarvae exhibiting far fewer A-bands than are actually visible in photomicrographs. The paralarval schematic illustrates the different appearance of cross sections of the fiber when shortened (section plane indicated by the dashed line) or stretched (at far right side of fiber). The greater the angle of obliquity, the greater the proportion of A-bands exhibited in a given cross section of the fiber. The angle of obliquity (22.8 deg. and 4.6 deg. in paralarval and adult circular fibers, respectively) is overestimated in each schematic. Thin filaments were not included in the adult schematic. Db, dense body; Mt, mitochondrial core; Tf, thick filaments.](Image 336x648 to 440x662)

![Fig. 5. Transmission electron micrographs showing the ontogeny of thick filament lengths. The thick filaments of adult superficial mitochondria-rich (SMR) (A) and central mitochondria-poor (CMP) (B) fibers were 3.7-fold and 2.5-fold longer, respectively, than the SMR (C) and CMP (D) thick filaments of the paralarvae. The arrows in A and B help delineate one thick filament. Scale bars, 0.5 μm in A, B and 0.25 μm in C, D.](Image 336x663 to 543x704)

![Fig. 6. Thick filament length in the two types of circular muscle fibers in paralarvae and adults. The thick filaments of the adult superficial mitochondria-rich (SMR) fibers were significantly longer than the SMR fibers of paralarvae, and also were significantly longer than the thick filaments of the central mitochondria-poor (CMP) fibers of adults and paralarvae ($P<0.0001$ for all comparisons; one-way ANOVA with Tukey's highly significant differences (HSD) post hoc test). The thick filaments of the CMP fibers of adults were significantly longer than the thick filaments of both the CMP or SMR fibers of paralarvae ($P<0.0001$ for all comparisons; one-way ANOVA with Tukey's HSD post hoc test). In paralarvae, thick filament lengths differed slightly between the SMR and CMP fibers ($P=0.05$; one-way ANOVA). The open circles indicate outliers that were at least 1.5 times higher than the interquartile range. The numbers above each box indicate the means ± the s.d. The data for adult Doryteuthis pealei are from Thompson et al. (Thompson et al., 2008), and are used with permission of the authors.](Image 336x706 to 543x723)
were ‘poorly organized’ relative to those of adults, and also cautiously suggested that the SMR fibers of paralarval *D. opalescens* were cross-striated (Preuss et al., 1997). We found no evidence to support the suggestion that either the SMR or CMP fibers of *D. pealeii* paralarvae are cross-striated (Figs 2, 3). Indeed, the photomicrograph in Preuss et al. (Preuss et al., 1997) of a paralarval SMR circular muscle fiber seems to display the alternating band pattern typical of obliquely striated fibers.

![Fig. 7. Boxplot comparing maximum unloaded shortening velocity (V$_{\text{max}}$) in brief tetanus (2 ms pulse width, 50 Hz, 200 ms duration) at 20°C for paralarval and adult *Doryteuthis pealeii* muscle preparations. The paralarval preparations (N=19) included the central mitochondria-poor (CMP) and superficial mitochondria-rich (SMR) circular muscle fibers and intact radial muscle fibers; the adult preparations (N=10) included either the SMR or CMP circular fibers plus the severed fragments of the radial fibers.](image)

Paralarvae preparations had a significantly higher V$_{\text{max}}$ than the CMP fiber preparations of adults (P=0.0007; one-way ANOVA). The numbers above each box indicate the means ± the s.d. $L_0$ is the preparation length that generated the peak isometric stress. The data for adult *D. pealeii* are from Thompson et al. (Thompson et al., 2008), and are used with permission of the authors.

Table 2. Temporal aspects of the contraction of paralarval and adult mantle muscle fibers

<table>
<thead>
<tr>
<th></th>
<th>SMR</th>
<th>CMP</th>
<th>Paralarvae</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_L$ (ms)</td>
<td>4.6±1.4</td>
<td>4.4±1.5</td>
<td>4.0±0.9</td>
<td>0.78</td>
</tr>
<tr>
<td>$T_F$ (ms)</td>
<td>143±28.9</td>
<td>143±57.7</td>
<td>126.8±28.1</td>
<td>0.001</td>
</tr>
<tr>
<td>$T_{50}$ (ms)</td>
<td>153±28.4</td>
<td>175±98.9</td>
<td>57.4±4.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>N</td>
<td>7</td>
<td>10</td>
<td>19</td>
<td></td>
</tr>
</tbody>
</table>

Comparison of the temporal aspect of the isometric contraction of paralarval and adult mantle muscle preparations in brief tetanus (2 ms pulse, 50 Hz, 200 ms duration). The means ± s.d. are listed. The P values for comparisons between the whole mantle paralarval preparations and the adult central mitochondria-poor (CMP) preparations are listed (one-way ANOVA). There were no differences between adult superficial mitochondria-rich (SMR) and CMP preparations (P>0.4 for all comparisons). $T_L$, latent period between the first stimulation and rise in force; $T_F$, time from the rise in force to the peak force; $T_{50}$, time from peak force to 50% peak force. The SMR and CMP data from adults are from Thompson et al. (Thompson et al., 2008) and are used with permission of the authors.
paralarvae but not in those of adults. Bone et al. noted crescent-shaped circular muscle cells in the mantle of *Allooteuthis subulata* and suggested that these represented early stages of new fibers formed from cell division (Bone et al., 1981). The frequent occurrence of such cells in the mantle of paralarvae in which growth and, presumably, rates of cell division are high (Moltschaniwskj, 1994; Moltschaniwskj, 1995; Moltschaniwskj, 1997), lends support to the hypothesis of Bone et al. (Bone et al., 1981).

**Thick filament length**

There was a significant increase in the lengths of the thick myofilaments of both the CMP and SMR circular muscle fibers (Figs 3, 5) through ontogeny from paralarvae to adults. This increase was similar to that found in the CMP and SMR fibers of another loliginid squid, *S. lessoniana* (Thompson and Kier, 2006), and suggests that such changes may be common among squids. The ontogenetic increase in the thick filament lengths of *D. pealei* and *S. lessoniana* are not unique among cephalopods (Kier, 1996), and Kier and colleagues have suggested that loliginid squids modulate muscle contractile properties by altering thick filament length alone (Kier, 1985; Kier, 1996; Kier and Schachat, 1992; Kier and Curtin, 2002; Thompson and Kier, 2006; Kier and Schachat, 2008; Thompson et al., 2008).

**Implications of thick filament length ontogeny for muscle function**

The shortening velocity of striated muscles, including the obliquely striated circular muscles, depends on a variety of factors: the lengths of the thick filaments and sarcomeres, the load on the muscle and the rate of cross-bridge cycling (e.g. Bárány, 1967; Josephson, 1975). In general, thick filament length is inversely proportional to unloaded shortening velocity and directly proportional to peak isometric stress (Millman, 1967; Josephson, 1975; Kier and Curtin, 2002).

In adult *D. pealei*, there is a strong correlation between thick filament length and contractile properties in the SMR and CMP circular muscle fibers (Thompson et al., 2008). The mean thick filament length of the adult SMR fibers (3.12 μm) is 1.75-fold greater than that of the adult CMP fibers (1.78 μm) (Thompson et al., 2008). Thus, if other aspects of the two fiber types are the same, and if the relationship between thick filament length and isometric stress is linear, the *P₀* of the SMR fibers should be 1.75 times greater than the *P₀* of the CMP fibers. Thompson et al. (Thompson et al., 2008) found that the *P₀* of the adult SMR fibers was approximately 1.5 times greater; correcting for differences in the size of the core of mitochondria between the two fiber types resulted in a *P₀* that was about 2 times greater. By similar reasoning, the *Vₐₘₓ* of the adult SMR fibers should be 1.75 times slower than the CMP fibers, and it was approximately 2 times slower (Thompson et al., 2008).

Assuming that other aspects of the circular muscles do not change during ontogeny, we therefore hypothesized that the circular muscles of paralarvae produce lower peak isometric stresses and higher unloaded shortening velocities than those of adults. Before analyzing this hypothesis in light of our results, we first discuss the limitations of our mechanical testing methods.

**Limitations of the mechanical testing methods**

The thin, deformable mantles of paralarvae thwarted our attempts to investigate the contractile properties of preparations composed solely of CMP or solely of SMR circular fibers. Thus, we studied mantle preparations that contained both types of circular muscle cells in addition to radial fibers. Electrical stimulation of the paralarval muscle preparations probably resulted in contraction of all muscle fibers, and this complicates the comparisons of muscle contractile properties. Nevertheless, several factors support the validity of our experimental approach. First, the thick filaments of the CMP and SMR fibers of paralarval *D. pealei* are roughly similar in length (Figs 3, 5). If, as our results and those from other studies suggest (Kier and Curtin, 2002; Thompson et al., 2008), the contractile properties of cephalopod obliquely striated muscles are determined largely by thick filament length, then the contractile properties of paralarval CMP and SMR circular muscle fibers are likely to be similar. Nonetheless, our experimental design masked any differences in contractile properties between the two fiber types.

Second, the radial muscle fibers are antagonists to the circular fibers and their activity therefore may have decreased both the speed of shortening and the peak isometric stress of the circular muscle fibers. We attempted to estimate the magnitude of this error in paralarvae as follows. We made mantle muscle preparations from two small adult *D. pealei* (90mm and 110mm DML) that had intact radial fibers and both SMR and CMP circular fibers. We then measured the contractile properties of these preparations using the same methods described earlier in this paper. By comparing the results with those of Thompson et al. (Thompson et al., 2008) for SMR and CMP preparations in which the radial fibers were severed and, therefore, inactivated, we were able to estimate the error introduced by having intact radial muscle fibers in the preparations. The mean *Vₐₘₓ* from the two intact adult preparations was 4.5±0.7 *L₀* s⁻¹, about 12% lower than the mean *Vₐₘₓ* for the CMP preparations of adult squid (Thompson et al., 2008). The mean *P₀* for the adult whole mantle preparations was 306±15 mN mm⁻² pcs, about 8.5% less than the value for the isolated SMR preparations from adults (Thompson et al., 2008). This suggests that our muscle mechanics data underestimate the true *Vₐₘₓ* and *P₀* by 8–12%.

Third, Lowy and Millman measured the contractile properties of the funnel retractor muscles of the octopuses *Octopus vulgaris* and *Eledone moschata* and the cuttlefish *Sepia officinalis* (Lowy and Millman, 1962). The funnel retractor is similar to the mantle in that (1) it is supported by a muscular hydrostat with muscle fibers oriented in two directions – parallel and transverse to the long axis of the muscle; (2) the parallel and transverse fibers are antagonists; and (3) the parallel fibers compose the majority of the muscle, just as the circular fibers compose the majority of the mantle musculature (Kier and Thompson, 2003). Lowy and Millman measured the contractile properties of the parallel fibers in intact funnel retractor muscles and preparations in which they pared the muscle down its long axis, thereby severing the transverse fibers (Lowy and Millman, 1962). They found no differences in the properties of the two types of preparations with the exception that the pared preparations produced higher forces per unit cross sectional area.

Despite these reassurances we nevertheless emphasize that the *Vₐₘₓ* and *P₀* data we report for paralarvae are conservative estimates.

**Ontogeny of circular muscle contractile properties**

Even with the conservative estimate of unloaded shortening velocity in the paralarvae, the data support the hypothesis that *Vₐₘₓ* is lower in adult SMR and CMP circular fibers than in the paralarval circular fibers. The mean *Vₐₘₓ* of the SMR and CMP fibers of adult *D. pealei* in brief tetanus was 2.4 and 5.1 *L₀* s⁻¹, respectively, where *L₀* was the preparation length that yielded the highest force (Thompson et al., 2008). The mean *Vₐₘₓ* of the adult SMR and CMP preparations was 3.8- and 1.8-fold lower, respectively, than the *Vₐₘₓ* of the paralarval preparations (9.1 *L₀* s⁻¹).

The difference in *Vₐₘₓ* between paralarvae and adults was less than that predicted based solely on the ontogenetic increase in thick
filament length. The thick filaments of adult CMP fibers were 2.5 times longer than those of paralarvae, yet the \( V_{\text{max}} \) of adult CMP fibers was only 1.8 times lower (Thompson et al., 2008). Several variables confound this comparison. First, paralarval preparations were composed of intact circular and radial muscle fibers whereas the radial fibers were severed in the adult preparations (Thompson et al., 2008). If the whole mantle preparations of adults described above provide a realistic effect of the radial fibers on \( V_{\text{max}} \), then we need to correct the \( V_{\text{max}} \) of paralarvae by 12%. Doing so raises the \( V_{\text{max}} \) to 10.2 \( L_0 \, \text{s}^{-1} \) and increases the speed of shortening of the paralarval preparations relative to adult CMP preparations to 2-fold. Second, the SMR fibers of the paralarvae had thick filament lengths that were 1.16 times longer than the CMP fibers (Figs 3, 5). If \( V_{\text{max}} \) scales linearly with thick filament length in the paralarvae, the slightly slower SMR fibers may have added a load to the CMP fibers; thus, lowering \( V_{\text{max}} \). Third, the paralarvae had numerous crescent-shaped and other unusually shaped circular muscle fibers, and the ratio of these to ‘normal’ fibers varied from 1:6 to 1:10 among the paralarval preparations. If these cells did not generate as much force per unit cross section as the ‘normal’ circular fibers, the greater the ratio of these cells in a preparation, the greater the load on the ‘normal’ cells and, therefore, the lower the \( V_{\text{max}} \). Indeed, there was greater variation in the paralarval than in the adult preparations, with \( V_{\text{max}} \) ranging from 4.3 to 14.8 \( L_0 \, \text{s}^{-1} \) (Fig. 5) in paralarvae. Although undetected damage to the preparations during dissection may account for some of this variation, we believe that most of it reflects natural variation in the contractile properties of the circular muscles. Paralarvae in the lab differed greatly in their swimming variation in locomotion.

Some of the paralarvae had a \( V_{\text{max}} \) that was nearly 3-fold higher than that of adults. Because thick filament length was relatively uniform among all the paralarvae (Figs 3, 5), this calls into question the notion that thick filament length alone explains the ontogeny of \( V_{\text{max}} \). The passive tension in the paralarval and adult CMP preparations was minimal at \( L_0 \) (data not shown). Thus, it is unlikely that elastic recoil during the slack tests contributed to the \( V_{\text{max}} \) data we present.

The \( P_0 \) we recorded for the paralarval preparations was much lower than that reported by Thompson et al. (Thompson et al., 2008) for the SMR and CMP preparations of adult \( D. \, \text{pealeii} \). It is, however, very similar to the \( P_0 \) (131±56 mN mm \(^{-2} \) psc) of the transverse muscle fibers of the tentacles of adult \( D. \, \text{pealeii} \) (Kier and Curtin, 2002). The tentacle muscle fibers are cross-striated (Kier, 1982) and, like the SMR and CMP circular fibers of paralarvae, have short (0.8 \( \mu \)m) thick filaments (Kier and Curtin, 2002).

**Alternative splicing of the MHC gene**

The dimensions of the myofilaments are but one factor that may affect the contractile properties of the SMR and CMP muscle fibers. The contractile properties of striated muscle fibers may be altered in a variety of ways, including, but not limited to, expression of different isoforms of the myofilament lattice proteins (e.g. Kendrick-Jones et al., 1976; Sweeney et al., 1988; Marden et al., 1998; Schiaffino and Reggiani, 1996) and the nematode *Caenorhabditis elegans* (Miller et al., 1999), or a single muscle fiber may express only one of the isoforms, as is often the case in the striated fibers of mammals and fish (Goldspink, 1998). We do not know if the two isoforms of MHC confer different contractile properties on the muscles in which they are expressed. It is quite possible that the two isoforms differ in ATPase activity given that the alternative splice site falls along one of the regions of the MHC gene that codes for the putative nucleotide binding region of the protein (Matulef et al., 1998), and changes in the structure of this region can affect ATP turnover rates in the MHC isoforms of scallops (Perreault-Micale et al., 1996). Despite these important gaps in knowledge, the molecular data we present do not illustrate an obvious difference in isoform expression pattern during ontogeny.

**Linking mantle muscle ultrastructure with jetting performance**

If the mechanics of the circular muscles change during ontogeny, an interesting question is raised: is there a change in circular muscle mechanics associated with ontogenetic changes in jet dynamics or jet propulsive efficiency? The answer is that yes, indeed, jet locomotion changes during ontogeny in a manner consistent with predictions based on the concurrent changes in mantle muscle structure and function we report. The rationale for this statement is provided below.

Many paralarval squids, including \( D. \, \text{pealeii} \), are planktonic and appear to spend much of their time maintaining vertical position in the water column using vertically directed jets (Boletzky, 1974;
Zeidberg and Hamner, 2002). To increase speed, as they would for prey capture or to undergo daily vertical migrations (Zeidberg and Hamner, 2002), paralarval *D. pealeii* increase the amplitude, but not the rate, of mantle contraction (Bartol et al., 2009a). In other words, paralarvae employ a relatively constant jet velocity across the range of speeds (5–27 DML s⁻¹) considered by Bartol et al. (Bartol et al., 2009a), varying speed by altering the total mass of water expelled per mantle contraction. By contrast, adult *D. pealeii* increase jetting speed by adjusting both the rate and amplitude of mantle contraction (Anderson and Grosenbaugh, 2005) (J.T.T. and K. R. Taylor, unpublished). Increasing the rate of mantle contraction requires application of greater pressure to the water in the mantle cavity, and this pressure is derived, ultimately, from active stress produced by the circular muscle fibers. The short thick filaments of the paralarval SMR and CMP circular fibers result in significantly lower peak stress production, and this limits the ability of paralarvae to produce high mantle cavity pressures.

This effect can be seen more clearly if the mantle is modeled as a pressurized circular cylinder. In a thin-walled cylinder, the relationship between the stress in the wall, the pressure in the mantle cavity and the radius of the mantle is given by a variation of Laplace’s Law (Fung, 1994):

\[ p = \frac{\sigma r_i}{r} \]  

where \( \sigma \) is the mean circumferential stress in the mantle wall (i.e. the stress generated by the circular muscle fibers), \( r_i \) is the radius of the inner edge of the mantle wall, \( r \) is the thickness of the mantle wall and \( p \) is the internal pressure (i.e. the pressure in the mantle cavity). We substituted measured values of mantle radius, mantle wall thickness and the peak isometric stress generated by the circular muscles of paralarval (119 mN mm⁻²) and the CMP circular fibers of adult *D. pealeii* (216 mN mm⁻²) (Thompson et al., 2008). We predict that a paralarval *D. pealeii* could generate a peak mantle cavity pressure of 12.7 kPa during jetting whereas an adult could produce a peak pressure of nearly 58 kPa. These predicted peak pressures would never be realized in vivo because the circular muscles produce much lower stresses during the isolotic shortening required of jetting (Milligan et al., 1997). In addition, Eqn 1 is for a closed cylinder, and fluid expelled from the open funnel aperture of a squid will prevent pressures from reaching those theoretical maxima. Nevertheless, the significantly lower peak isometric stress of the paralarval circular muscles imposes a constraint on thrust production (see Thompson and Kier, 2002) (J.T.T., P.S.K. and I.K.B., unpublished) and the strategies used to increase swimming speed (see Bartol et al., 2009a).

Paralarval squid swim in an intermediate Reynolds number (Re) fluid regime in which inertial forces dominate fluid dynamic drag but viscous forces are considerable. Within this environment, they utilize an intermittent or pulsed jet for propulsion. Squids form a jet pulse by first expanding the mantle radially and filling the mantle cavity. Once the mantle cavity is full, the circular muscles contract, thereby increasing the pressure in the mantle cavity and driving water out of the mantle cavity through the funnel. Repetition of this cycle results in a pulsed jet. Studies of mechanically generated pulsed jets at high (>1000) Re issuing into quiescent fluid have demonstrated the important role played by the formation of vortex rings at the leading edge of each jet pulse. Vortex rings provide additional thrust through nozzle exit over-pressure, i.e. fluid pressure above the local ambient pressure during jet ejection, which develops as additional input through over-pressure, pulsed jets can produce substantially more thrust than equivalent steady jets (Krueger and Gharib, 2005). Recent results have shown that paralarval *D. pealeii* also develop vortex-ring-like structures during jetting and hence may benefit from enhanced thrust that is useful for combating the drag challenges experienced at intermediate Re (Bartol et al., 2008; Bartol et al., 2009a).

To capitalize on over-pressure benefits provided by pulsing, it is essential to use short pulses. In particular, the ratio of the length of the ejected plug of fluid (L) to the nozzle diameter (D) should be small enough so that isolated vortex rings are formed with each pulse. If \( L/D \) is too large, the vortex ring will pinch off from the generating jet (Gharib et al., 1998) and the relative contribution of over-pressure related to the vortex ring formation will decline as the remainder of the pulse is ejected as a relatively steady jet (Krueger and Gharib, 2003). Not only do short jet pulses amplify the thrust benefit of pulsing but recent results from juvenile and adult *L. brevis* (Bartol et al., 2009b) and ‘Robosquid’ (Nichols et al., 2008) have shown that propulsive efficiency can also be improved by relying on short jet pulses during pulsed jetting. Indeed, paralarval *D. pealeii* seem to rely almost exclusively on relatively short jet pulses, which appears to be a contributing factor leading to their remarkably high propulsive efficiencies despite the intermediate Re at which they swim (Bartol et al., 2008; Bartol et al., 2009a).

In the context of swimming at intermediate Re and the considerable benefit pulsed jetting with short jet pulses appears to provide, the present results of increased \( V_{\text{max}} \) and short circular muscle relaxation times in paralarval *D. pealeii*, as compared with their adult counterparts, is particularly intriguing. Although we do not know the load on the circular musculature during jetting or how it scales with body size, the significantly higher \( V_{\text{max}} \) of the paralarvae undoubtedly contributes to their ability to produce the short jet pulses that are important for maintaining high propulsive efficiency. In addition, the higher \( V_{\text{max}} \) and shorter relaxation times may also facilitate the high jet pulsing frequencies that are often observed in paralarvae (I.K.B. and J.T.T., personal observation) (Bartol et al., 2009a), which allow for more continuous swimming and shorter coasting phase durations within each jet cycle. The ability to swim more continuously with short periodic refill periods during jet cycles is an important consideration at intermediate Re where the considerable viscosity inhibits coasting.

High jet propulsive efficiency actually may be more important for paralarvae than older life-history stages because their fins play such a minor role in propulsion relative to the contributions of the highly efficient fins of juveniles and adults (Bartol et al., 2008), requiring paralarvae to rely more heavily on their jet for propulsion. Thus, ultrastructural modifications of the circular muscle fibers over ontogeny may have evolved in response to the need for the rapid mantle contractions and short jet periods that lead to high propulsive efficiency.

**LIST OF ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>CMP</td>
<td>central mitochondria-poor circular muscle fibers</td>
</tr>
<tr>
<td>D</td>
<td>diameter of jet nozzle</td>
</tr>
<tr>
<td>DML</td>
<td>dorsal mantle length</td>
</tr>
<tr>
<td>( L )</td>
<td>length of the ejected plug of fluid</td>
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<td>( L_0 )</td>
<td>muscle preparation length that produced the peak isometric stress in brief tetanus</td>
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<td>MHC</td>
<td>myosin heavy chain</td>
</tr>
<tr>
<td>( p )</td>
<td>pressure inside the mantle cavity</td>
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<td>( P_0 )</td>
<td>maximum isometric stress in brief tetanus</td>
</tr>
<tr>
<td>pcs</td>
<td>physiological cross sectional area</td>
</tr>
</tbody>
</table>
radius of the inner surface of the mantle

Re

Reynolds number

RT-PCR

reverse transcriptase-polymerase chain reaction

SR

superficial mitochondria-rich circular muscle fibers

SMR


Ontogeny of muscle function in squid


