Gradients of Strain in the Hollow, Cylindrical Muscular Organs of Soft-bodied Invertebrates

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Abstract

The current paradigm of striated muscle function assumes that all muscle fibers within a single muscle shorten the same amount and produce the same force during a given behavior. I tested a mathematical model that predicts large and non-uniform length changes (i.e., strain) in the circumferential muscle fibers of cylindrical muscular organs, such as the mantle of squid. Sonomicrometry was used to measure dimensional changes in the mantle of adult Long-finned (*Doryteuthis pealeii*) and Oval (*Sepioteuthis lessoniana*) squid; videography and morphometric analyses were used in tandem to measure dimensional changes in the mantle of *D. pealeii* hatchlings. These experiments supported the predictions of the model, revealing (1) the existence of a gradient of strain (i.e., the fibers near the inner surface of the mantle experienced greater strain than those near the outer surface for a given mantle contraction) and a gradient of strain rate; (2) that the magnitude of the gradients of strain (for all animals tested) and strain rate (for adults and juveniles only) increased as jet amplitude increased; (3) and that the magnitude of the gradient of strain and strain rate increased with increasing relative mantle wall thickness, as occurs during growth. These findings challenge current theories of striated muscle function and call for a reanalysis of the evolution of muscle structure and function in cylindrical muscular organs.
Introduction

The hollow, cylindrical shape of the bodies and muscular organs of soft-bodied invertebrates is well suited to functions in skeletal support and locomotion (e.g. Trueman, 1975). However, this shape may result in a previously unrecognized cost – large non-uniformities in strain among the circumferential (i.e., circular) muscle fibers of the body wall. That is, as the circular fibers contract to decrease body diameter, fibers along the inner lumen surface undergo greater shortening (i.e., strain) than those along the outer surface. Because the circular muscle fibers near the inner lumen surface experience greater strain than the fibers near the outer surface over the same period of time, a gradient of strain rate must also exist. Gradients of strain and strain rate create several potentially serious problems for muscle fibers, including sarcomeres within the same muscle operating along different regions of the length-tension and force-velocity curves (see Pappas et al., 2002), the need for muscle fibers near the inner lumen surface of the organ to generate force over an especially wide range of sarcomere lengths, and challenges in “tuning” of elastic connective tissue fibers to compensate for variation in strains across the muscular wall of the organ.

The presence of a gradient of strain in a muscle is noteworthy because mechanisms have evolved, at least in the vertebrates, to prevent non-uniform shortening of fibers in a muscle or muscular organ (Rome, 2005; Arts et al., 2001). Although this phenomenon has never been investigated in invertebrates, several examples of non-uniform muscle fiber shortening in vertebrate striated muscles have been observed. However, the observed differences in shortening in vertebrates are caused by (1) regional anatomical structures that affect the ability of adjacent fibers to contract, such as a stiff
aponeurosis (Soman et al., 2004; Ahn et al., 2003; Pappas et al., 2002) or asymmetric tapering of the whole muscle (Ahn et al., 2003); (2) the independent function of various parts of the muscle such that different regions are acting as different muscles (Higham & Biewener, 2008); or (3) different muscle fiber orientations within the muscle (e.g., pectoralis of birds, Soman et al., 2004). The non-uniform shortening predicted for the circular muscles of animals with hollow, cylindrical bodies or muscular organs cannot be attributed to any of these causes. With the exception of non-uniform shortening caused by a stiff aponeurosis, (which affects shortening of fibers both in parallel and in series), all cases of observed non-uniform shortening in vertebrate muscle fibers are along the longitudinal axis of the muscle (i.e., between fibers linked in series). Unlike many of the previously observed instances of non-uniform shortening, the gradient of strain predicted for hollow, cylindrical muscular organs is perpendicular to the longitudinal axis of the muscle (i.e., between fibers in parallel).

The mantle of squids is a hollow, cylindrical organ that contracts and expands rhythmically to provide thrust for jet locomotion, and therefore is a good subject in which to investigate gradients of strain and strain rate. Squid produce a locomotory jet by contracting the circular muscle fibers of the mantle, thereby decreasing the diameter of the mantle cavity and pushing water through the funnel (Packard & Trueman, 1974; Ward & Wainwright, 1972). The distance traveled by the squid and speed of travel resulting from a jet are generally proportional to the amplitude and rate of the decrease in mantle diameter; however, this is not always the case since fin movements as well as the orientation and degree of contraction of the funnel can also affect locomotion. Some jets are preceded by a hyperinflation phase, during which the radial muscle fibers contract to
expand the diameter of the mantle beyond the resting diameter (Packard & Trueman, 1974). Hyperinflation serves to fill the mantle cavity with a greater volume of water than normal mantle expansion, potentially allowing for jets of greater thrust or duration. It can occur prior to any type of jet, but occurs most often in combination with large amplitude contractions.

Thompson et al. (2008) developed a model of mantle function during jetting (see Appendix 1) that makes three predictions: (1) gradients of strain and strain rate exist across the mantle wall; (2) for larger amplitude mantle contractions (i.e., higher velocity jets), the resulting gradients of strain and strain rate are greater; and (3) as the relative mantle wall thickness increases, as it does during growth, the magnitude of the gradients increases. My previous work focused on testing the first two hypotheses (Thompson et al., 2010); the focus of the current paper is a test of the third hypothesis. Analyzing the third hypothesis required a two-pronged approach. First, I used sonomicrometry to record dimensional changes in the mantle of adult and juvenile Long-Finned Squid, Doryteuthis pealeii and Oval Squid, Sepioteuthis lessoniana. By investigating non-uniform muscle fiber shortening in two species, my collaborators and I were able to determine the extent to which gradients of strain and strain rate as well as the resulting consequences may be more broadly applicable to other squid and to other organisms with a cylindrical body or organ. Second, I developed a method that combined high-speed videography and morphometric analyses to examine dimensional changes in the tiny mantles of D. pealeii hatchlings.

Methods

Strain Measurements in adult Doryteuthis pealeii and adult Sepioteuthis lessoniana
Two late-stage juvenile *S. lessoniana* were anesthetized using cold artificial seawater (Instant Ocean, Aquarium Systems Inc., Cleveland, OH) at 17°C for 3-7 minutes (Bower *et al.*, 1998) prior to surgery. Eight adult and juvenile *D. pealeii* were anesthetized using cold natural seawater at 3-5°C for 3-10 minutes. Sonomicrometry transducers were attached to the mantle at 1/3 of the dorsal mantle length (DML) from the anterior edge of the mantle (Figure 1A & B), with transducers on the exterior of the mantle sutured in place and the transducer at the inner surface of the mantle held in place using Vetbond (3 M, St. Paul, MN). A suture spanning the mantle wall was used during some experiments to stabilize the inner transducer while the Vetbond cured; this suture was removed prior to data collection. The leads for all three transducers were secured using a suture clip attached to the anterior edge of the mantle or with an additional stitch at the tail.

Swimming trials were conducted in a shallow tank filled with artificial seawater at 25°C for *S. lessoniana* and with natural seawater at about 17°C for *D. pealeii*. The analog output from the sonomicrometer (Triton Technology, Inc., San Diego, CA), which provided simultaneous measurements of mantle diameter and wall thickness, was digitized at 1,000 Hz and analyzed using WinDAQXL software (DATAQ Instruments, Inc., Akron, OH) (Figure 1C). Following the experiment, squid were over-anesthetized in 7.5% MgCl₂ • 6 H₂O mixed in a 1:1 volume ratio with seawater. This solution relaxed the mantle musculature (Messenger *et al.*, 1985) and has been used by others to determine resting mantle diameter (Thompson and Kier, 2001; 2002). Dorsal mantle length was measured and cross-sections at ⅓ DML were obtained from adult and juvenile squid to permit diameter and wall thickness measurements. Because adults and juveniles are cylindrical and therefore have relatively uniform mantle diameters at ¼ - ½ DML, the
diameter and wall thickness measurements are essentially independent of position along the mantle in this region. Therefore, these measurements were considered to be analogous to measurements at the ink sac, which is located at $\frac{1}{3} - \frac{1}{2}$ DML from the anterior edge of the mantle.

*Strain Measurements in Doryteuthis pealeii Hatchlings*

* D. pealeii egg fingers were incubated at 13 – 17°C. Healthy hatchlings were placed in a tank 10 x 6 x 10 cm at 17 – 20°C. High-speed video (125 frames/s; Troubleshooter, Fastec Imaging, San Diego, CA) was used to record the magnitude and rate of mantle diameter changes in the hatchlings during jetting (Figure 2). ImageJ software (National Institutes of Health, USA) was used to measure the diameter of the mantle at the ink sac, which is located $\frac{1}{3} - \frac{1}{2}$ of the dorsal mantle length (DML) from the anterior edge of the mantle. The pixel resolution of the cameras limited me to a maximum spatial resolution in the field of view of the camera of $\pm 0.0028$ mm$^2$, which represents an error of $\pm 0.28\%$ of the measurement of a mantle diameter of 1 mm, which is a typical diameter for hatchlings.

*Morphological Measurements in Doryteuthis pealeii Hatchlings*

Hatchlings fixed in 10% formalin in seawater for 96 hours (n=19) or glutaraldehyde (n=5) were stored in 70% aqueous ethyl alcohol (EtOH). In order to avoid added variation in the stage of development to our sample, hatchlings with a visible yolk sac or lacking eye pigmentation were excluded to ensure that they had not hatched prematurely (Arnold, 1965). DML measurements were made after replacing the EtOH solution using three washes with artificial seawater. Hatchlings were embedded in optimal cutting temperature compound (OCT) for sectioning with a cryostat. Cross-
sections of the mantle at the ink sac (⅓ - ½ DML) were obtained (Figure 3) at -18°C. Section thickness was 40 µm for formalin-fixed hatchlings and 45 µm for glutaraldehyde-fixed hatchlings. The sections were viewed on a compound light microscope using the highest magnification that permitted the entire cross section to remain in the field of view. The sections were sufficiently thin to ensure that cross-sections were perpendicular to the longitudinal axis of the mantle but were thick enough to prevent shearing of the tissue. Elliptical shape of the section or large variations in mantle wall thickness were indicative of a non-orthogonal orientation of the blade with respect to the longitudinal axis of the mantle, so sections demonstrating these features were not analyzed. Circumference (C) and wall thickness were measured from photomicrographs using ImageJ software. Mantle diameter (D) was calculated from circumference (D = C/π) rather than directly measured due to the irregular section shape that occasionally resulted from embedding artifacts.

**Calculation of Strain**

Using the model described by Thompson *et al.* (2008) (see Appendix 1 for details), circumferential strain at the outer surface (εout) and the inner lumen surface (εin) of the mantle were defined as:

\[ ε_{out} = \frac{r_i - r_f}{r_i} \]  

\[ ε_{in} = \frac{(r_i - t_i) - (r_f - t_f)}{r_i - t_i} \]

where \( r_i \) and \( t_i \) are the initial (i.e., resting) radius of the outer surface of the mantle and wall thickness, respectively, and \( r_f \) and \( t_f \) are the radius and wall thickness following contraction of the circumferential muscle fibers. Strain rate was calculated as the change
in strain divided by the duration of the exhalant phase of the jet. Since the mantle wall thickness could not be measured directly for the hatchlings from the video, wall thickness for a given observed mantle diameter was calculated from the linear relationship found between diameter and wall thickness, as described in Figure 4.

Statistical Analysis

The extent to which the fixative used (glutaraldehyde or formalin) unnaturally changes the wall thickness and diameter is unknown. Therefore, in order to avoid error that may be inherent when using two different fixatives, I performed an analysis of covariance (ANCOVA). The ANCOVA allowed me to determine whether the relationship between the diameter and wall thickness measurements of the formalin-fixed hatchlings was different from the relationship that resulted when considering both formalin- and glutaraldehyde-fixed hatchlings (Figure 4). I also found the 95% confidence interval for the slope of the best-fit line that represents all of the hatchlings. This confidence interval was applied to all subsequent calculations for the hatchlings wall thicknesses and circumferential strain (Figures 6 and 7).

The data shown in Figures 6A and 7A represent multiple jets from a given individual (e.g. 24 jets for the *S. lessoniana* data were recorded from only 2 squid). Therefore, not all of the data points are independent, which violates one of the assumptions of the analysis of covariance. However, assuming that the jets from a given individual are sufficiently independent and that similar jets would be observed from another individual of a similar size enabled me to use an ANCOVA for these data. Therefore, ANCOVA was used to determine the significance of the difference in slopes observed for the outer circumferential strain vs. the inner circumferential strain for all
three groups of animals (Figure 6).

I also used ANCOVA to determine if the slopes observed for the difference in strain between the inner and outer surfaces of the mantle differed between the adult and hatchling *D. pealeii* (Figure 7). In addition, the 95% confidence intervals for each line of best-fit for the adult squid were calculated. The 95% confidence intervals for the hatchlings represent the confidence interval as calculated from the data shown in Figure 4.

All the ANCOVA and confidence interval calculations were performed using VassarStats (Richard Lowry, http://faculty.vassar.edu/lowry/VassarStats.html).

**Results**

*Description of Jetting Behavior*

The adult *D. paeleii* and *S. lessoniana* squid exhibited very similar jetting behaviors including a broad range of swimming speeds and combinations of mantle pumping and fin movement. These squid often assumed a “bipod” position during which they rested on the bottom of the tank by balancing on the tip of the tail and the tips of the arms. While performing the bipod behavior, only slow, ventilatory mantle movements were employed. The squid also exhibited a variety of locomotory jetting behaviors. During normal jetting, overall speeds ranged from slow to moderate (i.e., <1 dorsal mantle length, DML, per second to <2 DML s\(^{-1}\)) and locomotion was powered by a combination of mantle contractions and fin movements. Escape jets involved vigorous contraction of the mantle, wrapping of the fins around the body, and high speeds (>3 DML s\(^{-1}\)). Escape jets were often preceded by hyperinflation of the mantle and accompanied by inking.
Newly hatched *D. pealeii* exhibited a considerably smaller repertoire of jetting behaviors, despite the employment of both mantle pumping and fin movements. The hatchlings never used the bipod stance. Hatchlings bobbed up and down in the water column of the tank as they alternated moderate jets and refilling the mantle cavity. Escape jets were also employed on occasion and sometimes included a hyperinflation phase. Ink discharges were either too small to be noticed or were not present in the hatchlings.

*Hatchling Diameter and WallThickness Measurements*

The diameter and wall thickness measurements for *D. pealeii* hatchlings fixed using glutaraldehyde (n=5) or formalin (n=19) are displayed in Figure 4. The relationship between the wall thickness and diameter appeared to be linear for the available range of sizes. The best-fit line for all of the data \( y = -0.0145x + 0.035 \) had an \( R^2 \) of 0.468 (Figure 4). Because the best-fit line for only the formalin-fixed hatchlings was not significantly different from the best-fit line for all of the data (ANCOVA; \( F_{1,40} = 0.09, p=0.76 \)), the line representing all of the data was used to calculate wall-thickness from diameter.

*Changes in the Diameter: Wall Thickness Ratio During Growth*

The thickness of the mantle wall increased with positive allometry relative to dorsal mantle length during growth \( (y = 0.013x^{1.13}, R^2 = 0.865; \text{Figure 5A}) \). The diameter of the mantle in anesthetized animals exhibited negative allometry relative to dorsal mantle length \( (y = 0.6x^{0.75}, R^2 = 0.950; \text{Figure 5A}) \). The ratio of mantle diameter : wall thickness decreased as dorsal mantle length (DML) increased, indicating that the relative
thickness of the mantle wall increased with body size (Figure 5B). DML varied very little among hatchlings.

**Gradients of Strain**

The circumferential strains measured for *D. pealeii* adults (N=8, n=375 jets) along the inner surface of the mantle ranged from -0.42 to +0.25, where negative strains indicate contraction of the mantle and positive strains indicate hyperinflation. This was comparable to the range experienced by *S. lessoniana* adults (N=2, n=24 jets; range: -0.39 to -0.0039); hyperinflation was not observed in *S. lessoniana* adults, hence the lack of positive values for circumferential strain in the reported range. For *D. pealeii* hatchlings (N=17, n=85 jets), the circumferential strains along the inner surface of the mantle ranged from -0.55 to +0.17 with no jets resulting in a circumferential strain between -0.18 and 0 (Figure 6). Thus, some of the strains along the inner surface of the mantle that the hatchlings experienced were larger than those experienced by the adults. However, the hatchlings had a striking lack of smaller strains, suggesting an absence of slow, low amplitude jetting.

The magnitude of strain experienced by the circumferential muscle fibers along the inner lumen surface of the mantle was greater than the strain experienced by the fibers along the outer lumen surface of the mantle for a given jet in *D. pealeii* adults, *S. lessoniana* adults, and *D. pealeii* hatchlings (Figure 6). The best fit lines for outer circumferential strain and the inner circumferential strain for each group of animals are shown in Figure 6B and have the following equations: (1) outer circumferential strain for all animals: \( y = x - 4.4 \times 10^{-17} \), \( R^2 = 1 \); (2) inner circumferential strain in adult *D. pealeii*: \( y = 1.40x - 0.00254 \), \( R^2 = 0.985 \); (3) inner circumferential strain in adult *S. lessoniana*: y
The inner circumferential strains for the hatchlings also exhibit an $R^2$ of 1.0 because these strains were calculated using the linear relationship between diameter and wall thickness (Figure 4). The 95% confidence intervals for the lines of best fit did not overlap (Figures 6B). In addition, the lines of best fit for the inner circumferential strain for all three animals groups were significantly different from the line of best fit for the outer circumferential strain (ANCOVA; $D$. pealeii adults: $F_{1,669}=1777.5$, $p<0.001$; $S$. lessoniana adults: $F_{1,405}=482.04$, $p<0.001$; $D$. pealeii hatchlings: $F_{1,356}=2565.07$, $p<0.001$; Figure 6B).

The difference in circumferential strain (i.e., the “gradient”) experienced along the inner and outer surfaces of the mantle for a given jet is shown in Figure 7. The slopes of the best-fit lines for each group of animals were positive ($D$. pealeii: $y = 0.316x - 0.0145$, $R^2 = 0.564$; Adult $S$. lessoniana: $y = 0.802x + 0.00196$, $R^2 = 0.999$; Hatchling $D$. pealeii: $y = 0.0605x - 1.182$, $R^2 = 1$) indicating that as jet size (i.e. magnitude of the outer circumferential strain) increased, the size of the gradient of strain rate also increased.

The magnitude of the gradient of strain for a given jet size was lower for $D$. pealeii hatchlings than adults and juveniles (Figure 7). Because adults and juveniles had lower diameter: wall thickness ratios than hatchlings (Figure 5), this indicates that as the relative thickness of the mantle wall increased, the gradient of strain also increased. In addition, for lower diameter: wall thickness ratios, the gradient of strain increased more
rapidly with jet amplitude as indicated by the higher slope for the best-fit line for the adult and juvenile *D. pealeii* data (slope: 0.316) than for the hatchling data (slope: 0.0605; Figure 7). The slopes for hatchlings and juveniles/adults were significantly different (ANCOVA, $F_{1,356} = 566.94$, $p < 0.001$; Figure 7B).

**Gradients of Strain Rate**

The circumferential strain rate along the inner surface of the mantle had a higher magnitude than that along the outer surface of the mantle (Figure 8) for all jets in the three groups of animals that I examined. In addition, the best-fit lines for the adult *D. pealeii* ($y = -1.033x + 0.0306$, $R^2 = 0.193$) and adult *S. lessoniana* ($y = -1.57x + 0.0038$, $R^2 = 0.978$) had negative slopes, which indicated an increase in the gradient of strain rate with larger jets (i.e. larger circumferential strains along the outer surface of the mantle). The best-fit line for the *D. pealeii* hatchlings had a positive slope ($y = 0.00357x + 0.0103$, $R^2 = 0.00205$), but the $R^2$ value was quite small, suggesting that there was no linear relationship between jet size and the magnitude of the gradient of strain rate for hatchlings. For a given jet size, the gradient of strain rate was greater in *D. pealeii* adults and juveniles than in *D. pealeii* hatchlings, meaning that the gradient of strain rate increased with greater relative mantle wall thickness.

**Discussion**

Gradients of circumferential strain (Figures 6, 7) and strain rate (Figure 8) were present for all observed jets in the mantles of adult *D. pealeii*, adult *S. lessoniana*, and hatchling *D. pealeii*. Gradients of strain and strain rate present challenges (described below) to many, if not all, life stages of *D. pealeii*. In addition, my work has demonstrated that gradients of strain and strain rate occur in more than one species of
squid, confirming the importance of these gradients in loliginid squids and suggesting broader applicability to the myriad organisms with hollow, cylindrical bodies.

Gradients of strain and strain rate create several potential problems for the motor system of the mantle. The first is related to the length-force relationship in striated muscles fibers. The force a muscle fiber produces varies with sarcomere length. Maximum force is produced at intermediate sarcomere lengths and force falls as the muscle fiber shortens or is extended beyond the sarcomere length range of this maximum-force plateau (Gordon et al., 1966). For all jets in the animals I studied, the circumferential muscle fibers along the inner surface of the mantle shortened to a greater extent than those along the outer surface. Thus, muscle fibers near the inner and outer surfaces of the mantle may operate over different ranges of sarcomere length and, therefore, potentially produce different levels of force during the same mantle contraction. This potential problem becomes more acute as the jet amplitude or relative mantle wall thickness increase (Figure 7). Furthermore, for a jet of a given size, adult squid experienced a larger magnitude gradient of strain than hatchlings (Figure 7), indicating that as relative wall thickness increases, as it does during growth from a hatchling to an adult (Figure 5), the magnitude of the gradient of strain increases. Thus, the motor system of the mantle must also be able to manage variations in the magnitude of the gradient of strain throughout ontogeny.

A second potential problem caused by gradients of strain is that circumferential muscle fibers near the inner surface of the mantle must shorten at higher rates than those near the outer surface during the same jet. This implies that circumferential fibers near the inner surface of the mantle experience lower loads for a given mantle contraction,
thereby allowing greater shortening velocity, or those muscle fibers have a higher intrinsic shortening velocity than the circumferential fibers near the outer surface of the mantle. The experiments I performed did not test either of these explanations but future work should address this issue.

The magnitude of gradients of strain rate (Figure 8) were more variable for a given jet amplitude than the gradients of strain (Figure 7). The overall increase in variability can likely be attributed to the fine control that squid have over the contraction of their muscle fibers (Otis & Gilly, 1990) such that by changing the rate of contraction, a given decrease in mantle diameter can function as a bipod pump to ventilate the mantle cavity or as a rapid escape jet. As was observed for gradients of strain, the magnitude of the gradient of strain rate increased with jet amplitude for adult *D. pealeii* and adult *S. lessoniana* (Figure 8). However, there did not appear to be any trend, either increasing or decreasing, relating the magnitude of the gradient of strain rate relative to jet amplitude for *D. pealeii* hatchlings, as indicated by the very low $R^2$ value (0.00205). The absence of a clear trend in these data may result from a lack of fine control over contraction rate in hatchlings and/or the violation of the assumption that length of the mantle remains constant during jetting.

*Does the mantle increase in length during jetting in hatchlings?*

The volume of the mantle tissue is constant during a jet (Kier and Thompson, 2003) and, thus, contraction of the circumferential muscle fibers would tend to elongate the mantle or increase the thickness of the mantle wall or both (Kier and Thompson, 2003). Thin, collagenous tunics enclose the mantle musculature, and their proposed function is to prevent length increases during mantle contraction (Ward and Wainwright,
Indeed, Ward (1972) and Packard and Trueman (1974) found mantle length increases during vigorous jetting in adult loliginid squids of less than 5% of the dorsal mantle length. Because many squids lack the longitudinal muscle fibers that could constrain mantle elongation during jetting (Ward and Wainwright, 1972), these studies supported the proposed function of the tunics. Newly hatched squid appear to have more extensible tunics than juveniles and adults (Thompson and Kier, 2001), and this may explain the absence of a clear relationship between the magnitude of the gradient of strain rate and jet amplitude in hatchlings. If the mantles of the hatchlings lengthened passively during the exhalant phase of the jet, the observed scatter in the relationship between the magnitude of the gradient of strain rate and jet amplitude can be, at least in part, attributed to variable degrees of lengthening for a given jet size. Despite the potential violation of one of the assumptions of the model (Appendix 1), the results (Figures 6, 7, 8) strongly support the conclusion that a gradient of strain and strain rate exist in hatchlings. Lengthening during jetting would tend to decrease the magnitude of a gradient of strain and strain rate because constant volume of the mantle could be maintained by both increasing mantle length and wall thickness as the diameter decreases.

Lengthening the mantle would seem to be energetically inefficient since the power produced by the contracting circumferential fibers would increase the length of the mantle rather than decrease the volume of the mantle cavity to expel water to allow jetting, thereby wasting energy that could have been used for locomotion. After observing the bulbous morphology of hatchlings (as opposed to the more elongated shape of adult squid) and the difference of jetting patterns between hatchlings and adults, I
suspect that lengthening during jetting could be occurring in the hatchlings. 
Unfortunately, the 2-camera video analysis used for this research was insufficient to control for all angles of movement in the hatchlings so mantle length could not be determined accurately. Lengthening of the mantle during jetting in hatchlings is an interesting phenomenon that should be investigated with a 3-camera high-speed videography system.

*Morphological and physiological mechanisms to accommodate gradients of strain and strain rate*

The data presented here raise many important questions about the mantle of squids as well as similarly shaped bodies and muscular organs. Particularly, how have the arrangement and function of the components of the motor system been changed to accommodate gradients of strain and strain rate? As mentioned previously, if the fibers along the inner surface of the mantle shorten to a greater extent and at a more rapid rate than the fibers along the outer surface of the mantle, then these fibers may operate along different portions of the length-tension and force-velocity curves. If true, this suggests that contrary to the current paradigm of muscle function (Biewener, 2003), power output and ATP usage vary across the mantle wall.

The potential problem of muscle fibers at different regions within the mantle wall producing different force during the same mantle contraction could be offset if the obliquely striated circumferential muscle fibers of squid have a length-force relationship with a broad maximum-force plateau, as observed in the obliquely striated muscle fibers of leeches (Miller, 1975). In a study of the circumferential muscle fibers of the loliginid squid, *Alloteuthis subulata*, Milligan et al. (1997) suggested that the muscle fibers had a
relatively narrow maximum-force plateau. Thompson et al. (2008) were unable to confirm their results in *D. pealeii*, instead observing a broader maximum force plateau, though not as broad as that observed by Miller (1975) for leeches. More work is needed to investigate this issue.

Another mechanism for dealing with gradients of strain may lie in the arrangement of the collagen fibers within the wall of the mantle. The squid mantle has several distinct networks of collagen fibers that serve important roles in storing and returning elastic energy during locomotion (Gosline and Shadwick, 1983). To serve these roles, the material properties and arrangement of the collagen fibers should be “tuned” to the kinematics of the mantle; i.e., contraction of the mantle should strain the collagen fibers such that they are loaded appropriately for elastic energy storage. The inner surface of the mantle and, consequently, the collagen fibers along the inner lumen surface, experiences substantially greater lengthening during hyperinflation than the outer surface. Thus, the collagen fibers near the inner surface of the mantle must be able to accommodate higher strains and store elastic energy maximally, which is especially challenging given that squid mantle collagen has relatively limited extensibility (Gosline and Shadwick, 1983; MacGillivray et al., 1999). One way that the squid could compensate for the variable demands on their collagen fibers throughout the mantle could be to vary the relative lengths of the collagen fibers with the position of the fiber in the mantle.

*Effects of Gradients of Strain and Strain Rate on Jetting Gaits*

If all the circular muscle fibers in the mantle have the same or similar lengths over which maximum force can be achieved, then which fibers are providing the most power
will change over the course of a jet, such that fibers near outer surface of the mantle may be capable of producing more power at the initiation of the jet whereas fibers near the inner surface may be capable of producing more power toward the end of the jet. In order to activate the fibers at the point in the jet when they are capable of producing the most power, there may be a wave of activation of the fibers starting at the outer surface of the mantle and traveling inward. The potential for waves of activation is intriguing and is a good topic for future research.

While no research has addressed the energetics of jet locomotion in the context of gradients of strain, Bartol et al. (Bartol et al., 2009A & 2009B) examined the effects of jet duration on the efficiency of locomotion. In general, hatchling D. pealeii tend jet more often than adults while using jets of shorter duration; this allows the hatchlings to maintain a high propulsive efficiency despite the relatively larger slowing effect of viscosity on the hatchlings, which prevents substantial coasting (Bartol et al., 2009A). Meanwhile, adults are able to perform ‘burst and coast’ locomotion and therefore tend to use longer duration jets, despite the lower propulsive efficiency associated with longer jets (Bartol et al., 2009B).

A wave of activation, as opposed to simultaneous activation, may provide the opportunity for longer duration jets. Since adults experience larger gradients of strain than hatchlings (Figures 6 and 7), I expect that adults may be experiencing a longer delay (i.e. longer wave) in the activation of the inner muscle fibers relative to the outer muscle fibers, if such waves exist. Therefore, adult squid would be capable of producing jets with a relatively longer exhalant phase. This may be advantageous for adults since they have relatively smaller funnel openings than hatchlings (Bartol et al., 2009B) and
therefore would need to produce a longer jet in order to expel a similar relative volume of water compared to hatchlings. Thus, adults may benefit from larger gradients of strain if such gradients result in a wave of activation that allows for a longer jet. However, this phenomenon is largely unexplored, so the advantages of gradients of strain may be overestimated here.

Concluding Remarks

The observation of non-uniform muscle fiber shortening in the mantle of squids raises questions about the role of gradients of strain and strain rate in the evolution of muscle structure and function. The ability of squid to adapt the arrangement of fibers within the mantle to address the challenges of gradients of strain and strain rate is not currently understood. In addition, the extent to which the mechanisms for managing gradients of strain and strain rate change throughout development of squids is yet uninvestigated. This research reveals the incomplete nature of our understanding of muscle function in hollow, cylindrical muscular bodies and organs.
Appendix 1

The mathematical model for the gradient of strain developed by Thompson et al. (2008) was based on the assumption that the mantle has a circular cross-section and is of constant volume during the jet (Ward, 1972). In order to maintain constant volume, as the diameter of the mantle muscle decreases during the contraction of the circumferential fibers, the wall thickness must increase.

To calculate the strain along the inner and outer edge of the mantle, we can start with the general equation for strain:

\[ \varepsilon = -\frac{C_i - C_f}{C_r} \]

Where \( C_i \) is the initial circumference of the muscle and \( C_f \) is the circumference of the muscle following contraction of the circular muscle fibers. The negative sign is indicative of a decrease in circumference. Using this, the strain along the outer surface (\( \varepsilon_{out} \)) of the mantle muscle can be calculated using the following equation.

\[ \varepsilon_{out} = -\frac{(2\pi r_i - 2\pi r_f)}{2\pi r_i} \]

Where \( r_i \) is the initial (resting) radius of the outer surface of the mantle and \( r_f \) is the final radius of the outer mantle. When simplified, this equals:

\[ \varepsilon_{out} = -\frac{r_i - r_f}{r_i} \]

Because the radius of the inner surface of the mantle can be calculated by subtracting the thickness of the mantle wall from the radius of the outer surface, the strain along the inner surface of the mantle can be calculated as follows.

\[ \varepsilon_{in} = \frac{[(r_i - t_i) - (r_f - t_f)]}{r_i - t_i} \]
Where \( t_i \) is the initial thickness of the mantle wall and \( t_f \) is the final thickness of the mantle wall following the contraction of the circumferential muscle fibers. The gradient of strain can be calculated from these values since it is the difference between inner strain and outer strain.

Sonomicrometry allowed me to measure instantaneous mantle wall thickness and diameter for the adult and juvenile *D. pealeii* and *S. lessoniana*. Thus, I was able to directly calculate the circumferential strain along the inner and outer surfaces of the mantle using the relationship stated above. The high-speed video allowed me to measure mantle diameter changes in *D. pealeii* hatchlings during jetting and therefore permitted the direct calculation of circumferential strain along the outer surface of the mantle.

Mantle wall thickness, however, could not be directly observed and so was calculated from the linear relationship between diameter and wall thickness (Figure 4) using the observed diameter. This calculated wall thickness was used to determine the circumferential strain along the inner surface of the mantle.

The mathematical model also outlines a way to predict the gradient of strain for a particular jet by calculating the predicted final mantle wall thickness. Using the measured initial wall thickness \( (t_i) \), initial radius of outer surface of the mantle \( (r_i) \), and final radius of the outer surface of the mantle \( (r_f) \), it is possible to calculate the predicted final wall thickness \( (t_f) \) for a single jet using the equation given below (MacGillivray *et al.*, 1999).

\[
t_f = r_f - \sqrt{r_f^2 - t_i(2r_i - t_i)}
\]

Using the model’s ability to describe and predict the function of the mantle and other cylindrical structures has lead to three qualitative predictions. Table 1 displays sample
calculations to demonstrate this point. For all displayed jets, the gradient of strain ($\varepsilon_{\text{in}} - \varepsilon_{\text{out}}$) is negative, indicating that the fibers along the inner surface of the mantle are predicted to shorten more than the fibers along the outer surface of the mantle; hence the first prediction of the model that a gradient of strain exists. The second prediction of the model is that for a larger amplitude jet (i.e., larger degree of contraction), the gradient of strain will be larger. This prediction can be determined by comparing the calculated magnitude of the gradient of strain for each group as jet amplitude increases from 5% to 15% to 30% of the resting radius. For both adults and hatchlings, the gradient of strain increases with jet amplitude. Comparisons between adults and hatchlings reveal that for a given jet amplitude, hatchlings experience a smaller magnitude gradient of strain than adults. Therefore, the model predicts that as relative wall thickness increases, the gradient of strain for a given jet amplitude will be larger (Table 1).
Literature Cited


Acknowledgements

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Table 1. Sample calculations using the model to predict gradients of strain in the mantle of squid.

<table>
<thead>
<tr>
<th>Group</th>
<th>Average Mantle Radius: Wall Thickness *</th>
<th>Measurement</th>
<th>Slow Jet 5% Contraction</th>
<th>Intermediate Jet 15% Contraction</th>
<th>Fast Jet 30% Contraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult</td>
<td>4 ± 1.2 (n = 48)</td>
<td>$\varepsilon_{\text{in}}$</td>
<td>-0.10</td>
<td>-0.32</td>
<td>-0.75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\varepsilon_{\text{out}}$</td>
<td>-0.05</td>
<td>-0.15</td>
<td>-0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\varepsilon_{\text{in}} - \varepsilon_{\text{out}}$</td>
<td>-0.051</td>
<td>-0.17</td>
<td>-0.68</td>
</tr>
<tr>
<td>Hatchling</td>
<td>21 ± 8.7 (n = 21)</td>
<td>$\varepsilon_{\text{in}}$</td>
<td>-0.055</td>
<td>-0.17</td>
<td>-0.34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\varepsilon_{\text{out}}$</td>
<td>-0.05</td>
<td>-0.15</td>
<td>-0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$\varepsilon_{\text{in}} - \varepsilon_{\text{out}}$</td>
<td>-0.0054</td>
<td>-0.017</td>
<td>-0.039</td>
</tr>
</tbody>
</table>

*Ratio of mantle radius: wall thickness was measured between 1/3 and 1/2 DML from the anterior edge of the mantle. Measurements were considered to be the resting radius: wall thickness ratio because animals were exposed to MgCl$_2$, a muscle relaxant, prior to measurements.

The percentages represent the percentage of mantle contraction during the jet, relative to the resting mantle radius.
Figure 1. Sonomicrometry Data Collection. (A & B) Cross-section of mantle approximately 1/3 of the total dorsal mantle length from the anterior edge of the mantle. The red ovals represent the placement of the sonomicrometry transducers. (A) For preliminary experiments transducers a & b measured diameter; c & d measured wall thickness. (B) For subsequent experiments, the a’-b’ and b’-c’ segments were recorded, measuring wall thickness (b’- c’) and allowing diameter to be calculated (a’ - b’ + b’ - c’). (C) Change in outer diameter (black trace) and change in mantle wall thickness (WT, blue trace) of a D. pealeii squid. Traces represent changes for ventilatory, resting contractions (1 & 2) followed by more vigorous, locomotory jets (3-8). Hyperinflation for a portion of a jet is denoted by *. Hyperinflation occurs when the radial muscle fibers contract to increase the diameter of the mantle beyond the resting diameter, as defined by the maximum diameter observed during resting mantle contractions. Decreases in diameter correspond to increases in wall thickness, as demonstrated by the red dashed line. These data were collected using sonomicrometry crystals, as indicated in (B) and underwent a low pass filter to eliminate high frequency waves attributed to electrical noise and not the movement of the mantle muscle.
Figure 2. Video analysis of hatchling jetting. Movements of *Doryteuthis pealeii* hatchlings were recorded using high speed video (recording rate = 125 frames/second). Each set of images (Ai & ii and Bi & ii) represents a jet from a different individual. The location of the ink sac is indicated for each hatchling shown here (Aii & Bii). The maximum diameter obtained prior to jetting (Ai & Bi) and minimum diameter obtained during a given jet (Aii & Bii) were measured and are shown here. Diameter was either measured laterally (A) or dorso-ventrally (B), depending on the orientation of the hatchling relative to the camera. Scale for each set of images is indicated by the solid black line.
Figure 3. Images of cross-sections of fixed *D. pealeii* hatchlings at the ink sac. Hatchlings were imbedded in Optimal cutting temperature compound (OCT) and sectioning was performed using a cryostat at -18 °C. (A) Cross-section of a formalin-fixed hatchling (10% formalin in seawater). Section thickness is 40 µm. (B) Cross-section of a glutaraldehyde-fixed hatchling. Section thickness is 45 µm. Scale for both images is indicated by a black bar, which represents 0.15 mm. Diameter was calculated from the circumference, which was measured using the polygon perimeter tool on ImageJ software. The wall thickness was measured from the thinnest portion of the mantle wall as close to the ventral midline as possible.
Figure 4. Wall thickness vs. diameter measurements of the mantle of hatchling *D. pealeii* at the ink sac. Hatchlings were fixed in 10% formalin in seawater (n=19) or glutaraldehyde (n=5) to allow varying amounts of *post-mortem* contraction and therefore obtain a wider range of observed diameter measurements. The solid line represents the best-fit line for all data and has the equation: $y = -0.0145x + 0.035$, $R^2 = 0.468$. The dashed lines represent the boundaries of the 95% confidence interval for the slope of the best-fit line. This confidence interval was applied to all data calculated from this equation. The equation of the best-fit line for the formalin-only data (not shown; $y = -0.020x + 0.041$, $R^2 = 0.374$) did not have significantly different slope from the line representing all of the data (ANCOVA: $F_{1,40} = 0.09$, $p=0.760$). Therefore, the equation for the line representing all of the data was used for subsequent wall thickness calculations.
Figure 5. Measurements of resting diameter and resting wall thickness at approximately 1/3-1/2 of dorsal mantle length (DML) in *Doryteuthis pealeii*. (A) Log [diameter or wall thickness] vs. log [DML]. The lines represent exponential best-fit lines and are described by the following equation. Diameter: \( y = 0.6x^{0.75} \), \( R^2 = 0.950 \). Wall thickness: \( y = 0.013x^{1.13} \), \( R^2 = 0.865 \). (B) Ratio of resting diameter: resting wall thickness vs. DML. The measurements for both panels were made *post mortem*; hatchlings (n=19) were fixed in 10% formalin in seawater for 96 hours prior to long-term storage in 70% aqueous ethyl alcohol (EtOH). DML measurements were made after replacing the EtOH solution with three artificial seawater washes. Diameter and wall thickness were measured from cross-sections made using a cryotome near the ink sac (1/3-1/2 DML). Adults and juveniles (n=31) were overanesthetized in a 1:1 solution of 7.5% MgCl₂ and seawater. Cross-sections were made at approximately 1/3 DML from the anterior edge of the mantle <1 hour after death.
Figure 6. Circumferential strain along the inner and outer surfaces of the mantle vs. circumferential strain along the outer surface of the mantle. Data represent measurements made for adult *Doryteuthis pealeii* (N=8), adult *Sepioteuthis lessoniana* (N=2), and *Doryteuthis pealeii* hatchlings (N=17). Circumferential strain along the outer surface was used as a proxy of the amplitude of the jet. Strain is arbitrarily negative to represent decrease in fiber length, as occurs during contraction, and positive to represent lengthening of fibers, as occurs during hyperinflation. (A) Each point represents strain during an individual jet and is either negative to indicate contraction (adult *D. pealeii* N=8, n=288; adult *S. lessoniana* N=2, n=24; hatchling *D. pealeii* N=17, n=72) or positive to indicate hyperinflation (adult *D. pealeii* N=7, n=87; hatchling *D. pealeii* N=4, n=13). (B) Best fit lines for outer circumferential strain for all jets (y = x - 4.4*10^{-17}, R^2 = 1) and inner circumferential strain for jets from each group (adult *D. pealeii*: 1.40x – 0.00254, R^2 = 0.985; adult *S. lessoniana*: y = 1.80x + 0.00196, R^2 = 0.999; hatchling *D. pealeii*: 1.06x – 1.18*10^{-5}, R^2 = 1). Best-fit lines for inner circumferential had significantly different slopes for all three groups of animals than for the outer circumferential strain (ANCOVA: p<0.001). Dashed lines represent 95% confidence intervals for the best-fit lines. Inset image magnifies a portion of the graph to give a better sense of the spread of the confidence interval. Please note that the R^2 = 1 for the best fit line for outer circumferential strain since the values are plotted against themselves and therefore must yield a perfectly straight line; the R^2 = 1 for the inner circumferential strain for *D. pealeii* hatchlings because these values were calculated from a linear relationship between diameter and wall thickness and therefore must also have a perfect relationship.
Figure 7. Difference in circumferential strain between the inner and outer surfaces of the mantle vs. the circumferential strain along the outer surface of the mantle. Circumferential strain along the outer surface of the mantle was used as a proxy for jet size. Strain measurements were made for adult *Doryteuthis pealeii* (N=8, n=288), adult *Sepioteuthis lessoniana* (N=2, n=24), and hatchling *Doryteuthis pealeii* (N=17, n=72). (A) Each point represents the contraction phase of an individual jet. (B) Solid lines represent best-fit lines for the data shown in panel (A). The slopes of all best fit lines are positive (Adult *D. pealeii*: $y = 0.316x - 0.0145$, $R^2 = 0.564$; Adult *S. lessoniana*: $y = 0.802x + 0.00196$, $R^2 = 0.999$; Hatchling *D. pealeii*: $y = 0.0605x - 1.182$, $R^2 = 1$). Dashed lines represent 95% confidence intervals for the best-fit lines. Slopes of the best-fit lines for *D. pealeii* adults and hatchlings were significantly different (ANCOVA: $p<0.001$). Please note that the $R^2$ value for the best-fit line for the hatchling data equals 1 because the circumferential strain along the inner surface was not measured directly but rather was calculated using a linear relationship and the measured changes in the outer diameter of the mantle.
Figure 8. Log of the gradient of strain rate vs. circumferential strain along the outer surface of the mantle for adult *D. pealeii* (N=8 animals, n=288 jets), adult *S. lessoniana* (N=2, n=24), and hatchling *D. pealeii* (N=17, n=72). The gradient of strain rate was calculated as the difference the circumferential strain rate between the inner and outer surfaces of the mantle. Circumferential strain along the outer surface of the mantle was used as a proxy for the magnitude of the jet, and in this instance, a negative strain indicates a decrease in fiber length. The y-axis was log-transformed to improve the clarity of presentation. Prior to the log transformation, linear best-fit lines were calculated for each group of animals. The slopes of the best fit lines were negative for adult *D. pealeii* and *S. lessoniana* and both had $R^2 > 0.1$; the slope for the best fit line for the *D. pealeii* hatchlings was positive and had an $R^2 << 0.1$ (adult *D. pealeii*: $y = -1.033x + 0.0306, R^2 = 0.193$; adult *S. lessoniana*: $y = -1.57x + 0.0038, R^2 = 0.978$; hatchling *D. pealeii*: $y = 0.00357x + 0.0103, R^2 = 0.00205$.)