The Functional Morphology and Ontogeny of the Nuchal Retractor Muscle in the Atlantic

Long-fin Squid (*Doryteuthis pealeii*)

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Abstract

The nuchal retractor muscle of the Atlantic Long-fin squid (*Doryteuthis pealeii*) appears to be multi-functional; i.e., it acts as a motor, strut, and brake during different locomotory behaviors. Previously, multi-function muscles have only been identified in vertebrates and arthropods, which makes muscle specialization in the nuchal retractor muscle of the squid particularly unique. My project involves an investigation of morphological differences in two putative muscle fiber types in the nuchal retractor muscle. Through paraffin-based histology, I have shown that the nuchal retractor muscle is obliquely striated and that there is a substantial difference in muscle fiber lengths within the nuchal retractor muscle. Furthermore, my analysis has shown that average cell area of muscle fiber cells is greater in the anterior regions of the nuchal retractor muscle. There are, however, more mitochondria-rich fibers in the posterior of the muscle; these fibers appear to be located around the periphery of the nuchal retractor muscle. Muscle fiber specialization would allow the squid to employ two different muscle fiber types to satisfy different mechanical roles. Lastly my research examined ontogenetic changes in the nuchal retractor muscle and has demonstrated that the nuchal retractor muscle is not present in newly hatched squid, but is present in juvenile squid.
**Introduction**

Striated muscles serve a variety of roles during locomotion and movement, including providing power for movement, acting isometrically as a strut or spring to stabilize joints and transmit force, and decelerating appendages and bodies or damping movement (reviewed in Dickinson et al., 2000). Although muscles of a single anatomical group (Ahn and Full, 2002) and even muscle synergists (Higham et al., 2008) can have different functions, there are few reports of individual muscles that serve as motors, struts, and brakes during locomotion (see Gabaldón et al., 2008, for an example).

To my knowledge, multi-function muscles have only been identified in vertebrates and arthropods that experience dynamic changes in relatively large inertial or gravitational loads during locomotion. Are complex jointed systems and high inertial/gravitational loading prerequisites for the evolution of multi-function muscles? Or have they also evolved in the comparatively simple muscular systems of aquatic soft-bodied invertebrates in which inertial and gravitational loads seem to be minimal? To address these questions, I investigated muscle specialization in the nuchal retractor muscle of squid.

The nuchal retractor muscle of the Atlantic Long-fin squid (*Doryteuthis pealeii*) appears to be multi-functional; i.e., it acts as a motor, strut, and brake during different locomotory behaviors (Thompson et al., 2015). The nuchal retractor muscle originates on the pen, a rigid internal structure that supports the mantle and acts as a site for muscle attachment in squid, and extends anteriorly along the dorsal midline of the nuchal region to its insertion on the ventral side of the nuchal cartilage (Williams, 1909; see Figures 1 and 2). The nuchal retractor muscle, in combination with the head retractor muscle, actuates the head during locomotion (see Figure 1), and also attaches the head to the pen (Williams, 1909). Interestingly, the nuchal retractor is the
only known muscle involved in locomotion in squid that is not supported by a muscular hydrostat (Thompson et al., 2014a). Instead, all of the muscle fibers in the nuchal retractor muscle run in the same direction, parallel to the long axis of the muscle. In contrast, the head retractor is a muscular hydrostat (Kier and Smith, 1985); i.e., it is composed of densely packed muscle fibers that run in longitudinal, transverse, and circumferential directions, which allows them to contract and elongate to perform a wide array of movements (Kier and Thompson, 2003). Muscular hydrostats rely on the fact that there is a constant volume of incompressible fluids and tissue, and are therefore able to transmit force through internal pressure instead of through a rigid skeletal system (Kier, 2012).

The nuchal retractor muscle fibers appear to be obliquely striated. Oblique striation has evolved independently multiple times in different species, which suggests that this muscle type must have a functional significance (Thompson et al., 2014b). Although the muscle fibers of the nuchal retractor muscle are in the same orientation, histological analysis of tissue samples completed previously in the Thompson lab suggests that the muscle contains two very different muscle fiber types (Figure 3). Different muscle fiber types have been identified previously in squid (Thompson et al., 2008). In the mantle, for example, there are different contractile properties of the mantle circular muscle fibers. It has been suggested that the structural differences among the circular muscle fibers allows the fibers to fulfill two distinct mechanical roles. Mitochondria-rich muscle fibers provide power for high-endurance, low-velocity swimming. In contrast, mitochondria-poor muscle fibers are used for briefer, high-velocity swimming (Thompson et al., 2008).

In the Fall of 2014 and Spring of 2015, I completed an investigation into the cellular detail of different regions of the nuchal retractor muscle. More specifically, I analyzed cell cross-
sectional area in different regions of the muscle. I found that cellular area was greater in the anterior region of the muscle. Furthermore, I analyzed how sizes of cores of mitochondria vary over the length of the muscle. I found that mitochondria-rich fibers are located in the posterior region of the muscle, while more mitochondria-poor muscle fibers are found in the anterior region of the muscle. These differences in cell cross-sectional area and mitochondrial abundance may provide insight into how different regions of the muscle might allow the squid to perform various functions (see Figures 5 and 6). Thus, I decided to analyze these two fiber types further in my research.

My project involves an investigation of morphological differences in two putative muscle fiber types in the nuchal retractor muscle. I am exploring the relative lengths of the muscle fibers in the nuchal retractor muscle and am describing structural differences in fiber type over the length of the muscle to define potential regional muscle fiber specialization. In addition, I am studying ontogenetic changes in the nuchal retractor muscle morphology to understand how changes in muscle structure might be related to ontogenetic differences in head movements.

**Materials and Methods**

I investigated nuchal retractor muscle morphology in Atlantic Long-fin Squid (*Doryteuthis pealeii*.) The animals were captured with squid jigs from lighted piers in South Bristol, ME, during the summers of 2014 and 2015. The squid were euthanized by decerebration and then fixed immediately *post mortem* in 3.75% formaldehyde in seawater for at least 48 hours. The fixed tissue was transferred to 70% ethanol, and then transported to Franklin & Marshall College at the conclusion of the summer for the histological studies.
D. pealeii embryos were obtained from Marine Biological Laboratories in Woods Hole, MA, in 1996. Within 24 hours of emergence from their egg cases, the newly hatched squid were anesthetized in a 1:1 solution of 7.5% aqueous MgCl\(_2\)•6H\(_2\)O and artificial seawater (Messenger et al., 1983) and then fixed in 3.75% formaldehyde in seawater. The hatchlings were stored in 70% ethanol for histological studies.

I performed gross dissections on adult and juvenile squid to examine the anatomy of the nuchal region of the squid, including the nuchal retractor muscle and nuchal cartilage. After documenting the anatomy carefully with high-resolution digital photographs, I performed dissections to isolate the nuchal region of the body that contains the head retractor, nuchal retractor, and nuchal cartilage (Figure 2). The isolated tissue blocks and the whole hatchlings were dehydrated in a graded series of ethanol, cleared in Hemo-D (Fisher Scientific, Pittsburg, PA), and embedded in paraffin (Paraplast Plus, McCormick Scientific, St. Louis, MO). The isolated tissue blocks and hatchlings were oriented to permit the cutting of cross and sagittal sections.

The resulting paraffin blocks were sectioned serially at a thickness of 10 µm using a rotary microtome, after which the sections were mounted on slides coated with Mayer albumin. The slides were stained using Picosirius, which differentiated between collagenous and muscular components of tissue sections (Sweat et al., 1964; protocol adapted from López-DeLeón and Rojkind, 1985). Slides were also stained using Milligan’s Trichrome (protocol in Kier, 1992), which differentiated between collagen, muscle, and nerve. The stained sections were viewed using brightfield microscopy.

Slides were made for cross sections and sagittal sections of the D. pealeii hatchlings. Slides were also made for cross sections and sagittal sections of D. pealeii juveniles.
Results

In the Fall semester, I completed gross dissections on adult and juvenile squid. These dissections allowed me to examine the nuchal region of the squid and to document the anatomy through high-resolution digital photographs.

Juvenile and adult squid had similar nuchal regions, however in adult squid all muscles were comparatively larger than in juvenile squid. In juvenile squid, the nuchal cartilage becomes smaller toward the posterior of the organism. Additionally the nuchal retractor muscle, which is quite large in the anterior region of the muscle, becomes smaller toward the posterior region of the muscle (Figure 7.)

I cross sectioned and examined hatchling *D. pealeii* at <24 hours old. There were no indications of a nuchal retractor muscle or a nuchal cartilage. I then examined hatchling *D. pealeii* in sagittal sections to confirm that the nuchal structures were not present. From this analysis, I was able to determine that the nuchal cartilage and nuchal retractor muscle were not present in *D. pealeii* at <24 hours old (Figure 8).

The nuchal cartilage and nuchal retractor muscle were both present in juvenile squid, as suggested by the large nuchal cartilage and large nuchal retractor muscle revealed from gross dissections of a juvenile squid. The nuchal cartilage is present directly below the joint pad in juvenile squid. It appears that the nuchal retractor muscle originates on the pen and inserts on the ventral side of the nuchal cartilage in the juvenile squid (Figure 9).

Discussion

The goals of my research were to investigate the morphological differences in two putative muscle fiber types of the nuchal retractor muscle. I described structural differences in
muscle fiber type over the length of the muscle to define potential regional muscle fiber specialization. Furthermore I have analyzed the nuchal retractor muscle through ontogeny by characterizing differences in the muscle in newly hatched, juvenile, and adult squid.

Although I made progress toward this goal, a few technical challenges hindered the project. For example, early attempts at embedding the hatchling squid failed because I was not able to orient the squid properly for the necessary sectioning. Thus, when a sample was sectioned, the squid was not in the correct orientation and analysis of the slides provided little insight into the morphology of the nuchal retractor cartilage and nuchal retractor muscle at that stage in development.

Eventually I was able to embed the hatchlings in the correct orientation by modifying embedding procedures and using a dissecting microscope to ensure proper orientation of the hatchling. However, this improvement came later in the Fall semester, which left me with little time to prepare both cross and sagittal sections.

In the Spring semester, I performed an analysis of the cross sections of *D. pealeii* hatchlings <24 hours old, and I was able to conclude that the nuchal cartilage is not present at that stage in development. However, the sample appears to be slightly tilted, which made it difficult for me to discern whether or not the hatchling has a nuchal retractor muscle (Figure 8B). Thus, I embedded *D. pealeii* hatchlings <24 hours old in Paraffin to permit sagittal sectioning of the tissue (Figure 8C). In conjunction with the cross sections, the sagittal sections illustrate that *D. pealeii* hatchlings <24 hours old do not have a nuchal cartilage or nuchal retractor muscle (Figure 8).

Cartilaginous structures often serve as a site of attachment for major muscle groups and thus develop prior to an organism’s locomotion (Cole and Hall, 2009). However, *D. pealeii*
hatchlings <24 hours old are motile without the development of a nuchal cartilage or nuchal retractor muscle, which suggests they are performing locomotion with different structures. Because they are not present, the nuchal cartilage and nuchal retractor muscle are not functionally significant to locomotion in *D. pealeii* <24 hours old.

However, in juvenile squid, the nuchal cartilage is quite large and is present immediately ventral the pen and joint pad (Figure 3). Furthermore the nuchal cartilage serves as an insertion point for the nuchal rector muscle (Figure 3). Thus, at this stage in development the squid is able to use the nuchal cartilage and nuchal retractor muscle for locomotion.

In both juvenile and adult squid, the nuchal regions appear to be quite similar, as suggested from the gross dissections. However, in adult *D. pealeii*, the nuchal cartilage and nuchal retractor muscle are comparatively larger than in the juvenile squid, because the body of the adult is larger itself (see Figures 4 and 7).

Previously in the Thompson lab, I completed an investigation into the cellular detail of the two muscle fiber types found in *D. pealeii*. My research illustrated that as cell size decreased toward the posterior region of the nuchal retractor muscle, average size of the cores of mitochondria within each fiber increased. These differences suggest that the contractile properties of the two fiber types may also differ, allowing the fibers to satisfy two distinct roles, as seen in the circular muscle fibers of the mantle of the squid (Thompson et al., 2008). The muscle fibers in the posterior region of the nuchal retractor muscle may be used to high-endurance, low-velocity movements (such as ventilating the mantle cavity) as they are rich in mitochondria and fatigue more slowly. While the muscle fibers in the anterior region of the muscle are likely utilized to perform briefer, high-velocity movements (such as jet escape) as they are relatively mitochondria-poor and fatigue more quickly.
The nuchal retractor muscle appears to be multi-functional (Thompson et al., 2015). Furthermore the two putative muscle fiber types in the nuchal retractor muscle allow the muscle to act as a motor providing power for movement, as a strut to transmit force, and as a brake to decelerate the head following locomotion. Having two distinct muscle fiber types allow the nuchal retractor muscle to fulfill three distinct mechanical roles.

Although I made progress toward my goals, there were some aspects of my research that I was not able to accomplish, as well as opportunities for future research that were presented during the semester that are interesting to pursue. I was not able to accomplish an analysis of the relative lengths of the two muscle fiber types in the nuchal retractor muscle. In the future, Image J will be utilized to measure the relative lengths of the two muscle fiber types. Exploring lengths and describing structural differences in fiber type over the length of the muscle will allow me to define potential regional muscle fiber specialization in the nuchal retractor muscle, which is particularly interesting to my research.

Furthermore, I would like to use electromyography to analyze head movement in hatchling *D. pealeii* at <24 hours old. Because it has been shown through my research that newly hatched *D. pealeii* at <24 hours old do not have a nuchal retractor muscle or a nuchal cartilage, it is uncertain whether or not they are able to actuate their head during locomotion. Both of these structures are essential to control head movement, therefore an absence of these structures implies that newly hatched *D. pealeii* at <24 hours old are not able to actuate their head. While it is not widely researched, it has been suggested that newly hatched *D. pealeii* at <24 hours old are able to perform jet locomotion. Therefore it is even more interesting to my research to determine whether or not newly hatched squid are able to actuate their head, or if they perform jet locomotion in a manner that is different than a juvenile and adult squid.
Lastly I am interested in examining the cellular detail of the two putative muscle fiber types through ontogeny. I will do this by detailing how average cross-sectional area of muscle fibers and how average sizes of cores of mitochondria change throughout development. Studying ontogenetic changes in the nuchal region of the squid will help me further understand how changes in muscle structure may relate to changes in head movement throughout development.
Figure 1. Images from a video recording of head retraction in *D. pealeii*. Panel A shows the head extended at 34 milliseconds after the video started recording. Panel B shows the head during retraction at 38 milliseconds after the video started recording. Panel C shows the head fully retracted at 44 milliseconds after the video started recording. The video was recorded in Summer 2014 in the Thompson lab.

Figure 2. Structures relevant to head actuation during locomotion. Panel A shows a lateral view of an Atlantic Long-fin squid (*Doryteuthis pealei*). Panel B shows a sagittal section of a formalin-fixed squid, cut posterior of the head, with the mantle removed. The nuchal retractor muscle is located out of view beneath the nuchal cartilage, which is labeled (NC). The head retractor muscle (HRM), the funnel retractor muscle (FRM), and the mantle (M) are labeled as well. Panel C shows a cross section of the squid that was sectioned from the dotted line in Panel B. The head retractor (HRM), nuchal retractor (NR), and nuchal cartilage (asterisk) are labeled. Panel A was recorded in the Summer of 2014 in the Thompson lab.
Figure 3. Images relevant to demonstrate two putative muscle fiber types found in the nuchal retractor muscle of *D. pealeii*. Panel A shows a lateral view of the juvenile Atlantic Long-fin squid (*Doryteuthis pealeii*). Panel B shows a sagittal section of the juvenile *D. pealeii*. The mantle (M), pen (arrow), nuchal cartilage (asterisk), head retractor muscle (HRM), and nuchal retractor muscle (NRM) are clearly labeled. Panel C is a magnification of the nuchal retractor muscle to demonstrate the two putative muscle fiber types. Scale bars, 1 cm; 1 mm.
Figure 4. Lateral view of dissection and photomicrograph for adult *D. pealeii*. The nuchal cartilage (NC), head retractor muscle (HRM), funnel retractor muscle (FRM) and nuchal retractor muscle (NRM), joint pad (JP), and pen (P) are clearly labeled. The nuchal retractor muscle originates on the pen and extends anteriorly to its insertion on the nuchal cartilage.
Figure 5. Images relevant to demonstrate the variety in average cell cross-sectional area and average sizes of cores of mitochondria in the nuchal retractor muscle. Panel A shows a cross section of the squid. The nuchal cartilage (asterisk), nuchal retractor muscle (NR), and head retractor muscle (HR) are all clearly labeled. Panel B is a magnified view of the nuchal retractor muscle. The grey areas are individual muscle fiber cells. The white cores in the center are cores of mitochondria. Scale bars, 1 mm; 10 μm.
Figure 6. Average cellular area (mm$^2$) and average mitochondrial area (mm$^2$) as a function of muscle region (dorsal, mid, ventral). Panel A is a photomicrograph of the nuchal retractor muscle and nuchal cartilage. This image shows how the nuchal retractor muscle is categorized into different regions (dorsal, mid, ventral) to facilitate analysis. Panel B shows the analysis for average cell cross sectional area. In the dorsal region of the nuchal retractor muscle, the anterior region of the muscle has significantly larger average cell area in comparison to the posterior region. In the mid region of the muscle, there is no difference in average cell area between the posterior and the anterior region. In the ventral region of the nuchal retractor muscle, the anterior region of the muscle has significantly larger average cell area in comparison to the posterior region. In the dorsal, mid, and ventral region of the nuchal retractor muscle, the posterior region of the muscle has significantly larger mitochondria than in the anterior region.
Figure 7. Lateral view of both juvenile and adult *D. pealeii*. Panel A shows the lateral view of a juvenile squid with mantle and pen removed. Panel B shows the lateral view of an adult squid with the mantle removed but the pen still intact. Nuchal cartilage (NC), head retractor muscle (HRM), and funnel retractor (FRM) are clearly labeled. Scale bars, 1 cm.
Figure 8. Newly hatched *D. pealeii* at <24 hours old do not have a nuchal cartilage or nuchal retractor muscle. Panel A shows an image of a newly hatched squid (*D. pealeii*) at <24 hours old. Panel B shows a cross section of the squid. Panel C shows a sagittal section of the squid. The pen, mantle, and joint pad (JP) are clearly labeled. If present, the nuchal cartilage and nuchal retractor muscle would be located directly below the pen.
Figure 9. Juvenile squid (*Doryteuthis pealeii*) have both a nuchal cartilage and a nuchal retractor muscle. Panel A shows a lateral view of the Atlantic Long-fin squid (*Doryteuthis pealeii*). Panel B shows a sagittal section of *D. pealeii*. The nuchal cartilage (NC), head retractor muscle (HRM), nuchal retractor muscle (NRM), and funnel retractor muscle (FRM) are all clearly labeled.
Literature Cited


