Morphological Description and Analysis of *Octopus rubescens* Testis and Sperm

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Abstract

Octopus rubescens is a small, yet abundant cephalopod found all along the West Coast. There is little known and relatively few studies done on the morphology of octopus sperm. We acquired a live *Octopus rubescens* testis and divided it into 4 sections to sample the morphological differences of spermatophore structure. The sections of the testis were stained with DAPI and Tubulin and imaged using immunofluorescence to determine the presence of nuclei and microtubules. The same sections of the testis were also imaged with scanning electron microscopy to compare cellular ultrastructure. We hypothesized that there is a gradient of morphological differences in spermatozoa structure that correspond to the maturity of the sperm. We found that there is a gradient of spermatogenesis with the most immature spermatophores in the wall of the testis and the most mature towards the center of the structure. The length of the sperm tail is very long compared to the size of the head which could be a distinguishing factor for sperm fitness.

1. Introduction

In many species, the competition sperm face to fertilize drives the morphology of the cells (Voight, 2009). Some organisms have rigorous mating rituals in which the male strives to prove his fitness to reproduce while other organisms have an internal selection process (García-Flores et al., 2019). Female octopuses have been known to mate with several males and hold onto their sperm bundles to internally select the fittest match (Voight, 2009). This delayed fertilization has been known to increase the competition between sperm loads, the most fit sperm will successfully fertilize the egg.

Male reproduction is scarcely studied in the order Octopoda; we know little about spermatophore morphologies in the testis. By understanding the morphologies of sperm, we can begin to understand what constitutes a fit and successful load. In the model organism, *Drosophila*, it has been found that the sperm with the longest tails are the ones to fertilize the egg (Snook & Karr, 1998). *Drosophila* is a genetic model to many systems; it is possible that invertebrates use long tails as a measure of sperm fitness. Other species within the Cephalopod class are known to have extremely long sperm; *Loglio pealeii* sperm have been found to be 1cm long (Kozloff, 1990).

Spermatogenesis is the process of differentiating spermatozoa into mature sperm due to meiotic cell proliferation (Wang et al., 2010). Stem cells are embedded in the wall of the testis in a concentric formation which will differentiate into germ cells. These will eventually grow a small tail while still having a round inflated head to become a spermatid. At this stage the head is filled with cytoplasm. After a period, the tail elongates and begins eliminating cytoplasm through the acrosomal complex via residual bodies. This process causes the nuclei to thin and retreat to the base of the head, at the neck of the sperm. Once the nucleus has shifted, tail has elongated, and the cytoplasm has been released the sperm has reached maturity (Gao et al., 20201).

These morphological changes such as expelling cytoplasm and extending the microtubule network are all done to achieve more efficient swimming which give the sperm advantages in competition (Gao et al., 2021). The aim of this research was to explain the morphological differences in different parts of the testis that represent different stages of sperm development in the most common species of octopus in the Pacific Northwest, *Octopus rubescens* (Voight, 2009). We hypothesized that there is a gradient of morphological differences in spermatozoa structure that correspond to the maturity of the sperm.

2. Methods

2.1 Animal and Tissues

Divers at the University of Washington captured many *Octopus rubescens* on Whidbey Island, WA (48.158518, -122.669359) and brought them back to Friday Harbor Laboratories (48.545007, -123.013604) for further studies. They suspected one of the animals was about to enter senescence, so we decided to euthanize and dissect it before it died naturally. The dissection met the requirements of the University of Washington Animal Care. We determined the male sex by looking for a hectocotylus on the R3 appendage. That night, we transferred the octopus into a dissecting tank kept on ice and titrated in 100% ethanol (EtOH) over a period of 20 minutes until it was anesthetized.

We made a longitudinal incision in the ventral side of the mantle exposing the mantle cavity and identified the testis by finding a white sac with a spiral coming from it in the posterior section of the cephalon. Next, we divided the sperm sac into 4 sections to take tissue samples sequentially from the genital pore to the testis.

Immediately following dissection, we put all the samples into a 2.5% glutaraldehyde and water solution and set for 48 hours. We removed the glutaraldehyde solution and chopped all the samples in half. Half of each sample was dehydrated in a graded EtOH series (30, 50, and 70%) to undergo antibody staining. The other half was dehydrated in a graded EtOH series (30, 50, 70, 90, 100%) to be dried with Critical Point. The samples were stored in a refrigerator at 4°C.

2.2 Immunocytochemistry (ICC)

We chopped the first set of samples into small pieces using a razor blade, rehydrated them using a graded EtOH series (70, 50, and 30%), and transferred them into a phosphate buffer

solution (PBS). To stain the nuclei, we diluted Diamidino-phenyl-indole (DAPI) from a stock solution to 1μL in 100μL PBS. We used the primary antibody, Anti-Tubulin, to visualize the microtubules after diluting from a stock solution to 1μL in 2500μL PBS. We used Goat Anti-Mouse IgG as the secondary antibody and diluted it from a stock solution to 1μL in 1000μL PBS. Each sample was washed in PBS 4x for 2.5 minutes each; DAPI and Tubulin solutions were added in equal amounts and mixed using a VWR Scientific Vortex-Genie 2. After 1 hour, we washed the samples with PBS 4x for 2.5 minutes. Goat Anti-Mouse IgG solution was then added to each sample. After 1 hour, the samples were washed again with PBS 4x for 2.5 minutes and placed in a refrigerator at 4°C. Slides were prepared by submerging each sample in PBS and glycerol. The samples were finally observed and photographed using a Nikon Eclipse E600 Epifluorescent Microscope and measured using ImageJ.

2.3 Scanning Electron Microscopy (SEM)

We took the second set of samples from 100% EtOH and dried them using a Samdri-790 Critical Point drying machine. Samples were then mounted on stubs and sputter-coated with a thin layer of gold palladium using a Cressington 108 Sputter Coater (model 6002-8). Finally, we placed the samples in an airtight container overnight and then observed and imaged them using a NeoScope JCM-5000 Scanning Electron Microscope and measured them using ImageJ.

3. Results

In *O. rubescens*, the testis occupied over a third of the mantle cavity suggesting that the organism spends a lot of its energy producing sperm. Our observations showed a very small amount of mature sperm in its testis which would require further study to better understand this

result. Both DAPI and Tubulin stained correctly and fluoresced in areas that we would have expected. The thin and long nuclei were seen at the base of the head, at the neck of the sperm.

Sample I was mostly ciliated sheath composed of microtubules and Sample IV had microtubules and nuclei embedded throughout the tissue. Sample III showed the highest concentration of sperm bundles which were rarely seen in the other samples. The length of the mature sperm tails in sample III and IV was roughly 300 μm in length.

Different stages of spermatozoa were seen across all samples including mature sperm, indicating that the gradient of spermatozoa development begins in the wall and ends in the center of the testis in *O. rubescens*. There are sperm bundles as well as individual free-swimming sperm present in the same area (Figure 2). Fluorescent images suggest that the further away from the seminiferous tubule the spermatozoa are, the larger the sperm will be (Table 1).

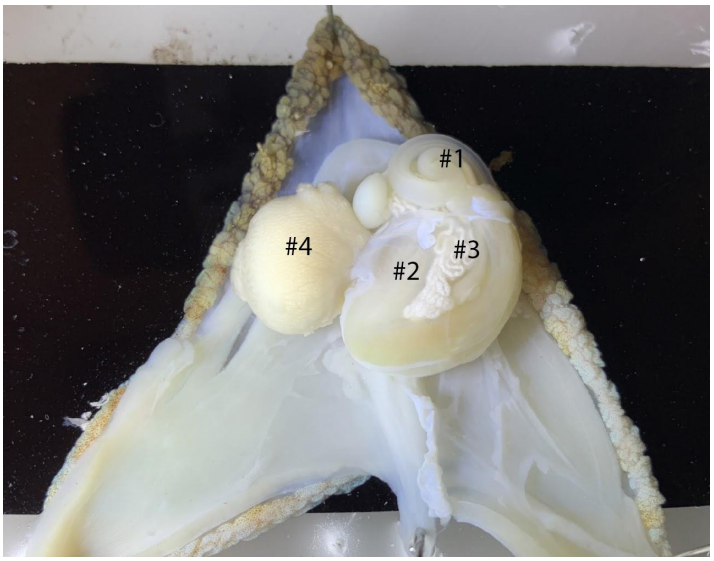
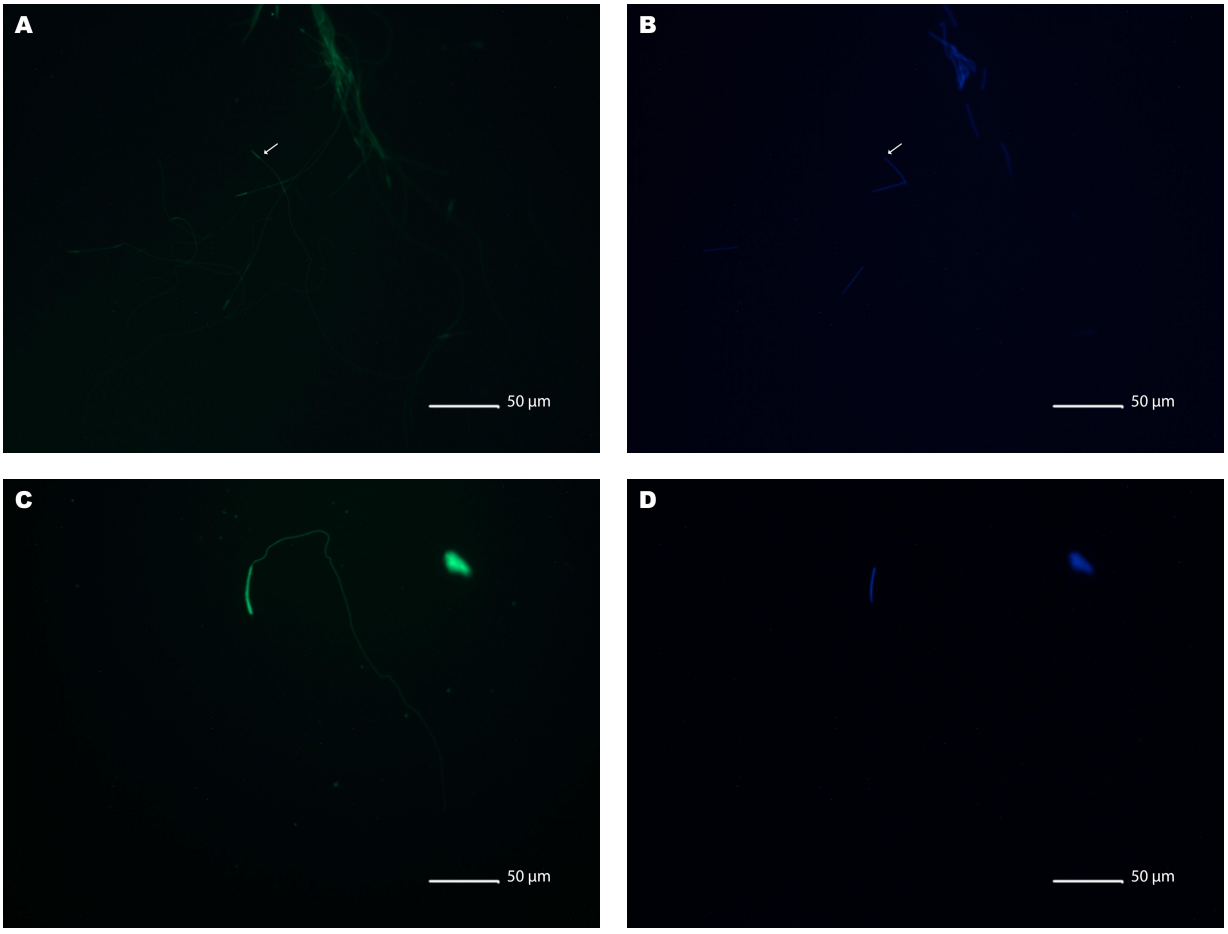


Figure 1. Ventral section of the *Octopus rubescens* mantle with exposed testis divided into 4 sections and labeled samples I-IV

There are many morphological differences in sperm as they develop. Sample IV shows most of the stages of sperm development from germ cells that resemble a head with no tail to a

spermatid that has the shape of a mature sperm with a large round head. Only tail structures are seen in sample III which could mean that sample III is more mature than sample IV. Thin long heads as well as long tails are seen in both sample I and II which show an abundance of mature sperm (Figure 4).



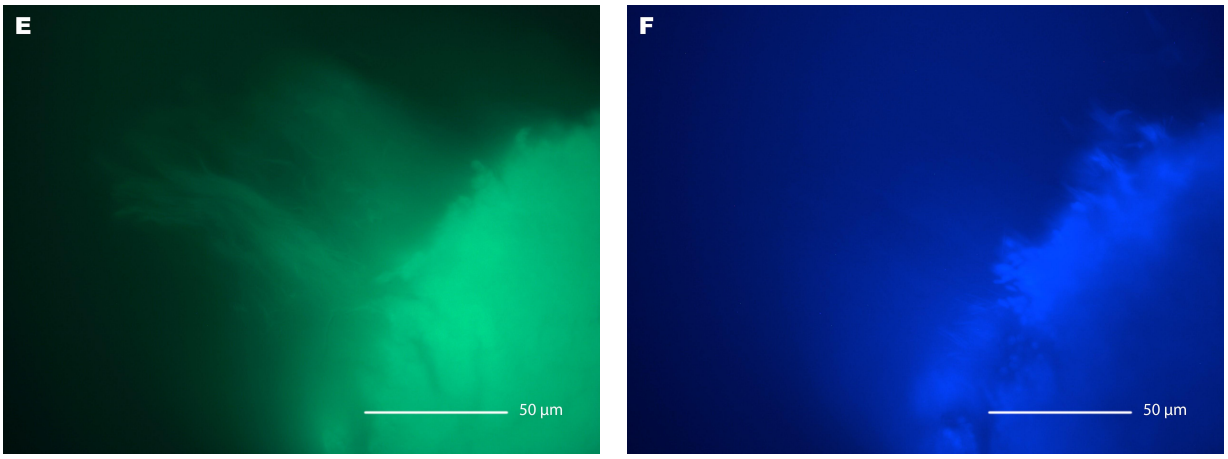


Figure 2. Fluorescent images of *Octopus rubescens* sperm stained with Tubulin and DAPI. Arrows correspond to the sperm that was measured and recorded in table 1. **A.** Bundle of sperm with microtubules stained with Tubulin in sample III. **B.** Bundle of sperm with nuclei stained with DAPI in sample III. **C.** Single sperm with microtubules stained with Tubulin in sample IV. **D.** Single sperm with nuclei stained with DAPI in sample IV. **E.** Sperm bundles with microtubules in sample III. **F.** Sperm bundle with nuclei in sample III.

Table 1. Sizes of mature sperm in each sample images with fluorescence

	Total Length (µm)	Length of Tail (µm)	Length of Head (µm)	Width of Head (µm)	Length of Nuclei (µm)	Width of Nuclei (µm)
Sample III	329.335	294.224	35.111	2.48	25.455	1.272
Sample IV	278.519	269.934	8.585	0.987	25.439	0.783

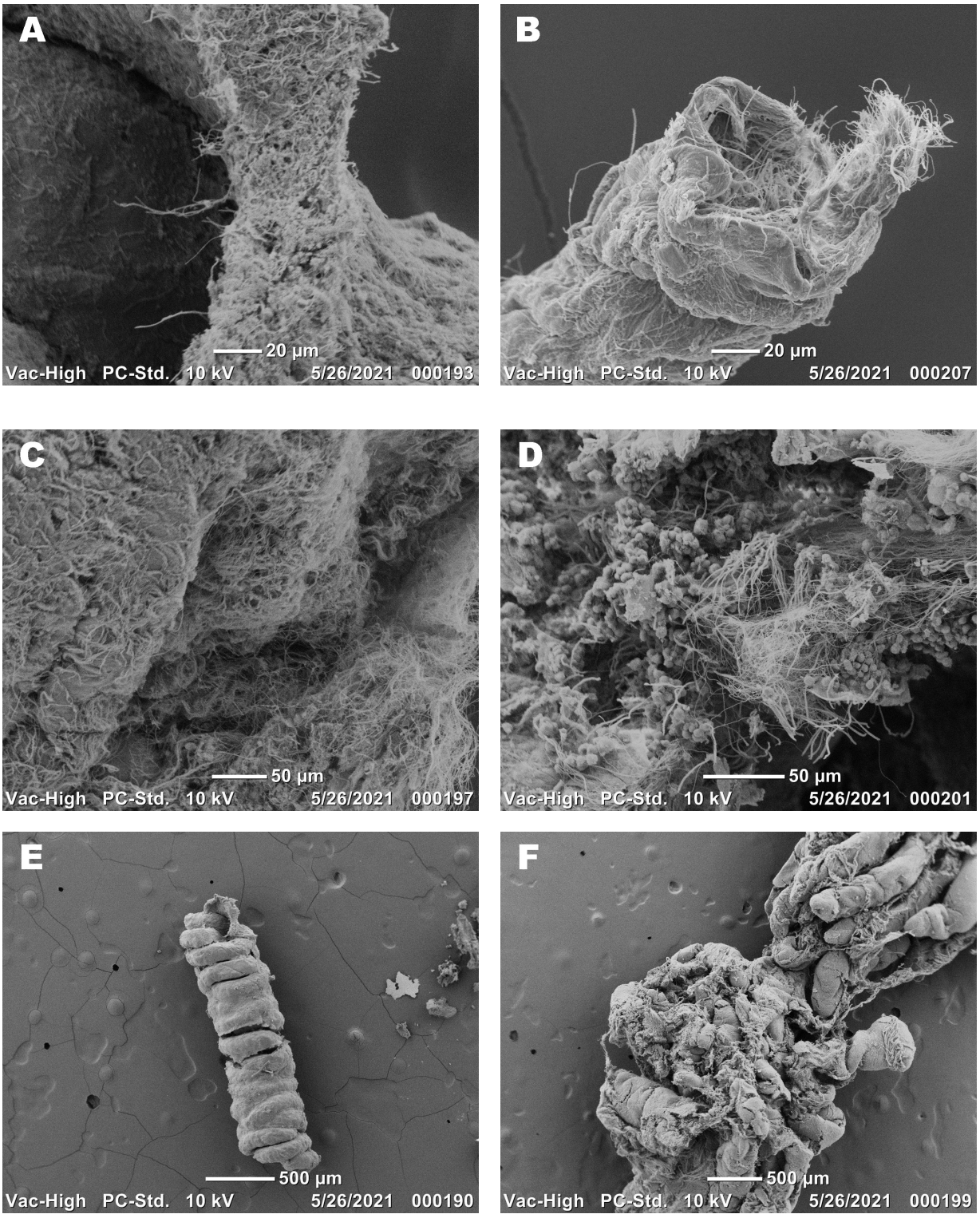


Figure 3. SEM images of each sample **A.** Mature sperm embedded in sheath in sample I **B.** Mature sperm on top of sheath in sample II **C.** Many tails embedded in the tissue of sample III

D. Spermatids embedded in tissue of sample IV **E.** Overall structure spermatophore from sample 1 near gonopore **F.** Overall structure of the seminiferous tubule from Sample IV

4. Discussion

These results support our hypothesis by showing a gradient of development of spermatophores in the testis. The most immature sperm are found deep in the wall of the testis and the most mature sperm are found concentrically lining the inner wall with the tails free floating in the inner cavity. This method of development is consistent throughout the species so far. Each sample does not represent a particular stage of development, just a different part of the testis. *O. rubescens* spermatophore morphology is similar to other species of cephalopods such as the Giant Pacific Octopus and the Common Octopus.

Long sperm was seen in *O. rubescens* which is consistent with the literature. Although it does not appear to have abnormally large sperm compared to the size of its body, the tail is very long. The tail makes up roughly 90% of the length of mature sperm and a long thin head is only seen in mature sperm as well. Evolutionary and reproductive pressures could have driven the morphology to change over time. Octopus sperm faces an abnormal amount of pressure to fertilize because of the female's ability to hold onto many different sperm loads at one time (García-Flores et al., 2019). This selective process could be the driving force that leads to the elongation of the sperm tail.

The heads are much larger in sample IV than sample I which suggests that the seminiferous tubules are the primary place that cytoplasm is expelled in order to advance the developmental process (Gao et al., 2021). We can also compare the round head seen in Figure 3D to the mature sperm seen in Figure 2C and D; both images are of sample IV and show the difference in head size. The final stage of spermiogenesis has the typical vertically compressed

head and the beginning stage has the round head. This evidence is consistent with our hypothesis and suggests that multiple stages of spermiogenesis are found in the same sample.

During immunofluorescence, mature sperm were found in sample III and IV which means that mature sperm could be found everywhere in the testis. The mature sperm tails were exceptionally long compared to the tails of the spermatids. Samples I and II are very similar; this could be the area where the mature sperm are being packaged up in the sheath and wait to be transferred into the female. The longer the tail the greater the chance the sperm has to out compete addition sperm (Gimenez-Bonafé et al., 2002). The less cytoplasm, more microtubules, and increased length of the tail all aid in the fitness or ability of the sperm to swim (Gao et al., 2021).

We were unable to compare the length of the tail between all samples because there were none left in sample I and II that were not embedded in the tissue. The sperm were most likely washed out during the staining process, but we expect the size to be relatively similar to the individuals in sample III and IV. Histology is the typical way to accurately identify stages of spermiogenesis. Doing a histological analysis could have added information which would have allowed us to compare internal cellular structures between stages. If we were to repeat this experiment, we would fix the samples in paraffin wax which would allow us to cut extremely thin cross sections. It was very difficult to get a thin enough cross section to see anything significant on the fluorescent microscope. Fluorescent microscopes require thin cross sections, approximately one cell thick, to get optimal image resolution. For our thick specimens, we would have benefited from doing confocal microscopy instead.

The morphology of *O. rubescens* sperm is similar to other octopods. We found that there is a gradient of spermiogenesis in the wall of the testis. Each stage has very distinct

morphological differences that were able to be seen with immunofluorescence and scanning election microscopy. Spermatids are embedded in basal membrane and packed on the inside of the tubules with the head still embedded in the tissue and the tail in the center in a concentric formation. The size and shape of the tail and nucleus change as development progresses which is seen throughout the testis.

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