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

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Abstract

The male reproductive system in cephalopods is a topic rarely studied in the field of fertilization biology. The changes the sperm cell undergoes during spermiogenesis varies slightly across the animal kingdom and unravelling the process as it occurs in individual species is vitally important for understanding evolution and fertilization strategies. This study aims to describe the structures involved in the spermiogenesis process of the cephalopod *Octopus rubescens*. To test the hypothesis that there will be a maturity gradient in the morphology of sperm developmental stages and overall sperm structure in *O. rubescens*, we dissected the testis of a male *O. rubescens* in senescence and divided the samples into 4 categories based on our proposed level of sperm maturity. After fixing the samples in glutaraldehyde and dehydrating them in ethanol (EtOH), we divided each sample in half. In one half, we stained the nucleus and microtubules to be imaged with fluorescent microscopy and dried and sputter-coated the other half to be imaged with scanning electron microscopy (SEM). The results of the fluorescent microscopy and SEM indicate a gradient of sperm maturity throughout the testis and that all maturation stages are present throughout the entire testis. The individual sperm were also observed to have considerably long tails which can be associated with sperm competition and selective pressures by the female during fertilization. This study broadens our understanding of the developmental biology of *O. rubescens* and cephalopods overall.

1 Introduction

Spermiogenesis is a complex process that is subtly different between different organisms. The immature spermatid undergoes many changes throughout the process including the formation of a flagellum, condensation of the nucleus, formation of the acrosome, and cytoplasm elimination (Sperry 2012). It is important to figure out this process as it occurs in individual species. Morphology of the spermatozoa and the structures associated with sperm production is essential for understanding fertilization and the evolution of reproductive processes in cephalopods.

Spermatogenesis is a highly consistent process in cephalopods, with a few extra steps added or removed based on the species observed (Gimenez-Bonafé et al. 2002). Sperm are produced in the seminiferous tubules of the testis where they mature and move to a duct in the left side of the mantle cavity. From there, the sperm concentrate into a rope-like structure and are packed into spermatophores to be stored in a structure called Needham's sac until being transferred into a female octopus (Wells 1978). During copulation, the spermatophores are moved to the hectocotylus arm and then placed into the mantle cavity of the female where they are stored in her oviducts. Because of this, fertilization can be delayed by the female, and competition between different sperm can occur. This selects for certain traits in the sperm structure that make it more likely to fertilize the egg (Mann et al. 1970).

The octopod *Octopus rubescens*, also known as the East Pacific red octopus, is a cephalopod in the family Octopodidae. Its distribution ranges from the Gulf of Alaska to the southern part of the Gulf of California, Mexico (López-Peraza et al. 2013). As a relatively small and easily collectible organism, it is an ideal candidate for laboratory investigations. Although many studies have focused on the behavioral responses (Warren et al. 1974, Onthank and Cowles 2011) and female reproductive system morphology of *O. rubescens* (Osborn 1995, López-Peraza et al. 2013), few studies have described the morphology of the male reproductive system in this animal. Doing so will provide a deeper understanding of spermatogenesis and fertilization strategies in cephalopods overall.

In this study, we hypothesize that there is a gradient of morphological differences in the spermatozoa structure of *O. rubescens* that correspond to the maturity of the sperm. The results of this study will broaden our understanding of the male reproductive system in cephalopods and contribute to the growing field of fertilization biology.

2 Materials and Methods

2.1 Animal and Dissection

Divers captured an *Octopus rubescens* in April 2021 near the Coupeville ferry terminal on Whidbey Island, WA (48.158518, -122.669359) and brought it back to Friday Harbor Laboratories (48.545007, -123.013604) for further studies. They determined the sex of the octopus to be male by identifying the hectocotylus arm on the R3 appendage. After capture, they confirmed that the octopus was in senescence by observing the animal become lethargic and refusing to eat. These are behaviors that typically indicate senescence in octopuses. Because of this, we transferred the octopus into a dissecting tank to be examined and euthanized.

For dissection of the animal, we titrated 100% ethanol (EtOH) into the dissecting tank for 20 minutes until the octopus was anesthetized. This was done in accordance with the University of Washington's animal care policies. Then, we made a longitudinal incision down the ventral side of the mantle exposing the mantle cavity. We identified the testis and other reproductive structures by locating a white sac with an obvious spiral toward the posterior end of the body. After dividing the sac into 4 distinct sections (Figure 1), we took tissue samples from each and set them immediately in a 2.5% glutaraldehyde in water solution for 48 hours. Sample 4 was hypothesized to be the least mature and sample 1 was hypothesized to be the most mature.

After that, we removed the glutaraldehyde solution and split each of the four samples in half. We dehydrated the first half in a graded EtOH series (30, 50, and 70%) to be used for antibody staining. The other half we dehydrated in a graded EtOH series (30, 50, 70, 90, 100%) to be dried and examined using scanning electron microscopy (SEM). We stored all samples in a refrigerator at 4℃.

2.2 Immunocytochemistry (ICC)

Using a razor blade, we chopped the first set of samples into small pieces. Then, using a graded EtOH series (70, 50, and 30%), we rehydrated the samples and transferred them into a phosphate buffer solution (PBS). To stain the nuclei, we used diamidino-phenyl-indole (DAPI), a fluorescent molecule that binds to DNA, which was diluted from a stock solution to 1μL in 100μL PBS. To stain the microtubules, we used the primary antibody Anti-tubulin which was diluted from a stock solution to 1μL in 2500μL PBS. We also used a diluted solution of the secondary antibody goat anti-mouse IgG taken from a stock solution to 1μL in 1000μL PBS. Before staining, we washed each sample in a PBS bath for 2.5 minutes for 4 rounds. We then added the DAPI and Anti-tubulin solutions in equal amounts and mixed the samples using a VWR Scientific Vortex-Genie 2. After 1 hour, we washed the samples with PBS for another 4 rounds at 2.5 minutes per round. We then added the goat anti-mouse IgG solution to each sample to sit for another hour. We washed the samples for the final 4 rounds with PBS and placed them in a refrigerator at 4℃. To prepare slides for fluorescent microscopy, we submerged each sample in a mix of PBS and glycerol. Finally, we observed and photographed the slides using a Nikon Eclipse E600 Epifluorescent Microscope and measured the images we took 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. We then mounted the samples on stubs and sputter-coated them with a thin layer of gold-palladium using a Cressington 108 Sputter Coater (model 6002-8). By keeping the stubs in an airtight container overnight we ensured that the prepared samples were fully dry. Lastly, we observed and imaged the samples using a NeoScope JCM-5000 Scanning Electron Microscope.

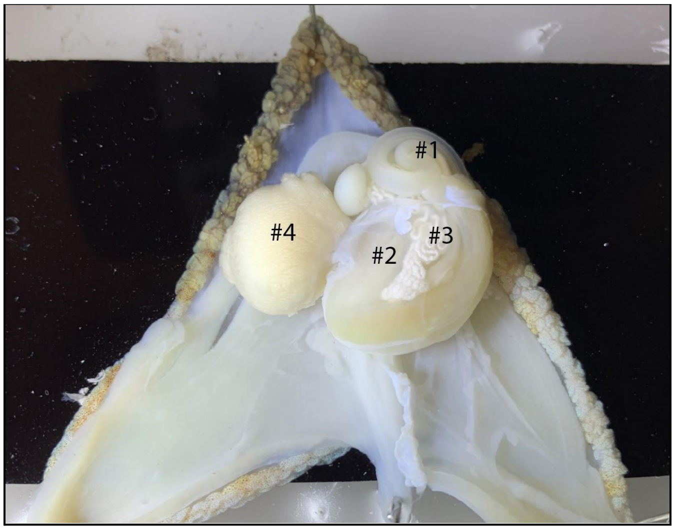

Figure 1. Dissection of the testis and associated structures divided into 4 sections and labeled.

3 Results

3.1 Analysis of Immunocytochemistry

As shown in Figure 2, we successfully stained the microtubule structures and nuclei which can be seen fluoresced in samples 3 and 4. Individual sperm appear long and thin with a distinct head and tail. Tubulin fluoresced green throughout the entire sperm cell which indicates the presence of microtubules throughout the whole structure. DAPI fluoresced blue only in the section directly behind the acrosome which can be associated with the location of the condensed nucleus. For all of the dimensions we measured for individual sperm, the sperm in sample 3 were overall larger than the sperm measured in sample 4 (Table 1). Samples 1 and 2 were void of sperm and therefore did not stain and fluoresce.

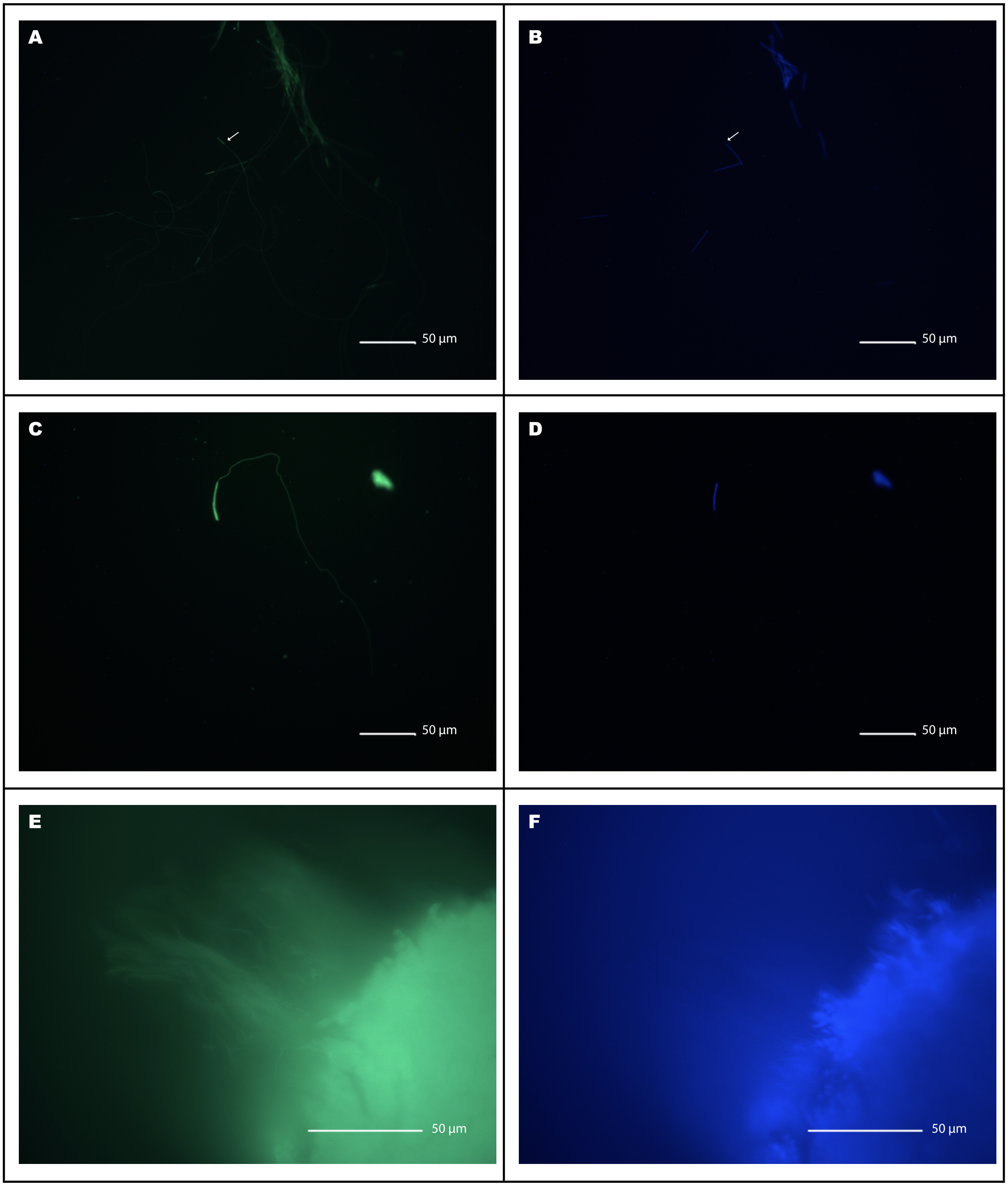

Figure 2. Fluorescent images of *O. rubescens* sperm stained with DAPI (blue) and Tubulin (green). **A.** Microtubules stained with Tubulin in sample 3. **B.** Nuclei stained with DAPI in sample 3. **C.** Microtubules stained with Tubulin in sample 4. **D.** Nuclei stained with DAPI in sample 4. **E.** Microtubules stained with Tubulin in sample 3 sperm embedded in testis **F.** Nuclei stained with DAPI in sample 3 sperm heads embedded in testis.

Table 1. Sizes of sperm in fluorescent images of samples 3 and 4 indicated with arrows (Figure 2A and 2B).

	Total Length (µm)	**Length of Tail (µm)**	**Length of Head (µm)**	**Width of Head (µm)**	**Length of Nuclei (µm)**	**Width of Nuclei (µm)**
Sample 3	329.34	294.22	35.11	2.48	25.46	1.27
Sample 4	278.52	269.93	8.59	0.99	25.44	0.78

3.2 SEM of Testis Structure

In all samples, we imaged and identified sperm throughout the testis. In sample 1, we observed sperm embedded into the walls of the testis (Figure 3A) which is arranged in a compact spiral structure (Figure 3E). In sample 2, we found an opening to the testis which allowed us to observe its fibrous structure (Figure 3B). Samples 3 and 4 had an abundance of sperm inside and around the testis which we observed in Figures 3C and 3D. In sample 4, we found small round structures which could possibly be developing spermatids exposed due to disturbance of the testis wall (Figure 3D). The testis in sample 4 was also much less compact and organized compared to the testis in sample 1 (Figures 3E and 3F).

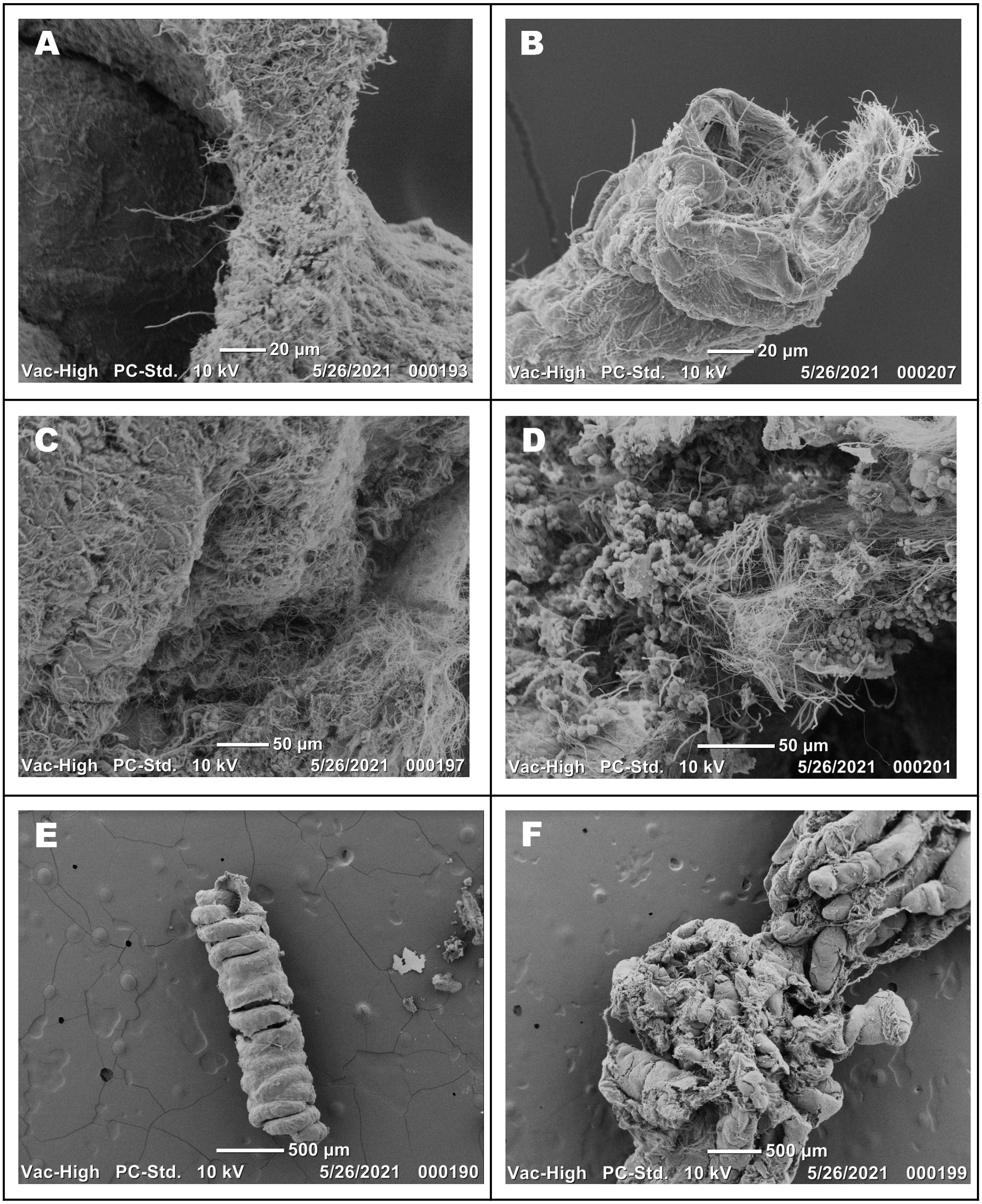
A
20 µm
Vac-High PC-Std. 10 kV 5/26/2021 000193
B
20 µm
Vac-High PC-Std. 10 kV 5/26/2021 000207
C
50 µm
Vac-High PC-Std. 10 kV 5/26/2021 000197
D
50 µm
Vac-High PC-Std. 10 kV 5/26/2021 000201
E
500 µm
Vac-High PC-Std. 10 kV 5/26/2021 000190
F
500 µm
Vac-High PC-Std. 10 kV 5/26/2021 000199

Figure 3. SEM images of all samples. **A.** Sample 1 testis cross-section **B.** Sample 2 testis cross-section **C.** Sample 3 exposed sperm **D.** Sample 4 exposed sperm **E.** Sample 1 testis structure **F.** Sample 4 testis structure.

4 Discussion

The overall sperm structure of invertebrates such as the octopus does not deviate much from mammals and other vertebrates (Sperry 2012). Aside from the characteristically thin shape and elongation along the acrosome-centriole axis (Gimenez-Bonafé et al. 2002), the morphology is fairly consistent with other taxa. The morphology of the sperm in *O. rubescens* is very similar to the results other morphological studies on octopods have found such as *O. tankahkeei* (Zhu et al. 2005, Gao et al. 2021), *O. ocellatus* (Yang et al. 2011), *O. vulgaris* (Gimenez-Bonafé et al. 2002), and *O. hubbsorum* (García-Flores et al. 2019). The microscopic and macroscopic structures of the male reproductive systems in those species are nearly identical to what we observed in this study.

The individual sperm observed via fluorescent microscopy in samples 3 and 4 (Figure 2) offer insight into the process of spermiogenesis in *O. rubescens*. In sample 3, the sperm head was longer and wider than in sample 4 (Table 1). In the later stages of sperm development, excess cytoplasm is eliminated in residual bodies and the sperm head condenses in size (Gao et al. 2021). The difference in the size of the sperm between samples suggests that our hypothesis of a gradient of maturity throughout the testis is correct. It is likely that the mature sperm in samples 1 and 2 were washed away during the PBS washes since the sperm were no longer attached to the testis walls. Therefore, we were unable to locate any mature sperm on those slides.

The testis of *O. rubescens* has a tubular and helical structure which is clearly observed with SEM imaging (Figure 3). The spermatids are embedded in the basal membrane and the mature sperm are packed on the inside of the tubules. In sample 4, we observed both immature spermatids and mature sperm cells in the same SEM image. Therefore, the different maturation stages of the sperm can be observed in any section of the tubules. These results are in agreement with the histological analysis of the testis of *O. rubescens* performed by López-Peraza et al. (2013). This also suggests that there is in fact a gradient of maturity throughout the testis.

In comparison to other cephalopods such as squid and cuttlefish, the length of octopus sperm, especially the tail, is significantly longer (Gimenez-Bonafé et al. 2002). Because female octopuses can store the sperm of multiple males (Quinteiro et al. 2011), competition for fertilization could have selected for longer sperm. For example, in the genetic model organism *Drosophila*, only long sperm are fertilization competent due in part to delayed fertilization in the female (Snook and Karr 1998). Preference by the female for longer sperm during fertilization likely resulted in that trait being selected for in *O. rubescens* as well. This finding highlights how evolutionary processes impact sperm morphology and development in this species of octopus.

Overall, this study offers a detailed description of the microscopic and macroscopic structures of the male reproductive system in *O. rubescens*. Given more time, we would have liked to perform a histological analysis of the testis in *O. rubescens* in order to further analyze spermiogenesis and spermatophore formation. Providing a comprehensive description of the whole male reproductive tract adds to the growing interest in octopus fertilization biology.

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