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Methods of Spermatogenesis in the Freshwater Mussels *Pyganodon lacustris* and *Venustochonda ellipsiformis* as Examined by Transmission and Scanning Electron Microscopy

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Note: All figure numbers refer to figures in Appendix

Abstract

As advances in scientific research allow scientists to better understand and protect animal species from extinction and other threats to their survival, there are still many threats to thousands of species which go unstudied. One of the largest oversights is that of invertebrate species, which make up 99% of animal diversity (Lydeard, et al., 2004). Invertebrates receive little attention when it comes to research that could preserve the species and their habitats. There is, however, some on-going research which aims to better understand invertebrate species which are in danger of extinction, such as freshwater mussels. Research into freshwater mussel physiology and anatomy has revealed much about the species, but there is still much more knowledge to gain. The purpose of this study was to continue the research into the methods of spermatogenesis in species of freshwater mussels. Both scanning and transmission electron microscopy were used to obtain images of sperm cells as they develop. The images of sperm cell structure from this study were compared to those from previous studies and found to be quite consistent.

Introduction

Despite the increase in public awareness of animal extinction, invertebrate species, which account for 99% of the animal kingdom, are usually the last to attract attention from both the public and scientific community alike. In particular, nonmarine mollusks are one of the most endangered groups of animals, yet receive very little publicity (Lydeard, et al., 2004). Nonmarine mollusks belong to the phylum Mollusca, which has the second largest number of described species of all phyla. The freshwater and terrestrial mollusks are in two classes, Bivalvia which includes clams and mussels, and Gastropoda which is comprised of snails, slugs, and limpets (Lydeard, et al., 2004). As freshwater is arguably the most unstable environment for organism survival and reproduction, the mollusk species which live in freshwater are in even greater peril. According to Lydeard, et al., there are approximately 7000 described freshwater mollusk species and a possible 3000 – 10,000 species which are still undescribed (2004). Of these described species, the 2002

International Union for the Conservation of Nature and Natural Resources (IUCN) Red List of Threatened Species listed a shocking 708 as being threatened; this is almost a quarter of all freshwater mollusk species (IUCN, 2003). The extremely high number of threatened species in this group of animals is possibly due to a lack of scientific research and study of these diverse species. Lack of knowledge about these animals prevents us from taking the proper measures to preserve and defend the species and their habitats.

By studying freshwater mollusk species, we can better understand and maintain these animals. One area that has been and continues to be the subject of research is the life cycle and habits of the freshwater mussel. In particular, the study of gonadal anatomy and gametogenetic cycles has put scientists on the right track to a deeper understanding of the freshwater mussels. Researchers in this area have been able to identify and describe the individual stages through which many freshwater mussel species produce mature sperm for reproduction. In 1988, Kotrla divided the spermato-genetic cycle of three freshwater mussel species into three phases: spermatocytogenesis, spermiogenesis, and spermatogenic cyst production. The first two phases were identified as being the typical means for spermatogenesis, while the final phase is considered to be the atypical method of spermatogenesis. Each of these stages is defined by the development and differentiation of the spermatocyte cells. While these stages do not apply to every species of freshwater mussel, they do give important insight into the developmental patterns of certain species.

In this study, species of freshwater mussels were viewed by both transmission electron microscopy (TEM) and scanning electron microscopy (SEM) in order to build upon previous research on the spermatogenetic cycles of the mussels. The purpose of this study was to continue the research of others and to identify the different structures of spermatocyte cells as they progress through spermatogenesis. The findings of this study were evaluated by comparison to existing data on the morphology of freshwater mussel spermatocyte cells.

Materials and Methods

Specimens of *Venustachonda ellipsiformis* and *Pyganodon lacustris* were obtained from freshwater lakes in Michigan by Dr. Richard Trdan of the Saginaw Valley State University Department of Biology. Following Shepardson's (S. Shepardson, personal communication, 2006) protocol, the specimens were dissected, then fixed in 2.5% glutaraldehyde and 1.0% OsO_4, both in 0.1 M Cacodylate buffer, for two and one hours, respectively. Each fixative was followed by three buffer rinses of 0.1M Cacodylate, pH 7.2 for 10 minutes each. Dehydration was done in a graded series of acetone in 10 minute increments. The *P. lacustris* specimens, which were intended for viewing by the TEM, were then embedded in Spurr's resin and acetone at a 1:1 ratio overnight and then in pure Spurr's in two stages for two hours each. The specimens embedded in Spurr's resin were cured at 70ºC for eight hours. They were then thin-sectioned using an ultra-microtome and placed onto a 300-mesh copper grid. The TEM specimens were positive stained in alcoholic uranyl acetate followed by lead citrate. These specimens were viewed on a JEOL 100CX TEM at 80kV.

The *V. ellipsiformis* specimens intended for viewing by the SEM were dehydrated as above and dried in a critical point dryer. They were then attached to a metal stub and coated with gold in a sputter coater at 40 amps and 50m Torr for 60 seconds. The specimens were viewed using a JEOL 5400 SEM at 200kV.

Results

The micrographs obtained by TEM proved to be the most useful for identifying and differentiating between stages of spermatogenesis in the freshwater mussel specimens (R. Trdan, personal communication, 2006). The TEM allowed for mature images of the sperm cells (see Figures 4, 5 & 6) as well as images of spermatids (see Figures 1, 2 & 3). We were also able to see the various developmental stages of the sperm cells, from early spermatocytogenesis (see Figure 1) all the way through to the mature spermatozoan (see Figure 6). The mature image of the spermatozoans showed a bullet-shaped nucleus, with two or more mitochondria at the posterior end (see Figures 4, 5 & 6). Some were still completely surrounded by cytoplasm (see Figure 4), while others, which were more mature, had very little cytoplasm (see Figures 5 & 6). Depending on the section of the spermatozoan, flagellum was sometimes present (see Figures 5 & 6). Sections through secondary spermatocytes also revealed the earlier stages of spermatogenesis. The earliest stage showed a generally circular shaped nucleus surrounded by cytoplasm (see Figure 1). In some cases a single cell was viewed to contain more than one nucleus (see Figure 1). As the spermatocytes matured, however, some nuclei became abnormally shaped (see Figure 3 – N1 & N2). The amount of cytoplasm varied from cell to cell (see Figures 2 & 3).

Images obtained from the SEM were not as functional for studying the developmental stages of the freshwater mussels. The SEM micrographs were helpful, however, in giving the general structure of the testicular tissue of male freshwater mussels. Figure 8 shows a

surface view of the testis, along with a cut through the tissue, giving a pseudo cross-section. The surface of the testicular tissue was observed to be covered in ridges (see Figure 7). One interesting finding was that of the cercarian stage of a parasitic trematode, which is common in freshwater mussels. It was noted in the tissue of the mussel (see Figure 9). Figure 10 shows the cercaria's tail in detail.

Discussion

Based on Kotrla's 1988 research, the images obtained from this study were very consistent with the previous findings on the structure of freshwater mussel sperm cell development. In Figures 1, 2 and 3, we see cross-sections of secondary spermatocytes, in which the cytoplasm is still a quite prevalent part of the cell. As the cell progresses through spermatogenesis, the cytoplasm will shrink in size, the nucleus will become more dense, and flagella will begin to form (Kotrla, 1988). Based on these characteristics, we can deduce that the cell in Figure 2 is slightly more mature than those shown in Figure 3. The nucleus in Figure 2 is round and quite dense, with very little cytoplasm. Conversely, the cells in Figure 3 have somewhat abnormally shaped nuclei and cytoplasm surrounding the cell. We can also infer that the cells shown in Figure 1 are less mature than those in both Figures 2 and 3, due not only to the amount of cytoplasm present but also to the fact that the two nuclei are still encased in the same cytoplasm.

When analyzing the TEM images of whole cells, we can use not only the size of the cytoplasm to approximate the maturity of the cell, but also the location of the mitochondria and the presence and length of the flagella. These characteristics can be used to separate the developing spermatid from the mature spermatozoa (Kotrla, 1988). A late spermatid, as in Figure 4, has the classic bullet-shaped nucleus but is still surrounded by a significant amount of cytoplasm. The mitochondria have migrated to the posterior end of the nucleus but the flagellum is not yet visible. The amount of cytoplasm present is what distinguishes the cell in Figure 4 as still being in the spermatid stage of development.

As the spermatogenetic cycle continues, we can see considerable changes in the structure of the sperm cell, resulting in the final stage of spermatozoa (Kotrla, 1988). As seen in both Figures 5 and 6, the cytoplasm surrounding the nucleus shrinks in size as development proceeds. Eventually the cytoplasm will diminish, and remain as a very thin layer around the nucleus (Figure 6). The presence of a flagellum is also a characteristic of the spermatozoan stage. However, the flagellum is not always seen in the micrographs, depending on where the thin-section is taken from. Figure 6 shows a mature spermatozoan, with a dense, bullet-shaped nucleus, two mitochondria located in the posterior, very little cytoplasm remaining, and a well-developed flagellum. This mature spermatozoan would travel out of the spermatogenic acini where it develops, into a duct where it can be released for fertilization (Kotrla, 1988).

As mentioned in the Results section, the images obtained from the SEM were not as easily compared to existing research. None of the developing sperm cells described above were located in the tissue which was viewed by SEM. However, the SEM was able to give a general overview of the testicular tissue. Perhaps with more time and further specimen collection and preparation, the SEM would prove extremely useful in examining the structure and development of sperm cells in freshwater mussels. As this study was intended to continue the research of others on the subject, the scope of this investigation was relatively small. As part of a larger, ongoing study of freshwater mussels, however, this research is helpful in providing further support for previous data on the spermatogenetic cycles of the freshwater mussel. As stated in the Introduction, nonmarine mollusks, including freshwater mussels, can benefit immensely from additional study. By continuing research into the reproductive systems of these species, we may be able to help these animals maintain population sizes and thrive in their natural habitats.

Acknowledgements

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References

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Lydeard, C., Cowie, R., Ponder, W., Bogan, A., Bouchet, P., Clark, S., et al. (2004). The global decline of nonmarine mollusks. *BioScience*, *54*, 321-330.

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Appendix

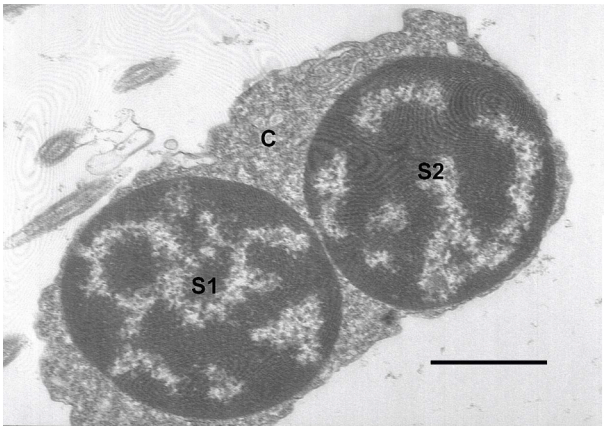

Figure 1. *P. lacustris*. Positive staining. Cross-section through secondary spermatocyte. **C**, cytoplasm; **S1**, nucleus of sperm cell 1; **S2**, nucleus of sperm cell 2. Micron bar = 1 μm.

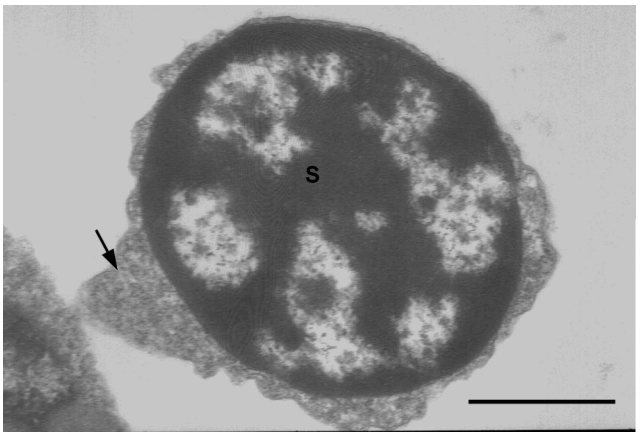

Figure 2. *P. lacustris*. Positive staining. Cross-section through secondary spermatocyte. **S,** sperm cell nucleus; **arrow**, cytoplasm. Micron bar = 1μm.

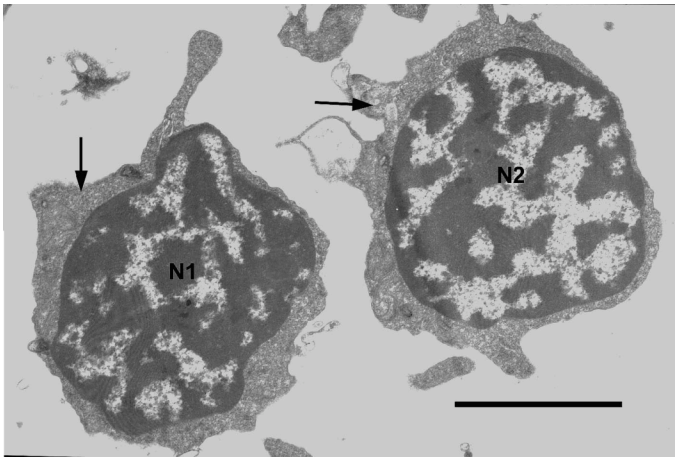

Figure 3. *P. lacustris*. Positive staining. Cross-section of two secondary spermatocytes. **N1**, nucleus of sperm cell 1; **N2**, nucleus of sperm cell 2; **arrows**, both point to cytoplasm. Micron bar = 2μm.

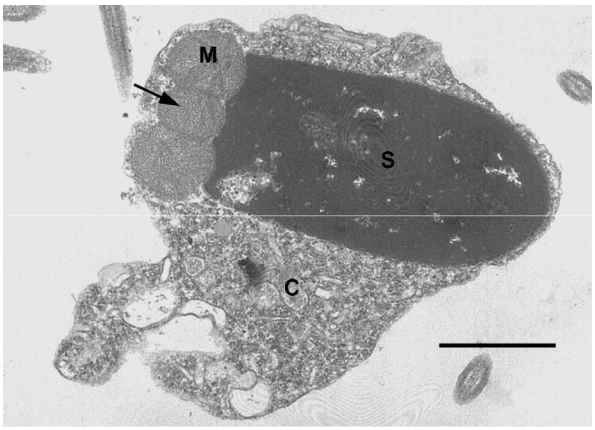

Figure 4. *P. lacustris*. Positive staining. Late spermatid – note amount of cytoplasm surrounding the nucleus. **S**, sperm cell nucleus; **M**, mitochondria; **C**, cytoplasm; **arrow**, third mitochondrion. Micron bar = 1μm.

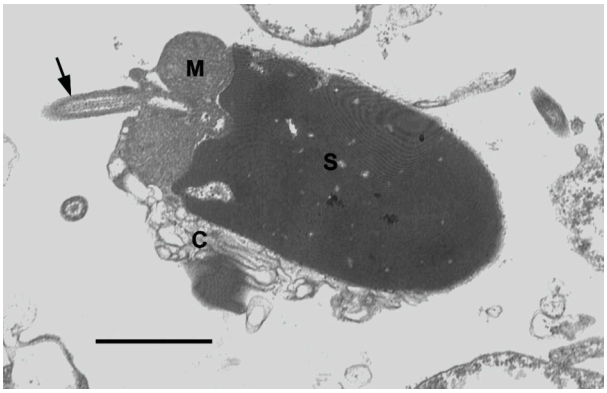

Figure 5. *P. lacustris*. Positive staining. An early spermatozoan with developing flagellum. **S**, sperm cell nucleus; **M**, mitochondrion; **C**, cytoplasm; **arrow**, flagellum. Micron bar = 1μm.

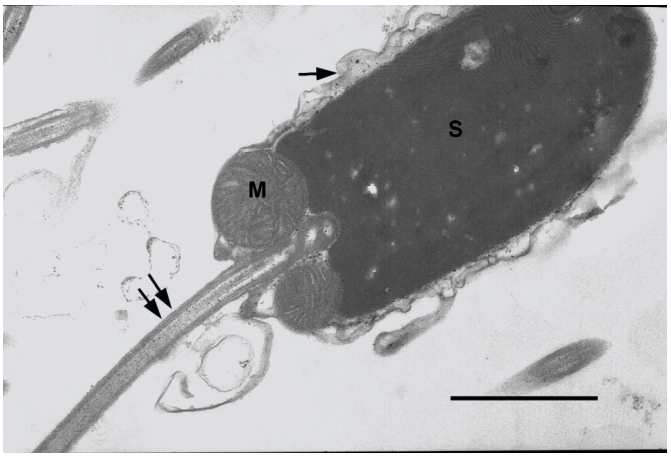

Figure 6. *P. lacustris*. Positive staining. Mature spermatozoan with flagellum. **S**, sperm cell nucleus; **M**, mitochondrion; **arrow**, cytoplasm; **double arrow**, flagellum. Micron bar = 1μm.

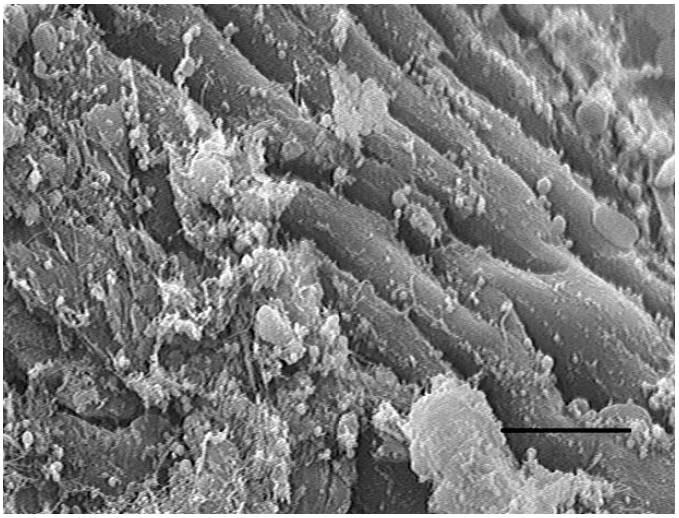

Figure 7. *V. ellipsiformis*. Heavy metal shadowing. Testicular tissue – note ridges formed by tissue. Micron bar = 30µm.

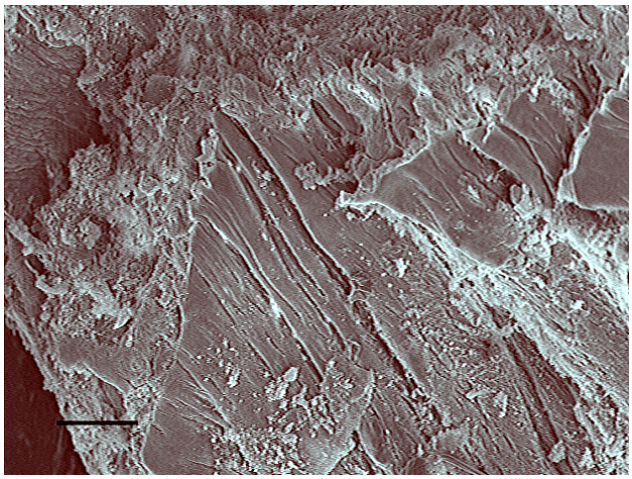

Figure 8. *V. ellipsiformis*. Heavy metal shadowing. Testicular tissue showing cut area of cross-section. Micron bar = 200µm.

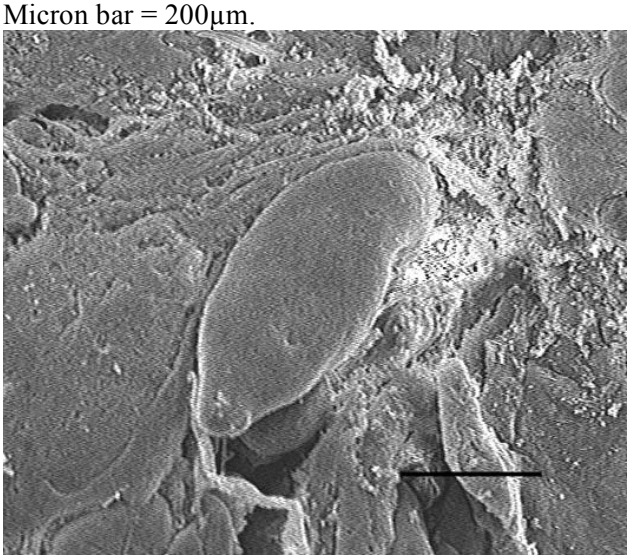

Figure 9. *V. ellipsiformis*. Heavy metal shadowing. The oval structure in the center is the nematode cercaria in testis. Micron bar = 300µm.

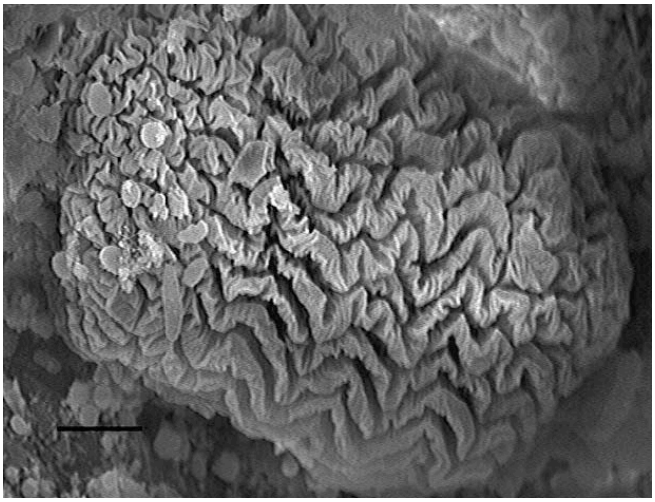

Figure 10. *V. ellipsiformis*. Heavy metal shadowing. Tail of the nematode cercaria. Micron bar = 20µm.