Analysis of Reproductive Structures of *Venustachona ellipsiformis* and *Pyganodon lacustris* by Scanning and Transmission Electron Microscopy

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Note: All figure numbers refer to figures in Appendix

Abstract

Samples of *Venustachona ellipsiformis* and *Pyganodon lacustris* were examined using both scanning and transmission electron microscopy (SEM and TEM respectively). TEM analysis of the testes of *P. lacustris* resulted in observation of various stages of the typical pathway of spermiogenesis as well as a morula of the atypical pathway of spermiogenesis. SEM analysis of the female gill and testes of *V. ellipsiformis* showed various structures of each organ in great detail. Gill bars and particulate matter were noted on the female gill. In a tear on the female gill, juvenile forms of the clam lifecycle glochidia were also observed. The tail section of a cercaria parasite was viewed on an SEM of the testes of *V. ellipsiformis*. The appearance of some artifacts (slight charging and edge effects) on some of the SEM micrographs was also noted.

Introduction

Venustachona ellipsiformis and *Pyganodon lacustis* belong to the family Unionidae under the phylum Mollusca ("Catalogue of Life," 2007). Both *V. ellipsiformis* and *P. lacustris* are dioecious species; however, some members are known to be hermaphroditic, producing both spermatozoa and oocytes. While oogenesis occurs at different rates during the year, it follows a distinct pathway (Kotrla, 1988). Spermiogenesis in freshwater clams occurs in typical and atypical fashions. The typical pathway involves the production of spermatocytes and the differentiation of spermatids into spermatozoans, a process similar to other invertebrates. Atypical spermiogenesis is usually seasonal in occurrence and involves the differentiation of spermatozoa from spermatogenic cysts (called morulae) produced in follicle cells (Kotrla, 1988). Morphology of spermatozoans produced by both pathways is identical.

The gills are a vital structure in clams, allowing them to retrieve oxygen from the water. Along with oxygen retrieval, gills are also places where eggs are fertilized in sexual reproduction (Wilbur, 1984). In some species of freshwater clams, female members incubate their young in the gills and release them as free-swimming juvenile forms of the lifecycle. These immature forms in certain species of freshwater clams are called glochidia. Glochidia are released to parasitize fish in order to complete development (Wilbur, 1984).

With the use of both scanning and transmission electron microscopes (SEM and TEM respectively), this experiment analyzed the morphology of the reproductive structures in *V. ellipsiformis* and *P. lacustris*. The testes of *P. lacustris* were examined by TEM. Female gills and testes of *V. ellipsiformis* were examined by SEM analysis.

Materials and Methods

All techniques for sample preparations were accomplished by following a lab preparation sheet provided by Sally Shepardson. Samples of *Venustachona ellipsiformis* for SEM analysis were taken from the Sugar River at M30 in Michigan. Male and female samples were kept on ice until extraction of testes, ovaries, and male and female gills were performed. Samples of *Pyganodon lacustris* for TEM analysis were acquired from East Twin Lake in Lewiston, Michigan. They were kept on ice until extractions of the testes were performed. The extractions of reproductive organs from both species of clams were done at room temperature under ice in a quick fashion, to preserve the structures of the reproductive organs.

Once the organs were extracted from the *V. ellipsiformis*, all samples were fixed in a 2.5% glutaraldehyde solution in 0.1M cacodylate buffer at pH 7.2 for two hours. After primary fixation, the samples were washed three times in 0.1M-cacodylate buffer at 10-minute intervals. The samples were then fixed in a 1.0% Osmium tetroxide solution in cacodylate buffer for one hour and washed three times in 0.1M cacodylate at 10-minute intervals. Samples were then dehydrated in a graded series of acetone washes at 10-minute intervals.

The samples of *V. ellipsiformis* were processed for SEM analysis by critical point drying after complete dehydration of the samples. Samples were mounted on an aluminum stub using colloidal graphite. Gold was sputter coated onto the specimens at the following settings: 50mTorr, 40mAmps for 60 seconds of gold application. The samples were then viewed on a JEOL 5400 Scanning Electron Microscope at 20kV.

Testes specimens of *P. lacustris* were provided by Sally Shepardson and processed in the same manner. The only difference was that after the dehydration steps, the specimens were embedded in Spurrs resin with 100% acetone in a 1:1 ratio overnight, followed by pure Spurrs resin twice for two hours. After the second saturation, the samples were cured in a 70°C oven overnight. The specimens were then thin sectioned on an ultramicrotome, placed on a copper grid and stained for TEM analysis on a JEOL 100CX Transmission Electron Microscope at 80kV.

Results

TEM analysis of testes shows the different phases in the spermiogenesis of P. lacustris

TEM analysis of the testes sample of *P. lacustris* showed the different stages of typical spermiogenesis. Most prevalent in the sample were representatives of the various degrees of maturation of spermatids. In the development to spermatozoa, spermatocytes begin to lose their nucleolus and the chromatin begins to condense. The spermatocyte then becomes what is known as a spermatid (see Figure 1). As the spermatid matures, further condensation of chromatin occurs. This was observed in the nucleus by the appearance of a solid color due to the staining. The gradual disappearance of the cytoplasm was also observed as spermatid matured (see Figure 2). In the late spermatid stage, the nucleus becomes bullet shaped and is stained more solidly. The cytoplasm around the nucleus is almost absent as the mitochondria shift toward the base of the nucleus. The flagellum extends from the nucleus, between the mitochondria and outward (see Figure 3). The spermatozoa stage (not shown) is the fully developed sperm cell that is ready to fertilize an egg.

Another observed structure of interest was the spermatogenic cyst that appears in the atypical spermogenetic pathway (see Figure 4). Morulae have sacs that contain nuclear material which undergoes meiosis. Spermatozoa develop within these sacs and upon maturation, the sacs rupture. The fully developed spermatozoa leave to fertilize an egg.

SEM analysis shows the ultrastructure of the female gill of V. ellipsiformis and an immature stage of clam development

SEM analysis of the female gill of *V. ellipsiformis* gave great detail of the fine structures used in oxygen uptake and reproduction. Gill bars were observed as many rows of parallel filaments (see Figure 5). Unknown objects were also observed on the gills; however, since most bivalves are filter feeders, it is most

likely particle matter for food. The slight "sticky" appearance of the particle matter (see Figure 5) gives further evidence of food particles, since bivalves are known to secrete mucus to capture the particles (Hickman et al., 2004).

Upon further examination of the SEM samples, tears were observed on the surface of the female gill. These tears exposed the appearance of small "clam-like" organisms, which turned out to be an immature form of the clam life cycle called glochidia (see Figures 6 and 7). As mentioned previously, these juvenile clams rupture from the female and parasitize fish in order to complete development (Wilbur, 1984).

SEM analysis of the testes of V. ellipsiformis reveals a trematode parasite on specimen

One observation of importance was the discovery of a "tire-tread" appearing structure in the SEM of the testes. According to Dr. Richard Trdan, an expert in the field of mollusks, the structure was classified as a tail of a cercarian (personal communication, November, 2006). Cercariae are known to be parasites to bivalves, especially in the gonads (Wilbur, 1984). However, the top part of the parasite wasn't observed (see Figure 8). This may be because it was hidden or cut off from the tail. The structure in front of the tail appears to be a type of connective tissue belonging to the testes.

Discussion

With the help of the TEM and appropriate staining, various stages in the spermiogenesis of *P. lacustris* were observed. Examples of both atypical and typical pathways were seen in this experiment. While much is still unknown about the atypical pathway and why it occurs, further TEM analysis might be able to shed light on the subject.

SEM analysis of the female gill showed the fine ultrastructure of the gill. The cilia on the gill bars pulse, generating currents to guide water over the surface of the gills, maximizing gas exchange. Another interesting aspect of the female gill is its use as a reproductive organ. As spermatzaoans travel through the water, some female members of the clam species will place their eggs on the gill to maximize the chance of fertilization. These fertilized eggs are then incubated until the glochidial stage, upon which they parasitize fish and complete their development shortly after.

One observation worth noting was the appearance of some artifacts on the SEM micrographs. The main artifacts were edge effects and some charging on the glochidium (see Figure 7). Insufficient gold sputter coating on the sample could have caused this. However, a decent micrograph with good detail was still obtained.

The electron microscopes were vital in determining the structures that make up both the testes and female gill. With the data gathered from the structural analysis of both, further insight on their development was determined.

Acknowledgements

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Appendix

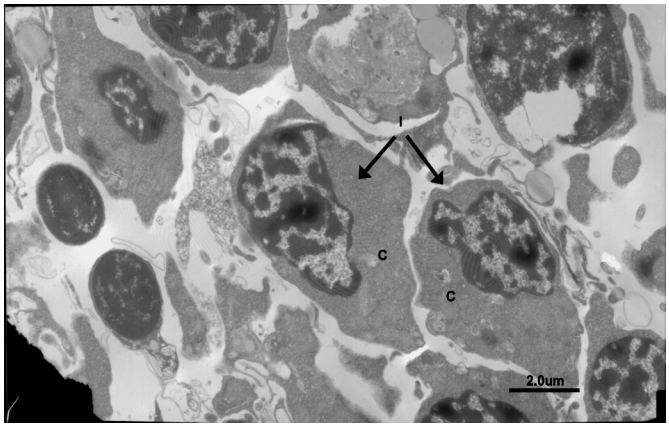

Figure 1. *P. lacustris*. Positive staining with uranyl acetate and lead citrate. **I,** early stage of spermatids next to each other; condensing of chromatin is seen in the irregular nucleus. **C,** cytoplasm of the early spermatids. Micron bar = 2.0µm.

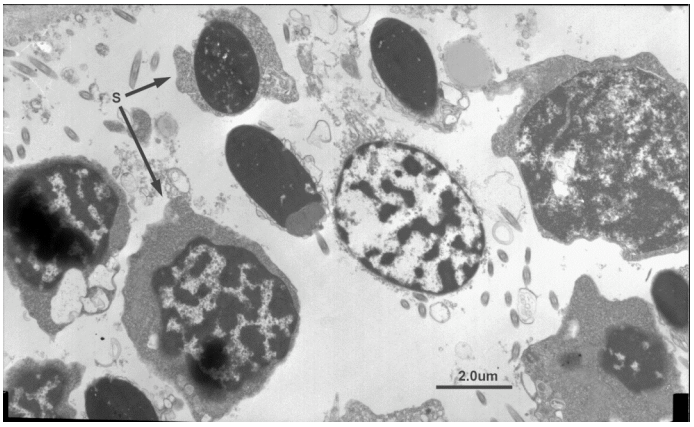

Figure 2. *P. lacustris*. Positive staining with uranyl acetate and lead citrate. **S, l**ate stages of spermatid formation. Lower spermatid shows considerable condensation of chromatin and loss of cytoplasm. The one above shows almost complete condensation of chromatin; cytoplasm disappearance is also present. Micron bar = 2.0µm.

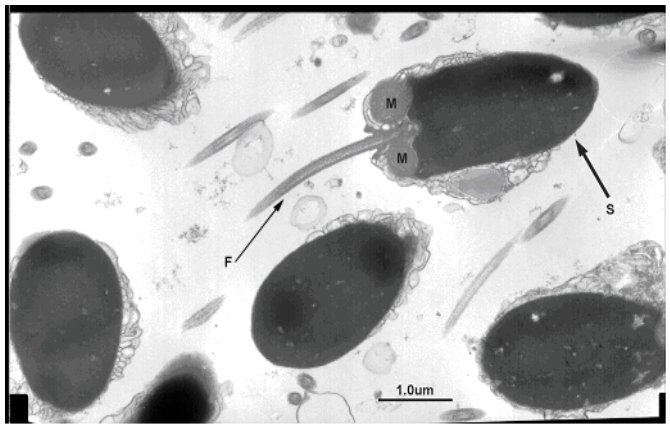

Figure 3. *P. lacustris*. Positive staining with uranyl acetate and lead citrate. **S, m**ature spermatid (immature spermatozoan). Nucleus is bullet shaped and almost stained solid. Cytoplasm from the earlier stages is almost absent. **M,** mitochondria clustered around the base of the nucleus. One is also seen in the last of the cytoplasm. **F, f**lagellum is seen extending from the nucleus, past the mitochondria and outward. Micron bar = 1.0µm.

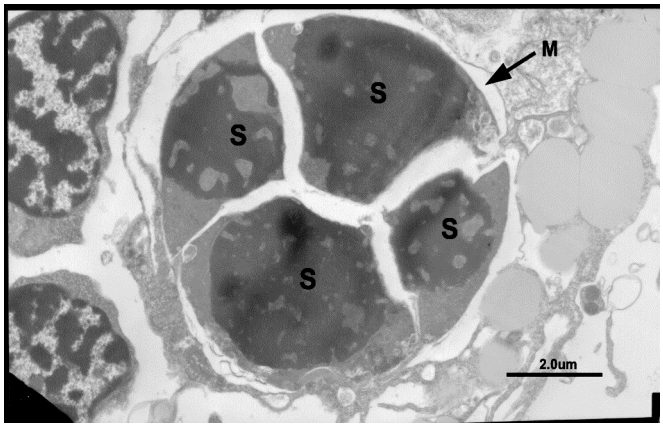

Figure 4. *P. lacustris*. Positive staining with uranyl acetate and lead citrate. **M,** spermatogenic cyst in atypical pathway called Morula. **S, n**ucleus area where developing spermatids are being formed. Micron bar = 2.0µm.

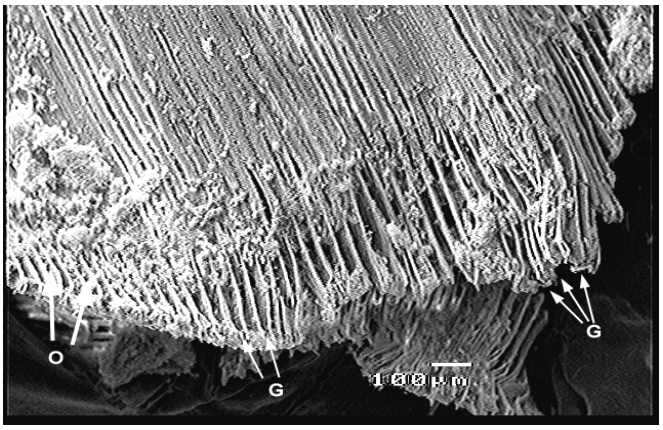

Figure 5. ***V. ellipsiformis*****.** Gold sputter coating. **G,** gill bars of the female gill. Numerous gill bars maximize surface area to volume ratio, increasing the clam's capacity for gas exchange in the water. **O, u**nknown objects are seen in between and on top of the gill slits. Since many clams are filter feeders, it is likely that these objects are food particles entangled in the mucus secreted by the clam. Micron Bar = 100.0µm.

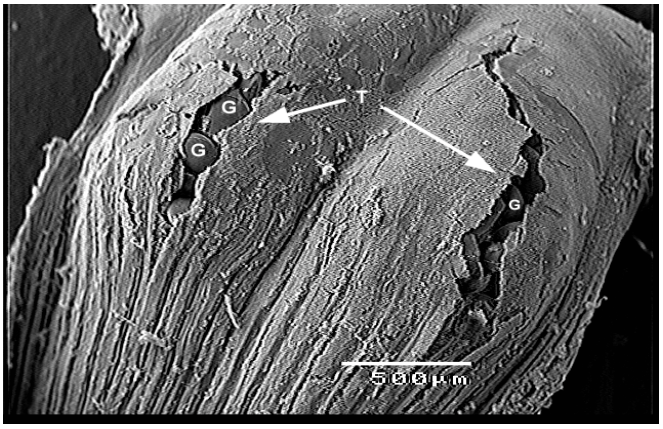

Figure 6. ***V. ellipsiformis*****.** Gold sputter coating. **T, t**ears observed in the female gill, exposing areas where glochidia (**G**) are kept until released. Micron bar = 500.0µm.

Figure 7. ***V. ellipsiformis*****.** Gold sputter coating. **G,** numerous glochidia are observed in one of the tears in the female gill. **E, e**dge of torn female gill. It is important to note that there is some charging and edge effect seen on the samples in areas that appear bright white. Micron bar = 100.0µm.

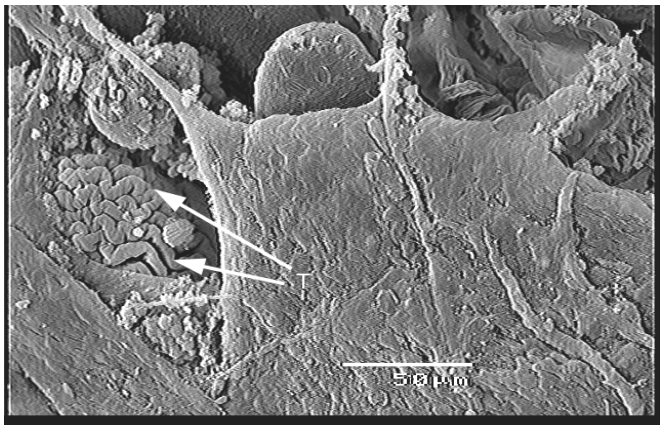

Figure 8. ***V. ellipsiformis*****.** Gold sputter coating. **T,** tail of cercarian parasite inside tissue of testes. Micron bar = 50.0µm.