

**The Microscopic Anatomy of the
Freshwater Sponge,
*Ephydatia muelleri***

Submitted in Partial Fulfillment of the Requirements
for Graduation with Honors to the Department of Biology and Chemistry
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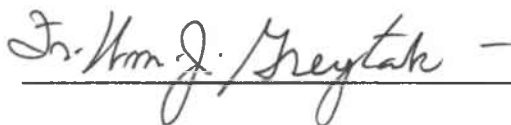
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Abstract

Ephydatia muelleri is a freshwater sponge abundantly found in the lakes and streams of Western Montana. In this study, the microscopic anatomy of *E. muelleri* in Salmon Lake, a lake located ca. 35 miles east of Missoula, MT, is described for specimens taken at ca. two week intervals between the months of May and August, 1994. The major cells (pinacocytes, choanocytes, and archaeocytes) are described. Although no sperm forming structures were noted, an egg and embryo indicate that sexual reproduction occurred.

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Introduction and Literature Review

Ephydatia muelleri (Phylum Porifera, Family Spongillidae) is a freshwater sponge that is found in the lakes of western Montana. This sponge grows throughout the spring and summer and almost disappears during the winter. It overwinters as gemmules, a seed-like structure, which germinates once warmer conditions return.

The original focus of this project was to examine the process of gametogenesis and embryonic development of *Ephydatia muelleri*, but the focus was later expanded to include a description of the major cell types present in the different areas of the sponge.

Functional Processes of *Ephydatia muelleri*

Demosponges, such as *Ephydatia muelleri*, consist of an elaborate system of canals, containing the feeding cells and surrounded by a supporting matrix, termed the mesohyl (Bergquist, 1978). The cells of these canals include three types of pinacocytes (exopinacocytes, endopinacocytes, and basopinacocytes), choanocytes, porocytes, and cone cells. There are two types of canals: incurrent and excurrent. The incurrent canals are responsible for carrying water from the external environment into the sponge to the choanocyte chamber where algae and bacteria are filtered from the water and engulfed (Simpson, 1984). The incurrent canals take water in through small openings on the surface called ostia, which are made of one or more porocytes (modified exo- or endopinacocytes) (Simpson, 1984). Incurrent canals enter the into a choanocyte chamber through an opening termed a prosopyle (Bergquist, 1978). Excurrent canals, on the other hand, return water back to the external environment (Simpson, 1984). Both of these

canals are lined by endopinacocytes (Simpson, 1984). The canal system provides a large surface area enabling the greatest amount of nutrients to be removed from the water (Frost, 1991).

Pinacocytes form net-like cellular structures inside of these incurrent canals which function to catch large particles that would otherwise clog the prosopyles and prevent water from entering the choanocyte chamber (Weissenfels, 1992). The choanocyte chambers create a current of water, with the use of flagella, through the canals so that food particles can be extracted from the water (Weissenfels, 1992). The choanocyte chamber is made of several choanocytes that are joined in a spherical pattern. Each choanocyte has a single flagellum and many long microvilli, which create a collar around the base of the flagellum (Simpson, 1984). Through the active movement of the flagella, food particles that are suspended within the water move through the incurrent canals (Frost, 1991). The beating of the flagella creates a suction effect and a low pressure in the incurrent canal which serves to pull the water into the sponge (Weissenfels, 1992). Low pressure also serves to draw the water through slits between the microvilli of the collar (Weissenfels, 1992). It has been shown that the microvilli between two adjacent cells are very close together, with only $0.1 \mu\text{m}$ separating them (Frost, 1991). Through these processes, the food particles can be filtered from the water. This material is taken up into the cells through a process of phagocytosis (Frost, 1991). Some food particles that may be too large to enter the ostia can be taken up by the exopinacocytes (Frost, 1991).

Now that the water has been filtered, it is expelled out of the cell. There is a high

pressure in the choanocyte chamber and throughout the excurrent canal, so the expelling of the water is achieved due to the buildup of this pressure (Weissenfels, 1992). Water first enters a large opening called an apopyle which leads from the choanocyte chamber to the excurrent canals (Weissenfels, 1992). An apopyle is made up of a ring of cone cells (Weissenfels, 1987). Each cone cell has two flagella that extend off into the apopyle opening (Weissenfels, 1987). It is thought that the apopyles function to prevent a backflow of water into the choanocyte chamber (Weissenfels, 1987). The excurrent canals lead to a large chamber termed the atrium, and the water is expelled through openings on the surface of the sponge, called oscula (Bergquist, 1978). Wastes and unphagocytosed materials are expelled far enough away from the sponge that the material is unlikely to be picked up again by incoming water (Frost, 1991).

Phagocytosed material is now capable of interacting with the cells of the mesohyl. The mesohyl contains archaeocytes, cystocytes, and sclerocytes, along with fibers and other connective tissues (Bergquist, 1978). The nutrients that are filtered out of the water are transferred from a choanocyte to archaeocytes that are present in the mesohyl. The archaeocytes take up the nutrients through phagocytosis (Frost, 1991). These cells are mobile in the mesohyl, and they will take the nutrients to other cells of the sponge, (Simpson, 1984). After digestion is complete, archaeocytes position themselves by the excurrent canals, and there they release the undigested material through a process of reverse phagocytosis (Frost, 1991). Now this material can move into the excurrent flow of water to be moved out of the sponge (Frost, 1991).

Cystocytes are also present in the mesohyl. These cells secrete a ground

substance that makes up a part of the mesohyl (Simpson, 1984). Other cells, termed spongocytes, will secrete fibrils which will give the cell the support it needs to function (Simpson, 1984).

Sponges have the special property of being able to reproduce sexually as well as asexually. During sexual reproduction in *Ephydatia muelleri*, one sponge is responsible for the production of the sperm and another will be responsible for the production of the eggs (Frost, 1991). The reproductive period passes during the months of November and July (Simpson, 1984). The winter starting month is due to the production of young oocytes which pass through the winter in greatly reduced sponges (Saller, 1988). Once the temperature of the water rises high enough in the spring, the sponges are able to begin production of sperm to start fertilization (Saller, 1988). Eggs and sperm originate from different cells that are already present in the sponge. Eggs develop from archaeocytes (Saller, 1988). The archaeocyte begins to increase in size and is now called an oocyte. This increase in size is the first growth phase of the oocyte (Saller, 1988). The oocyte becomes surrounded by a follicle layer, composed of pinacocytes, during the second growth phase (Saller, 1988). The oocyte then takes up nurse cells (trophoblasts) by phagocytosis. These nurse cells provide the oocyte with nutrients (Saller, 1988). As the oocyte continues to grow, the nucleus takes up a position at the periphery of the cell (Saller, 1988). After the oocyte reaches its full size, the nucleus moves back to the center of the egg (Saller, 1988). At this point, the egg is ready to be fertilized.

Sperm cells are known to arise from choanocytes (Bergquist, 1978). According to Paulus who studied spermatogenesis in *Ephydatia fluviatilis*, a species closely related to

E. muelleri, the choanocytes undergo a differential cell division, and then they are called spermatagonia. When all the cells of a choanocyte chamber have been converted to spermatagonia, the chamber is called a spermatocyst. As the cyst develops, the spermatagonia lose their nucleoli and become non-nucleolated primary spermatocytes. Each primary spermatocyte develops a flagellum and continues to undergo a reduction in size and become more compact in the process. At this point, the cell is called a secondary spermatocyte. The secondary spermatocytes give rise to spermatids which grow in the spermatocyst and become arranged so that the heads are directed towards the outside of the spermatocyst and the flagella point into the center. Because of this head to tail arrangement, the individual spermatocysts are able to fuse. The spermatids become separated from one another and are able to move individually. These are spermatozoa, which are ready to fertilize the egg. It is thought that the spermatocyst is in contact with an excurrent canal, so that when the spermatozoa are released, they can immediately move into the canal and pass out of the sponge. The sperm cells are taken into the incurrent canals of other individuals. The sperm cells are trapped by the choanocytes and transferred to the eggs where fertilization will take place (Bergquist, 1978).

The fertilized egg undergoes cleavage (Saller, 1988). Cleavage is not consistent in different sponges of the same species; the same stages of cleavage can have different arrangements of cells as cleavage progresses (Saller, 1988). After cleavage and gastrulation, a larva is produced that undergoes many internal and external changes (Frost, 1991). The nutrients that the larva will need to grow are provided by the surrounding trophoblasts (Frost, 1991). The larva eventually become covered with

flagellated epithelial cells which help the larva swim on its own once it gets released into the excurrent canal. The larva will swim until it lands on a suitable substratum, where it will metamorphosize into an adult (Frost, 1991).

Asexual reproduction occurs by two means. The first is for the sponge to undergo fragmentatation. From fragments, a whole new sponge can be generated (Bergquist, 1978). The other way that asexual reproduction occurs is through the complex process of gemmulation. The capacity for asexual reproduction through the formation of gemmules gives sponges an added advantage in being able to survive the harsh conditions of the freshwater lakes and streams. Gemmules arise from a cluster of archaeocytes that have protective spongin coats applied to them (Bergquist, 1978) This coat usually consists of three collagen layers (Frost, 1991). In *Ephydatia muelleri*, specialized spicules known as gemmoscleres are produced in the gemmules (Frost, 1991). The coat is continuous except for a small opening which is called a micropyle, where cells exit during germination in the spring (Frost, 1991).

Materials And Methods

During the months of May through August of 1994, *Ephydatia muelleri* was collected from the outlet of Salmon Lake, a lake approximately 35 miles east of Missoula, MT (T15/NR14/WS8). Samples of approximately 1cm square were cut from the sponge and were immediately placed in Bouin's fixative for a period of at least 4 hours. After fixation, the samples were washed six times with 0.2M sodium phosphate buffer, pH 7.2/ 6.9% (w/v) sucrose (wash buffer). Each wash lasted 10 minutes. The identity of the species was confirmed at this time by removing a gemmule and examining its gemmoscleres.

The sponge pieces were then cut into cubes approximately 0.5 cm on side, and placed in a 5% hydrofluoric acid solution in order to dissolve the spicules present. The duration of this step was varied from 0.5 to 2 hr, but even two hours proved insufficient to dissolve all spicules completely. After the hydrofluoric acid treatment, the tissue was washed 2X in wash buffer (10 min each) and dehydrated in a series of ethanol solutions (50%, 70 %, 2X at 95%, and 2X at 100%), and finally a xylene solution (2X). Tissue was left in each solution for one hr. The tissue was next infiltrated with a two changes of liquid paraffin (Paraplast) at 55°C. Next the tissue was embedded, which entailed placing the tissue into metal molds containing a small amount of paraffin on the bottom. Additional melted paraffin was poured into the molds until the tissue was completely immersed. The paraffin blocks were allowed to cool for at least 2 hr in order to achieve the best stability for sectioning.

After mounting the blocks onto a rubber support, a razor blade was used to cut the face of the block into a trapezoidal shape which would help in creating a ribbon during sectioning. The base of the trapezoid was aligned parallel to and facing the blade edge.

Sectioning was performed with a Spencer microtome. The thickness of the sections was varied from 6-10 μm . When a ribbon of several sections was collected, it was placed on a few drops of water in the middle of a gelatin coated slide. The gelatin on the slides helped to ensure adhesion of the sections to the slides during the staining process. The water was then allowed to evaporate by placing the slide on a warming tray.

Staining, with Delafield's Hematoxylin and Eosin Y, was carried out according to protocols described by Sheehan and Hrapchek (1973) and Raphael (1976). The stained sections were examined and photographed with a Nikon Optiphot microscope equipped with a Nikon AFX camera unit.

Results and Discussion

The cell types described below were identified in sections prepared from sponge samples collected at two week intervals.

Epithelial Cells

The pinacocytes line most of the external and internal surfaces of the sponge. The exopinacocytes are found on the outside of the sponge and comprise the outer boundary of the sponge. These cells are highly flattened, and in transverse sections, they are thicker towards the middle where the nucleus is found (Fig. 1). These cells measure ca. $10\mu\text{m}$ long and ca. $4\mu\text{m}$ wide at their thickest points. As in all pinacocytes (Simpson, 1984), nucleoli were not found in the cells. These cells have an affinity for eosin, causing them to appear red. The cells seem to contain granules, making the cell appear nonhomogeneous. The cells do not appear to overlap, but do contact one another at their edges. These cells can be found extending all the way around the outside of the sponge.

Endopinacocytes are found deeper within the sponge, lining the surfaces of the incurrent and excurrent canals. These cells are also thin, but appeared somewhat thicker than the exopinacocytes, ca. $6\mu\text{m}$ at their thickest part (Fig. 2). They are likewise eosinophilic and form a continuous layer throughout the wall of the canal.

Sections were not taken from the basal surface of the sponges, so basopinacocytes were not found in the micrographs.

The choanocytes aggregate to form the choanocyte chambers (Fig. 3, 4, 5).

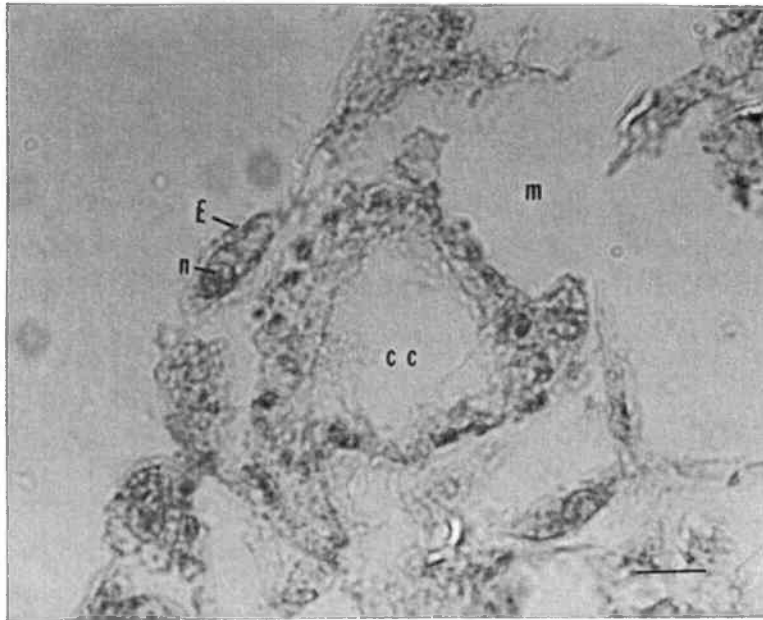


Fig. 1. Probable exopinacocyte with the nucleus causing a thickening in the cell. E, exopinacocyte, lying next to a choanocyte chamber; m, mesohyl; n, nucleus, cc, choanocyte chamber. Bar=10 μ m.

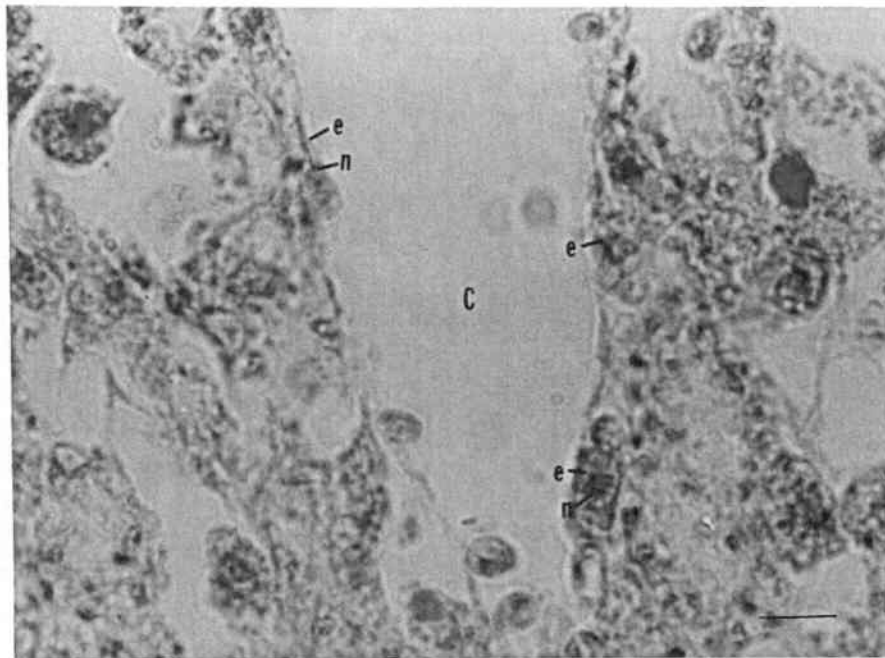


Fig. 2. Endopinacocytes lining the inner portion of a canal. e, endopinacocyte; n, nucleus; c, canal. Bar=10 μ m.

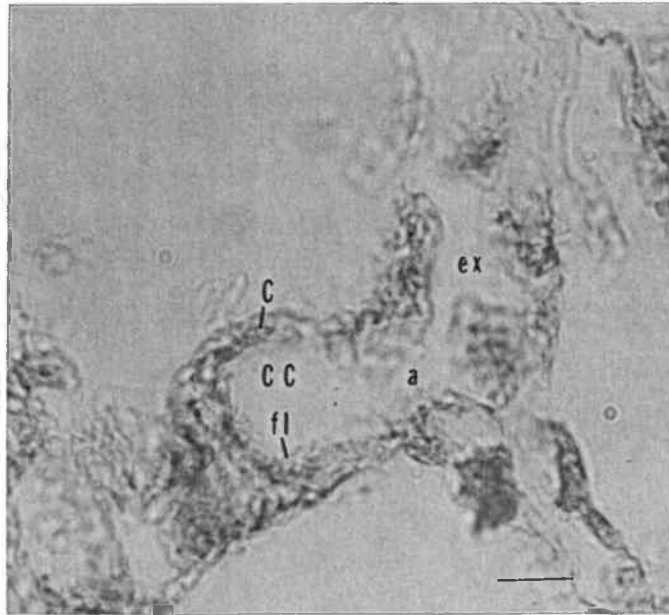


Fig. 3. Choanocyte chamber that is opening into an apopyle. cc, choanocyte chamber; c, choanocyte; a, apopyle; fl, flagella; ex, excurrent canal. Bar=10 μ m.

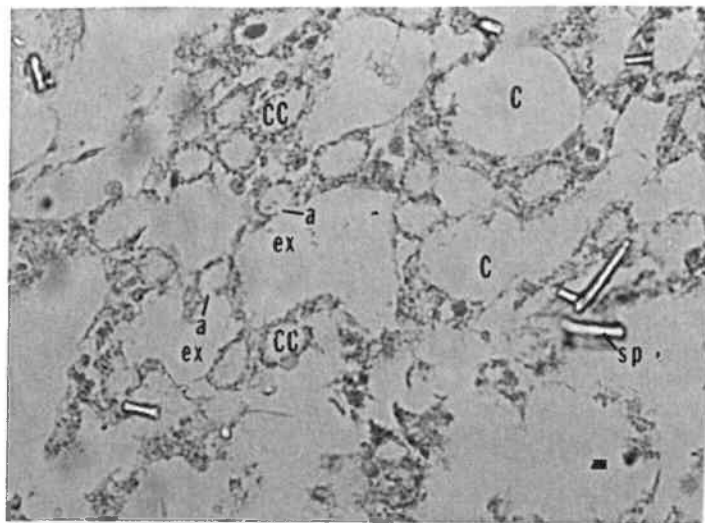


Fig. 4. An overview of the sponge, showing the many canals running through the tissue as well as the choanocyte chambers. c, canals; ex, excurrent canals; cc, choanocyte chambers; sp, spicule fragment; a, apopyles. Bar=10 μ m.

In agreement with the literature (Simpson, 1984), the choanocyte has a prominent, centrally located nucleus but lacks a nucleolus. The cells comprising the chamber are relatively uniform in shape and size, with the average diameter being $5\mu\text{m}$, and the overall diameter of the chamber was ca. $33\mu\text{m}$. Flagella extend into the center of the chamber (Fig. 1, 3, 5).

The apopyle is the opening that leads from the choanocyte chamber into the excurrent canal. Such an opening is seen in Figs. 3 and 4. The apopyle is identified by its size, which is bigger than the prosopyle, the opening that leads from the incurrent canal to the choanocyte chamber.

Cells of the Mesohyl

Archaeocytes are found throughout the interior of the sponge. These nutrient transporters are rounded cells that have a centrally located nucleus. They are described as having a prominent nucleolus (Simpson, 1984), and this organelle can be distinguished in the cell adjacent to the choanocyte chamber in Fig. 5. The cells have a granular, eosinophilic cytoplasm with a diameter ca. $9\mu\text{m}$. These cells are often situated in the vicinity of a choanocyte chamber.

Cystocytes, found in only a few species (Simpson, 1984), are common in the mesohyl of *Ephydatia muelleri*. This cell is positioned throughout the mesohyl. These cells produce the ground substance of the mesohyl (Simpson, 1984). A large eosinophilic inclusion in the cytoplasm can be easily made out (Fig. 6). The inclusion pushes the nucleus and the rest of the cytoplasm to the outside portion of the cell. A distinct

nucleolus could not be seen in these cells.

The egg has a nucleus that is pushed to one side of the cell (Fig. 7). Granules are seen in the cytoplasm of the egg. These are probably nurse cells, also known as trophocytes, that have been engulfed by the egg. The egg measures about $100\mu\text{m}$ in diameter. This egg was seen in tissue collected on June 16.

Spermatocysts were not observed.

After fertilization takes place, embryogenesis begins. There are several cell layers that compose the probable larva (Fig. 8). All of these cells have an affinity for eosin. The larva is a very large structure. It was observed in the July 17 sample. There is a layer of flattened follicular cells that surround the entire structure. In the center of the larva is an opening, called the larval cavity. It appears there is another layer of continuous cells lining the inside. The area around the larva consists of diffuse tissue.

A cluster of archaeocytes surrounded by a spongin coat is visible in Fig. 9. This is most likely a forming gemmule. The overall size of this structure is ca. $40\mu\text{m}$. This section is from a sample collected on July 17. The gemmule is much larger than the surrounding cells. Since the cells inside the coat are archaeocytes, they also have an affinity for eosin.

Although spermatocysts were not identified, the larvae present imply that such structures must have been present at some time. Possibly they were present close to the basal surface, a region not surveyed. The presence of an egg in mid-June and larvae in mid-July is consistent with the observation of others (Simpson, 1984).

Overall, this project achieved some level of success, with cells such as exo- and

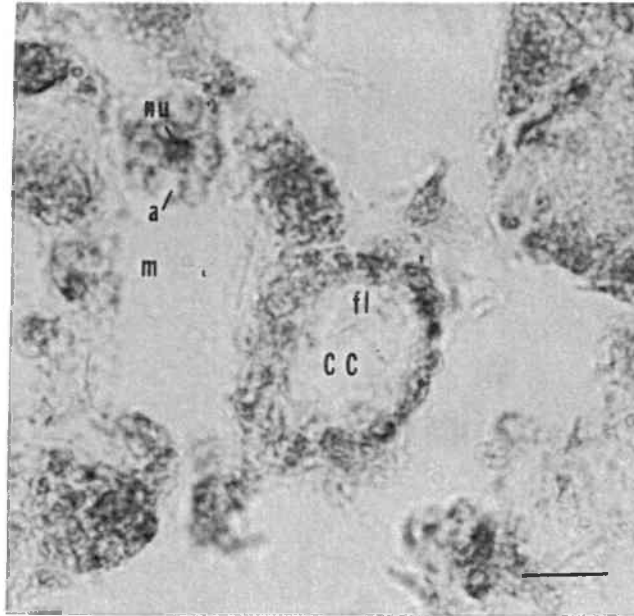


Fig 5. Archaeocyte close to a choanocyte chamber. a, archaeocyte; cc, choanocyte chamber; m, mesohyl; fl, flagella in chamber; nu, nucleolus, of archaeocyte. Bar=10 μ m.

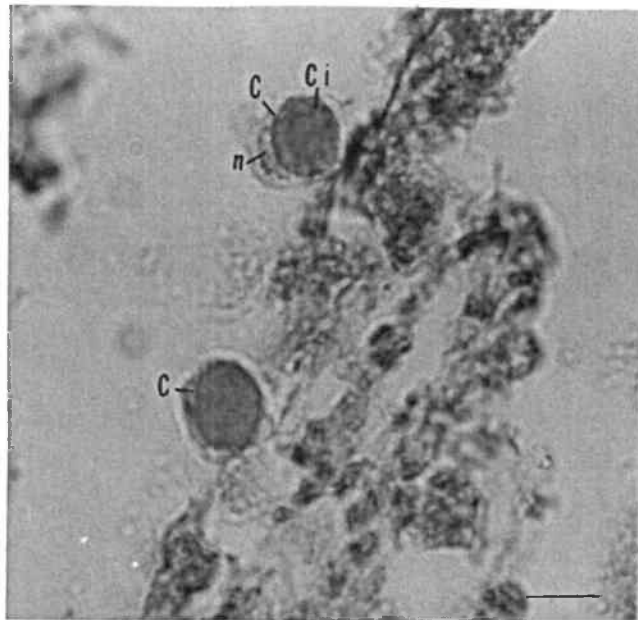


Fig. 6. Cystocytes showing the cytoplasmic inclusion. c, cystocyte; ci, cytoplasmic inclusion; n, nucleus. Bar=10 μ m.

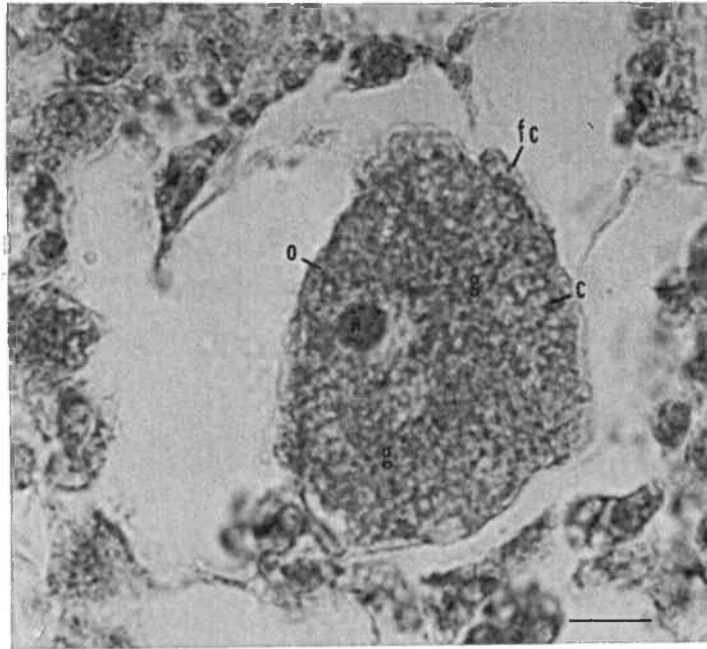


Fig. 7. A possible, developing oocyte, surrounded by the follicular cells. o, oocyte; n, nucleus; g, granules; fc, follicular cells; c, cytoplasm. Bar=10 μ m.

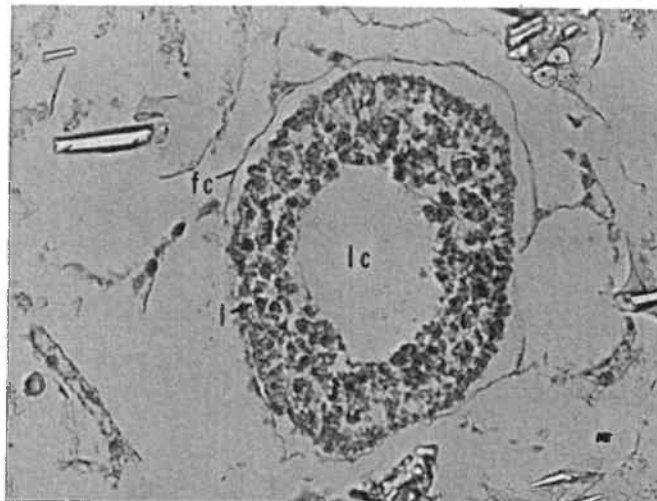


Fig. 8. A possible developing larva, with the larval cavity forming in the middle. This structure is surrounded by a layer of follicular cells. l, larva; lc, larval cavity; fc, follicular cells. Bar=10 μ m.

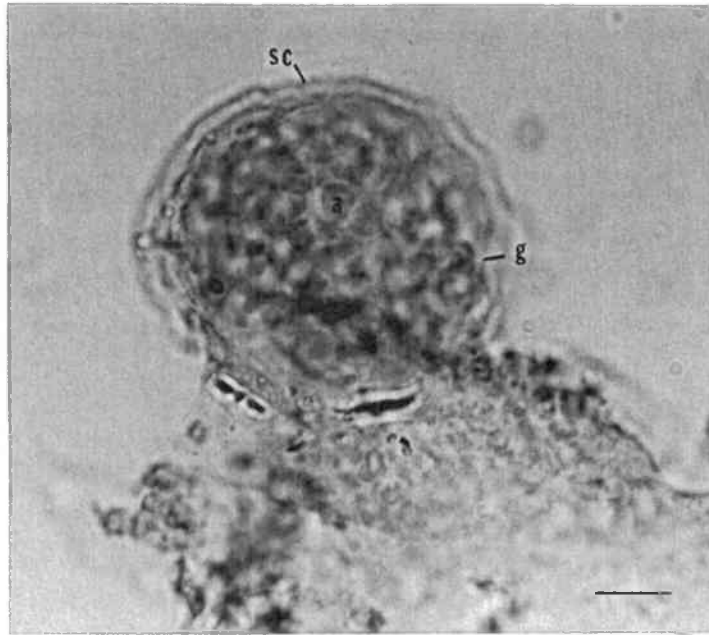


Fig. 9, Several archaeocytes bound inside a spongin coat, forming a gemmule. a, archaeocyte; sc, spongin coat; g, gemmule. Bar=10 μ m.

endopinacocytes, choanocytes, and cystocytes were identified. Fortunately, some reproductive structures were seen, such as an oocyte and a developing larva. Although not all structures of sexual reproduction could be found, the results obtained do indicate that structures such as spermatocysts must have been around at some point. These identifications lead to a better understanding of the structure of the sponge. But as with any project, problems will be encountered and these problems must be noted as well as the results.

Technical Considerations

A major problem encountered was failure of the HF to completely dissolve the spicules. If this project were repeated, I would recommend using the hydrofluoric acid at a higher concentration, or possibly a longer treatment than the two hours that I used. If these spicules could be totally removed, it would help ease the sectioning process.

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Appendix A

Procedure for the modified hematoxylin and eosin Staining (Sheehan and Hrapchek, 1973; Raphael, 1976)

1. Deparaffinize in 3 changes of xylene, 3 min each.
2. Hydrate through graded alcohol as follows:
 - A. 100 % Ethanol, 2 min
 - B. 95 % Ethanol, 2 min
 - C. 80 % Ethanol, 2 min
 - D. 70% Ethanol saturated with lithium carbonate, 2 min
 - E. 70% Ethanol, 2 min
 - F. Gently running tap water, 1 min
 - G. Distilled water, 1 min
3. Stain in Delafield's hematoxylin, 10-12 min
4. Dip in tap water, 10 sec
5. Decolorize in 1% concentrated HCl-Ethanol (1% concentrated HCl in 80% Ethanol) mixture, dip 2-3 times, 10 sec each.
6. Rinse in 2 changes of tap water, 20 sec each.
7. Intensify blue color by dipping in 1% lithium carbonate, 30 sec
8. Wash in tap water, 2 changes, 3 min each.
9. Counterstain in 1% aqueous eosin Y, 1-2 min
10. Rinse in tap water, 30 sec
11. Dehydrate in absolute Ethanol, 3 changes, 30 sec each.
12. Pass through 2 changes of xylene, 20 sec each.
13. Mount in Permount