Growing Local Freshwater Sponge Gemmules to Adulthood for Preliminary Utilization as Gray Water Filters

by

Gabriel Barton

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Thesis Committee:

Dr. Cole Easson, Thesis Director

Dr. Kim Sadler, Thesis Committee Chair

Dr. Jessica Arbour, Second Reader

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APPROVED:

_____

Dr. Cole Easson, Thesis Director
Associate Professor, Biology

_____

Dr. Kim Sadler, Thesis Committee Chair
Professor, Biology

_____

Dr. Jessica Arbour, Second Reader
Professor, Biology

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Abstract

Freshwater sponges are multicellular sessile heterotrophs, extracting their nutrients from their surrounding water environment through a process called filter feeding. As the river current flows through their internal canals, they collect essential minerals and ions to grow such as magnesium, potassium, sodium, and nitrogen. During unfavorable environmental conditions, these sponges produce gemmules within their adult tissue to survive these adverse conditions. When conditions become favorable for growth, gemmules will then release totipotent archeocytes, which will differentiate into specialized cells needed for growth to produce another adult sponge. Locally collected sponge gemmules were subjected to three treatments to stimulate hatching and measure their growth *in vitro*. Overall, these results provide the first records of hatching conditions for Tennessee sponges. The sponge *Ephydatia fluviatilis* proved to be the most robust species, hatching in a wide variety of *in vitro* conditions. The three other species used in this study showed more mixed results. Strekal's medium in larger petri dishes were the optimal conditions for sponge hatching and growth in these experiments. The results of sponge feeding trials were not determinant of an optimal treatment, and further research is needed in this area. The foundational knowledge generated in this study will serve as a steppingstone for future research to potentially use sponges as a living filter for gray water reduction.

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CHAPTER I: INTRODUCTION

Freshwater sponges are sessile invertebrate multicellular animals that comprise the taxonomic phylum Porifera (Copeland et al., 2019). They can be found on the fully submerged underside of rocks and tree branches. Sponges were long classified as plants due to their sessile nature, lack of specialized organs, and visible plant-like features. However, upon closer examination, sponges were determined to be animals that gather their nutrients from organic molecules derived from their environment, thus classifying them in the taxonomic kingdom of Animalia (Copeland et al., 2019).

Sponges are known to inhabit the submerged dark underside of rocks or tree branches. Sponges will also face the stream's current to facilitate their filtering process. Additionally, sponges will form symbiotic relationships with other organisms wherever they may plant themselves. Field observations of sponges showed them living on the same rocks inhabited by algae, arthropods, and gastropods.

Sponges gather their nutrients through filter-feeding. Water flows through their internal canals called ostia and out their osculum (Figure 1). Sponges filter minerals, ions, and various microscopic organisms and then metabolize their collection through specialized cells called choanocytes (Watts, 2020).

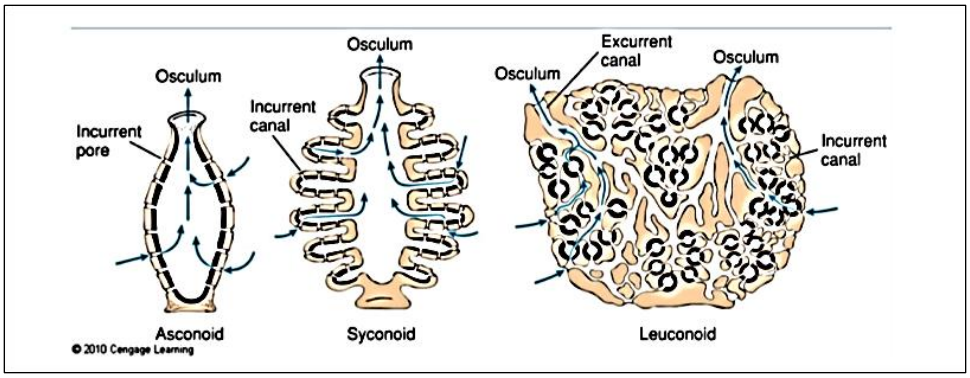

FIGURE 1. ADULT SPONGE ANATOMY.

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The Asconoid and Synconoid may be familiar shapes associated with sponges, however the research presented here focuses on the leuconoid structure.

Freshwater sponges can reproduce both sexually and asexually, however the focus of my thesis will be on the asexual abilities of freshwater sponges. Asexual reproduction can occur in the form of budding or gemmule formation. Through budding, a piece of the adult sponge tissue will pinch off from the main body and implant itself elsewhere in the stream of water and continue to grow (Copeland et al., 2019). Gemmule formation, however, is a much more complex process.

Gemmules are produced when environmental conditions become unfavorable to the sponge, such as decreasing temperature, desiccation, anoxia, and eutrophy (Copeland et al., 2019). Some cells of an adult sponge tissue are totipotent, meaning they can change their morphology and function to better serve the survival of the sponge. The totipotent cells, called archeocytes, aggregate together to form gemmule capsules outlined by spicules. Within the capsule, an inner membrane forms an additional layer of protection for the archeocyte cells that will differentiate as needed once a new sponge begins to

grow from the gemmule (Figure 2). During gemmule formation, thousands of gemmules are produced per sponge (Copeland et al., 2019).

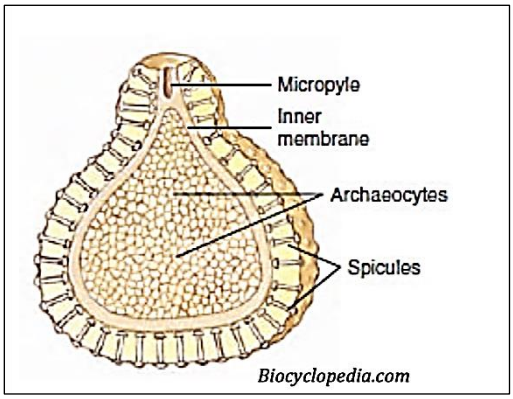

FIGURE 2. ANATOMY OF GEMMULE.

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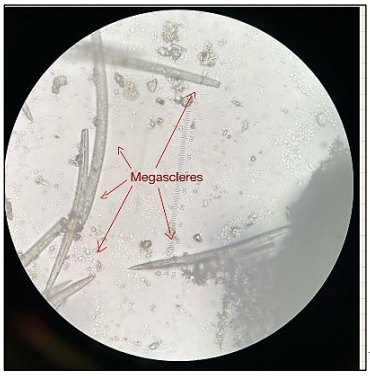
The inner cells (archaeocytes) are surrounded by a protective inner membrane along with spicules. Once the gemmule implants itself in more favorable conditions, the archaeocytes will diffuse out of the micropyle (also called foreman).

In addition to spicules providing a protective layer for the gemmule, the spicules that compose them are also used to identify sponge species. In freshwater sponges, spicules can be divided into three groups: megascleres (>100µm), microscleres (~20µm, but not always present), and gemmuloscleres (3-20µm). Further, megascleres, microscleres, and gemmuloscleres can be subdivided by their shape, elongation, and texture. For example, *Ephydatia fluviatilis,* a species commonly referred to in this thesis, has smooth elongated megascleres, no microscleres, and short dumbbell-shaped

gemmuloscleres (Figure 3). On the other hand, *Racekiela ryderi*, another common species, has spikey and thick megascleres, no microscleres, but elongated apple-core shaped gemmuloscleres. Given this great amount of specificity, each sponge species has a certain combination of each spicule type, giving rise to an abundant amount of sponge diversity.

a. 

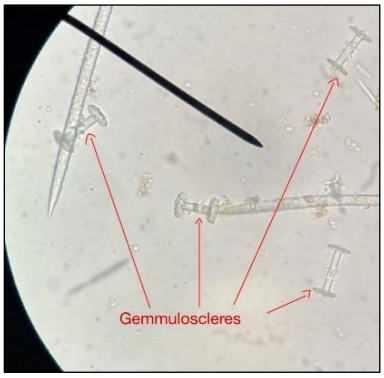
b. 

FIGURE 3. MEGASCLERES AND GEMMULOSCLERES.
(a) *Ephydatia fluviatilis*, (b) *Racekiela ryderi.*

This research seeks to take advantage of the sponge's ability to filter feed and rapidly reproduce. As part of a larger project, this thesis presents data of *in vitro* growth of sponge gemmules producing living tissue capable of filtering gray water.

CHAPTER II: MATERIAL AND METHODS

A. Field Collection

Four species were used in the current study. Three species were collected from waterways in Tennessee: *Ephydatia fluviatilis, Ephydatia muelleri, and Racekiela ryder*i. (Figure 4) and one species was purchased from Carolina Biological Supply (*Spongilla lacustris*). All species except *E. muelleri* were collected or obtained prior to the current study. *Ephydatia muelleri* was collected as part of the current project from the Elk River in October of 2023 (Figure 5) in Lynchburg, TN where previous field research (Copeland, 2019) located this species (Figure 5).

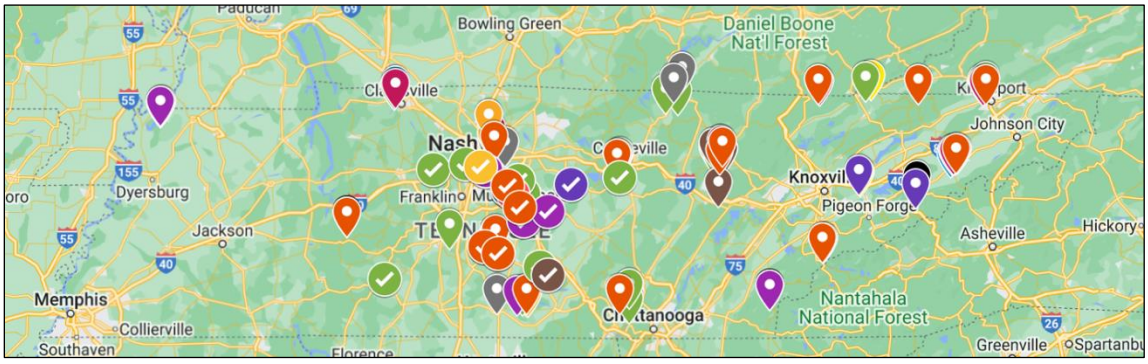


FIGURE 4. MAP OF FRESHWATER SPONGE FINDINGS IN TENNESSEE

Different colored markers indicate specific sponge species findings. Check marks represent where the Easson lab has visited. The collection site of interest is directly south of Nashville at the Elk River as denoted with a brown checkmark pinpoint.

FIGURE 5. ELK RIVER

All sponge specimens collected from Elk River were transported in sterile conical tubes filled with river water on ice (Figure 6). Collected sponges were identified by their microscopic spicule combinations. Adult sponge tissue was avoided as much as possible while trying to collect the gemmules to ease the process of separating the gemmules from the tissue once back in the lab. Elk River water was also collected in buckets for bacterial sampling.

FIGURE 6. FIELD IMAGES OF *EPHYDATIA MUELLERI*
Freshwater Sponges found on the underside of rocks near the banks of the Elk River, some measuring up to 14 cm in length.

B. Identification

The gemmules collected from the Elk River were separated from their adult tissue to analyze their spicule composition. Gemmule clusters were laid out on a paper towel covered petri dish and softly ground with another paper towel on top of it in a circular motion. Using light pressure, gemmules were separated from the adult tissue. De-ionized (DI) water from a squirt bottle was then used to flush them free from adult tissue. Of the thousands of gemmules collected, four or five were separated for identification. The remaining cleansed and separated samples were retained in a DI water solution and stored at 4°C.

To identify the species, a small sample of the gemmules was crushed and soaked in bleach for one minute. By crushing the gemmules, the spicules can be observed under the

microscope. A dichotomous key (Thorp and Rogers, 2015) was used to determine the identity of the samples to the lowest taxonomic level possible.

In addition to this species, other gemmules used throughout the project include those previously preserved and identified in the Easson Laboratory: *Ephydatia fluviatilis (E. fluviatilis), Racekiela ryderi (R. ryderi),* and *Spongilla lacustris (S. lacustris).* All species included here were put through identical growth treatments.

C. Protocols

While current research on *in vitro* sponge growth is limited, Nichols (2023) and Leys et al. (2019) did publish protocols regarding the initial first ten days of gemmule incubation and hatching. The instructions listed thereof have been regarded for all experimentation.

Sterilization

Gemmules were first sterilized by submerging them in a 1% solution of Hydrogen Peroxide (H_2O_2) for five minutes in a 10 cm petri dish. This process will cause the unviable gemmules to "pop" and float to the top of the solutions surface. The H_2O_2 will enter the gemmule by way of diffusion through the spicule membrane and micropyle only if archeocytes are insufficient enough to halt this invasion. Gemmules that are viable for the experiment will remain at the bottom of the petri dish (Figure 7).

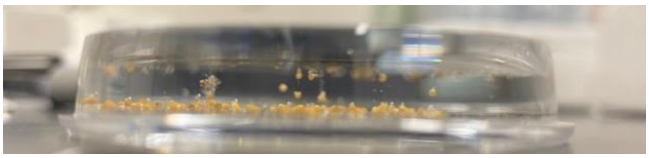


FIGURE 7. STERILIZATION

Gemmules submerged in a 1% solution of H_2O_2 for five minutes in a 10 cm petri dish. Viable gemmules rest at the bottom of the dish while unviable hollow gemmules float to the top.

After 5 minutes of sterilization, the petri dish was decanted into a waste beaker so the floating gemmules can easily be discarded while the viable gemmules at the bottom remain in the dish. The dish was then flushed multiple times with cold DI water to wash out any remaining H_2O_2. The dish was then filled with enough DI water to fully submerge all the gemmules until they are ready to be placed into their proper solutions for incubation.

Incubation

Following sterilization, the gemmules were placed in different solutions for 48 hours as part of their incubation period based on previous protocols (Leys et al., 2019, Nichols 2023).

The first proposed incubation solution consisted of a 10X concentration of magnesium sulfate heptahydrate, calcium carbonate, sodium silicate nonahydrate, and potassium chloride as listed below. Table 1 shows the quantities added to a 500 mL bottle

to make stock solution that would last for the entire experiment. The solution was labeled as Strekal's medium and will be addressed as such for the remainder of this thesis. Strekal's medium contains the basic necessary ions and minerals needed for eukaryotic tissue growth (Strekal, 1974; Leys et al. 2019).

TABLE 1. STREKAL'S INGREDIENTS

Compound	Amount (mg)
magnesium sulfate heptahydrate ($MgSO_4$ $7H_2O$)	108.33
calcium carbonate ($CaCO_3$)	5.04
sodium silicate nonahydrate ($NaSiO_3$ $9H_2O$)	28.42
potassium chloride (KCl)	7.55

Another incubation solution suggested was a 1X concentration of Antibiotic: Antimycotic (Anti: Anti) derived from a 100X stock. This solution contains 10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Gibco Amphotericin B. The antibiotics penicillin and streptomycin prevent bacterial contamination due to their combined action against gram-positive and gram-negative bacteria. Amphotericin B prevents fungal contamination of cell cultures due to its inhibition of fungus and yeast (Nichols 2023; Fisher, 2023).

Lastly, a third incubation solution was tested. Given the results of the first two incubation methods as will be discussed in Chapter III of the thesis, a 2:1 ratio of Strekal's and Anti: Anti was used. This solution, however, was not attempted until the results of the separate incubation solutions of the Strekal's and Anti: Anti were observed.

Trial 1 (November 2023)

An initial trial was conducted with *E. fluviatilis* and *E. muelleri*. Two samples of each species were used. One sample of either species was incubated in Strekal's medium, and the other sample was incubated in Anti: Anti (Figure 8). Gemmules from *E. muelleri* and *E. fluviatilis* were placed in either 12-well plates or 10cm petri dishes. The average well in the 12-well plates was 2 cm in diameter. No visible growth is expected to occur during the 2-day incubation period, rather a change takes place on the microscopic level: the gemmule begins to recognize its environment and prepares to release its archeocytes (Copeland et al., 2019). However, the gemmules should be planted enough to its surface that there should be no visible rocking movement when the petri dish/12-well is handled. Following their incubation, all samples were changed to Strekal's medium for the next 10 days. Microscopic pictures were obtained every 48 hours of all samples and analyzed in ImageJ. Lateral growth data was gathered by taking pictures through a dissecting microscope on 4X magnification. The pictures were analyzed with ImageJ to compare their growth at a calibrated scale of 646.1184 pixels per 0.5 mm.

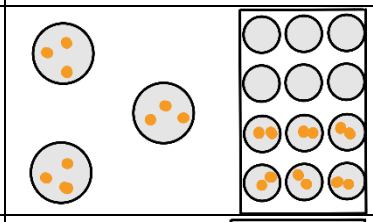
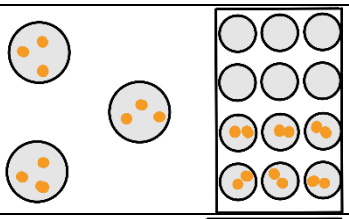
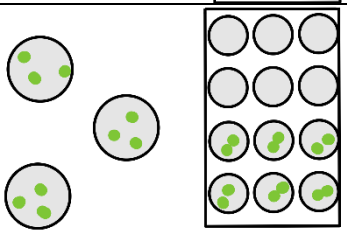
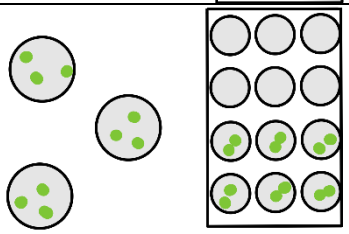
	Strekal's	Anti: Anti
E. fluviatilis		
E. muelleri		

FIGURE 8. TRIAL 1 SCHEMATIC

fluviatilis and *E. muelleri* tested in Strekal's and Anti: Anti incubation. Different colors represent different species. Two gemmules were placed in each well in the 12-well dish. Approximately 15-20 gemmules were placed in each petri dish.

Trial 2 (January 2024)

A second trial was conducted using the two previously mentioned species along with *Racekiela ryder*i (*R. ryderi*) and *Spongilla lacustris* (*S. lacustris*). Given the results from the first trial (as outlined in chapter III: Results), three experimental groups were formed. The first was a control group of Strekal's incubation followed by Strekal's post-incubation. The second included the species incubated in a 2:1 solution of Strekal's: Anti-Anti followed by Strekal's post-incubation. Lastly, the species were also incubated in the 2:1 Strekal's: Anti-Anti solution but followed by filtered Elk River water post incubation (Figure 9). For simplicity's sake, the following identification codes were used for each trial:

Group A) gemmules incubated in Strekal's and post-incubation solution of Strekal's.

Group B) gemmules incubated in 2:1 Strekal's: Anti-Anti and post-incubation solution of Strekal's, changed every 48 hours.

Group C) gemmules incubated in 2:1 Strekal's: Anti-Anti followed by post-incubation in filtered Elk River water.

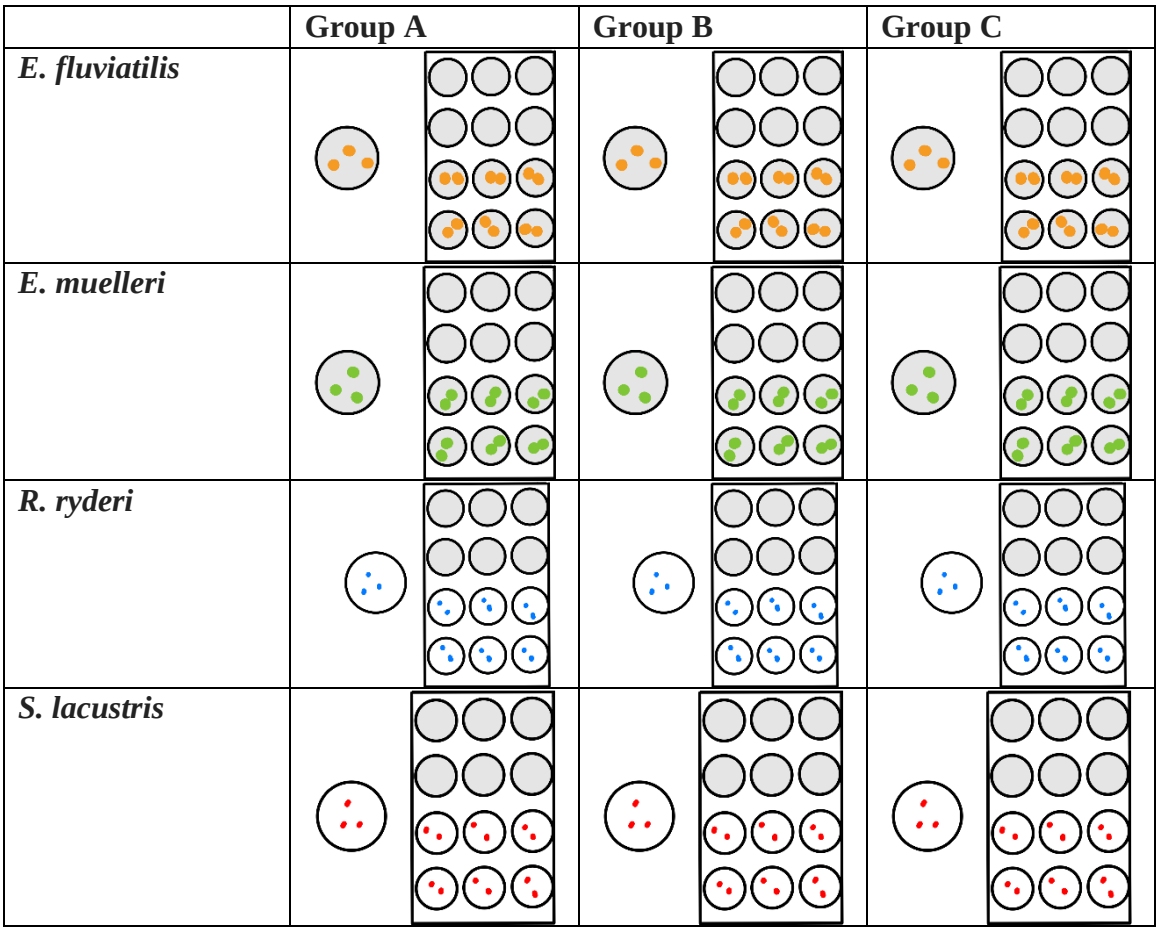


FIGURE 9. TRIAL 2 SCHEMATIC

E. fluviatilis, *E. muelleri*, *R. ryderi* and *S. lacustris* tested in groups A, B, and C. Different colors represent different species. Two gemmules were placed in each well in the 12-well dish. Approximately 15-20 gemmules were placed in each petri dish.

Trial 3 (March 2024)

A third trial was conducted, testing *E. muelleri* and *E. fluviatilis.* The goal of this trial was to assess the potential to extend gemmule growth in the lab by feeding the sponge either heat-killed bacteria or fish fry food.

This research phase took place about two months after determining the proper incubation and post-incubation conditions. Both species were treated identically: 48-hour Strekal's incubation followed by Strekal's post-incubation. The species were split into two groups following their 10-day growth. Group 1 was fed heat-killed bacteria collected from Elk River while Group 2 was fed *Liquifry* fish food (Figure 10). Growth was tracked every 48 hours using ImageJ.

Tryptic Soy Agar (TSA) plates and Plate Count Agar (PCA) plates were both prepared to grow the bacteria. The plates were inoculated and left to incubate for 48 hours. Miller broth was then prepared to grow the bacteria in solution. Two 1000 mL glass jars were used to make the broth. Both broths were autoclaved before bacterial inoculation. The TSA and PCA plates were swabbed of their bacteria and inoculated in the Miller broths and left to grow for 24 hours. The TSA grown bacteria successfully reproduced to make a thick foggy broth, however, the PCA grown bacteria did not reproduce to make a successful broth. The TSA bacteria Miller broth was autoclaved once more to prevent further growth and to follow protocols. It was then stored at 4°C for future use.

The broth of TSA bacteria was pipetted into five 1ml snap caps and centrifuged for 1 minute and 6000 G. This created a pellet of bacteria in each snap cap at the bottom

of the broth. Then 1 ml was pipetted out of the snap cap and a fresh sample of broth replaced it. The snap caps were then centrifuged in the same manner. This was done five times in order to create a large enough pellet of bacteria to diffuse in a 10 ml sample of Strekal's, creating a 10X bacterial feeding solution. A different concentration was made for the *Liquify*. The fish-food came in the form of powder that had to be diluted. The dilution was 1 ml mixed with 10 ml of Strekal's, creating a 10X. 1 ml of this 10X solution was then placed in another 10 ml of Strekal's, creating a 1X solution.

Administering the feeding solutions of either the bacteria or fish food occurred during daily solution changes. This meant that 10% of the solution change would now come from the bacteria or fish food instead of the Strekal's. For the specimen in the petri dishes, at least 10 ml of solution was needed at all times (500 µl per gemmule); 9 ml would come from the 10X Strekal's and the last ml would come from the bacterial dilution or fish food. In the 12-well plates, 1 ml was needed per well; 900 µl of Strekal's and 100 µl of food. For all trials, ImageJ was used to track radial tissue growth and the rate at which growth was achieved.

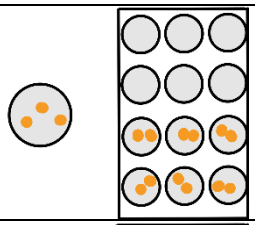
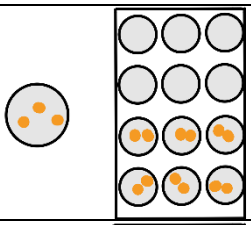
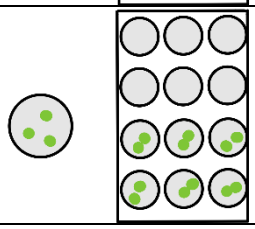
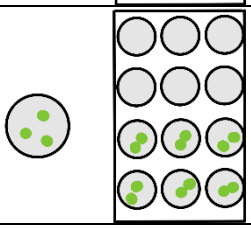
	Bacteria Fed	Fish food
E. fluviatilis		
E. muelleri		

FIGURE 10. TRIAL 3 SCHEMATIC

E. fluviatilis and *E. muelleri* tested for their growth beyond the 10-day incubation period with different sources of nutrients. Different colors represent different species. Two gemmules were placed in each well in the 12-well dish. Approximately 15-20 gemmules were placed in each petri dish.

CHAPTER III: RESULTS

Trial 1 Results

The first attempted gemmule growth treatment was performed on *E. fluviatilis* stored in the Easson lab and *E. muelleri* collected from the Elk River in Lynchburg, TN. Gemmules are not expected to show visible change during incubation, rather a molecular change is occurring that prepares the gemmules to release their archeocytes to facilitate tissue growth. Microscopic change is expected to be seen only after an additional 48 hours of sitting in a fresh solution of Strekal's. Samples of *E. fluviatilis* and *E. muelleri* were incubated in their respective solutions. Their medium was then changed to just Strekal's at 48 hours and maintained for the remainder of their growth. Their growth patterns are listed below. Table 2 shows the percent recovery of gemmules before and after sterilization, each species response to either Strekal's or Anti-Anti incubation.

TABLE 2. RESPONSE TO STREKAL'S AND ANTI: ANTI INCUBATION FOR TRIAL 1

	Ephydatia fluviatillis	***Ephydatia muelleri***
Percent Recovery after sterilization	90%	80%
Response to Strekal's Medium Incubation	Plated	No change
Response to Anti-Anti incubation	No change	No change

Ephydatia fluviatilis surprisingly plated in only 48 hours in the Strekal's medium. Plating occurs when the gemmules stay in its position when the petri dish or 12-well container is moved. Plating is the first successful step of hatching a gemmule *in vitro* (Figure 11).

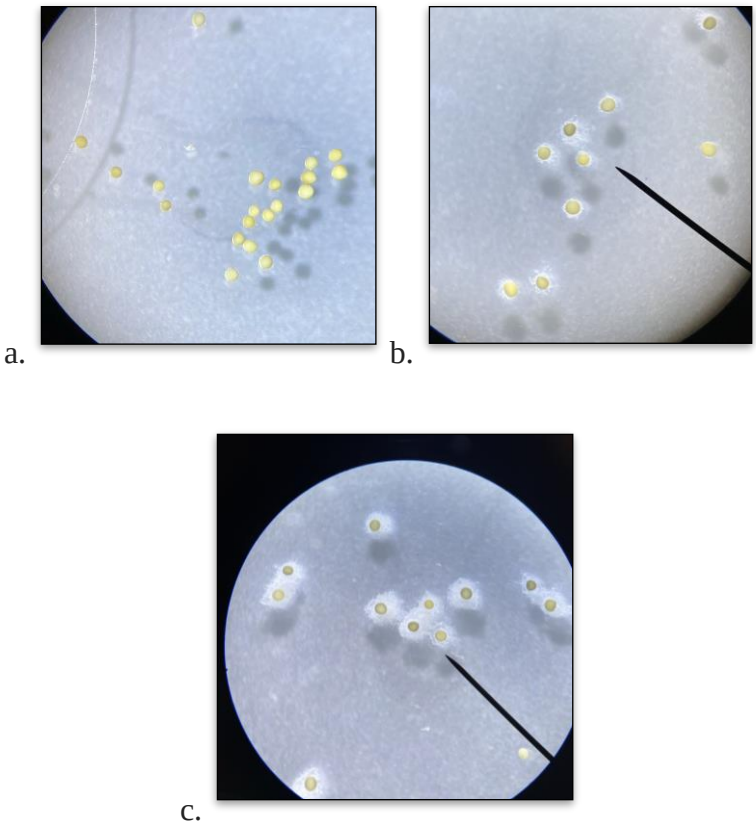


FIGURE 11. E. FLUVIATILIS ACROSS 6 DAYS OF GROWTH

(a) after 48 hours of incubation, (b) after 48 hours of growth in fresh medium (c) another 48 hours of growth in medium.

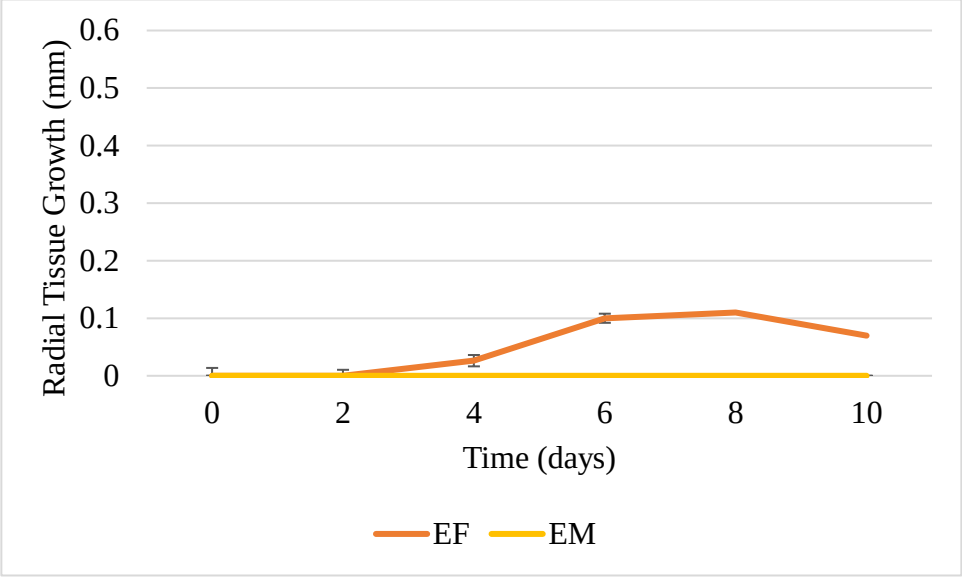


FIGURE 12. STREKAL'S INCUBATION GROWTH

Ephydatia muelleri and *Ephydatia fluviatilis* growth over ten days after previously incubated in Strekal's medium.

No growth was seen in either species incubated in the Anti-Anti solution after changing their solutions with Strekal's. However, when incubated in Strekal's and its medium continually changed with Strekal's, *E. fluviatilis* was able to successfully produce tissue. On the other hand, *E. muelleri* showed no growth despite the protocols given were specifically outlined for its species (Figure 12).

This initial lack of hatching by *E. muelleri* was hypothesized to be due to a lack of vernalization in this species of gemmules, as they were freshly collected from the environment just after formation. Vernalization is a period of resting gemmules need when being transferred to a new environment (Jepps, 1946). *Ephydatia fluviatilis* had

been resting in the Easson laboratory at 4°C for over a year while *E. muelleri* was immediately tested after being removed from its natural habitat.

Trial 2 Results

Following this procedure, a second trial of gemmules were tested using the previous species plus *Racekiela ryderi (R. ryderi)* and *Spongilla lacustris (S. lacustris)* provided by the Easson lab. These new species had been stored at 4°C for more than 6 months. The incubation results for these trials are shown in Table 3.

TABLE 3. TRIAL 2 RESULTS

	Ephydatia fluviatillis	***Racekiela ryderi***	***Ephydatia muelleri***	***Spongilla lacustris***
Percent Recovery after sterilization	90%	72%	80%	94%
Viable after incubation?	Yes	Yes	No	Yes
Response to Strekal's Medium Incubation	Plated	Plated	No change	Plated
Response to Strekal's: Anti-Anti incubation	Plated	Burst	No change	Plated

E. fluviatilis, R. ryderi, and S. lacustris successfully plated in both the Strekal's and Strekal's: Anti-Anti 2:1 solution. To determine which incubation is truly better for gemmule growth, continued observation was carried out as their solutions were changed every 48 hours for the next ten days. The success of each species was measured by gemmule tissue radial growth and rate at which growth occurred. Using an online software called *ImageJ*, outward tissue growth was measured over time in days. See Figure 13 –15 to view the relationship between incubation medium, species growth, and rate.

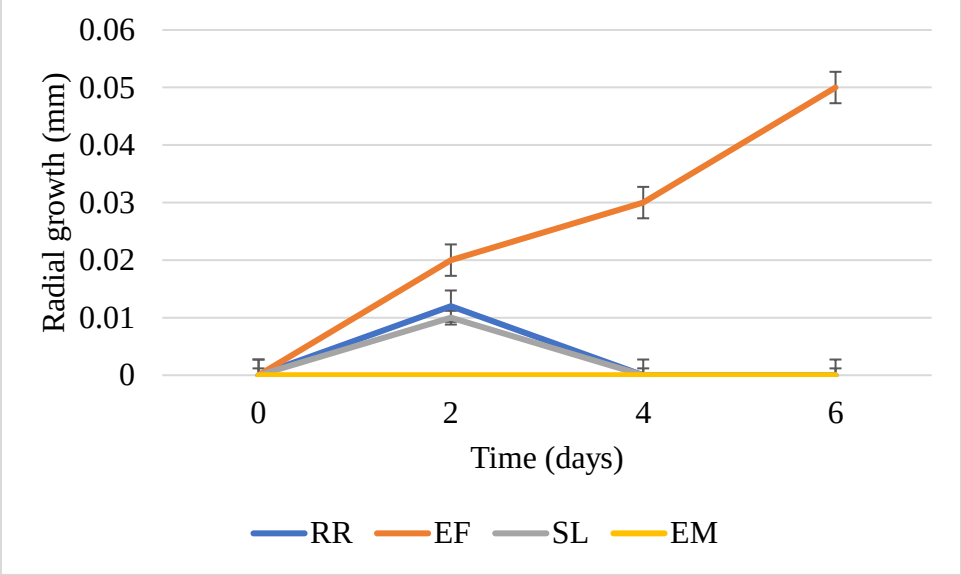


FIGURE 13. GROUP A GROWTH

Ephydatia fluviatilis, R. ryderi, E. muelleri, and *S. lacustris* growth patterns after incubation in Strekal's (day 0) followed by post incubation solution changes of Strekal's. A slight decrease is shown for RR and SL, indicating tissue deterioration due to insufficient conditions for the gemmules.

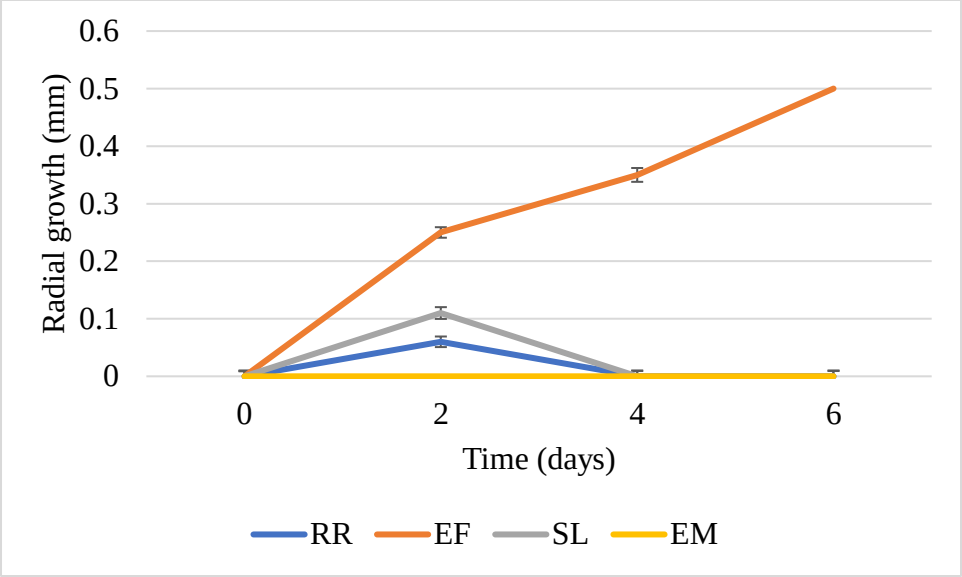


FIGURE 14. GROUP B GROWTH

Ephydatia fluviatilis, R. ryderi, E. muelleri, and *S. lacustris* growth patterns after incubation in 2:1 Strekal's: Anti (day 0) followed by post-incubation solution changes Strekal's. E. fluviatilis continues to grow and the same pattern is shown for R. ryderi and S. lacustris with tissue decay after 2 days. *E. muelleri* fails to plate or grow.

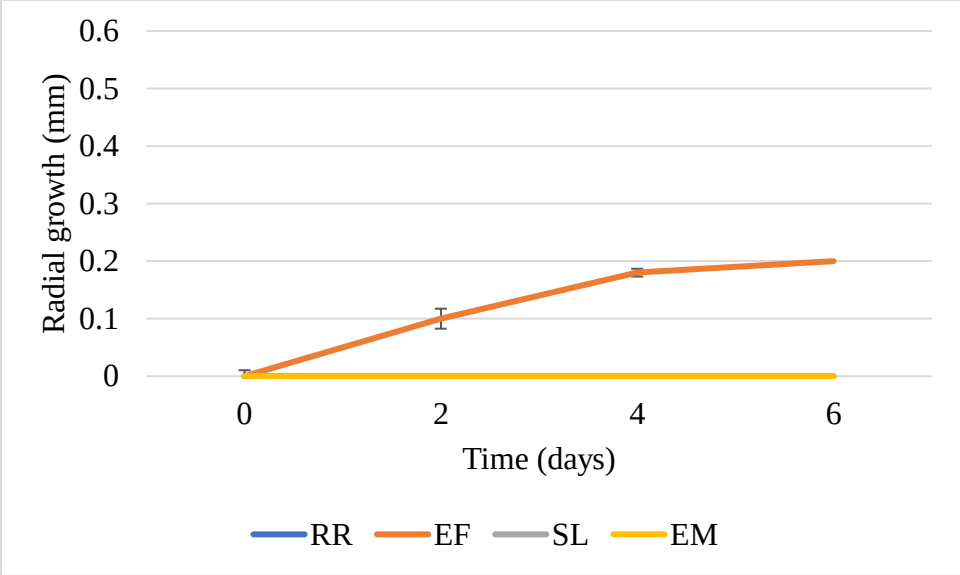


FIGURE 15. GROUP C GROWTH

Ephydatia fluviatilis, R. ryderi, E. muelleri, and *S. lacustris* Gemmules incubated in 2:1 Strekal's: Anti followed by solution changes of filtered Elk River water. Growth was only seen in Strekal's. No growth observed for any of the other specimen.

The results do indeed show Strekal's incubation to be more beneficial for gemmule growth than Anti: Anti or the 2:1 Strekal's: Anti-Anti solution. Additionally, the results showed *E. fluviatilis* to be the most successful sponge specimen to endure any solution.

Trial 3 Results

After determining the best possible incubation and post-incubation conditions, the next phase of research was investigating the proper nutrients to feed the sponges to continue growing. After ten days of consecutive solution changes every 48 hours, the sponges will start to deteriorate if not fed (Nichols, 2023). According to protocols from Nichols (2023), two options were proposed to feed the sponges: heat-killed *E. Coli* or *Liquifry* fish food.

Ephydatia fluviatilis and *E. muelleri* were both incubated in Strekal's 10X for 48 hours followed by a solution change with fresh Strekal's changed every 48 hours following their incubation. The results of sterilization and plating are shown in Table 4.

TABLE 4. TRIAL 3 INCUBATION

	Ephydatia fluviatilis	*Ephydatia muelluri*
Percent Recovery after sterilization	80.6%	78%
Viable after incubation?	Yes	Yes
Response to Strekal's incubation	Plated	No change

Following incubation, both species were treated with Strekal's solution every 48 hours. In preparation for feeding, samples of each species were divided into treatments and labeled as "Bacteria fed" or "fish food." As determined from phase 1 of the experiment, the best fitting post-incubation solution is Strekal's. The growth of each species was tracked after 10 days of Strekal's changing solution. The results of radial growth were gathered from *ImageJ and* were analyzed (Figure 16). The outcome observed from *E. fluviatilis* is not surprising as it has been this way consistently. However, *E. muelleri* now grew at a much faster rate and larger radius than *E. fluviatilis.* There is a slight decrease shown in the tissue growth of *E. muelleri*. An additional observation gained from these trials was the relative abundance of hatching and growth achieved, dependent upon the gemmule being plated in a 2 cm diameter 12-well or a 10 cm petri dish, especially for *E. muelleri.* Table 5 shows the percentage of gemmules that hatched and grew from either a 12-well or petri dish. These results may suggest gemmule growth is more favored in larger surface areas.

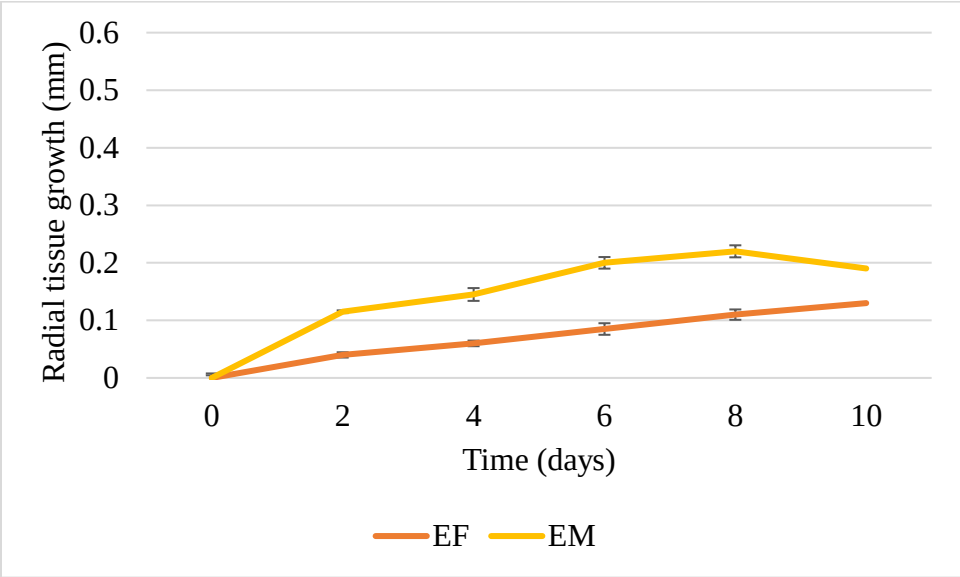


FIGURE 16. TRIAL 3, RADIAL GROWTH

Ephydatia fluviatilis and E. muelleri in Strekal's incubation and Strekal's post-incubation solution.

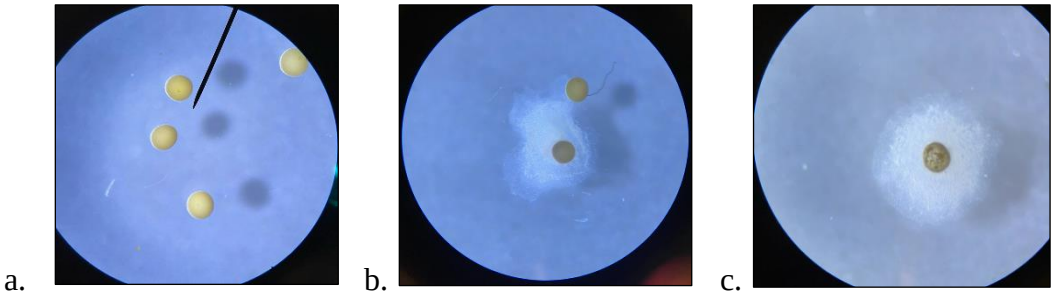


FIGURE 17. *E. MUELLERI* POST INCUBATION

(a) 2 days post incubation (b) and 4 days post incubation (c) all after their vernalization period.

TABLE 5. TRIAL 3: HATCH PERCENTAGES

	12-well	Petri Dish
Ephydatia fluviatilis	76%	87%
Ephydatia muelleri	3%	47%

The results of the feeding trials, shown in figures 18-21, do not show an obvious trend in growth patterns for either treatment. *E. fluviatilis* is seen to have growth in the first 24 hours of being fed either bacteria or fish food. However, in the next 24 hours there is a slight decrease for the group being fed bacteria compared to a stalemate for the group being fed fish food. Figure 18 shows the same trend for the first 24 hours with initial growth when introduced to any kind of feeding source. Nonetheless, the results for the next 24 hours are quite the opposite to *E. fluviatilis*: bacteria fed sponges experience an increase in growth, while the fish food fed sponges have a decrease in radial growth. Considering these outcomes, it is unclear what sponges prefer for their diet as they continue to grow *in vitro.*

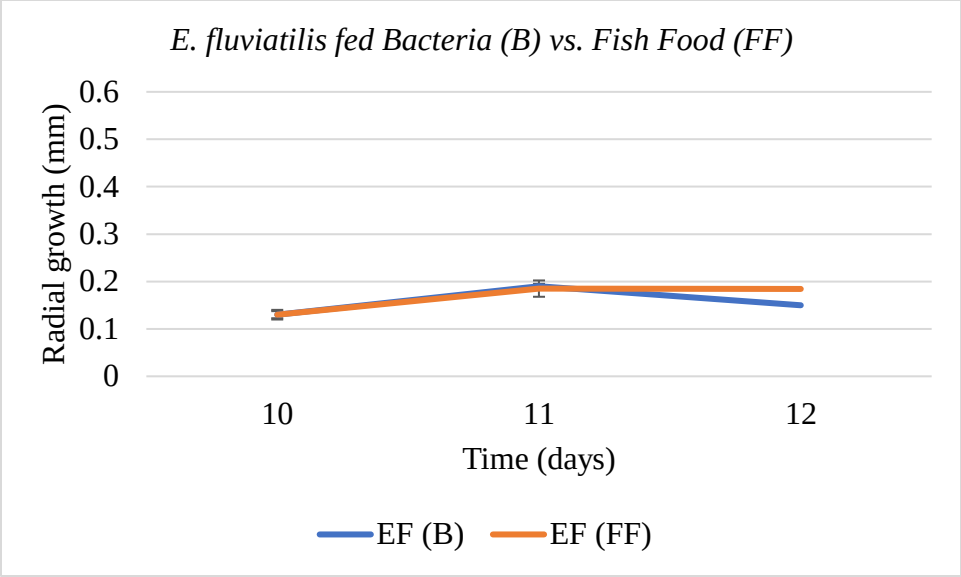


FIGURE 18. E. FLUVIATILIS TRIAL 3 AFTER 10 DAYS OF GROWTH

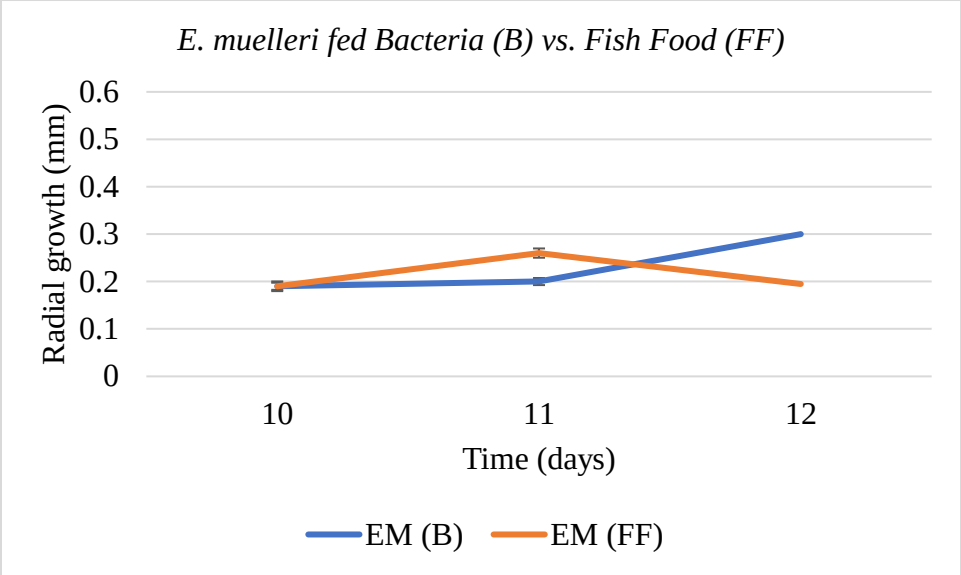


FIGURE 19. *E. MUELLERI* TRIAL 3 AFTER 10 DAYS OF GROWTH

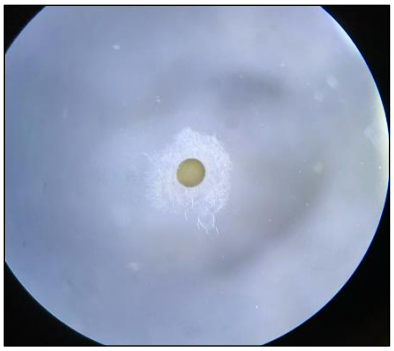
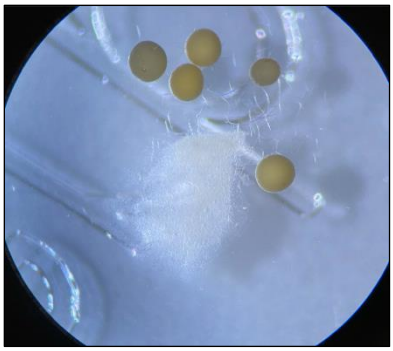
a.  b. 

FIGURE 20. TRIAL 3 GROWTH, BACTERIA

(a) *Ephydatia fluviatilis* and (b) *E. muelleri* 2 days after being fed bacteria from the Elk River

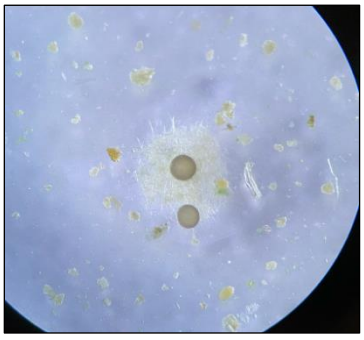
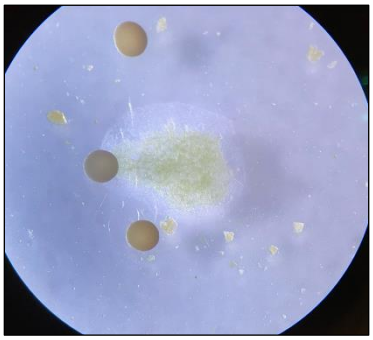
a.  b. 

FIGURE 21. TRIAL 3 GROWTH, FISH FOOD

(a) *Ephydatia fluviatilis* and (b) *E. muelleri* 2 days after being fed Liquifry Fish food

CHAPTER IV: DISCUSSION AND CONCLUSIONS

The results of growing sponges from gemmules *in vitro* have revealed several findings regarding Tennessee sponge growth. Strekal's medium is clearly the best incubation and post-incubation solution for *E. fluviatilis* and *E. muelleri*. Additionally, the larger petri-dishes appear to facilitate better hatching and growth than smaller 12-well plates. While not all species hatched successfully in the current study, a significant factor in gemmule plating and hatching appears to be their vernalization period (Jepps, 1946, Jetton et al. 1987). If a sponge does not have its proper resting period in a chilled environment (simulated winter), it will not be able to grow under controlled conditions as seen from the results of this research.

Protocols for gemmule growth either suggested Strekal's medium (Strekal 1974) or Antibiotic: Antimycotic medium (Anti: Anti) for incubation (Nichols, 2023; Leys 2019). The Strekal's medium contains essential minerals and ions, such as calcium, magnesium, and potassium, needed to facilitate cell permeability which facilitates activity (Strekal, 1974). The Anti: Anti possesses penicillin, streptomycin, and Gibco Amphotericin B. The antibiotics penicillin and streptomycin prevent bacterial contamination due to their combined action against gram-positive and gram-negative bacteria. Amphotericin B prevents fungal contamination of cell cultures due to its inhibition of fungus and yeast (Nichols 2023; Fisher, 2023). The current study observed no hatching using the Anti-Anti solution suggesting this is not a viable protocol for freshwater sponges in Tennessee. The protocols for sponges hatching have been developed in more northern latitudes (e.g., northeast USA and southern Canada; Leys et al. 2019, Strekal 1974) or for sponges at higher elevations (e.g., western Colorado, USA;

Nichols 2023). The results of the current study suggest that some Tennessee sponges respond well to the Strekal's medium, but others (*R. ryderi* and *S. lacustris*) may require additional or different cues for proper hatching of viable gemmules. Future studies could explore factors such as temperature and water flow manipulation.

When growing the gemmules, they were either placed in a 12-well or petri dish based off the protocols provided by Nichols (2023) and Leys (2019). Trial 3 of the current study revealed 44% more *Ephydatia muelleri* gemmules hatched in petri dishes than in 12-wells. While more evidence and data are needed to make a concrete conclusion, the current situation suggests larger surface area promotes gemmule hatching. At present the reason for this is unclear, but it may be due to a number of factors including competition (Niklas, 1994), current flow, and amount of medium administered.

A particularly interesting result from the current study was that *E. muelleri* only hatched after it was kept at 4℃ for five months. This observation led us to hypothesize that gemmule vernalization was an important factor in the hatching of sponge gemmules. Vernalization is shown to be a key factor in the growth of gemmules in previous studies (Jepps,1946) and thus, we hypothesize that *E. muelleri* was not able to hatch until it was able to go through its simulated "winter" cycle. *E. muelleri* was collected in late Fall (October 2023), and it did not begin to respond to treatment until March of 2024. This period coincides perfectly with the yearly length of winter Tennessee experiences, and this "pause" in hatching ability is likely a key adaptation for freshwater sponges in temperate climates.

The results of feeding trials in the current study were inconclusive in discovering whether one food source was better than the other. For each food source, the current

study was able to maintain or slightly increase lateral growth, but further research is needed to draw any firm conclusions. One important factor to consider is the proper concentration of food in each container. For trial 3, sponges were fed a diet recommended by Nichols (2023) and Leys (2023). However, the exact dilutions of either food source was not provided within the protocols. The dilutions proposed in chapter II: Materials and Methods were based on relevance to the dilution of Strekal's medium (10X). However, because neither of the diets were successful, the concentration of each must be tested on by gemmules to find the right dose. Determining the optimal concentration range for feeding sponges *in vitro is* essential to fulfill the broader goal of this thesis to grow gemmules to adulthood to be used as living filters to aid in gray water reduction.

Overall, *E. fluviatilis* in Tennessee seems to be the most durable sponge species encountered even though the published protocols all use the congener *E. muelleri*. Future research will consist of *E. fluviatilis* as the sole candidate for growth and discovery of its proper diet. While the proper feeding conditions were not fully discovered, more research is intended to be carried out beyond this thesis. This thesis serves as a steppingstone for greater research intended to aid in sponges to be used as a living filter to reduce wastewater treatment and clean gray water.

CHAPTER V: REFERENCES

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