Glycogen determination of the effect of salinity on byssal thread production in two Oregon mussels: *Mytilus trossulus* and *Mytilus californianus*

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Abstract

Mussels are able to withstand a variety of environmental changes, including salinity. This experiment took two local *Mytilus* species and exposed them to various salinities in order to find a lower limit for the production of byssal threads. A glycogen assay was also preformed on *Mytilus trossulus* to determine if constant byssal thread production uses more energy reserves than one-time byssal thread production does. The *M. trossulus* that were exposed to 100% and 75% seawater treatments had smaller glucose stores in animals that had their byssal threads cut, as opposed to animals that did not have their byssal threads cut. Control *M. trossulus* individuals produced on average more byssal threads in 100% seawater than they did in 75% and 50% seawater, with 50% animals producing the least number of byssal threads.

Introduction

Regardless of their environment, mussels of all shapes and sizes must attach to a substrate to stay alive. Attachment is achieved by means of a byssus, which is an extracellular, collagenous secretion of the foot (Bell & Gosline 1997). Brown (1952) describes the byssus in three parts: (1) the root, (2) the stem, and (3) the byssal threads. The root is a part of the foot and is also attached to the byssus retractor muscles, and ventrally from the root extends the stem which supports each byssal thread which branches off the stem in many directions before attaching to the substrate. The byssal thread is made up of "conchiolin", which is a molecular complex that contains collagen-type fibrils tanned by quinone (Van Winkle 1970). The foot is used to apply the byssal thread to the substrate, and when the foot retracts, the conchiolin is instantly hardened by the surrounding seawater.

Not every byssal thread is the same. Wave exposed mussels like *Mytilus californianus* have stronger threads than less exposed mussels like *Mytilus trossulus*. Even individuals within a species can be found to have differing strengths of byssal threads depending on the amount of wave action they are exposed to (Bell & Gosline 1997) as well as the mussel size (Denny 1985).

Van Winkle (1970) conducted a study that looked at multiple environmental factors that could potentially influence byssal thread production, including salinity. I decided to investigate this concept further by looking at two local species *Mytilus trossulus* and *Mytilus californianus*.

I chose to look at their glucose energy reserves because glucose is a product of glycogen, and glycogen is the primary energy storage molecule in bivalves (Patterson, 1999, quoting Bayne, 1997). I worked with the mantle tissue because that seems to be the easiest tissue to dissect out, and it also was the tissue of choice according to Patterson (1997 & 1999).

The purpose of this study was to further my understanding of how byssus production works, as well as determine what the lower salinity limit of byssal thread production is for each species. I hypothesize that the animals in the 100% treatment will have more byssal threads and higher glucose stores because they are in a less stressed environment than the animals in the 75% or 50% treatments. Also, I hypothesize that the animals that have to constantly produce byssal threads will have lower glucose levels than animals that only have to maintain their byssal threads.

Methods

HYPO-SALINITY EXPERIMENT

20-30 similar sized individuals of both *M. californianus* and *M. trossulus* were collected on 25 October 2007 from the Outer Boat Basin 'B' Dock in Charleston Harbor, Charleston, Oregon. The other *M. trossulus* were collected by Alix Laferriere early in October near the Betty Kay dock in the Inner Boat Basin in Charleston Harbor, Charleston, Oregon, and the other *M. californianus* were collected on the same day, but from the Boat House Dock of OIMB, in Charleston, Oregon. They were kept in a saltwater table for two weeks before experimentation began. On the start day of testing, 3 individuals of both species were flash frozen using liquid nitrogen, and then stored in a -80°C freezer for a later glycogen assay. Three different salinities (100%, 75%, and 50% seawater) were tested for each species, giving a total of six water baths necessary. The water solutions were made using seawater from the flow table and RO water in 3:1 or 1:1 ratios for the 75% and 50% baths respectively. The 75% bath had a salinity of 25.5 ppt. and the 50% bath had a salinity of 17 ppt. The 100% bath used straight seawater, and had a salinity of 34 ppt. Each bath was set up with 12 individuals in three rows of four. Baths were arranged so that the left 6 animals were the test group and the right 6 were the control group. An air stone was added to each bath to prevent anoxia.

The experiment was allowed to run for three weeks, and water was completely changed half way through the experiment. Byssal thread numbers were counted in every 1-3 days over the duration of the experiment. After every recording, the test animals had their byssal threads cut and were replaced in their original position. The control animals were left uncut, but also replaced in their original positions. At the end of the experiment, three *M. trossulus* from each salinity treatment were dissected and at least 50 mg of mantle tissue were removed from each.

SAMPLE PREPERATION

To prepare the samples, I followed a protocol similar to that of Carr and Neff (1984). 50 mg of mantle tissue was dissected out of each animal, placed into microcentrifuge tubes, and frozen using liquid nitrogen. The samples were then homogenized in 1,000 uL of sodium citrate buffer with plastic pestles. The homogenate was then incubated at 90°C for 3 minutes, and rehomoginized. This step was done twice. Homogenates were centrifuged at 13,000 rpm for 5 minutes and the supernatant was aliquoted for protein quantification and for the glucose assay. The samples chosen for glucose assay were digested in amyloglucosidase. Two samples (one control and one test) were chosen at random to run "undigested." This was done by adding another 400 uL to a third tube. "Undigested" samples were run as a negative control in order to see if there was any glucose in the sample before the addition of amyloglucosidase. All samples were then put into an incubator for 2 hours at 55°C before being centrifuged at 13,000 rpm for another 30 minutes. 333.3 uL of supernatant from each sample was removed and stored in a separate microcentrifuge tube. All samples sat in the refrigerator overnight (due to time restraints).

PROTEIN QUANTIFICATION

The tubes with 20 uL were used to run a protein quantification assay. 100 uL of Reagent A was added to each tube. The tubes were then vortexed for 3 seconds before having 800 uL of Reagent B added and vortexed again. Samples were allowed to sit at room temperature for 15 minutes before reading the absorbance in a spectrophotometer at 750 nm. From the standards a standard curve (R^2 value of 0.9918 and equation y =

0.3335x + 0.0864) was made, showing the ug/mL of protein, and the unknown concentrations were determined from the standard curve.

GLUCOSE DETERMINATION ASSAY

Glucose levels were determined by use of a Glucose (GO) assay kit (#GAGO-20 which was purchased from Sigma-Aldrich). A standard curve (R^2 value of 0.9925 and equation y = 0.0067x + 0.1258) was created according to the protocol included in the kit (the protocol volumes were cut by 1/3), except that sodium citrate buffer was used instead of water. Samples had 666.7 uL of Assay Reagent added to them to start the reaction (each sample was done in 30 second intervals in order to allow enough time to prepare for the next sample).The samples were incubated for 30 minutes at 37°C. After incubation, each sample had 666.7 uL of 12 N H_2SO_4 added to stop the reaction (again in 30 second intervals). Absorbance values were obtained for each sample using a spectrophotometer set at 540 nm, again in 30 second intervals. Unknown glucose concentrations were determined from the standard curve.

Results

For individuals that were cut, *Mytilus trossulus* produced more byssal threads than *Mytilus californianus* did in each treatment. For both species, the 75% treatments had the highest average byssal thread production per sampling time, and the 100% treatment had the next highest average number (Fig. 1). Uncut *Mytilus trossulus* produced on average 3 more byssal threads in the 100% treatment than they did in the 75% treatment, and both treatments were at least double the amount produced in the 50% water bath (Fig. 2). Uncut *Mytilus californianus* individuals clustered on one side of the tub, so counting byssal thread numbers was very difficult therefore there is no data to report for them.

Over the course of the experiment, there were some fatalities. Four (one test and three control) *M. trossulus* from the 100% treatment, two (one each) from the 75% treatment, and two (both test) from the 50% treatment died. *M. californianus* had no fatalities in the 100% and 75% treatments, but four (two each) from the 50% treatment died.

Only *Mytilus trossulus* samples were quantified in the glycogen assay. In the 100% and 75% treatments, the glucose concentration was lower in the animals that had the byssal threads cut on a regular basis. The opposite was true for the 50% treatment. The control (no experimentation) animals had a much higher glucose concentration than any of the experimental animals did (Fig. 3).

Discussion

Cutting byssal threads proved to be more difficult than I originally imagined. Animals rolled off their watch glasses and ended up attached to other mussels or the side of the container. This made counting the number of byssal threads difficult because sometimes I was unsure which byssal thread went with which animal. This problem occurred mostly in the uncut animals, so it wasn't as crucial I get exact numbers until the end of the experiment. Also, there were mass spawning events that occurred in all three of the *Mytilus californianus* tubs, which meant that the water needed to be changed, therefore the other five tubs required a water exchange as well. I don't believe there was anything I could have done to prevent this phenomenon.

The byssal thread numbers were close to what I expected. I thought that animals that were cut would produce similar numbers of byssal threads between each time point. This was difficult to determine since their byssal threads weren't cut every day. For

future experimentation, I would suggest cutting the byssal threads on a daily basis in order to see if they do in fact produce similar numbers of byssal threads per day. The animals that weren't cut generally increased their number of byssal threads between each time point, as expected. A more precise method of counting their byssal threads would be needed to ensure more accurate data.

The animals collected from 'B' Dock were found in water with a salinity of 23 ppt. Animals from the Betty Kay dock and the Boat House Dock were found in 26 ppt and 32 ppt respectively. I did not expect *M. californianus* in the 75% treatments to produce more byssal threads than in the 100% treatments because they were kept at a salinity that is lower than the salinity in their normal environment. The fact that the *M. trossulus* in the 75% treatment produced more byssal threads did not come as a shock because they were collected from a similar salinity. Since *M. trossulus* are commonly found on the docks in the Outer and Inner Boat Basins, I would expect them to have better production than *M. californianus* through all three treatments, and this was the case. However, I also expected *M. californianus* to have greater production in the 100% treatment since that is the normal salinity of its environment, and this wasn't the case (Fig. 1).

I expected the uncut organisms to have higher glucose concentrations, and other than the 50% treatment, that was true (Fig. 3). As for the 50% treatment, I am at a loss as to why the glucose concentration values are the opposite of the other two treatments. Repeat testing, if not further testing would be needed to determine if this is just an anomaly or if this really is how the animals are in the wild.

Overall, the experiment went as expected. More precise counting and consistent counting would be needed in order to draw more concrete conclusions however the data that was collected is accurate. If I were to repeat this experiment, I would collect all animals from the same site, and I would also try to get animals of similar sizes. Measuring the mussel size is another way to make more comparisons between the data, and I suggest doing this in future experiments. The only major hold up that I encountered occurred during the glycogen assay, and that was completely my fault. I accidentally spilled all my samples and was forced to repeat the assay with diluted solutions. I don't believe this caused any error because all of the solutions were lost therefore they all had to be diluted in the same manner, so the data that was collected can still be compared between samples. I do recommend keeping all pellets and left over supernatant from all assays just in case spillage occurs.

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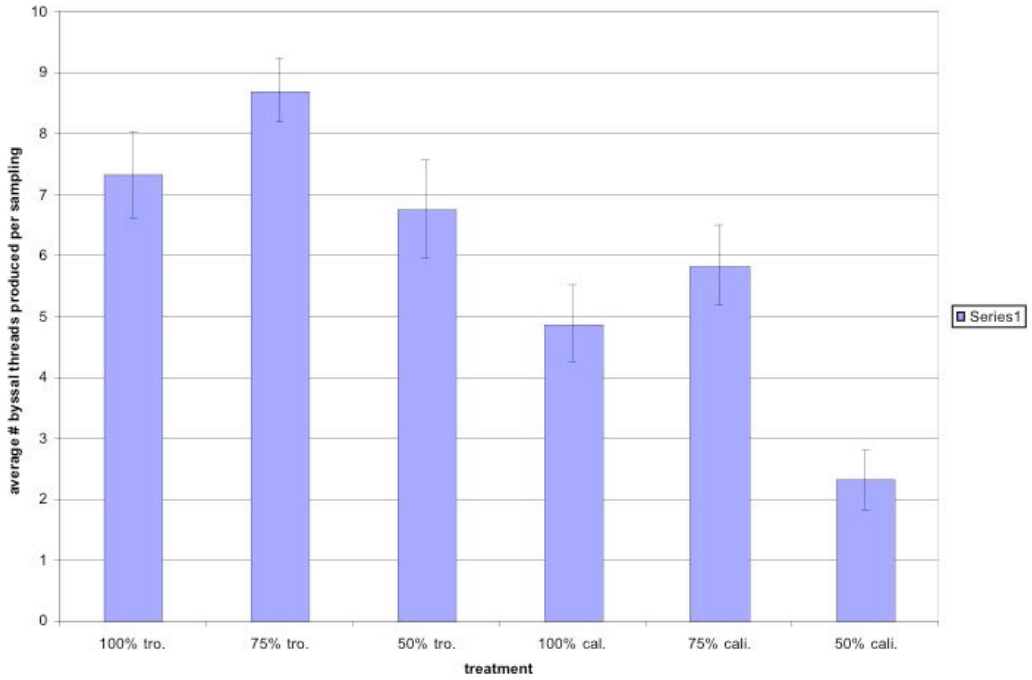
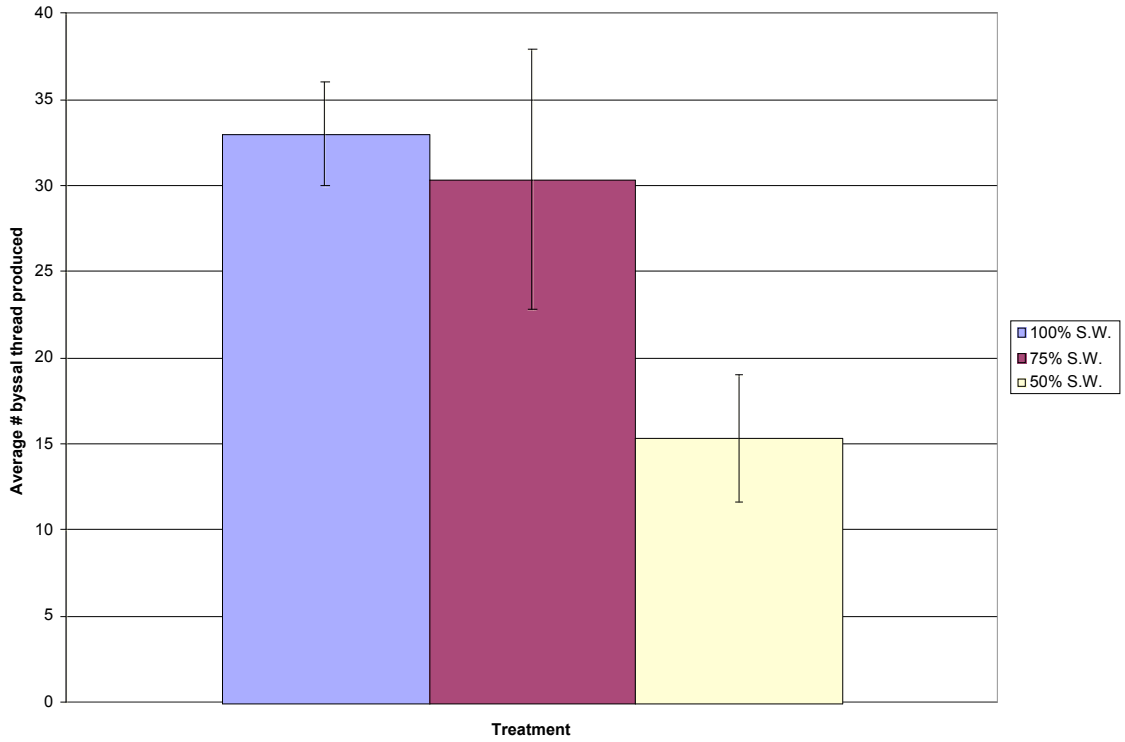
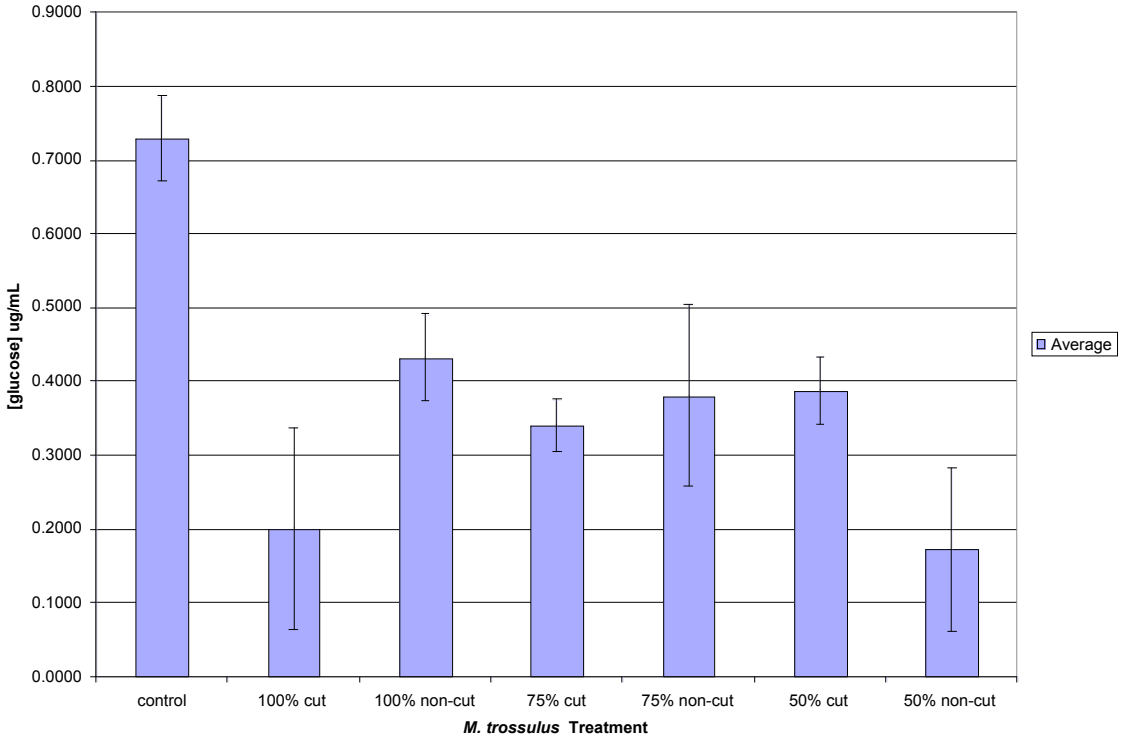


(Fig. 1) This figure is for the cut animals in each treatment. The figure shows average number of byssal threads produced over the entire population within each treatment per sampling time. Note *M. trossulus* consistently produced more byssal threads than *M. californianus* did in each salinity treatment.



(Fig. 2) The data presented here is the average final number of total byssal threads produced by *M. trossulus*. The data for *M. californianus* isn't presented because there was clustering of control animals during testing, and determination of which byssal threads belonged to which individual was difficult.



(Fig. 3) This figure shows the average concentration of glucose for the three different treatments. The control data are from animals that never experienced the experimentation process. They were flash frozen at the start of experimentation in order to note any differences between untested and tested animals. Note that the concentration of glucose in the control animals is much higher than any of the tested groups. The 50% treatment is different from expected, and the reason for that is unknown. Note in the 100% and 75% treatments that the cut organisms had less glucose than non-cut organisms. This is consistent with the hypothesis.

Protocol for Khoury Hickman's experiment

1. Collect 36 mussels of both *Mytilus californianus* and *Mytilus trossulus*.
2. Prepare three separate water baths for each species. One bath will be 100% seawater (34 ppt), the second bath should be 75% seawater (25.5 ppt.) and the third bath should be 50% seawater (17 ppt). Each bucket should have an air stone.
3. Three animals from each species should be flash frozen and then kept at -80°C until the end of the experiment.
4. Keeping all animals separate, place 12 individuals into each bucket according to species (each bucket will be either *M. Trossulus* or *M. Californianus*).
5. As often as possible (with at least 24 hours in between sampling) check the number of byssal threads produced on all animals.
6. Cut the byssal threads on half of the animals in each bucket.
7. Allow experiment to run for 3 weeks and check/cut as often as you can.

SAMPLE PREPERATION

8. Prepare the samples by dissecting 50 mg of tissue from each organism you plan to test.
9. Homoginize the tissue samples in 20 volumes of sodium citrate buffer, heat them at 90°C for 3 minutes, rehomogenize, and reheat at 90°C for another 3 minutes.
10. Rehomogenize a third time before centrifuging the samples at 13,000 for 5 minutes.
11. Separate supernatant into two microcentrifuge tubes (one with 20 uL and the other with 400 uL).
12. Incubate samples for 2 hours at 55°C
13. Centrifuge them at 13,000 for 30 minutes, and then place them into the refrigerator overnight.

PROTEIN QUANTIFICATION

14. Create known concentrations of BSA according to the chart provided in the protein quantification assay protocol.
15. Perform a protein quantification assay using the 20 uL samples from the day before.
16. Add 100 uL of reagent A to each, and then vortex the samples.
17. Add 800 uL of reagent B to each, and vortex immediately afterward.
18. Incubate at room temp for 15 minutes.
19. Run a spec on the samples at 750 nm.
20. Using the standards created in step 14, make a graph that allows determination of sample concentration from their absorbance values.

GLUCOSE SPECTROPHOTOMETRIC ASSAY

21. Create standards according to the protocol that came with the Sigma glucose kit.
22. Using the 400 uL samples, aliquot out 333.3 uL of supernatant for testing.
23. Add to that 666.7 uL of assay reagent to start the reaction. Let the reaction run at 37°C for 30 minutes.
24. Every sample after the first should be started in 30 second intervals and ran for the same amount of time as the first sample.
25. Stop the reaction by adding 666.7 uL of 12 N H_2SO_4. Do this in 30 second intervals as well, that way each sample was incubated for 30 minutes total.
26. Run each sample and the standards through the spectrophotometer at 540 nm
27. Create a standard curve and determine the glucose concentration for each unknown from their absorbance value.