Temperature effects on byssal thread production in the mussel, *Mytilus trossulus*

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Abstract

Mussels attach themselves to hard substrates by extruding thin, strong, and flexible hair-like attachments known as byssal threads, which are anchored in place with small adhesive plaques. While many studies have examined how multiple abiotic variables affect mussel attachment strength, few have performed extensive single variable tests within a controlled lab setting. In this study, we investigated the effects of temperature on byssus production in the native Northern Pacific intertidal blue mussel, *Mytilus trossulus*, and expected to see a decline in production with increasing temperatures (specifically, between 18°C-25°C). We also hypothesized that threads produced in warmer temperatures would show a visual decline in quality, either through thinning, degradation, or tearing. Mussels were placed in a temperature controlled flume for 24 hours. Six different trials were run at 10°C, 14°C, 18°C, 20°C, 22°C, and 25°C. Byssal threads were counted for the duration of each trial to determine production and rate of attachment. Threads produced under these different temperature regimes were analyzed under an SEM to visually compare differences in quality. Overall, there was a significantly negative correlation between thread production and increases in temperature, with the highest amount occurring at 18°C (11.25 threads $\pm$2.0 s.e.m.) and the lowest at 25°C (0 threads). Acclimatization and rate of thread production was also negatively affected by temperature increases. In addition, we saw a significant difference in percentage of mussel attachment between temperatures, with up to 93% attached in the 10°C and 18°C treatments and 50-0% in the 20°C-25°C treatments. However, visual analyses of thread quality differences were inconclusive. Our findings indicate that climatic temperature increases may negatively affect both the range of *M. trossulus* within Northern Pacific rocky intertidal zones as well as economically setback the aquaculture of the speciess.

Introduction

The rocky intertidal is a dynamic and challenging environment to live in. Faced with constant fluctuations in water salinity, tide level, wave force, current velocity, and temperature many organisms within this zone have evolved adaptations to tolerate the ebb and flow of their surroundings. One such adaptation can be seen in select bivalves, which create strong, yet flexible, byssal thread attachments to fasten themselves to hard substrates and withstand the wave-swept forces around them. Of the byssus producing bivalves is the mussel, *Mytilus trossulus*, which is both an important foundation organism within the intertidal zone as well as a popular species in aquaculture.

Similar to other mussel species, *M. trossulus* produces collagenous byssal threads by extending a muscular foot lined with a groove from which the threads are molded, extracted, radiate outward, and attach with small, adhesive plaques to a hard surface (Tamarin and Keller, 1972). Byssal threads keep the mussel tethered to a rocky substrate and prevent it from washing away in the surf, thus acting as a lifeline. In this way, a mussel's survival is dependent on the condition and/or the amount of threads it produces (Young, 1985; Moeser and Carrington, 2006). Weak threads with little tenacity and produced in too few a number can lead to a higher amount of mussel dislodgement and mortality (Moeser and Carrington, 2006).

With global climatic change and rising ocean temperatures an ongoing concern, organisms within the intertidal zone are especially vulnerable (Helmuth et al., 2002; Carrington et al., 2009). Mussel mortality increases in response to temperature stress (Carrington et al., 2009), and byssus production in particular is also susceptible to environmental stressors (Young, 1985). Numerous studies have shown that mussel byssal thread tenacity is affected by seasonality, wave force, and temperature (Moeser and Carrington, 2006; Carrington et al., 2009; Waite and Broomell, 2012), with at least one byssus protein known to be temperature dependent (Waite and Broomell, 2012). The amount of threads a mussel produces as well as the overall attachment rate could be equally susceptible to temperature shifts.

An increase in mussel displacement can not only affect the biodiversity of rocky intertidal areas but also significantly impact the aquaculture of mussels within the Mytilus genus. Mussels are culturally grown using different methods, but all rely on the ability of the bivalve to produce threads and attach firmly to a substrate (Lutz et. al. 1991). As an industry worth $6.6 million per year in the US, a decline in byssus formation and mussel attachment could thus be economically problematic for Mytilus aquaculture (FAO, 2010).

While studies have been done both in the field and lab to test multiple variables on Mytilus byssal thread production and strength, few have tested a single variable, such as temperature, on byssus production alone. The purpose of our experiment was to determine the water temperature(s) in which there is a marked decline of byssal thread production in the mussel, *M. trossulus*, and whether those produced show a difference in visual quality. We hypothesized that there would be fewer byssal threads made and an overall lower percentage of mussel attachment at higher temperatures, with a decline occurring between 18-25°C. In addition, we suspected that byssus produced at higher temperatures would be thinner and more degraded than that produced at lower temperatures.

Materials and Methods

Animals

A native intertidal mussel of the Northern Pacific, *Mytilus trossulus* is one of three closely related species that make up the *Mytilus edulis* complex of blue mussels

(Fig. 1). This species is well-adapted for cooler marine climates and can form dense beds in the rocky intertidal (Fields et al. 2012). For our experiment, *M. trossulus* mussels were collected on October 12th, 2012 at low tide in Argyle Creek on San Juan Island, WA, USA. This tidal creek serves as a link between two saltwater habitats and is part of the Argyle Lagoon Biological Preserve, where average water temperatures fluctuate between 12-20°C. The specimens collected ranged in length from 40-50 mm (Table 1) and were checked for byssal thread activity (mussels with few to no threads were not utilized). Mussels were then placed in a protected cage underneath a dock ("dock box") at the University of Washington Friday Harbor Laboratories and remained there when not in use for a treatment trial. This allowed for optimal food availability and water flow as well as minimized stress and damage.

Experiment Design

Following the procedure outlines by Moeser et al. (Moeser et al., 2006), we measured thread production as a function of temperature in a small flume. To control for flow and temperature, a single circulating flow tank (2.22 m long, 0.16 wide, and 0.19 m high) was used for all treatment trials throughout the experiment and is described in prior studies by Vogel (Vogel, 1981). The flume was a predominantly closed system, which we adapted to contain additional minimal flow-through to ensure aeration and proper nutrient content (Fig. 2). In addition, two air stone bubblers were used for maximum gas exchange. A motor (Minarik model RG510A) controlled water flow at a constant 0.06 m/s rate while a honeycomb restrictor evenly spaced flow through the working section of the flume.

There were six temperature treatments with one trial per treatment: 10°C, 14°C, 18°C, 20°C, 22°C, and 25°C. Each trial lasted 24 hours and, to minimize experimental variability, their order was selected haphazardly. A separate cooling/heating unit (Neslab RTE10) adjusted water temperature within the flume. Prior to the start of each trial, water temperature was also set and stabilized.

To hold the mussels within the flume and create a mussel "bed", a rack was constructed from plastic egg crate material (or ceiling tile) and attached to a clear acrylic sheet bottom with hot melt adhesive. A trial consisted of twelve mussels, which were carefully selected from the dock box for thread activity, cleaned of any barnacles and/or limpets, and measured (length, width, and height). Byssal threads were cut and removed to both promote new byssus growth within our controlled treatments. Each mussel was then adhered with cyanoacrylate to thin plastic zip ties, which were evenly spaced within the mussel rack and suspended 6.5 mm above the acrylic sheet (Fig. 3). The rack was then placed in the flume and flush along the bottom, with mussel anterior facing downstream to control for orientation effects. Mussels selected for a trial remained out of water for one hour, from the time they were removed from the dock box to the time they were placed in the flume. Each trial used twelve different mussels (for a total of 72 mussels in the experiment) to prevent any lingering effects between treatments. We considered each mussel to be a treatment replicate and thus independent from one another.

Data Collection

Once mussels were placed in the flume, a trial began and continued for a total of 24 hours. To track byssal thread production and attachment rate, mussels were checked and threads counted every hour for the first six hours and every four hours after that until the 22^{nd} hour. After 24 hrs, a final thread count was conducted. A byssal thread was only counted if the adhesive plaque attached to the acrylic sheet base or to another mussel (Fig. 1), at which point a mussel would be classified as "attached". Threads were cut from the active mussels, and a sample from each was saved for comparison analysis under a scanning electron microscope (SEM, JEOL5000) to determine visual differences in thread quality for each temperature trial. At the end of each trial, average water flow velocity was manually measured with fluorescent dye.

Statistics

All mussels, whether attached or not, were included in the statistical analyses and mean thread counts. Regression and one-way ANOVA analyses were used to determine correlation between temperature and mean byssal thread production, temperature and overall mussel attachment, and mussel attachment rate over 24 hours. In addition, a Tukey-Kramer post-test was conducted to track significant differences between treatments. To track significant differences in thread production rates between temperatures, we applied an ANCOVA to cumulative thread production and temperature, time, and temperature with time. Two-way ANOVA analyses were also performed to determine if mussel sizes within temperature treatments had an effect on byssal thread production. All statistical analyses were performed with JMP and R software.

Results

Thread Quantity

Overall, there was a significantly negative correlation between amount of byssal threads produced and increasing temperature ($F_{5,66}$=3.503; p=0.007). Mussels in the coldest (10°C) and middle (18°C) temperature treatments produced the highest number of threads, averaging 11.25 and 15.5 threads per mussel. However, those in the warmest treatments (22°C and 25°C) produced the fewest (Fig. 4), averaging 2.08 and 0 threads per mussel. We applied a 2-order polynomial regression to track an overall trend in production (r^2=0.6338; $y = -0.1036x^2 + 2.9114x - 8.784$), which indicated a peak between 14°C and 18°C but a decline after 18°C. A Tukey post-test analysis further revealed that there was a significant difference between thread numbers in 18°C and 22°C as well as 18°C and 25°C. Regressions of thread numbers against mussel length, width, and height were statistically insignificant except for mussel height at 20°C (Table 1). In this treatment, the mussel that produced the most threads also had the largest height, but was not the largest mussel in that treatment (as determined by length, width, and height). Two of our temperature treatments (14°C and 18°C) also had at least one mussel spawn during the beginning hours of the trial. These mussels also produced the highest number of threads within their temperature treatments, at 3-4 times more than the mean.

For all temperature treatments except 25°C (in which no threads were made) there was a significantly positive correlation between cumulative threads produced and time (Fig. 5). However, the rates between treatments varied, with mussels producing threads earlier in the 10°C, 14°C, and 18°C treatments than in 20°C and 22°C (Fig. 6). Our

ANCOVA results showed significant differences in these thread production rates between temperatures ($p<0.0001$).

Mussel attachment (Fig. 7) was also significantly different between the temperature treatments ($F_{5,66}=9.900$; $p<0.0001$), with a greater amount of attachment occurring at 10°C, 14°C, and 18°C (75-92%) than at 20°C, 22°C, and 25°C (0-50%). Post-analysis tests revealed that the greatest difference occurred between 10°C and 25°C, as well as 18°C and 25°C ($p<0.0001$ for both). While the number of threads produced at 14°C was lower than at 20°C, there was a higher amount of mussel attachment within that treatment (75% vs. 50%). This was reflected in the post-analysis, with mussel attachment between 14°C and 25°C more significantly different than between 20°C and 25°C ($p<0.0002$ vs. $p=0.0286$). Attachment between hours of all six temperatures differed, but not significantly so ($F_{10,55}=1.828$; $p=0.077$).

Visual Thread Quality

Byssal threads for each temperature treatment trial were also examined under SEM to evaluate visual differences in quality. Threads were compared at both the plaque and distal (middle thread) regions for any tears, degradation, and/or thinning. Comparisons of plaques between temperature treatments did not reveal any significant differences in visual quality (Fig. 8). Some plaque curling and distortion occurred, which was likely due to the drying process before SEM analysis took place. Similar comparisons of thread distal regions reveal the inner thread fibers in most threads produced at 10°C, 14°C, 18°C, and 20°C (Fig. 9). SEM images of threads from the 22°C treatment presented what may be minimal cuticle damage in some of the thread samples, but ultimately all visual thread quality comparisons were too variable within treatments and thus inconclusive. Threads produced by the spawned mussel at 14°C were compared separately against normal threads within that same treatment. Analysis under SEM showed that some of these threads were thinner than those produced by non-spawned mussels.

Discussion

Byssal thread production of *Mytilus trossulus* was highest in the 10°C and 18°C treatments, with a suggested peak between 14°C and 18°C, whereas a significant decline occurred in temperatures above 18°C. This trend supports previous studies (Carrington, 2002b; Moeser et al., 2006; Lachance et al., 2008) in which the amount of threads produced seemed negatively impacted by an increase in temperature. At 10°C, 14°C, and 18°C threads were made earlier in the trials, between hours 1-3, before hitting a second spike of production between hours 10-12. However, thread production in 20°C and 22°C treatments took much longer, with significant thread counts not occurring until hours 10-22. No threads were made in our warmest temperature treatment, implying that *M. trossulus* byssal thread production is greatly inhibited at and above 25°C.

In the rocky intertidal, a healthy and productive mussel bed relies on all mussels to be attached to the substrate. Therefore, our study also closely monitored the percentage of attachment (defined as the point at which a mussel produces its first attached byssal thread) between temperatures. We saw a significantly higher percentage of overall mussel attachment between 10°C and 18°C, with a drop of 42% from 18°C to 20°C. None occurred at 25°C, which complements past assumptions that Mytilus byssus attachment becomes abnormal above 24°C (Pearce, 1969). The rate of attachment was increasingly slower with an increase in temperature as well. Significant attachment at 20°C did not occur until hour 10 (40% attached) while at 22°C this same amount of attachment was not seen until hour 22. This suggests that *M. trossulus* acclimation takes much longer in temperatures above 18°C and is of particular concern for both rocky intertidal mussel beds as well as Mytilid aquaculture, which requires consistency and predictability in byssus attachment.

It is possible that with longer treatment trials (such as 48-hrs instead of 24-hrs) mussels would have become more acclimated and active, however it was clear that those in the 22°C and 25°C treatments were stressed and unwell, as nearly all remained closed throughout the duration of the trial. High levels of mussel activity, such as foot extension or filter feeding, was observed immediately at 10°C- 18°C but seen less often at the higher temperatures. Two mussel fatalities also occurred within the 22°C treatment but none of the other temperature trials.

Analysis of byssal threads from each temperature treatment under SEM showed slight differences in visual quality between a select number of threads from 22°C and those in the colder treatments, but ultimately any differences were minor, too variable, and inconclusive. Thread quality is known to be dependent on biotic as well as abiotic conditions, and both mussel stress and increased water temperatures may affect byssus quality and thread formation (Moeser and Carrington, 2006; Lachance et al., 2008; Waite and Broomell, 2012). Our experiment was unable to detect a visual decline in byssal thread quality with increased temperatures; however, differences in the molecular structure of the threads were not tested and may show different results.

While we did not see an overall pattern of thread degradation with an increase in water temperature, we observed that some of the threads produced by the spawned mussel at 14°C were thinner than those produced by the other mussels within that same treatment. In addition, the highest amount of threads produced at both 14°C and 18°C were made by spawning mussels, which produced between 3 to 4 times more than the mean thread amount. This may support hypotheses of Carrington, who suggested that gamete production dictates thread amount and condition, with thread production and tenacity increasing after spawning stages due to an increase in energy availability (Carrington, 2002). While this may explain the number of threads these two spawned mussels produced, it is not clear whether the thinning of these threads was also a result of gamete production or the rate at which they were produced.

Mussel attachment strength is dependent on the number of byssal threads a mussel produces or the quality/strength of threads (Moeser and Carrington, 2006; Brazee and Carrington, 2006). In this way, an intertidal mussel such as *M. trossulus* can lower its risk of displacement by producing many threads or limiting itself to fewer threads of better tenacity and thickness. As our experiment indicates a negative correlation with thread production and temperature, mussel attachment strength can remain unchanged if there is a positive correlation with thread quality and temperature. We were unable to see a definite difference in thread quality between temperatures through visual analysis, but other studies currently being conducted indicate that high temperatures decrease byssus strength (L. Newcomb, unpublished results), which may lead to a greater risk of *M. trossulus* displacement in the field at warmer temperatures.

While precautions were taken to avoid error and curb any source of variability, our study did rely on pseudoreplication in the flume design. Preliminary tests showed no tank effects on thread formation; however, future studies would be best with at least two identical flume set-ups. In addition, a more refined heating system would allow for greater temperature control and permit 1°C increases in temperature. Our ANOVA analyses comparing the amount of threads produced with mussel size per temperature treatment were mostly insignificant, indicating that thread production within our experiment was not influenced by mussel size. This was not true at 20°C, where an ANOVA comparing thread production with mussel height was minimally significant ($p=0.039$). The mussel that produced the most threads within that treatment also had the greatest height, but it was not the largest mussel (as determined by length, width, and height) in that trial. With a larger mussel sample size (18-22) per temperature treatment, comparisons of thread production with mussel size per temperature likely would have all been insignificant, as we expected. While many studies have focused on multiple variables that may hinder byssus production and mussel attachment, more single variable testing in a controlled lab environment is warranted and needs to be further explored.

As we continue to see a slow rise in oceanic temperatures, questions remain about how these climactic shifts may impact organisms within the intertidal zone. A decline in *M. trossulus* byssus production and attachment in warmer temperatures could lead to high displacement and mortality for the species. As *M. trossulus* is also an important foundation species, creating habitat and supplying prey for other organisms, a decline in its abundance could also lead to a decrease in rocky intertidal biodiversity (Altieri and Whitman, 2006). In addition, this may lead to further habitat takeover by a closely related and invasive non-native mussel, *Mytilus galloprovincialis*. This warm tolerant species is most abundant at higher temperatures and has replaced *M. trossulus* in much of its southern range along the Pacific coast of North America as it continues to encroach northward (Braby and Somero, 2006).

A decrease in byssus production and attachment rate could also have implications for Mytilus aquaculture. While *M. galloprovincialis* continues to be the dominant species farmed in the industry, *M. trossulus* is also cultivated by mussel farms in the Pacific Northwest. Mytilus cultivation techniques vary, but all rely on reliable attachment rates (Lutz et. al. 1991). As the results from our experiment showed a significant decline in overall attachment as well as rate of attachment at temperatures above 18°C , an increase in water temperatures could potentially be an economic deterrent for the use of *M. trossulus* in aquaculture.

In conclusion, our experiment indicates that temperatures above 18°C can negatively affect the amount of threads produced, the time it takes to produce them, and the overall percentage of attachment in *Mytilus trossulus*. We also found that the species is ill-equipped for temperatures exceeding 25°C. While we were unable to detect a visual difference in thread quality between 10°C-25°C, the effects of temperature on the molecular properties and overall structure of Mytilus byssal threads remain unanswered and should be investigated.

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Table 1: Summary of mussel size data (mm)

Temperature (°C)	Mussel Length	P Value	Mussel Height	P Value	Mussel Width	P Value
10	44.58±2.75	0.723	21.33±1.56	0.516	16.92±1.62	0.253
14	42.08±2.47	0.916	19.50±2.65	0.562	14.83±0.83	0.322
18	42.17±3.76	0.950	19.42±3.82	0.557	14.25±3.55	0.499
20	44.50±2.47	0.877	21.83±2.52	0.048*	17.5±2.24	0.296
22	41.50±3.29	0.258	20.25±1.29	0.362	16.08±1.51	0.312
25	44.25±3.28	N/A	20.83±1.34	N/A	17.08±0.79	N/A

Values for mussel data are means ± s.d.
n=12 per temperature
P-values compare threads produced vs. mussel sizes per temp (*=significance)

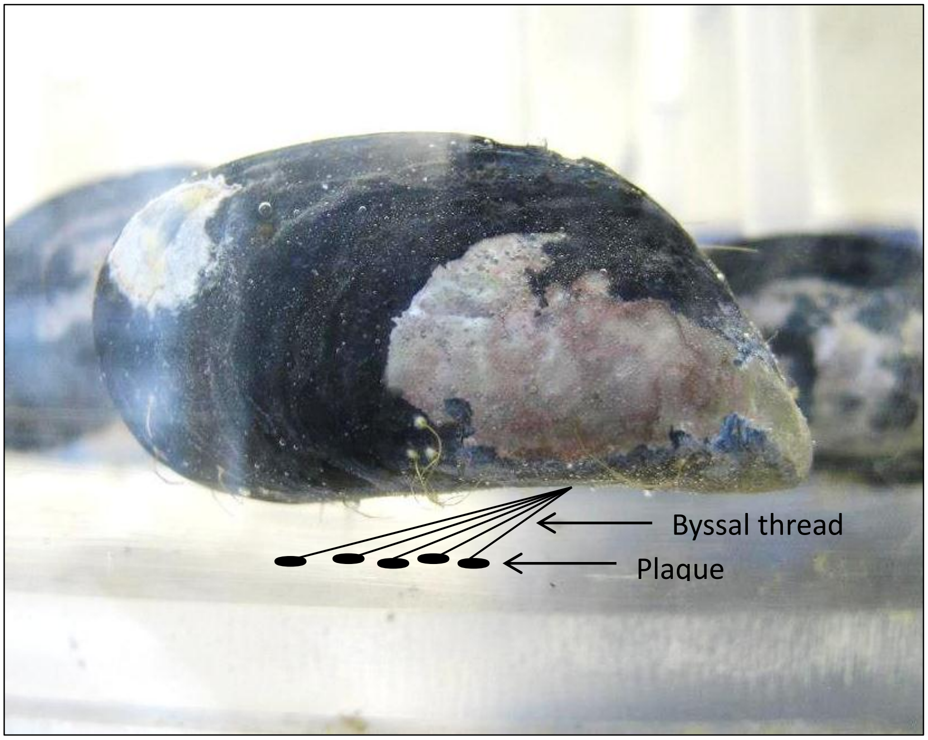

Fig. 1: Diagram of the blue mussel, *Mytilus trossulus*, illustrating how byssal threads attach to a substrate with small adhesive plaques. In our experiment, only threads that attached were counted for thread production and percentage of mussel attachment.

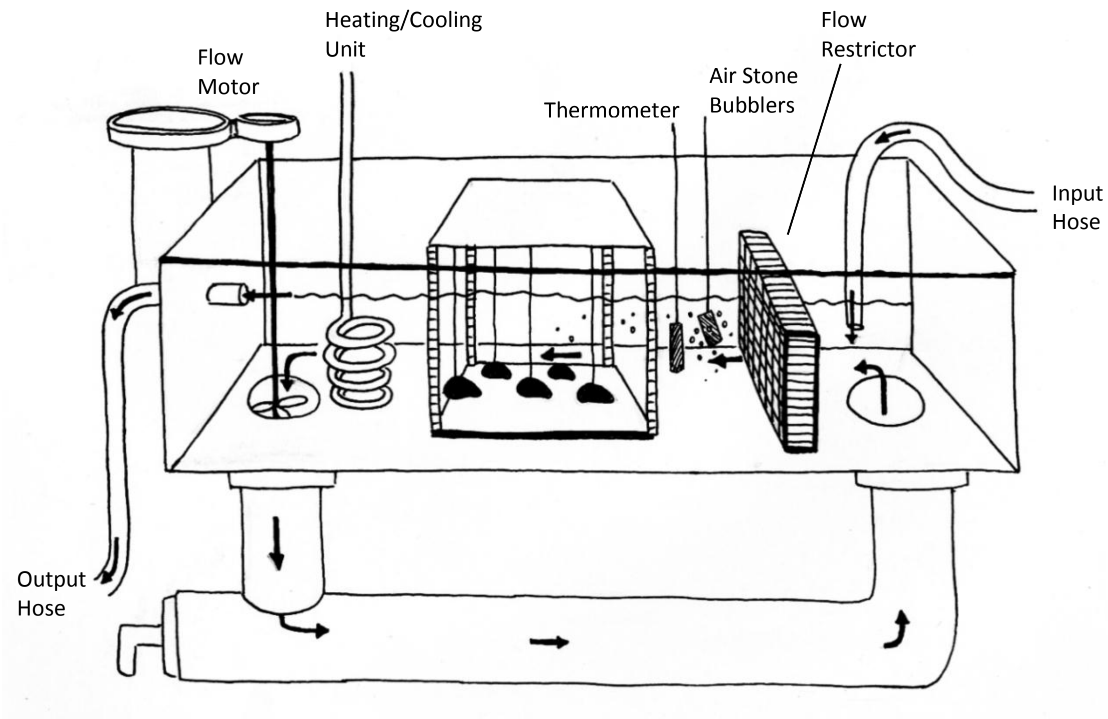

Fig. 2: Flume layout and design. A single circulating flow tank, (measuring 2.22 m X 0.16 m X 0.19 m in the working section) was used for all temperature treatment trials. Water flow was controlled by a Minarik motor (model RG510A) and evenly spaced by a honeycomb flow restrictor. Flow averaged 0.06 m/s for all trials. A separate cooling/heating unit (Neslab model RTE10) placed downstream in the flume was used to control water temperature, which was recorded next to the mussel bed. To maximize water oxygen content, two airstone bubblers were placed near the mussel rack and a minimal flow-through of seawater was created with an input and output hose.

Fig. 3: Mussel rack design. Constructed with acrylic sheeting, plastic egg crate material, and hot melt adhesive, the mussel rack held 12 randomly chosen active mussels that were evenly spaced with anterior ends facing downstream to create a mussel "bed". Mussels were attached to thin plastic rods with cyanoacrylate and suspended 6.5 mm above the acrylic sheet bottom.

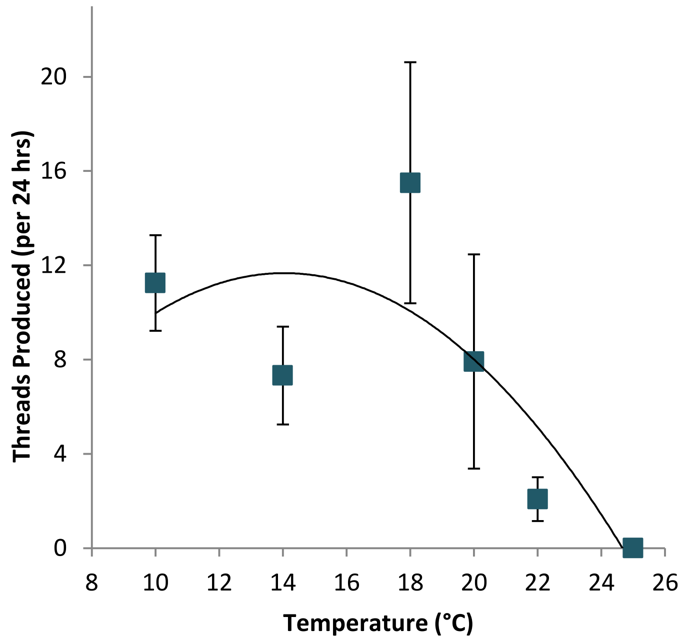

Fig. 4: Threads produced per 24 hr temperature treatment trial. Thread counts are means (n=12 per temperature) ± standard error of the mean (s.e.m). We found significant differences of the amount of threads produced between temperatures ($F_{5,66}$=3.503; p=0.007). A 2-order polynomial regression fit the data well ($y = -0.1036x^2 + 2.9114x - 8.784$; r^2=0.6338). The highest amount of thread production occurred in the coldest (10°C) and mid-temp (18°C) trials, while the least (no threads produced) was in our warmest temperature treatment of 25°C.

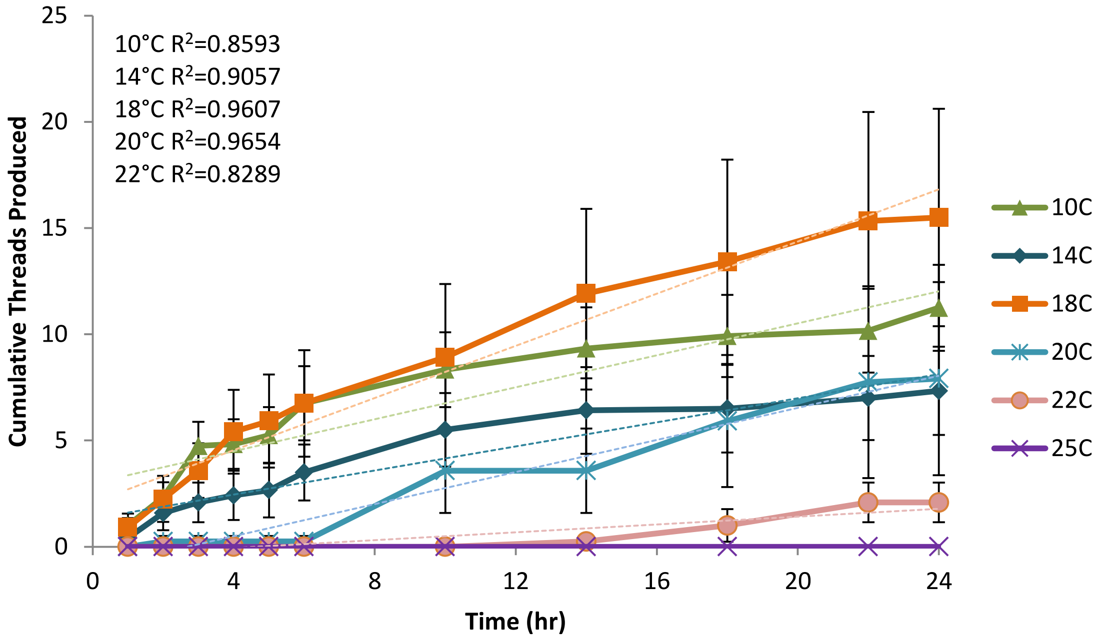

Fig. 5: Linear regressions for cumulative threads produced over 24 hrs per temperature treatment trial. Thread counts are means $\pm$ s.e.m (n=12 per temperature). Each dotted line linear regression (except for 25°C, where no thread production occurred) was significant ($p<0.0001$). An ANCOVA applied to cumulative thread production against temperature ($p<0.0001$), time ($p<0.0001$), and temperature with time ($p<0.0001$) showed significant differences in thread production rates between temperatures.

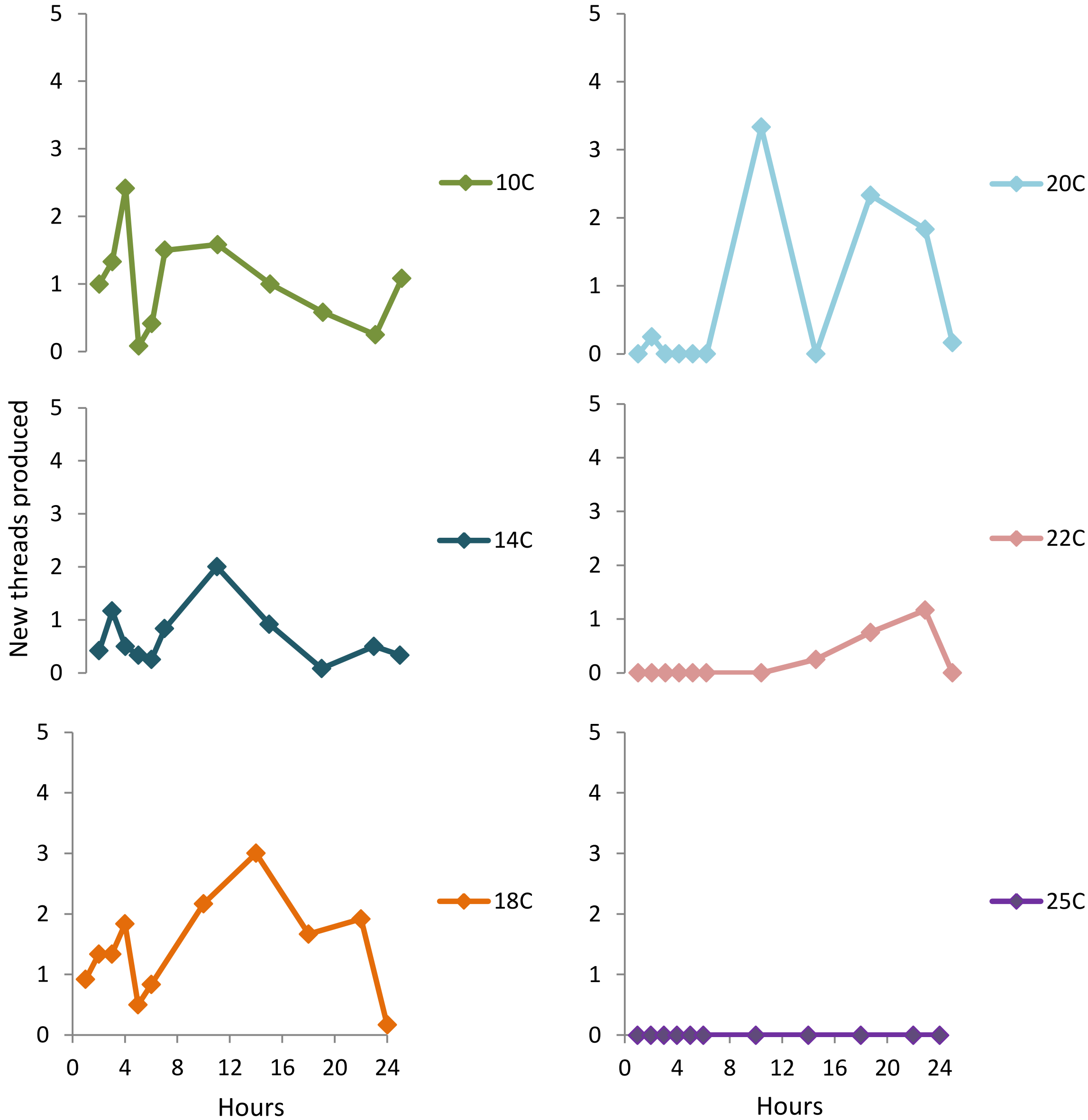

Fig. 6: The amount of new threads produced throughout each 24 hr temperature treatment trial. Thread counts are means (n=12 per temperature). Mussels in temperatures 10°C, 14°C, and 18°C generally showed trends of early thread production from hours 1-4, with another spike around hour 10. We observed that mussels took longer to produce threads in the warmer temperatures of 20°C and 22°C, where threads were not made until hours 10-22. No threads were produced in the warmest treatment (25°C).

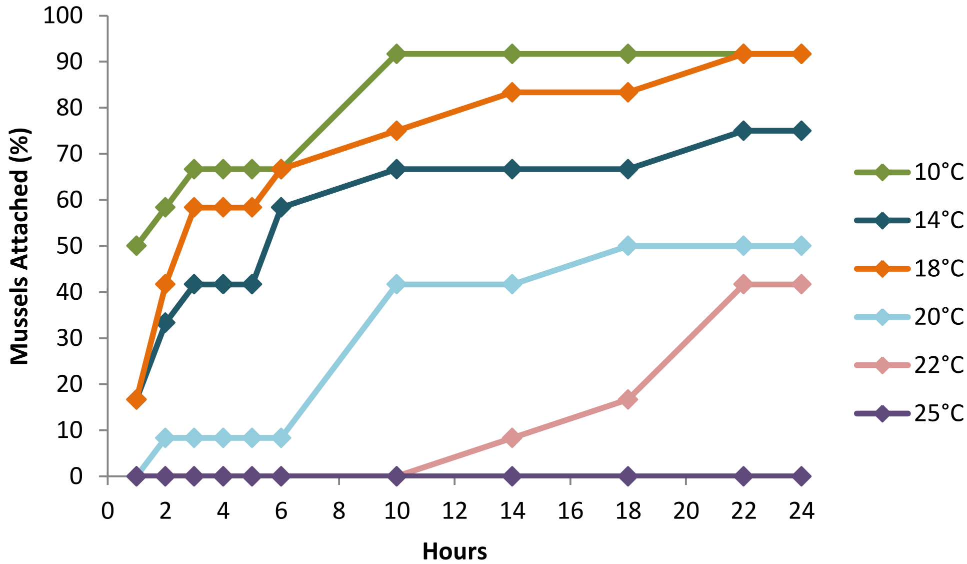

Fig. 7: Percentage of mussel attachment during each 24 hr temperature treatment trial. A mussel was counted as "attached" if one or more byssal threads were attached to the acrylic sheet bottom or to another mussel. Each trial consisted of 12 different mussels, ranging in size from 40-50 mm long. We found a significantly higher percentage of attachment ($F_{5,66}$=9.900; $p<0.0001$) in the cooler to mid-range temperatures (75-91%) than in the warmer trials (0-50%). Overall, mussels took longer to acclimate, or never acclimated, to the warmest temperatures. Attachment in the cooler to mid-range temperatures generally occurred in hours 1-3, while in the higher temperatures it occurred between hours 10-22.

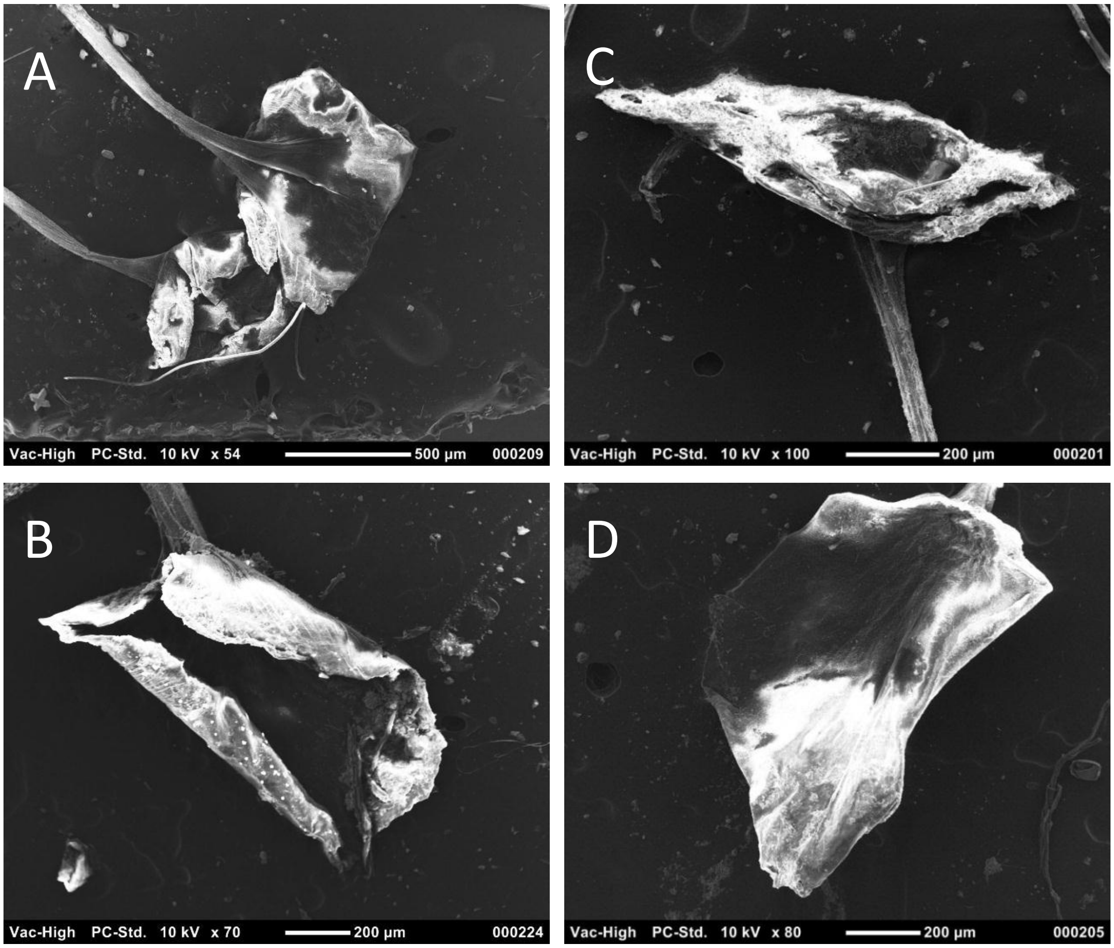

Fig. 8: Byssal thread plaques. SEM comparisons of plaques between 4 of 6 temperature treatments: (A) 10°C, (B) 18°C, (C) 20°C, and (D) 22°C. No distinct degradation could be seen in any of the samples observed. Plaque curling/distortion was likely caused by the drying process.

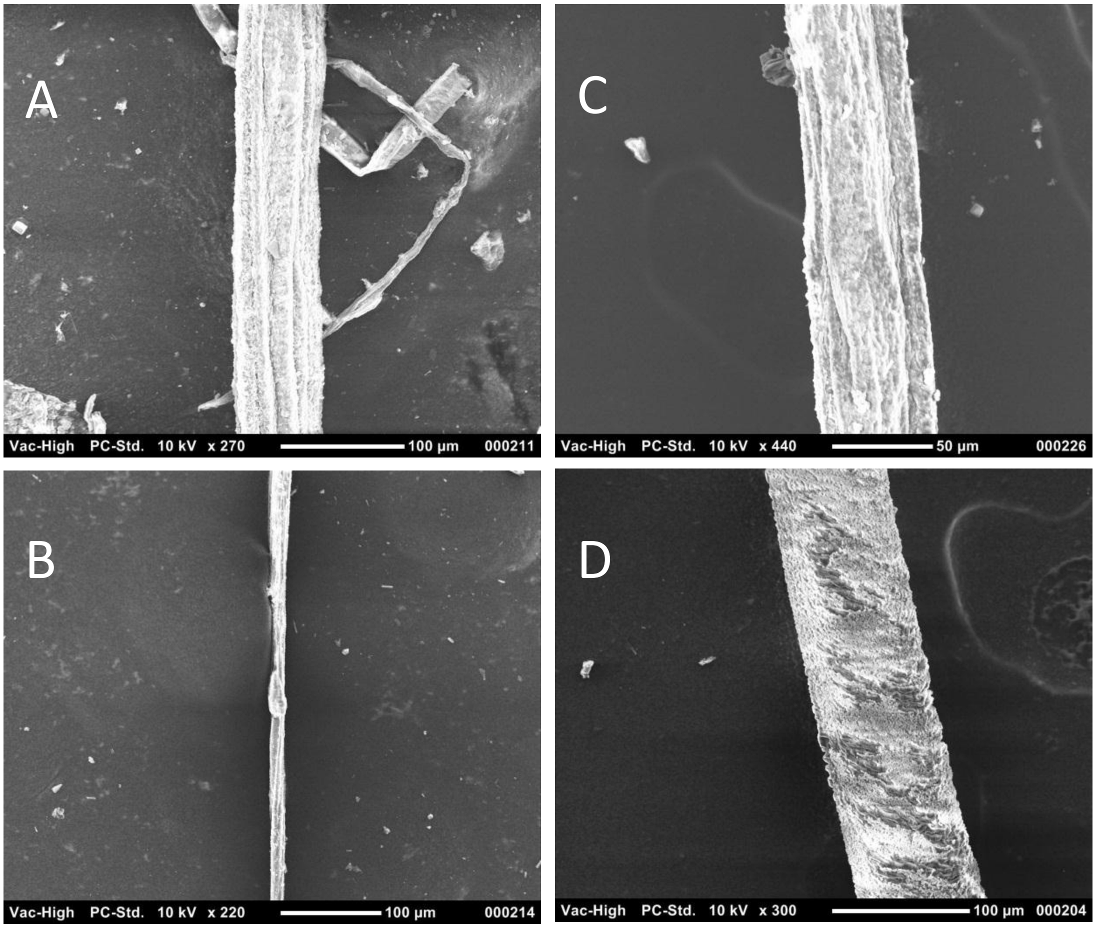

Fig. 9: Byssal threads. SEM comparisons of threads between 4 of 6 temperature treatments: (A) 10°C, (B) a spawning mussel's thread at 14°C, (C) 18°C, and (D) 22°C. The fibrous thread core was distinctly visible on most threads at 10°C, 14°C, 18°C, and 20°C. Cuticle thread damage may have occurred to a lesser degree at 22°C, but ultimately we were unable to detect significant differences in thread quality between temperature treatments due to the amount of variability within treatments.