

Elevated temperature and ocean acidification alter mechanics of mussel attachment

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**Abstract**

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Global climate change by way of warming ocean temperatures and ocean acidification threatens the survival of marine organisms. For mussels, survival is tightly tied to byssal threads they form that anchor them to substrates, from the rocky intertidal to mussel aquaculture lines. Weakened byssal threads increase the likelihood of “fall-off” or “sloughing” from rocks and aquaculture lines, disturbing intertidal communities and reducing aquaculture yields. Seasonal variation in mussel attachment strength suggests environmental conditions may alter byssal thread strength and production. In this dissertation, I explore the role of rising temperature and ocean acidification on the mechanical performance of mussel byssal threads to improve predictions of what environmental conditions may precede fall-off events. In Chapter 1, I expose mussels (*Mytilus trossulus*) to a range of pH (7.3 – 8.2, total scale) and temperature conditions

(10 – 25°C) in a full factorial cross. Elevated temperature dramatically weakens mussel attachment: mussels produce 60% weaker and 65% fewer threads at 25°C in comparison to 10°C. The effects of temperature are strongest in the proximal region of the threads. Low pH (7.3) strengthens the plaque region of the thread by 20%, but has no effect on overall byssal thread strength. Since a thread is only as strong as its weakest region, these stressors do not act synergistically with each other; the strongest negative effect dominates, in this case temperature. Chapter 2 expands on the impacts of temperature on attachment strength, finding species-specific temperature effects. In the northeast Pacific, the warm-adapted mussel *M. galloprovincialis* and cold-adapted mussel *M. trossulus* compete for space on shore. While the attachment strength of these two closely related species does not differ from 11 - 18°C, at temperatures from 18 - 24.5°C, *M. trossulus* attachment strength decreases while *M. galloprovincialis* attachment strength increases. At temperatures greater than 18°C *M. trossulus* produces fewer and weaker byssal threads with attachments that were up to 93% weaker than *M. galloprovincialis*. Chapter 3 follows up these laboratory studies using the field setting of a mussel farm to examine correlations between ocean conditions and attachment strength in *M. trossulus*. In the field, weak attachment strength in *M. trossulus* is best predicted by high temperature >14°C and low pH, <7.5. Similar to the lab study, the effects of these two stressors are independent of each other. Altogether, these results find mussels and the communities they support are vulnerable to multiple aspects of future ocean conditions. Warming oceans may increase the competitive advantage of *M. galloprovincialis* in more northern latitudes at the expense of native *M. trossulus* populations. Monitoring these conditions near farms can signal periods where attachment is expected to be weak in order to best adapt farming practices.

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## Chapter 1

### **Elevated temperature weakens mussel attachment independent of pCO<sub>2</sub>**

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## 1.1. Abstract

Rising ocean temperature and pCO<sub>2</sub> are current threats to marine organisms, compromising survival, growth, and the materials organisms make to persist in their environment. For mussels, survival is tightly tied to the byssal threads they form to anchor themselves. Long-term exposure to elevated pCO<sub>2</sub> has been implicated in weakening byssal thread strength. We expand on this recent finding by examining how the additional stressor of temperature, combined with different pCO<sub>2</sub> exposure times, influences byssal thread strength. Field collected mussels (*Mytilus trossulus*) produced threads in controlled temperature and pCO<sub>2</sub> treatments for a set duration, then threads were counted and pulled to failure to determine strength. We found elevated temperature dramatically weakens mussel attachment: mussels produced 60% weaker and 65% fewer threads at 25°C in comparison to 10°C. Contrary to our expectations, byssal threads showed a subtle positive response to short-term pCO<sub>2</sub> exposure. Elevated temperature and pCO<sub>2</sub> act on different regions of the thread; their impacts are therefore independent of each other. Our tests of the effects of pCO<sub>2</sub> exposure duration on byssus strength were inconclusive, with no pCO<sub>2</sub> effect detected at either exposure of 3 d or 32 d. Altogether, these results show mussels and the communities they support will be vulnerable to multiple aspects of future ocean conditions, but temperature may have the most important impacts.

## 1.2. Introduction

Ocean conditions are changing at rates unprecedented in geological history and are projected to have wide scale and mostly negative effects on marine organisms (Doney, 2010; Kroeker et al., 2013). Coastal marine environments experience highly variable conditions on a daily and seasonal basis with changes in tidal height, productivity, sunlight, and riverine inputs. For example, coastal organisms experience changes in dissolved pCO<sub>2</sub> over a single tide cycle that are greater than the most extreme open ocean predictions for 2100 (Wootton et al. 2008, IPCC, 2013). Global climate change and ocean acidification may cause the already highly variable conditions to become more extreme (Duarte et al., 2013; Hofmann et al., 2011). Organisms that could once tolerate these extremes may now be pushed beyond their physiological tolerance (Hoegh-Guldberg, 1999).

These changes in environmental conditions can have a wide range of effects on organisms at different life stages (Byrne, 2011). Building strong materials, such as shells, calcified skeletons, and spines, are functionally important for organisms to persist in their environment. However, changing ocean conditions can weaken these materials (Collard et al., 2015; Gaylord et al., 2011; Madin et al., 2008; O'Donnell et al., 2013, Newcomb et al. 2015). Changes in performance may be due to the direct interaction of the environment and a biological material (Waite and Broomell, 2012). Alternatively, growing and living in adverse water conditions may be energetically costly, potentially reducing the resources devoted to building strong and durable materials (Ivanina et al., 2013; Michaelidis et al., 2005).

For mussels, an ecologically and economically critical species, survival is dependent on their attachment structure, collagen-like byssal threads. These threads tether mussels to solid substrate, providing protection from dislodgement by predators and waves. A thread's ability to



yield, extending without incurring greater load, enables the recruitment of multiple threads to share the load, keeping a mussel anchored (Bell and Gosline, 1996). Multiple studies have shown this attachment structure weakens seasonally, likely due to low thread number and/or quality, resulting in mussel dislodgement or fall-off events (Carrington, 2002; Carrington et al., 2009; Lachance et al., 2008; Moeser and Carrington, 2006). This strong seasonal pattern suggests adverse water conditions may reduce mussel attachment strength.

One link between environmental conditions and byssal thread strength was identified by O'Donnell et al. (2013), where elevated pCO<sub>2</sub> (greater than 1200 µatm) lowered byssal thread strength in the mussel *Mytilus trossulus*. Carrington (2002) and Carrington et al. (2009) find strong correlations between elevated temperature and weak attachment strength. Elevated temperature is known to have strong impacts on mussel physiological performance, including increased metabolism and other cellular processes until reaching a lethal point (Braby and Somero, 2006; Somero, 2002), and increased activation of heat-shock proteins and depressed heart rate at temperatures above 20°C (Braby and Somero, 2006; Byrne, 2011; Tomanek and Zuzow, 2010). The ability of elevated temperature to alter thread strength has not been studied in isolation nor in combination with pCO<sub>2</sub>.

Previous studies examining these two stressors find when acting concurrently, temperature and pCO<sub>2</sub> do not always affect bivalves in a manner that can be deduced from their individual effects. For example, elevated pCO<sub>2</sub> did not change oyster survival at 22°C, but increased oyster survival at 27°C (Ivanina et al., 2013). This same study found synergistic effects of elevated temperature and pCO<sub>2</sub> on carbonic anhydrase activity; these two stressors together exerted a stronger negative effect on calcification than either did alone. Intermediate temperatures (16 – 20°C) offset negative effects of elevated pCO<sub>2</sub> (1200 µatm) on mussel growth

whereas cold (12°C) and warm (24°C) temperature did not (Kroeker et al., 2014). It is therefore important to test elevated temperature and pCO<sub>2</sub> together to understand their combined effects on byssal thread strength.

In addition to the concurrent stress of elevated temperature, the time mussels are exposed to elevated pCO<sub>2</sub> conditions may impact the magnitude and direction of their response. When exposed to elevated pCO<sub>2</sub> conditions over a relatively long time, some organisms are able to acclimate to these conditions and improve performance (Form and Riebesell, 2011). Alternatively, extended exposure to stress can weaken the overall energetic condition of the organism, which in turn can affect key processes such as the formation of biomaterials (Carrington et al. 2015). Because coastal organisms such as mussels experience acute exposure to high pCO<sub>2</sub> on the scale of hours to months (Wootton et al. 2008), the effect of exposure time to elevated pCO<sub>2</sub> on byssal threads warrants further consideration.

The goal of this study is to evaluate how pCO<sub>2</sub> alters byssal thread strength in combination with elevated temperature and exposure time. We expose mussels (*M. trossulus*) to a range of temperature and pCO<sub>2</sub> conditions over a short duration (3 d) to determine the effects of temperature on byssal thread strength. We uncover a mechanism by which temperature and pCO<sub>2</sub> act as concurrent stressors and explore how the duration of elevated pCO<sub>2</sub> exposure mussels experience impacts thread strength. Our results find (1) elevated temperature weakens thread strength, (2) elevated temperature and pCO<sub>2</sub> act independently on byssal thread strength, and (3) exposure duration to elevated pCO<sub>2</sub> did not change the response of byssal threads.

### 1.3. Methods

#### 1.3.1. General Experimental Mesocosm Setup

Mussels (*M. trossulus*) were collected from Argyle Creek, San Juan Island, WA (48.52° N, 123.01° W) and held in a mesh box submerged under the dock at Friday Harbor Laboratories (FHL), San Juan Island, WA for up to 14 d until the beginning of the experiment. Mussels were housed in experimental organismal mesocosms in the Ocean Acidification Experimental Laboratory (OAEL) at FHL as described in O'Donnell et al. (2013) and Timmins-Schiffman et al. (2012). Briefly, manipulations of temperature and pH were made in a 150 L water reservoir, which supplied water to eight 3.5 L containers at a turnover rate of 50 ml min<sup>-1</sup>. Submersible pumps (model number P396, Annex Depot, Sacramento, CA) provided mixing in the chambers at 3.8 L min<sup>-1</sup>. The bottom of each chamber was lined with pebbles, collected from an FHL beach and autoclaved and sterilized to provide a substrate for byssal thread attachment.

pH and temperature were monitored continuously in each water reservoir with a Durafet pH and temperature electrode probe (Honeywell Durafet III pH electrode, Fort Washington, PA). Probe pH was calibrated prior to each trial with dissolved inorganic carbon (DIC) using an IR analyzer and total alkalinity (TA) measurements, taken following SOP3b from Dickson et al. (2007). pH was calculated on the total scale in CO<sub>2</sub>Calc v1.2 based on DIC and TA using the following constants: CO<sub>2</sub> K1, K2 from Mehrbach et al. (1973), KHSO<sub>4</sub> from Dickson (1990) and total Boron from Lee et al. (2010). Certified reference materials from the Dickson laboratory were run on DIC and TA to ensure accuracy. During the experiment, pH and temperature were recorded every minute by a Honeywell dual input analytical analyzer (UDA 2182, Fort Washington, PA). Daily measurements of salinity (Hach Sension5, Loveland, CO) and temperature (Fluke 1523 Reference Thermometer, Hart Scientific, Everett, WA) were made in

the specimen chambers. Average pCO<sub>2</sub> for each treatment was calculated from average pH over the 3 day trial and the measured alkalinity. Error in pH and alkalinity measurements were propagated to average pCO<sub>2</sub> using the methods of Ellison et al. (2000) and sensitivity coefficients of Dickson and Riley (1978).

### *1.3.2. Byssal Thread Mechanical Testing and Morphology*

At the end of the experiment, mussels and the rocks to which they had attached with byssus were removed from the specimen chambers. Byssus was dissected from each mussel and air-dried overnight before storing ~ 20 days in an air-tight plastic bag prior to testing. Byssus was rehydrated in seawater prior to testing, a method that does not alter the properties of the byssal threads (Brazee, 2004). The number of byssal threads each mussel produced was counted, and one byssal thread was haphazardly chosen for mechanical testing following the procedure of Bell and Gosline (1996). An individual thread was clamped with submersible pneumatic grips on either end by holding the proximal byssal stem between cardstock with cyanoacrylate glue and affixing the distal plaque with attached rock to an aluminum T-bar with 5 minute epoxy. Using an Instron 5565 tensometer (Norwood MA, USA), a thread was extended until failure at a rate of 10 mm min<sup>-1</sup> in a temperature controlled water bath (3130-100 BioPuls Bath, Instron, Norwood, MA, USA). Tests were performed in seawater with a pH of 7.8 and the relevant treatment temperature. The tensometer measured force (thread strength, ±10<sup>-5</sup> N) and extension (± 10<sup>-5</sup> mm) every 0.1 sec.

Pull to failure mechanical tests provided estimates of thread breaking strength, yield strength, extensibility, and initial stiffness (Bell and Gosline, 1996). Yield strength was determined by the point where the initial slope of the force extension curve decreased by 40%.

Extensibility, a measure of how much a thread extends before breaking, was calculated by dividing thread extension at failure by initial length. Initial stiffness was determined from the initial slope of the force extension curve. The location of failure (proximal, plaque, and/or distal region) was noted and threads were retested to quantify the breaking strength of each remaining region. Tests that broke at the grips were considered underestimates, and these samples were either retested if possible, or discarded.

The cross sectional area of the proximal region was measured to evaluate morphological differences among treatments. The proximal region has an elliptical shape; area was estimated by measuring the major and minor axes of the ellipse (Brazee and Carrington, 2006). The major axis was estimated by measuring thread diameter 3 mm from the plaque to the nearest 0.001 mm using a dissecting microscope. The minor axis was measured by flattening the proximal region between two thin metal sheets. The distance between the sheets was photographed with a dissecting scope and measured to the nearest 0.001 mm with imageJ v 1.49 (Rasband, 2008). Proximal breaking stress ( $\text{N mm}^{-2}$ ), a material property, was calculated as proximal breaking strength divided by proximal area. Threads were air-dried, mounted on carbon tape, and sputter coated with Au/Pd for imaging in FEI Sirion XL30 SEM (Hillsboro, OR) to explore differences in thread surface structure.

### *1.3.3. Scaling up to whole mussel attachment strength*

Whole mussel attachment strength was estimated for mussels pulled parallel and normal to the substrate using two models developed in Bell and Gosline (1996). These models assume a mussel is anchored with 50 threads arranged in a circle. The parallel model assumes a dislodgement force parallel to the substrate (e.g., drag); threads on the upstream side are the first

in tension, yield and extend until they reach maximum strain and break, while more threads are recruited into tension until they have all broken. The normal model assumes a dislodgement force perpendicular to the substrate (e.g. lift); all threads are engaged and extend until they reach their maximum force. A key modification to the model made here was to incorporate the variation in number of threads produced across treatments. Mussels in the treatment with the greatest mean number of threads produced were assigned the maximum value of 50 threads (so as to compare the additional effect of thread number with thread strength alone), while the thread number for mussels in the other treatments was scaled down in proportion to the mean number of threads produced.

#### *1.3.4. Mussel Condition*

Mussel body condition was measured following Moeser et al. (2006) to assess possible changes among treatments. Following dissection to remove byssus, gonadal and somatic tissue were separated and dried to a constant weight at 60°C. Body condition ( $\text{g cm}^{-3}$ ) was calculated as total tissue dry weight divided by shell length cubed. Gonad index was calculated as gonad dry weight divided by the total tissue dry weight (Carrington, 2002).

#### *1.3.5. Experiment 1: Short-term exposure to elevated temperature and $p\text{CO}_2$*

Following the general methods listed above, this experiment was run in May 2012. Mussels were acclimated to their treatment temperatures in ambient pH (~7.8) over 9 d, ramping temperature up no more than 2°C per 1 d, and fed a maintenance level of Shellfish Diet 1800 (6 g  $\text{l}^{-1} \text{ day}^{-1}$ , Reed Mariculture, Campell, CA, USA).

Treatments were established with one of three temperatures (10°C, 18°C, or 25°C) and one of four pHs (7.3, 7.6, 7.8 or 8.1) for a total of twelve independent treatments. These ranges span local marine conditions (Feely et al., 2008; Murray et al. 2015; Nishizaki and Carrington, 2014; Wootton et al., 2008). Mussels were starved during the 3 d treatment trials to avoid changes in specimen chamber water chemistry due to food addition and to keep the chambers and byssal threads clean from possible fouling.

Before transfer to their experimental conditions, the byssus was cut. Mussels were maintained in their experimental treatments for 3 d to produce new byssal threads following the acclimation period, sufficient time for mussels to produce mature byssal threads (Bell and Gosline, 1996) while minimizing the effect of treatment on mussel condition. Three trials were conducted in succession to replicate treatments over time, increasing sample size (n=8 x 3) for each temperature\*pCO<sub>2</sub> treatment.

#### *1.3.6. Experiment 2: Long versus short-term exposure to elevated pCO<sub>2</sub>*

This second experiment was run in September 2013, following the general methods described above, to test the effects of pCO<sub>2</sub> exposure time on byssal thread strength. Mussels were randomly sorted (n=16) into one of six pH treatments ranging from 7.3 to 8.1 at 12°C and two exposure time treatments of 3 days and 32 days. While in their treatments, mussel were fed a maintenance level of Shellfish Diet 1800 (6 g l<sup>-1</sup> day<sup>-1</sup>, Reed Mariculture, Campell, CA, USA). For the 32 d treatment, existing threads were cut on day 29 in order to compare 3 d old threads among treatments. Strength was measured only in the plaque, the only thread region known to be sensitive to pCO<sub>2</sub> (O'Donnell et al., 2013).

### *1.3.7. Statistics*

Statistical tests were carried out in R version 2.13.0 (R Development Core Team, Vienna, Austria, 2011, packages: plotrix, RStudio, sciplot). Thread breaking strength (overall, proximal, plaque), distal yield strength, extensibility, and thread number produced were analyzed with a linear mixed effect models (lmem) with pCO<sub>2</sub> and temperature as fixed factors and trial as a random factor. Proximal region long axis, short axis, and breaking stress were compared among temperature treatments with lmem with temperature as a fixed factor and trial as a random factor because only temperature effects were significant in this region (see results). The effect of treatment on the region in which threads broke was tested with a Pearson's chi-squared test. Mussel condition index and gonad index were analyzed with lmem on means from each treatment with pCO<sub>2</sub> and temperature as fixed factors and trial as a random factor for experiment 1 and ANCOVA on means from each treatment with pCO<sub>2</sub> as covariate and exposure time as fixed factor for experiment 2. The influence of pCO<sub>2</sub> and exposure time on plaque strength was measured with an ANCOVA on means from each treatment, with pCO<sub>2</sub> as covariate and exposure time as fixed factors. The effect of treatment was considered significant with an  $\alpha \leq 0.05$  for all linear mixed effects model and ANCOVA tests. In order to run a post-hoc test we excluded the random effect of trial, and were detected differences among treatments using a Tukey's HSD post-hoc test.

## **1.4. Results**

### *1.4.1. Experiment 1*

Treatments reached within 5% of their targeted temperature and 2% of targeted pH values (Table S1). The pCO<sub>2</sub> for replicate treatment conditions varied 1% - 32% from the mean



of all trials; data from each trial were therefore not pooled but instead considered independent data points for statistical analyses.

Byssal threads produced at 25°C were 60% weaker than threads produced at 10°C and 18°C (Figure 1.1A, Table 1.1, lmem,  $p < 0.001$ ). pCO<sub>2</sub> did not influence whole thread breaking force (Table 1.1, lmem,  $p = 0.2$ ). The breaking strength of the proximal region was 70% weaker at 25°C in comparison to 10°C and 18°C (Figure 1.1B, Table 1.1, lmem,  $p < 0.001$ ) and the effect of pCO<sub>2</sub> was not significant (Table 1.1, lmem,  $p = 0.7$ ). Plaques produced at 25°C were 60% weaker than plaques produced at 10°C and 18 °C (Figure 1.1C, Table 1.1, lmem,  $p < 0.001$ ). Elevated pCO<sub>2</sub> increased plaque strength across all temperatures; plaques from the high pCO<sub>2</sub> treatment (2273 – 2838 μatm) were 20% stronger than plaques produced in the lower pCO<sub>2</sub> treatments. (Figure 1.1C, Table 1.1, lmem,  $p < 0.05$ ). Yield strength, a property of the distal region, was 60% weaker at 25°C, and was not influenced by pCO<sub>2</sub> (Figure 1.1D, Table 1.1, lmem, Temperature:  $p < 0.001$ , pCO<sub>2</sub>:  $p = 0.8$ ). Temperature and pCO<sub>2</sub> did not significantly interact for any of these mechanical properties (Table 1.1).

Threads produced at 25°C were 30% less extensible than threads produced at 10°C and 18°C (Figure S1.1A, Table S1.3, lmem,  $p < 0.001$ ). pCO<sub>2</sub> did not effect thread extensibility (Table S1.3, lmem,  $p = 0.3$ ). Similarly, threads at 25°C were 67% less stiff than threads made at 10°C and 18°C (Figure S1.1B, Table S1.3, lmem,  $p < 0.001$ ). pCO<sub>2</sub> did not effect thread stiffness (Table S1.3, lmem,  $p = 0.06$ ). As with breaking strength, there was no significant interaction of temperature with pCO<sub>2</sub> on thread extensibility or stiffness (Table S1.3). Figure 1E illustrates representative force-extension curves summarizing these collective differences in the mechanical properties of byssal threads made at 25°C versus 10°C and 18°C; high temperature threads were less stiff, did not yield, and were weaker and less extensible.

The proportion of proximal breaks increased with temperature (Figure 1.2, Pearson's Chi Squared,  $p < 0.5$ ), from 40% at 10°C to 60% at 25°C. Distal breaks were infrequent and only occurred at 25°C. pCO<sub>2</sub> did not affect the breaking location of the threads (Figure 1.2B, Pearson's Chi Squared  $p = 0.3$ ).

Mussels grown in 25°C produced 65% fewer threads than mussels in 10°C and 18°C (Figure S1.1C, Table S1.3, lmem,  $p < 0.001$ ). Neither pCO<sub>2</sub> nor the interaction of temperature and pCO<sub>2</sub> influenced thread number (Table S1.3, lmem,  $p = 0.5$  and  $0.2$ , respectively).

The major axis of the proximal region did not differ among temperature treatments (lmem,  $p = 0.08$ ), but at 25°C the minor axis was 27% than at 10°C, indicating that at 25°C, the proximal region of byssal threads are flatter (Table 1.2, lmem  $p < 0.001$ ). Proximal breaking stress was also 47% lower at 25°C (Table 1.2, lmem  $p < 0.05$ ). SEM images of proximal regions from 25°C suggest malformation in the outer cuticle, the proteinaceous coat on the outside of the thread shows an extra fringe extending from the major axis. This fringe was absent from all byssal threads formed at 10°C and 18°C (Figure 1.3).

The model for force to dislodge a mussel when pulled normal to the substrate estimates a 68% reduction in force at 25°C relative to 10°C and 18°C (Figure 1.4). When pulled parallel to the substrate, mussel attachment was 64% weaker at 25°C relative to 10°C and 18°C (Figure 1.4). When differences in thread number among temperature treatments were incorporated, the normal and parallel models for 25°C predict an 88% and 87% percent weaker attachment, respectively (Figure 1.4).

Reproductive condition and overall mussel condition did not differ among treatments (Table S4). The one exception was the 25°C treatment in the second trial, where condition index

was 30% lower, most likely due to spawning in the treatment (Table S1.4, lmem temperature:  $p < 0.05$ ).

#### *1.4.2. Experiment 2*

pH conditions were reached within 1% of targeted conditions for the 3 d treatment and 3% for the 32 d treatment (Table S1.5). Overall, neither pCO<sub>2</sub> nor exposure time effected plaque strength (Figure 1.5, ANCOVA, pCO<sub>2</sub>:  $p = 0.8$ , time:  $p = 0.9$ , pCO<sub>2</sub>\*time:  $p = 0.6$ ). pCO<sub>2</sub> and exposure time did not affect mussel condition index or gonad index (Table S1.6).

### **1.5. Discussion**

The major finding of this study is mussel byssal attachment strength decreases dramatically at 25°C. At this elevated temperature, byssal threads were weakened at all regions: proximal, distal, and plaque. Mussels with weaker byssal threads will have less available to resist a dislodgement force. Additionally, thread extensibility was also reduced in mussels at 25°C. Lower extensibility means fewer threads can be recruited to share the load to keep a mussel in place (Bell and Gosline 1996). Exacerbating these effects, mussels produced fewer threads at 25°C, reducing the number of load-bearing tethers. Altogether, mussels at 25°C produce fewer, weaker and less extensible byssal threads, resulting in an attachment estimated as 88% lower than mussels at 10°C and 18°C.

The observed 60% decrease in thread strength due to elevated temperature was both morphological and material. Although temperature weakened the entire thread, its effects were most pronounced in the proximal region. The ratio of proximal to plaque breaks increased from 1:1 to 3:2 at elevated temperature. The proximal region became the most likely region of failure

under elevated temperature, making it the weakest link in the thread. This region is reduced in cross sectional area at 25°C, providing less load bearing material. Moreover, the material itself was weaker, evidenced by a reduced breaking stress at 25°C. While the nature of this material change in the proximal region was not explored in detail, we did observe malformed cuticle coatings, with a wide fringe along the side of the thread. It is well known that fluid viscosity increases with temperature (Gosline et al., 2002; Wainwright et al., 1976), and it is possible that this altered morphology results from liquid collagen precursors leaking out of the groove of the mussel's foot before they solidify. High temperature may also change the shape of the groove itself. It is also possible that the changes in morphology are not a result of increased viscosity with temperature but due to changes in post-translational modification of proteins due to high temperature (Waite et al. 2005). Malformation has been observed in coral and squid larvae under elevated temperature (Polato et al., 2010; Rosa et al., 2012) and oysters and pteropods under elevated pCO<sub>2</sub> (Bednaršek et al., 2012; Waldbusser et al. 2015). The presence of malformed byssal threads may be a useful visual tool to identify temperature stress in mussels, similar to the way malformation has been used as a bioindicator of pCO<sub>2</sub> stress in pteropods (Bednaršek et al., 2012).

Unlike the proximal region, the adhesive plaque was influenced by both elevated temperature and pCO<sub>2</sub>. It is unknown whether the plaque material has altered chemistry and/or structure. It is possible that elevated temperature alters the function of plaque proteins, such as mefp3 and mefp5, that are involved in adhesion to the substrate surface (Lee et al., 2011; Waite et al., 1998). The strength of the plaque may also be influenced by the structure of the adhesive that is in contact with the rock as well as the biofilm on the rock (Lee et al., 2011). For example, a more foamy adhesive would reduce contact area, thereby weakening attachment. Further study

of plaque composition and structure under elevated temperature and pCO<sub>2</sub> would distinguish between these potential mechanisms.

In addition to changing thread strength, elevated temperature decreased the number of threads mussels produced. Threads may require more time to mold under elevated temperature, or perhaps mussels choose to wait out adverse conditions and make threads under more favorable conditions that result in stronger attachment. Living at 25°C is energetically costly to *M. trossulus* (Braby and Somero, 2006), thus energy reserves may be shifted away from byssus production towards other processes (Carrington et al., 2015). Previous studies on *M. edulis* have shown thread production increases as temperature ranges from 2°C to 18°C (Mooser et al., 2006; Young, 1985), likely due to temperature speeding up the reactions that lead to thread formation. Our results reported here suggest 25°C exceeds the point where temperature has a positive influence on thread production for *M. trossulus* and the temperature optimum for this species may lie between 18°C and 25°C. Mussels used in this study were collected from a location that experience temperatures as high as 25°C only on warm summer days (Nishizaki and Carrington 2014), suggesting temperature may play a role in defining mussel distribution limits.

Elevated pCO<sub>2</sub> was found to affect only plaque strength, as seen in O'Donnell et al. (2013). This result was most evident in the lower two temperature treatments in Experiment 1, where elevated pCO<sub>2</sub> increased plaque strength by 20% after 3 d of exposure. This result contradicts that of O'Donnell et al. (2013) who found that plaque strength decreased 40% in mussels exposed to high pCO<sub>2</sub> for 40 d. Because of this discrepancy between the two studies, we examined the interaction of exposure time and pCO<sub>2</sub> on byssal thread plaques. We found plaque strength was unaffected over a range of six different pCO<sub>2</sub> treatments in exposures of 3 and 32 d. Thus in three different experiments on the effects of pCO<sub>2</sub>, we have three different results: a

decrease in plaque strength (O'Donnell et al., 2013), an increase in plaque strength (Experiment 1), and no change (Experiment 2). Another consideration is that the Experiment 1 control treatment threads were 50% weaker than those in O'Donnell et al. (2013). These results suggest some other factor, as yet unidentified, modulates the susceptibility of byssal thread plaques to elevated pCO<sub>2</sub>. This factor could include uncontrolled differences among experiments such as fouling, nutrient levels, and/or initial mussel condition.

Regardless of the direction or magnitude of the pCO<sub>2</sub> effect, synergistic or additive effects of elevated temperature and pCO<sub>2</sub> are not the only options to consider when evaluating the impacts of multiple stressors on byssal threads. Instead, this study finds that depending upon the conditions byssal threads are made, one factor dominates. This is due to the structure of the threads comprising regions connected in series. Like links of a chain, byssal threads are only as strong as their weakest region, the “weak link” on the thread (Figure 1.6). The factor with the strongest effect on a link will therefore dictate where a byssal thread breaks. More modest effects of other environmental variables on other links will not be readily observable. For example, even though elevated pCO<sub>2</sub> increased plaque strength in Experiment 1, elevated temperature caused byssal threads to break in the weakened proximal region before any increase in plaque strength could be realized. In general, the stressor with the greater effect, in this case temperature, will dictate thread strength.

In summary, we find elevated temperature weakens mussel attachment strength by reducing both the quality and quantity of the byssal threads produced. Further, this study uncovers the mechanism by which elevated temperature and pCO<sub>2</sub> act as concurrent stressors on byssal thread strength. The two stressors operate independently because they act upon different regions of the byssal thread. This important finding allows us to extrapolate these results to

ocean conditions to predict how combinations of stressors will influence mussel attachment strength. We can apply this framework to evaluate how other stressors will act in combination if we know the region of the thread they act upon. Strong attachment is integral to a mussel's survival and these results show elevated temperatures predicted by global climate change pose a threat to this ecologically and economically important species. Our understanding of the mechanism behind how these concurrent stressors can weaken mussel byssus allows us to predict sensitive periods for mussel attachment and allow us to better understand how increases in global temperature and  $p\text{CO}_2$  will impact future mussel populations.

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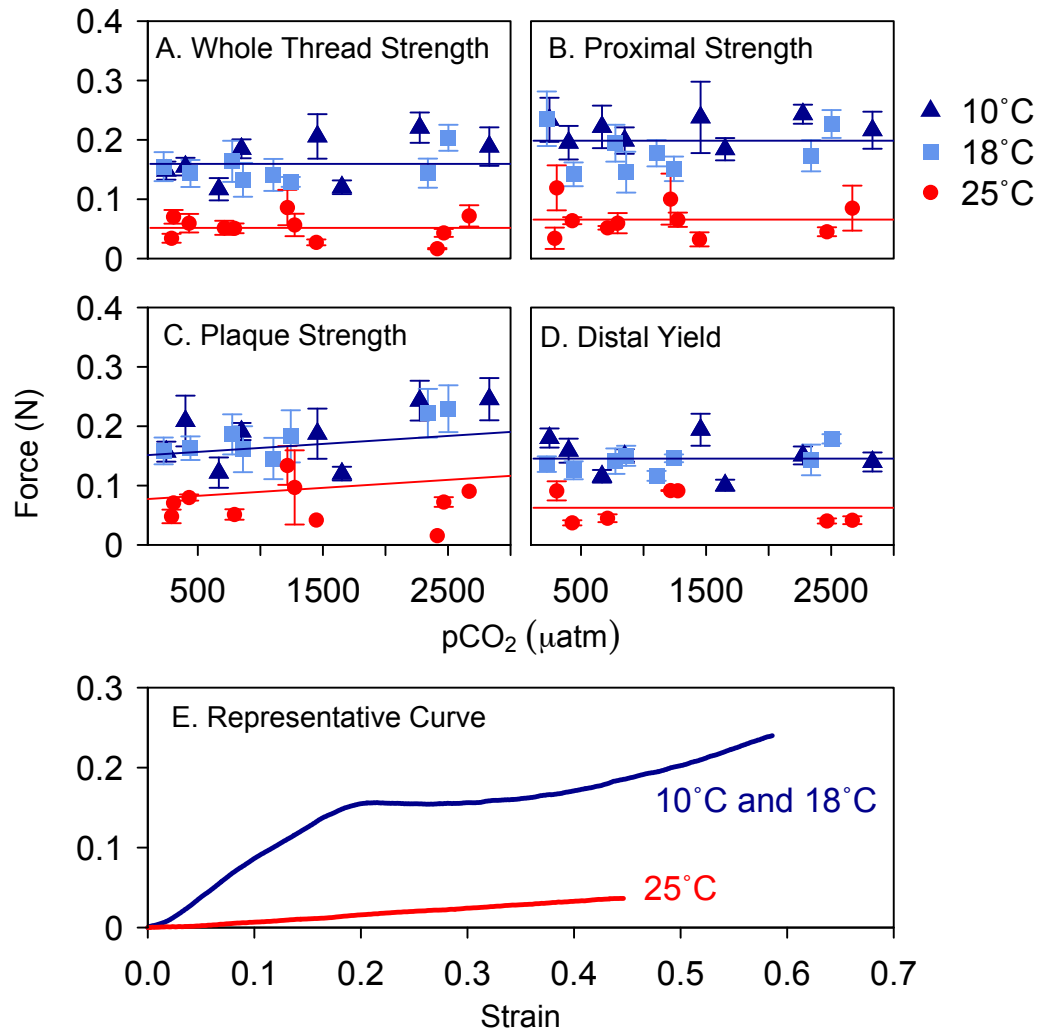
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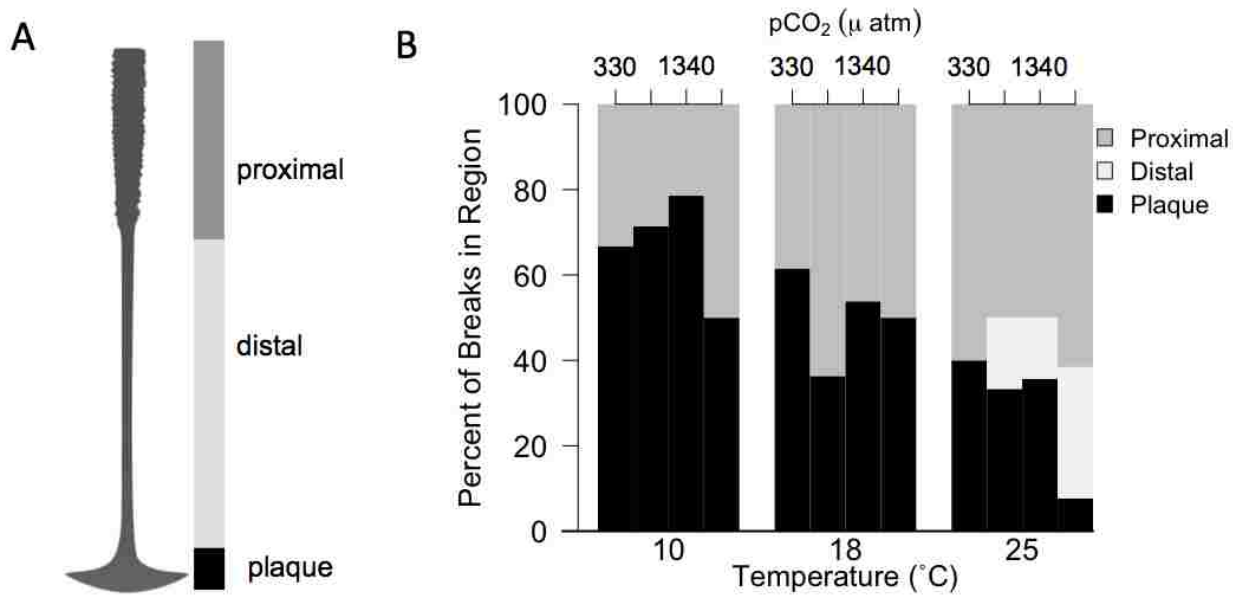
## 1.7. Tables and Figures



**Figure 1.1.** Byssal thread strength as a function of temperature and pCO<sub>2</sub> for (A) whole thread breaking strength, (B) proximal region breaking strength, (C) adhesive plaque breaking strength, and (D) distal yield strength. See Table 1.1 for statistical summary. Thread strength decreased at 25°C for all regions (ANCOVA,  $p < 0.001$ ). Plaque strength was the only region sensitive to pCO<sub>2</sub>, with a weak positive response (ANCOVA,  $p < 0.05$ ). Lines represent linear fit of strength versus pCO<sub>2</sub>. For all panels, 10°C and 18°C were not statistically different and data were therefore pooled to form one line. Symbols are means  $\pm$  SEM; N reported in Table S2. (E) Representative force versus extension curve of a mussel byssal thread at high (25°C) and low (10°C or 18°C) temperature. A low temperature thread is initially stiff, extends and yields in the distal region then stiffens until it breaks in either the plaque or proximal region. Threads formed at high temperature (25°C) are less stiff, do not yield, and are weaker and less extensible.

**Table 1.1.** Summary of statistical analyses of elevated temperature and pCO<sub>2</sub> on byssal thread mechanics. Linear mixed effect models were run with pCO<sub>2</sub> and temperature as fixed factors and trial as a random factor on each of the dependent variables: whole thread strength, plaque strength, proximal strength, and distal yield. Temperature and pCO<sub>2</sub>\*Temperature models are compared to the null model of pCO<sub>2</sub>. N= 8 -11 pCO<sub>2</sub> levels per temperature treatment (see Table S1.2).

			<b>Whole Thread Strength (N)</b>	<b>Proximal Strength (N)</b>	<b>Plaque Strength (N)</b>	<b>Distal Yield (N)</b>
random effect of trial		stdev	0.000	0.000	0.000	0.000
ANOVA on lmem	Temperature	$\chi^2$	45.9	50.0	35.2	28.9
		d.f.	2	2	2	2
		p	<b>&lt; 0.001</b>	<b>&lt; 0.001</b>	<b>&lt; 0.001</b>	<b>&lt; 0.001</b>
	pCO <sub>2</sub>	$\chi^2$	2.0	0.13	4.4	0.09
		d.f.	1	1	1	1
		p	0.16	0.7	<b>&lt; 0.05</b>	0.8
	Temperature*pCO <sub>2</sub>	$\chi^2$	4.2	0.32	4.8	3.2
		d.f.	2	2	2	2
		p	0.12	0.9	0.9	0.2
Tukey HSD	10°C - 18°C		0.5	0.9	0.06	0.9
	10°C - 25°C		<b>&lt; 0.001</b>	<b>&lt; 0.001</b>	<b>&lt; 0.001</b>	<b>&lt; 0.001</b>
	18°C - 25°C		<b>&lt; 0.001</b>	<b>&lt; 0.001</b>	<b>&lt; 0.001</b>	<b>&lt; 0.001</b>



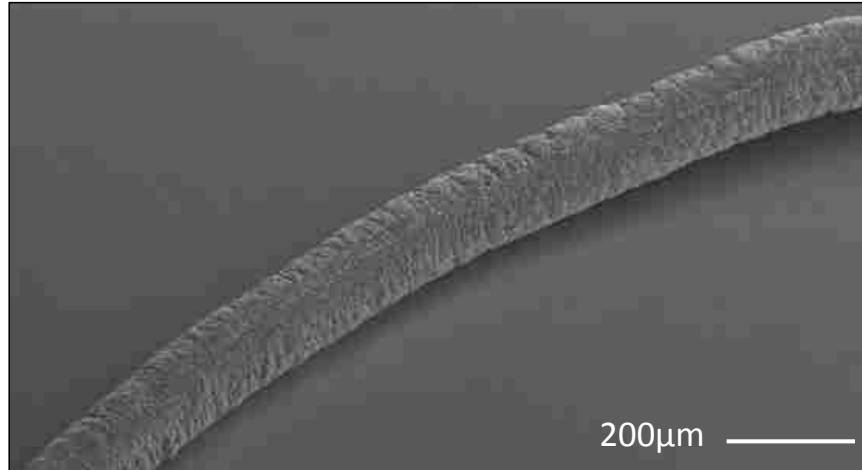
**Figure 1.2.** (A) Diagram of a byssal thread, with morphologically and chemically distinct proximal, distal, and plaque regions. (B) Percentage of failures in each region for each temperature and pCO<sub>2</sub> treatment. With increased temperature, the location of failure is biased toward the proximal region (Pearson's Chi Squared,  $p < 0.05$ ). Distal breaks were infrequent and only observed in the 25°C treatments. pCO<sub>2</sub> did not affect break location (Pearson's Chi Squared,  $p = 0.3$ ).

**Table 1.2.** Increased temperature alters proximal region morphology and material strength. Compared to threads made at 10 or 18 °C, those made at 25°C showed no difference in major axis (lmem, p = 0.08), but were thinner in the minor axis (lmem, p < 0.001). Proximal region breaking stress, calculated as proximal breaking strength divided by proximal area, was reduced at 25°C (lmem, p < 0.05). Significantly different treatments are bolded.

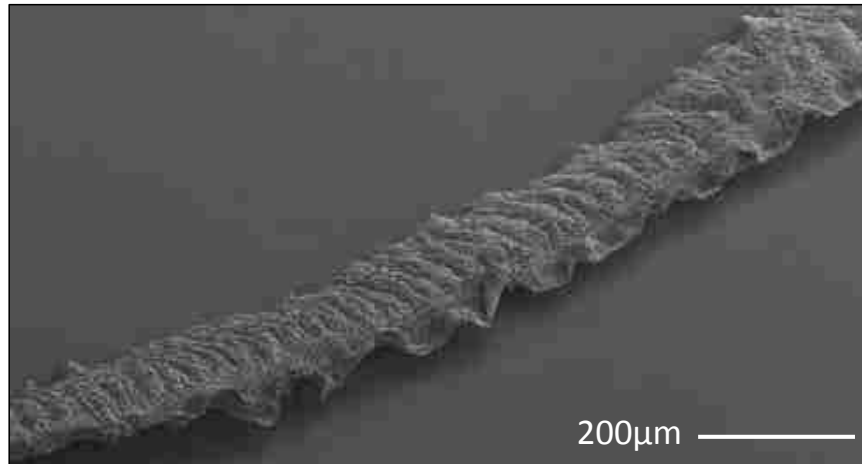
Temperature	Proximal Major Axis (mm)	Proximal Minor Axis (mm)	Proximal Stress (N mm <sup>-2</sup> )
10°C	0.127 ± 0.005	0.036 ± 0.002	12.75 ± 1.59
18°C	0.138 ± 0.005	0.034 ± 0.002	15.31 ± 2.17
25°C	0.121 ± 0.005	<b>0.023 ± 0.002</b>	<b>6.73 ± 0.90</b>



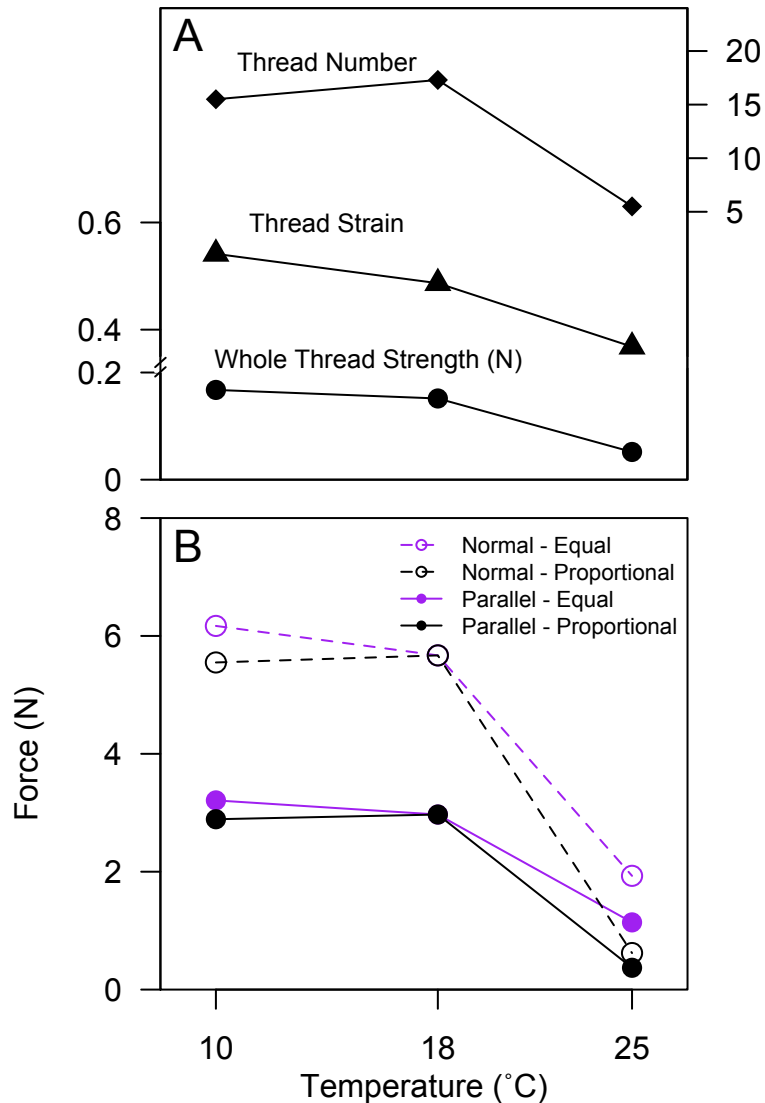
A. 10°C and 18°C



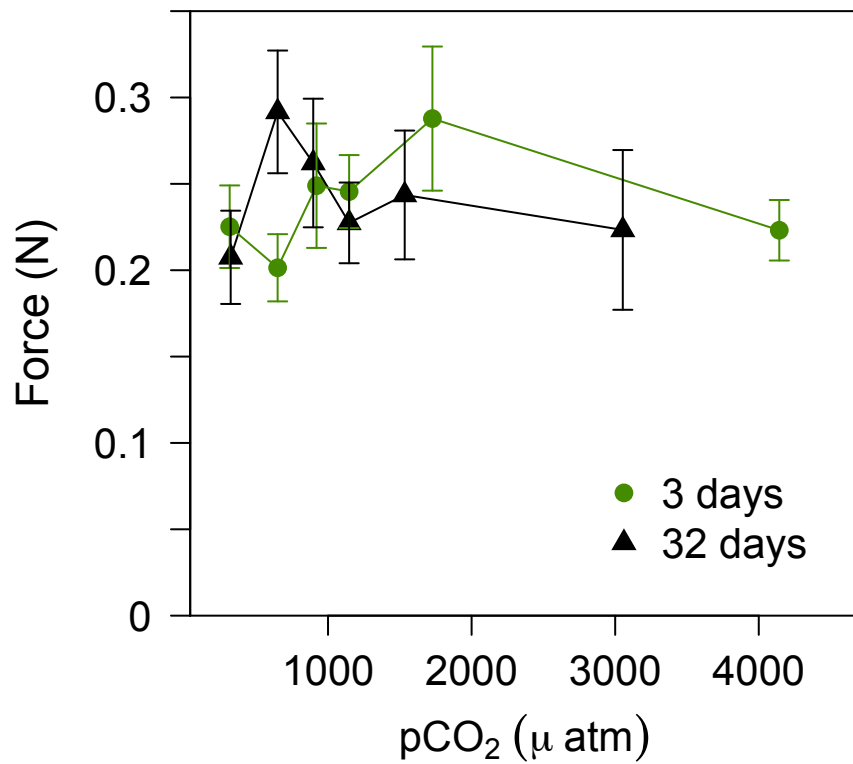
B. 25°C



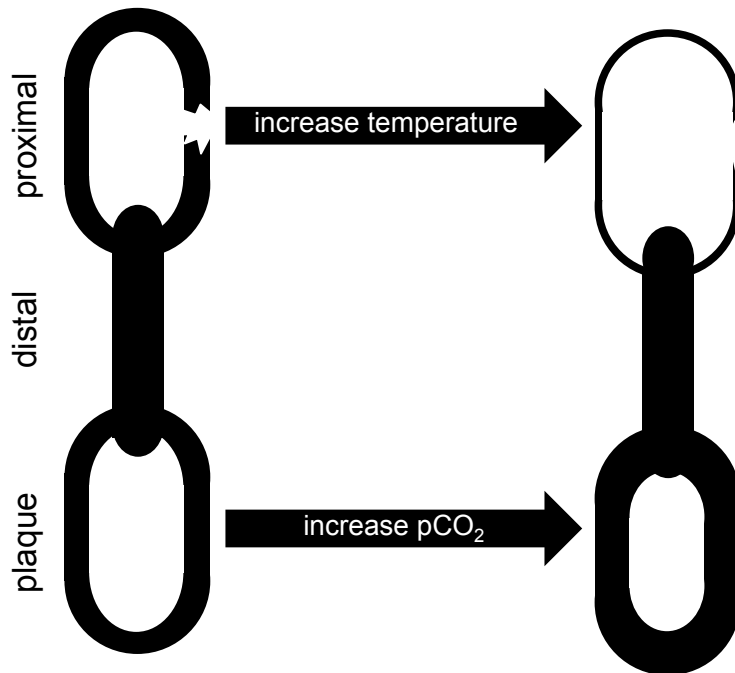
**Figure 1.3.** SEM images of proximal regions of byssal threads produced in (A) 10°C and (B) 25°C. Threads in 25°C are flatter and appear malformed, with an added fringe of cuticle along the length of the thread.



**Figure 1.4.** (A) Model Inputs: Thread number, thread extensibility, and whole thread strength are depressed at 25°C. (B) Model Outputs: The force to dislodge a whole mussel as predicted by simulating a pull parallel or normal to the substrate (modified from Bell and Gosline 1996). Models were run with either equal thread number or proportional thread number (incorporating differences in thread production among treatments). Whole mussel attachment decreases dramatically (at 25°C) under all scenarios.



**Figure 1.5.** Byssal thread plaque strength as a function of pCO<sub>2</sub> following exposure times of 3 and 32 days. Plaque strength is independent of pCO<sub>2</sub> and exposure time (ANCOVA, pCO<sub>2</sub>: p = 0.8, time: p = 0.9, pCO<sub>2</sub>\*time: p = 0.6, N=16).



**Figure 1.6.** The mechanism behind elevated temperature and pCO<sub>2</sub> as independent stressors. The three thread regions are connected in series, like links of a chain, to keep a mussel attached. Elevated temperature weakens all regions but shifts the location of failure to the weakest link, the proximal region. Elevated pCO<sub>2</sub> alone strengthens the plaque, but in combination with elevated temperature no pCO<sub>2</sub> effect is seen because threads have already broken at the weakest link, the proximal region. Once one stressor breaks a thread, the effect of the other stressor is masked and has no apparent effect on thread strength.

## **1.8. Acknowledgements**

We thank Jennifer Ruesink, Carolyn Friedman, Hilary Hayford, Rebecca Guenther, Molly Roberts, Jaquan Horton, and Nicki LeBaron for insightful discussions that improved the quality of this manuscript. Michelle Herko provided valuable assistance in the OAEL. Funding was provided by the National Science Foundation (EF104113 to E.C.), L.A.N. was supported by a National Science Foundation IGERT Program on Ocean Change Fellowship (1068839), and several grants from UW Friday Harbor Laboratories and UW Biology (Edmondson Award, Richard and Megumi Strathmann Fellowship, Stephen and Ruth Wainwright Fellowship, Patricia Dudley Fellowship, and Carrington Student Travel Fellowship). Data are archived under project #2250 at [www.bco-dmo.org](http://www.bco-dmo.org).

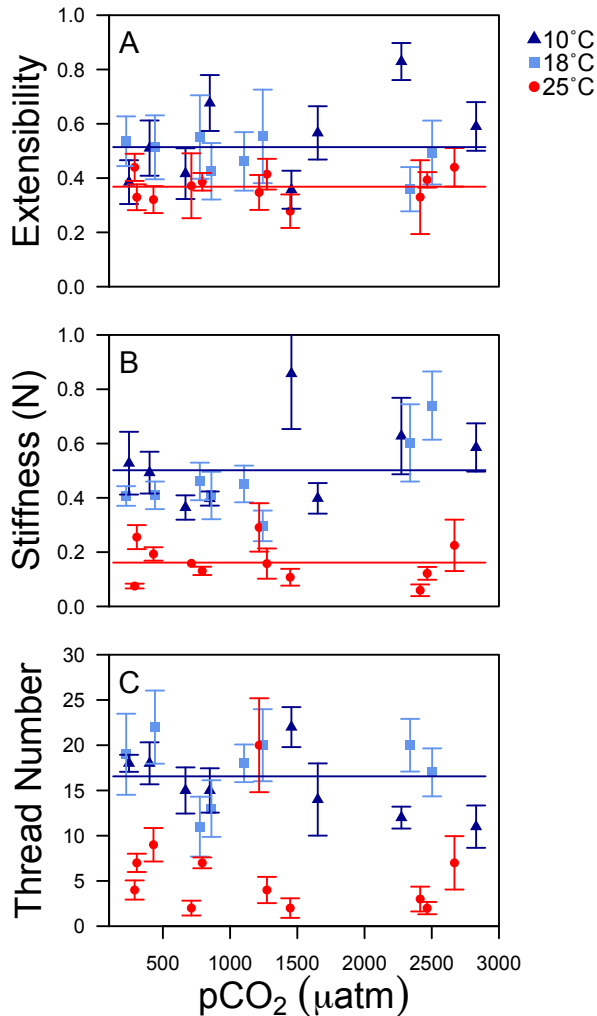
## 1.9. Supplementary Tables and Figures

**Table S1.1.** Water conditions in the different treatments in all trials. Temperature and pH are mean  $\pm$  SD from monitoring every minute in the water reservoirs. pH is reported on the total scale. Total alkalinity (Talk) represents the mean of 7-9 samples taken from randomly selected chambers in different reservoirs and is reported for all treatments across a trial. Dissolved inorganic carbon (DIC) was measured once in a reservoir.  $p\text{CO}_2$  was calculated from the average pH and Talk with error propagated according to Ellison et al. (2000) using sensitivity coefficients from Dickson and Riley (1978). Missing error terms are due to equipment malfunction.

pH Target Total Scale	Trial	Temperature °C		pH Total Scale		Talk $\mu\text{mol kg}^{-1}$	DIC $\mu\text{mol kg}^{-1}$	$p\text{CO}_2$ $\mu\text{mol kg}^{-1}$	
7.3	1	10.3	$\pm$ 0.4	7.22	$\pm$ 0.02		2168	2830	$\pm$ 54
7.6	1	10.3	$\pm$ 0.6	7.50	$\pm$ 0.01		2092	1456	$\pm$ 26
7.8	1	10.1	$\pm$	7.82	$\pm$		2025	668	$\pm$ 11
8.1	1	9.9	$\pm$ 0.7	8.02	$\pm$ 0.00		1908	401	$\pm$ 7
7.3	1	17.9	$\pm$ 0.3	7.32	$\pm$ 0.07	2093 $\pm$ 9.8	2142	2339	$\pm$ 75
7.6	1	17.9	$\pm$ 0.8	7.63	$\pm$ 0.09		2081	1104	$\pm$ 41
7.8	1	17.9	$\pm$ 0.2	7.73	$\pm$ 0.07		2064	861	$\pm$ 26
8.1	1	18.1	$\pm$ 0.4	7.99	$\pm$ 0.00		1941	441	$\pm$ 7
7.3	1	24.9	$\pm$ 0.1	7.28	$\pm$ 0.02		2111	2669	$\pm$ 51
7.6	1	23.8	$\pm$ 0.3	7.60	$\pm$ 0.05		2022	1216	$\pm$ 31
7.8	1	25.0	$\pm$ 0.1	7.77	$\pm$ 0.00		1953	793	$\pm$ 14
8.1	1	24.7	$\pm$ 0.2	8.00	$\pm$ 0.01		1867	430	$\pm$ 7
7.3	2	10.0	$\pm$ 0.1	7.31	$\pm$ 0.44		2186	2274	$\pm$ 385
7.6	2	10.1	$\pm$	7.44	$\pm$		2113	1652	$\pm$ 24
7.8	2	10.0	$\pm$ 0.1	7.72	$\pm$ 0.04		2013	849	$\pm$ 17
8.1	2	10.1	$\pm$ 0.1	8.20	$\pm$ 0.07		1882	248	$\pm$ 7
7.3	2	17.9	$\pm$ 0.0	7.29	$\pm$ 0.01	2085 $\pm$ 6.8	2259	2503	$\pm$ 38
7.6	2	17.9	$\pm$ 0.1	7.58	$\pm$ 0.02		2040	1243	$\pm$ 20
7.8	2	17.9	$\pm$ 0.0	7.77	$\pm$ 0.01		1985	775	$\pm$ 11
8.1	2	17.8	$\pm$ 0.1	8.23	$\pm$ 0.06		1898	227	$\pm$ 6
7.3	2	24.9	$\pm$ 0.0	7.32	$\pm$ 0.01		2123	2414	$\pm$ 37
7.6	2	24.9	$\pm$ 0.0	7.53	$\pm$ 0.05		2077	1447	$\pm$ 34
7.8	2	24.9	$\pm$ 0.0	7.81	$\pm$ 0.06		1962	712	$\pm$ 18
8.1	2	24.9	$\pm$ 0.0	8.14	$\pm$ 0.01		1821	290	$\pm$ 4
7.3	3	24.9	$\pm$ 0.0	7.31	$\pm$ 0.00		2128	2466	$\pm$ 40
7.6	3	24.9	$\pm$ 0.3	7.58	$\pm$ 0.00	2085 $\pm$ 8.4	2115	1274	$\pm$ 20
8.1	3	23.8	$\pm$ 2.1	8.12	$\pm$ 0.10		1807	306	$\pm$ 12

**Table S1.2.** Number of byssal threads tested for material properties (whole thread strength, plaque strength, proximal strength, and distal yield) for each temperature and pCO<sub>2</sub> treatment. Sample size per treatment fell below the target of 8 mussels in some treatments due to mortality and low thread production.

<b>Temperature °C</b>	<b>pCO<sub>2</sub> µatm</b>	<b>Material Properties Sample Size</b>
10	2830	8
10	2274	6
10	1456	7
10	1652	7
10	849	6
10	668	7
10	401	8
10	248	7
18	2503	8
18	2339	8
18	1243	5
18	1104	8
18	861	5
18	775	6
18	441	6
18	227	7
25	2669	6
25	2466	6
25	2414	2
25	1447	3
25	1274	6
25	1216	5
25	793	4
25	712	2
25	430	5
25	290	4
25	306	8



**Figure S1.1.** (A) Byssal thread extensibility as a function of pCO<sub>2</sub> for each temperature treatment. Extensibility varied with temperature (Table S1.3, lmem,  $p < 0.001$ ); threads at 25°C were less extensible than threads at 10°C and 18°C (Table S1.3, Tukey HSD,  $p < 0.05$ ). There was a slight interaction between temperature and pCO<sub>2</sub>, and no effect of pCO<sub>2</sub> on extensibility (Table S1.3, lmem,  $p < 0.05$  and  $p = 0.3$  respectively). (B) Byssal thread stiffness as a function of pCO<sub>2</sub> for each temperature treatment. Stiffness varied with temperature (Table S1.3, lmem,  $p < 0.001$ ), but there was no interaction of temperature and pCO<sub>2</sub> and no effect of pCO<sub>2</sub> on stiffness (Table S1.3, ANCOVA,  $p = 0.06$  and  $0.051$  respectively). (C) Mean number of threads produced as a function of pCO<sub>2</sub> for each temperature treatment. Elevated temperature reduced thread production at 25°C (Table S1.3, lmem,  $p < 0.001$ , Tukey HSD:  $p < 0.001$ ) and there was no interaction between temperature and pCO<sub>2</sub> and no pCO<sub>2</sub> effect on thread number (Table S1.3, lmem,  $p = 0.5$  and  $p = 0.2$  respectively). Error bars represent  $\pm$  SEM. N reported in Table S1.2.



**Table S1.3.** Summary of statistical analyses of elevated temperature and pCO<sub>2</sub> on byssal thread mechanics. Linear mixed effect models were run with temperature and pCO<sub>2</sub> as fixed factors and trial as a random factor on each of the dependent variables: thread extensibility, thread stiffness, and thread number. N= 8 -11 pCO<sub>2</sub> levels per temperature treatment (see Table S1.2).

			<b>Thread Extensibility</b>	<b>Thread Stiffness</b>	<b>Thread Number</b>
random effect of trial		stdev	0.03	0.02	2
ANOVA on lmem	Temperature	$\chi^2$	14.0	32.8	26.8
		d.f.	2	2	2
		p	<b>&lt; 0.001</b>	<b>&lt; 0.001</b>	<b>&lt; 0.001</b>
	pCO <sub>2</sub>	$\chi^2$	1.1	3.4	1.6
		d.f.	1	1	1
		p	0.3	0.06	0.2
	Temperature*pCO <sub>2</sub>	$\chi^2$	7.4	5.6	1.5
		d.f.	2	2	2
		p	<b>&lt; 0.05</b>	0.051	0.5
Tukey HSD	10°C - 18°C		0.5	0.6	0.7
	10°C - 25°C		<b>&lt; 0.01</b>	<b>&lt; 0.001</b>	<b>&lt; 0.001</b>
	18°C - 25°C		<b>&lt; 0.05</b>	<b>&lt; 0.001</b>	<b>&lt; 0.001</b>

**Table S1.4.** Mussel condition and gonad indices. Only mussels in the second trial at 25°C showed decreased condition index (Tukey HSD,  $p < 0.05$ ). Neither temperature nor pCO<sub>2</sub> influenced Gonad Index.

Temperature (°C)	Trial	Condition Index (g mm <sup>-3</sup> * 10 <sup>6</sup> )	Gonad Index
10	1	3.12 ± 0.17	0.13 ± 0.01
10	2	3.03 ± 0.13	0.15 ± 0.01
18	1	3.21 ± 0.13	0.12 ± 0.01
18	2	2.79 ± 0.13	0.12 ± 0.01
25	1	3.17 ± 0.14	0.13 ± 0.01
25	2	2.14 ± 0.08**	0.09 ± 0.01
25	3	2.76 ± 0.15	0.16 ± 0.01
p – value	pCO <sub>2</sub>	0.2	0.2
	temperature	< <b>0.05</b>	0.3
	pCO <sub>2</sub> *temperature	0.8	0.3
	stdev of trial	< 0.001	0.01

**Table S1.5.** Water chemistry conditions in Experiment 2. Temperature and pH are means ( $\pm$  SD) of readings taken every minute during the experiment. Total alkalinity (Talk) is reported as the average of three of the six reservoirs because our manipulation did not change alkalinity and all treatments were supplied from the same header tank. Dissolved inorganic carbon (DIC) and Talk are means of 5 replicates in the 32 d treatment and 1 replicate in the 3 d treatment.  $p\text{CO}_2$  is calculated from Talk and pH, error was propagated to  $p\text{CO}_2$  according to Ellison et al. (2000) using the sensitivity coefficients of Dickson and Riley (1978).

<b>pH Target</b>	<b>Days</b>	<b>Temperature °C</b>		<b>pH Total Scale</b>		<b>Talk <math>\mu\text{mol kg}^{-1}</math></b>	<b>DIC <math>\mu\text{mol kg}^{-1}</math></b>	<b><math>p\text{CO}_2</math> <math>\mu\text{mol kg}^{-1}</math></b>	
7	3	11.64	$\pm$ 0.34	7.06	$\pm$ 0.6		5141	4143	$\pm$ 987
7.45	3	11.58	$\pm$ 0.12	7.43	$\pm$ 0.01		2328	1727	$\pm$ 21
7.6	3	11.74	$\pm$ 0.45	7.60	$\pm$ 0.04	2103	1355	1147	$\pm$ 21
7.7	3	11.54	$\pm$ 0.16	7.69	$\pm$ 0.02		1224	920	$\pm$ 12
7.8	3	11.61	$\pm$ 0.27	7.83	$\pm$ 0.03		624	649	$\pm$ 10
8.1	3	11.72	$\pm$ 0.23	8.11	$\pm$ 0.02		321	315	$\pm$ 4
7	32	12.08	$\pm$ 0.54	7.19	$\pm$ 0.1		2345 $\pm$ 33	3053	$\pm$ 135
7.45	32	12.24	$\pm$ 0.68	7.48	$\pm$ 0.1		2174 $\pm$ 28	1534	$\pm$ 66
7.6	32	12.29	$\pm$ 0.56	7.60	$\pm$ 0.03	2097 $\pm$ 13	2128 $\pm$ 24	1147	$\pm$ 27
7.7	32	12.04	$\pm$ 0.42	7.70	$\pm$ 0.03		2084 $\pm$ 25	897	$\pm$ 21
7.8	32	12.15	$\pm$ 0.61	7.83	$\pm$ 0.02		2029 $\pm$ 23	649	$\pm$ 14
8.1	32	12.15	$\pm$ 0.4	8.10	$\pm$ 0.02		1920 $\pm$ 22	322	$\pm$ 7

**Table S1.6.** Condition and gonad indices of mussels in Experiment 2. ANCOVA found no effect of pCO<sub>2</sub> or exposure duration on either index.

pCO <sub>2</sub> (µatm)	Exposure (days)	Condition Index (g mm <sup>-3</sup> * 10 <sup>6</sup> )		Gonad Index	
315	3	3.891	± 2.8	0.19	± 0.02
649	3	3.676	± 3.6	0.15	± 0.01
920	3	4.003	± 1.8	0.16	± 0.01
1147	3	4.202	± 3.4	0.19	± 0.01
1727	3	4.560	± 3.3	0.17	± 0.02
4143	3	4.455	± 3.8	0.18	± 0.02
322	32	4.180	± 3.5	0.16	± 0.02
649	32	3.787	± 2.7	0.14	± 0.02
897	32	4.395	± 3.9	0.16	± 0.02
1147	32	3.687	± 3.4	0.18	± 0.01
1534	32	3.788	± 2.6	0.15	± 0.02
3053	32	3.766	± 3.8	0.17	± 0.03
p-value	pCO <sub>2</sub> :	0.4		0.7	
	Exposure:	0.2		0.3	
	pCO <sub>2</sub> *Exposure:	0.9		0.3	

## Chapter 2

### **The attachment strength of congener mussel species responds in opposite directions to elevated temperature**

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Keywords: ecomechanics, *Mytilus trossulus*, *Mytilus galloprovincialis*, non-native species, climate change, material properties

## 2.1. Abstract

One predicted biological consequence of temperature increase due to global climate change is shifts in species ranges and latitudinal distributions. Cold-adapted species may suffer range contractions, while warm-adapted species may experience range expansions. In the northeast Pacific, two competing species include the warm-adapted mussel *Mytilus galloprovincialis* and cold-adapted mussel *M. trossulus*. The persistence of these congeners on wave swept rocky shores is closely tied to their attachment strength, which weakens with high temperature in *M. trossulus*. We compared attachment strength of *M. trossulus* and *M. galloprovincialis* exposed to temperatures ranging from 11 to 24.5°C. We find these species attach equally strong at temperatures between 11 and 18°C. However, from 18 to 24.5°C, the native *M. trossulus* produces fewer and weaker byssal threads with attachments that were up to 93% weaker than *M. galloprovincialis*. Warming oceans could therefore increase the competitive advantage of *M. galloprovincialis* in more northern latitudes at the expense of native *M. trossulus* populations.

## 2.2. Introduction

Surface ocean waters have been warming at a rate of 0.09 - 0.13°C per decade since 1971 and are predicted to continue to warm an additional 0.5 – 2°C by 2060<sup>1</sup>. This warming could challenge many marine organisms because temperature is intricately tied to physiological performance. Every species has a range of temperatures at which they perform optimally<sup>2</sup>. Species adapted to warm water conditions or living far from their upper temperature limit will survive in this warmer climate, potentially expanding their range. Other species, living closer to their upper temperature limits will not be able to survive in warmer waters, contracting their range<sup>3-5</sup>. These range expansions are further complicated by competition among species: when a species expands its range it will encounter new species<sup>6</sup>. In order to make these range shift predictions, we need a physiological understanding of how a species and its competitors respond to warmer temperatures.

We use this ecophysiological framework to explore how two mussel congeners that compete with each other for space on rocky shores respond to elevated temperature. *Mytilus galloprovincialis*, native to the Mediterranean Sea, competes with a closely related bay mussel *M. trossulus*<sup>7</sup>. Since its introduction to the northeastern Pacific, *M. galloprovincialis* has replaced *M. trossulus* as the dominant mussel south of San Francisco Bay, CA<sup>8,9</sup>. This dominance has been explained by superior physiological performance of *M. galloprovincialis* in warm temperatures, a product of its warmer home range. For example, *M. galloprovincialis* has been shown in numerous physiological studies to outperform *M. trossulus* at temperatures above 20°C through its ability to maintain its heart rate<sup>10</sup>, reduce heat-shock proteins activation<sup>11</sup>, and produce superior functioning enzymes<sup>12</sup>. Additionally, its energetic performance under elevated temperature allows a greater scope-for-growth than *M. trossulus*<sup>13</sup>.

While these studies focus on growth, a common proxy for reproductive output, the other component of fitness is survival<sup>14</sup>. Mussel survival is closely tied to the attachment structure, byssal threads<sup>15</sup>. A weak attachment increases the probability wave forces will dislodge a mussel from the shore while a strong attachment will allow a mussel to persist<sup>16,17</sup>. The competitive dominance of mussel species in a region can be mediated by attachment strength. For example, in South Africa, two mussel species *Perna perna* and *M. galloprovincialis* compete for space. *Perna perna* has superior attachment strength, and thus outcompetes *M. galloprovincialis* in high wave areas. In low wave areas, no longer restricted by attachment ability, *M. galloprovincialis* outcompetes *P. perna* due to greater gamete production<sup>17</sup>. These results impart the importance of considering attachment strength when evaluating how rising temperatures may modulate competition between *M. trossulus* and *M. galloprovincialis* in the northeastern Pacific.

Mussels attach to hard substrates with many collagen-like fibers known as byssal threads<sup>18</sup>. A mussel's overall attachment strength is a product of both the number of threads the mussel forms to anchor itself and the quality of those threads<sup>19</sup>. High quality threads are strong, extensible, and yield before breaking. This yield confers the thread's extensibility, which enhances the collective strength of the byssal attachment<sup>19</sup>. Byssal thread quality depends on environmental conditions<sup>20,21</sup>, including temperature<sup>14</sup>; Chapter 1 showed elevated temperature weakened the byssal thread strength of *M. trossulus* by 60% at temperatures above 25°C. However, because *M. galloprovincialis* typically performs at its optimum under higher temperatures than *M. trossulus*, the warm-adapted species may respond differently with regards to byssus production under warm temperatures.

In this study we test the hypothesis that elevated temperatures will decrease the attachment strength of *M. trossulus*, which is native to cooler waters, and will increase the



attachment strength of *M. galloprovincialis*, which is native to warmer waters. We use these results to examine which locations along the west coast of North America, due to temperature and its effects on byssus production and other aspects of performance, would favor one species over the other. We extend these predictions to ask how future warming scenarios could expand or contract the latitudinal range of each species.

### **2.3. Attachment strength of congener species under elevated temperature**

The Mediterranean mussel *M. galloprovincialis* showed superior attachment strength under high temperature; thread strength and extensibility remained constant from 11°C to 24.5°C while thread production increased (Figure 2.1). For *M. trossulus*, thread strength, extensibility, and thread production all decreased with increased temperature. These differential effects of elevated temperature on thread quality and quantity dramatically affected overall attachment, with *M. galloprovincialis* estimated to be 93% stronger than *M. trossulus* at 24.5°C (Figure 2.1).

More specifically, thread strength and extensibility decreased significantly with temperature for *M. trossulus* (by 75% and 60%, respectively, from 11 to 24.5°C) but not for *M. galloprovincialis* (Figure 2.1A-B, Table 2.1). Additionally, *M. trossulus* produced 64% fewer threads over the range 11 to 24.5°C, following the same pattern as thread strength (Figure 2.1C, Table 2.1). In contrast, elevated temperature did not affect thread strength but increased thread production in *M. galloprovincialis* by 61% (Figure 2.1C, Table 2.1). Incorporating the effects of temperature on individual thread strength, extensibility, and production into a model that predicts the force to dislodge the collective byssus attachment, we find the two species diverge dramatically beyond 18°C; with rising temperature the strength of *M. galloprovincialis* increases by 62% while *M. trossulus* decreases by 89% (Figure 2.1D, Table 2.1).

## 2.4. Insight into mechanical differences between species

These species-specific effects of temperature on thread material properties are due to changes in specific regions of the byssal thread. Each byssal thread comprises three structurally and functionally unique regions that work together to give a thread its overall extensibility and strength: a stretchy proximal region, a strong and stretchy distal region (characterized by its yield), and an adhesive plaque that glues the thread to the substrate<sup>18</sup>. Proximal strength decreased linearly with temperature for *M. trossulus* (by 76%, 11 versus 24.5°C) while it remained constant across all temperatures for *M. galloprovincialis* (Figure 2.2A, Table 2.1). Plaque strength decreased with temperature for *M. trossulus* (by 64%, 11 versus 24.5°C) while it increased linearly across that same temperature range for *M. galloprovincialis* (by 41%, 11 versus 24.5°C; Figure 2.2B, Table 2.1). Distal yield force was independent of temperature for *M. galloprovincialis* while yield force decreased with elevated temperature for *M. trossulus* (73%, 11 versus 24.5°C; Figure 2.2C, Table 2.1). Threads yielded before they broke for *M. galloprovincialis*, but for *M. trossulus* at temperatures above 22°C, threads broke before they could yield causing their lower extensibility.

Although elevated temperature weakens all regions of the thread, it was the proximal region that became the weak link in the tether (Figure 2.2D, Table 2.1). For *M. trossulus*, this is due to elevated temperature decreasing proximal strength to a greater magnitude than it decreases plaque strength. For *M. galloprovincialis*, the proximal region becomes the most common location for breakage because elevated temperature strengthens the adhesive plaque.

Here we find a structure, the byssal thread, shared between two closely related species does not perform equally across a range of temperatures. This mechanical difference suggests an underlying difference in protein structure and composition. *Mytilus galloprovincialis* has greater

concentrations of histadine cross-linking than other *Mytilid* mussels, which may to confer greater stability to the thread<sup>22</sup>. This stability may be what counteracts the increases in material viscosity with temperature that may plague *M. trossulus* threads. Additionally, there are known protein differences between *M. trossulus* and *M. galloprovincialis* byssal threads<sup>23</sup>. The proteins found only in the threads of *M. galloprovincialis* may enable *M. galloprovincialis* to thrive under elevated temperature, driving these performance differences.

Additionally, we observed a lower condition index for *M. trossulus*, a measure of tissue mass per mussel size, in warmer water (Table S2.1). Both weaker threads and lower body weight suggest mussels under elevated temperatures direct resources away from thread production and towards other vital organismal processes<sup>14,24</sup>. However, for *M. galloprovincialis*, which experiences temperatures into 25°C in its native range in the Mediterranean Sea, warmer temperatures increased thread formation with no ill effects on body condition or thread strength. Moreover, unlike *M. trossulus*, *M. galloprovincialis* increased allocation to reproductive tissues with elevated temperature (Table S2.1).

## **2.5. Disturbance in mussel beds and species competition**

We compared attachment strength between these two species because of its importance in a mussel's survival on the shore<sup>17</sup>. Mussels must be stronger than the strongest force (wave or predator) they encounter to avoid dislodgement. In a theoretical mussel assemblage on shore, the attachment strength of the population is normally distributed about the mean (blue line, Figure 3). Any mussels that are weaker than the maximum force encountered in an environment (shaded box, Figure 2.3) will detach, creating a disturbance where bare patches appear in mussel beds<sup>24</sup>.

Applying the results of this study to this framework, *M. trossulus* and *M. galloprovincialis* are equally strong at temperatures below 18°C and would have similar strength distributions and probabilities of dislodgement. Elevated temperature (> 18°C) will shift the normal distribution of *M. trossulus* strength to the left (lower strength), causing a greater proportion of mussels to be weak enough to dislodge. The same elevation in temperature will instead shift the *M. galloprovincialis* distribution to the right (higher strength), out of the risk of dislodgement (red lines, Figure 2.3). In this manner, warmer temperatures will make aggregations of *M. trossulus* more prone to dislodgement and *M. galloprovincialis* less prone to dislodgement. Either of these outcomes have the potential to dramatically alter the disturbance regime (patch size and number) on rocky shores,<sup>24</sup> which in turn can change community dynamics in this ecosystem<sup>25,26</sup>. For example, after *M. galloprovincialis* was introduced to South Africa and established populations on the shore, competition for space led to a decline in limpet populations<sup>27</sup> while the abundance of organisms that eat mussels such as whelks and oyster catchers increased<sup>28</sup>. Greater disturbance rates in *M. trossulus* beds could increase available space, allowing colonization of other turf-forming species such as coralline algae<sup>29</sup> as well as open up space for *M. galloprovincialis* colonization<sup>30</sup>.

## 2.6. Effects on species range

*Mytilus galloprovincialis* outcompetes *M. trossulus* in the areas the two species co-occur by outgrowing and smothering *M. trossulus* populations<sup>7</sup>. Based on their performance under the range of temperatures we tested, we predict *M. galloprovincialis* and *M. trossulus* to be equal competitors at temperatures below 18°C and *M. galloprovincialis* to hold a competitive advantage over *M. trossulus* at temperatures above 18°C (two-way ANOVA comparing all

thread parameters in 11-18°C versus 20 – 24.5°C, Table 2.1). Other studies comparing the physiological responses of these two species to elevated temperature show a similar temperature threshold where *M. galloprovincialis* performance becomes superior<sup>10,11</sup>. Therefore, we used this criterion of 18°C to define where along the west coast of North America *M. galloprovincialis* has the potential to dominate over *M. trossulus* under current and future conditions. This model assumes: (1) no other limiting environmental factors, (such as salinity, which is known to limit *M. galloprovincialis* in certain areas<sup>30</sup>), (2) recruitment to new areas is not limiting for either species and (3) that the species with the stronger attachment (as predicted from local temperature) will outcompete the other.

Using daily mean sea surface temperatures from 2010 to 2014 (<http://www.esrl.noaa.gov>) we mapped locations where for at least 1 day, *M. galloprovincialis* would experience more favorable conditions for attachment than *M. trossulus* (Figure 2.4A). We find under present conditions, *M. galloprovincialis* is favored from San Diego to just south of San Francisco Bay, CA. The reported northern range of significant presence of *M. galloprovincialis* is south of San Francisco Bay<sup>31</sup>. Additionally, Figure 2.4A identifies the Strait of Georgia, Canada as a potential northern haven for *M. galloprovincialis* with warmer temperatures than the surrounding area. A recent study finds *M. galloprovincialis* comprises 30% of the mussels in this region<sup>32</sup>. This reported range for *M. galloprovincialis* matches well with range we predict (Figure 2.4A), supporting our hypothesis that temperature is one factor controlling the northern encroachment of *M. galloprovincialis*.

We then estimated how future warming of 2°C will affect the range of *M. galloprovincialis* (Figure 4B). With 2°C warming, the predicted distribution of *M. galloprovincialis* expands extensively, at the expense of *M. trossulus*. Specifically, this range

extends north to the northern California coast, the majority of the Oregon and Washington coast, and south to cover a greater proportion of the Strait of Georgia. This result indicates many regions are close to a tipping point where just slightly warmer conditions will favor the establishment of the non-native mussel.

## 2.7. Study Implications

Warming ocean temperatures could facilitate the introduction of *M. galloprovincialis* in the northeastern Pacific through a number of mechanisms, including attachment strength. Currently, *M. galloprovincialis* is only present in pockets north of San Francisco Bay; mussel beds in this area are primarily the native bay mussel *M. trossulus*<sup>31,33</sup>. The presence of *M. galloprovincialis* in these northern latitudes may increase the rate they outcompete the dominant *M. trossulus* populations when temperatures warm<sup>7</sup>. Our results find even a slight increase in water temperature can increase this trend over time.

Regardless of the mechanism by which *M. galloprovincialis* may outcompete *M. trossulus* under elevated temperature, warmer ocean waters weaken *M. trossulus* attachment and increase its risk of dislodgement. For mussel aquaculture, an industry that relies on strong mussel attachment, the ability of *M. galloprovincialis* to perform well under warming conditions may prove advantageous. Currently *M. galloprovincialis* is the more commonly farmed mussel along the West Coast of the United States and its ability to thrive as water temperature warms bodes well for the industry. Additionally, *M. galloprovincialis* and *M. trossulus* can form hybrids; breeding programs could take advantage of this compatibility to capture the most desirable qualities of each species (e.g., taste, shell appearance, thread temperature tolerance).

In conclusion, this study demonstrates the differential effects of ocean warming on the attachment strength of *M. galloprovincialis* and *M. trossulus*. The results demonstrate that performance of a shared structural material, byssus, across a range of conditions is species-dependent, even within a genus. Because byssus strength defines the ability of a mussel to remain attached, increasing seawater temperatures will likely have opposite effects on dislodgement risk, ultimately facilitating the range expansion of *M. galloprovincialis* into areas currently occupied by *M. trossulus*. These results stress that the impacts of climate change on organismal eco-physiology, and more specifically, material performance, can be species specific. Further, an understanding of the effect of environmental stressors on key biological materials, from tethers to skeletons to spines, can yield important insights into the ability of species to persist in a warming climate.

## **2.8. Methods**

We collected mussels in March 2014 from aquaculture lines at Penn Cove Shellfish in Penn Cove, Whidbey Island, WA (48.22°N, 122.71°W) and transported them to Friday Harbor Laboratories, where they were placed in temperature-controlled aquaria set to one of 6 temperature treatments ranging from 11 – 24.5°C. Each manipulation of temperature was made in a 150 L water reservoir, which supplied water to eight replicate 3.5 L specimen chambers with a turnover rate of 50 ml min<sup>-1</sup> (see Chapter 1). Submersible pumps (model number P396, Annex Depot, Sacramento, CA) provided mixing in the chambers at 3.8 L min<sup>-1</sup> and mussels were drip fed Shellfish Diet 1800 at a weight maintenance ration (1% day<sup>-1</sup>, Reed Mariculture, Campbell, CA, USA). Tanks were kept saturated in oxygen with a venturi injector and held at a pH of about 8.0 (total scale) and salinity of 30 during the experiment.

We acclimated mussels (N=16 per treatment) from 10°C to their randomly assigned temperature treatments over 10 days by raising temperature no more than 2°C per day. We then held the mussels at their temperature treatments for four days, allowing mussels to produce byssus on small pebbles at the base of each chamber. After four days we removed all mussels and their newly formed byssus from aquaria, dissected mussels to measure condition and gonad indices<sup>34</sup>, counted the number of threads produced, and stored threads dry for later material testing.

We tested threads in seawater for breaking strength (N), distal region yield (N), and extensibility in an Instron 5565 Tensometer (Instron, Norwood, MA, USA) fitted with submersible pneumatic grips (3130-100 BioPuls Bath, Instron, Norwood, MA, USA). We tested single threads as in ref. 20; the stem of the thread was clamped in cardstock while the rock attached plaque was epoxied onto an aluminum t-bar. Threads were extended normal to the rock surface at a rate of 10 mm min<sup>-1</sup>. We recorded the region where the thread first broke, and then re-tested to measure the strength (N) of each remaining region in isolation. We modeled whole mussel attachment strength as the force (N) to pull a mussel normal to the substratum as a function of mean thread breaking force, thread extensibility, and thread number per treatment<sup>17</sup>.

We used ANCOVA to test for the effect of temperature as covariate and species as fixed factor on each byssus material property (whole thread strength, extensibility, number of threads, force to dislodge, proximal strength, distal yield strength, and plaque strength). To identify the 18°C temperature threshold, we group temperature as high (>18°C) or low (<18°C) and ran a two-way ANOVA with species and temperature as factors. Thread breakage (failure) typically occurred in the proximal region or plaque. We used a binomial logistic regression using



generalized linear models to test for the effect of temperature on thread breaking location for each species. All statistical tests were performed in R (version 3.1.0).

We used sea surface temperature data for the west coast of North America ([http://www.esrl.noaa.gov/psd/cgi-bin/db\\_search/DBListFiles.pl?did=132&tid=47456&vid=2423](http://www.esrl.noaa.gov/psd/cgi-bin/db_search/DBListFiles.pl?did=132&tid=47456&vid=2423)) to calculate the number of days a 0.25 decimal degree sized coastal region had an average daily temperature that exceeded 18°C, the threshold where *M. galloprovincialis* produces a stronger attachment than *M. trossulus*. Using ArcGIS (version 10.3.1), we mapped the number of days calculated from present day conditions (2010 – 2014) as well as from present day conditions with a hypothetical 2°C warming.

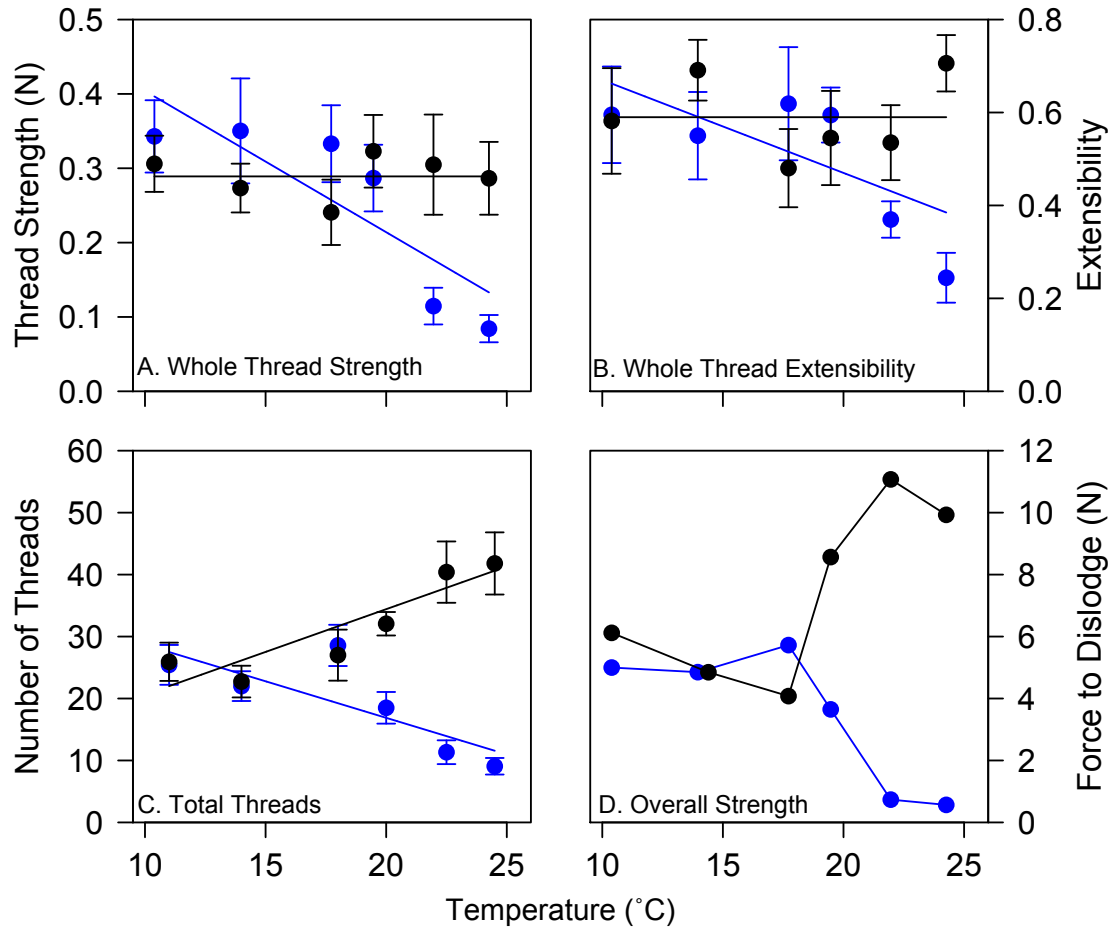
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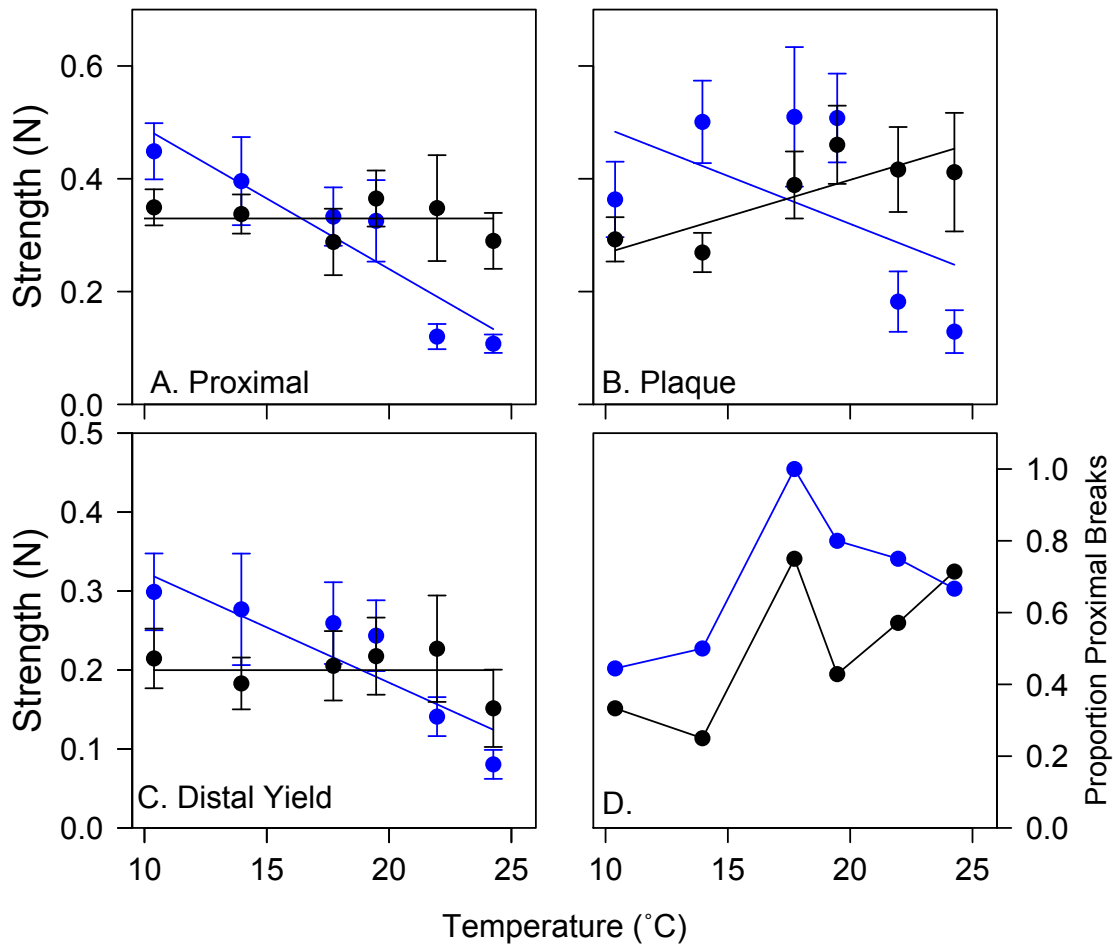
## 2.10. Tables and Figures



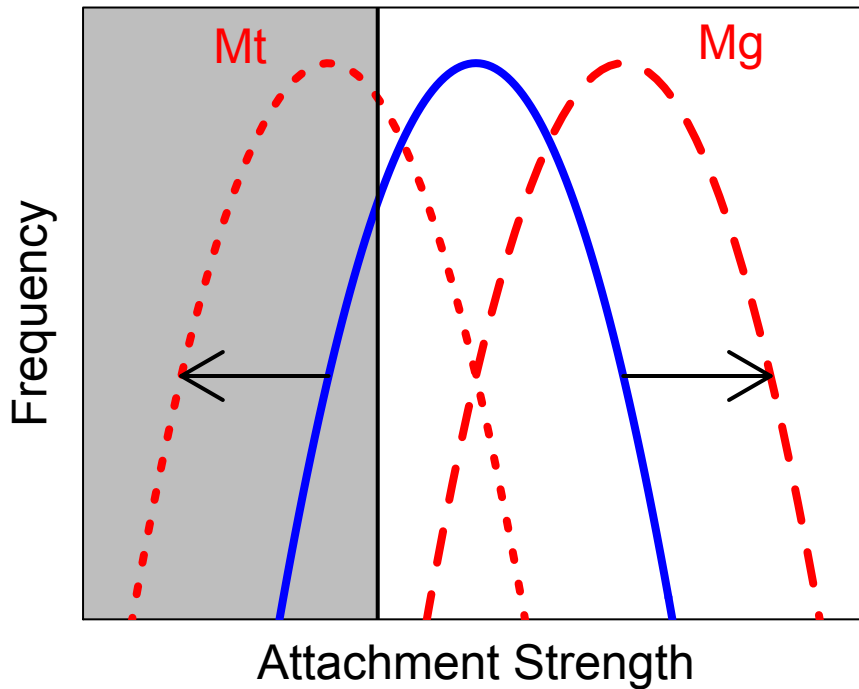
**Figure 2.1.** Effect of temperature on attachment strength of two mussel species. A, *Mytilus trossulus* (blue) thread strength decreases with elevated temperature while *M. galloprovincialis* (black) thread strength is constant across all temperature treatments (Table 2.1). B, Extensibility also decreases with elevated temperature for *M. trossulus* but not for *M. galloprovincialis* (Table 2.1). C, Elevated temperature altered thread production in both species: *M. trossulus* responded negatively while responded *M. galloprovincialis* positively. The difference in thread production between the two species is most evident above 18°C (Table 2.1). D, Using the means of three parameters in A-C to model the overall force to dislodge a whole mussel, *M. galloprovincialis* overall attachment strength increases with temperature while *M. trossulus* decreases (Table 2.1). Symbols are mean  $\pm$  standard error.

**Table 2.1.** Summary of ANCOVA (all temperatures) and two-way ANOVA (grouped < 18°C and >18°C) analyses of the effects of temperature and species on byssus attachment. T = temperature, S = species.

			Whole Thread Strength (N)	Extensibility	Thread Number	Force to Dislodge (N)	Proximal Strength (N)	Plaque Strength (N)	Yield Strength (N)
ANCOVA	T	F	10.2	3.7	0.3	0.05	18.1	0.6	3.1
		df	1	1	1	1	1	1	1
		p	< <b>0.01</b>	0.4	0.5	0.8	< <b>0.001</b>	0.5	0.08
	S	F	1.3	3.6	42.3	14.9	1.3	0.03	7.2
		df	1	1	1	1	1	1	1
		p	0.3	0.06	< <b>0.001</b>	< <b>0.01</b>	0.3	0.8	< <b>0.01</b>
	T*S	F	10.6	4.3	39.1	10.9	11.7	10.4	5.3
		df	1	1	1	1	1	1	1
		p	< <b>0.01</b>	< <b>0.05</b>	< <b>0.001</b>	< <b>0.05</b>	< <b>0.001</b>	< <b>0.01</b>	< <b>0.05</b>
	residual df			81	81	81	8	80	66
two-way ANOVA (11-18°C vs. 20-24.5°C)	T	F	5.9	2.3	4.95	0.9	8.6	0.5	9.9
		df	1	1	1	1	1	1	1
		p	< <b>0.05</b>	0.13	< <b>0.05</b>	0.37	< <b>0.01</b>	0.5	< <b>0.01</b>
	S	F	0.4	3.4	35.5	33.1	<b>0.9</b>	0.03	2.6
		df	1.1	1	1	1	1	1	1
		p	0.3	0.06	< <b>0.001</b>	< <b>0.001</b>	0.3	0.9	0.11
	T*S	F	14.8	4.3	<b>8.7</b>	<b>36.0</b>	12.14	12.4	<b>10.0</b>
		df	1	1	1	1	1	1	1
		p	< <b>0.001</b>	< <b>0.05</b>	< <b>0.01</b>	< <b>0.001</b>	< <b>0.001</b>	< <b>0.001</b>	< <b>0.01</b>

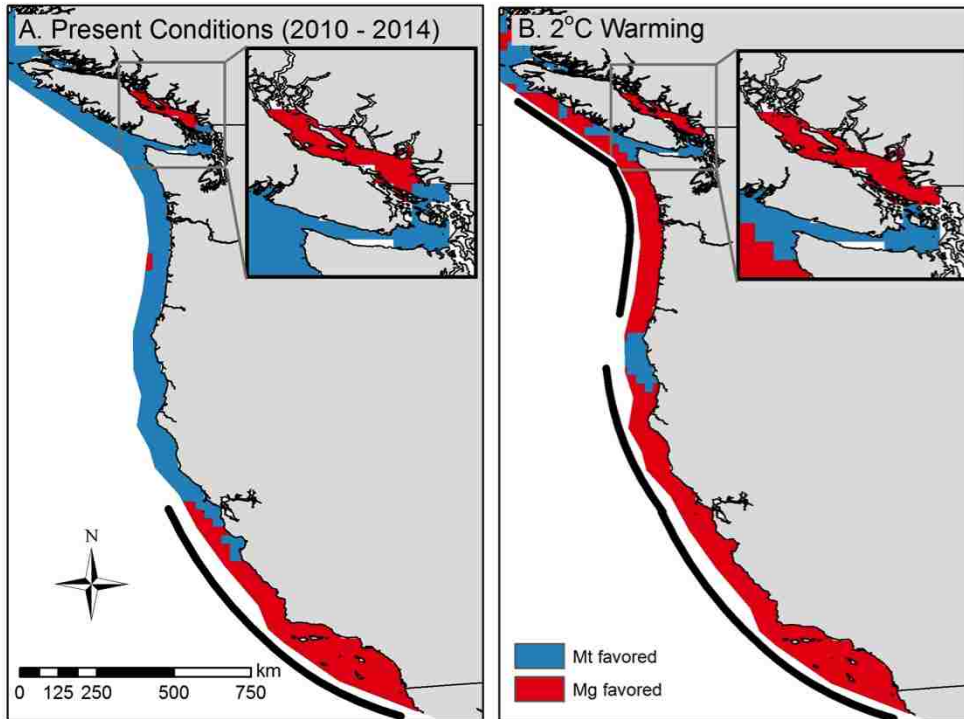


**Figure 2.2.** Interactive effects of temperature and species on the strength of isolated byssal thread regions. A, Proximal strength decreases with temperature in *M. trossulus* (blue symbols) but is unaffected by temperature in *M. galloprovincialis* (black symbols, Table 2.1). B, Plaque strength decreases with temperature for *M. trossulus*, but increases for *M. galloprovincialis* (Table 2.1). C, Distal yield decreases with temperature for *M. trossulus* but does not change for *M. galloprovincialis* (Table 2.1). D, Increasing temperature increases the proportion of proximal breaks for both species (GLM,  $p < 0.05$ ). Symbols are mean  $\pm$  standard deviation.



**Figure 2.3.** Effect of temperature on dislodgement risk for both species. The blue curve represents a normal distribution of attachment strengths in a mussel population at temperatures 10 – 18°C, identical for *M. trossulus* and *M. galloprovincialis*. The vertical black line represents the maximal force a mussel will encounter in nature, due to a wave, predator, etc. The proportion of the attachment distribution that falls below this force (overlapping the shaded box) represents the population’s risk of dislodgement. Elevated temperatures (above 18°C) weaken *M. trossulus* (Mt) shifting its normal distribution to the left and increasing the proportion of mussels dislodged. *Mytilus galloprovincialis* (Mg) strength, on the other hand, will increase at temperatures above 18°C, shifting its distribution to the right and out of danger of dislodgement.





**Figure 2.4.** Geographical map of the frequency of mean daily seawater temperatures over 18°C. Locations with at least 1 day (red) favor *M. galloprovincialis* (Mg) attachment, while those with 0 days (blue) favor *M. trossulus* (Mt). (A) Calculations for present day conditions (averaged over 2010 – 2014) and (B) present day conditions with 2°C warming. Warming of 2°C extends northward the latitudinal range where conditions favor *M. galloprovincialis* (black lines).

## **2.11. Acknowledgements**

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## 2.12. Supplementary Table

**Table S2.1.** Condition and gonad indices for each mussel species at the end of each temperature treatment. *Mytilus trossulus* had a greater condition index (CI) and gonad index (GI) than *M. galloprovincialis* (ANCOVA,  $p < 0.001$  for both CI and GI). CI decreased with temperature for both species and there was no interaction with temperature (ANCOVA,  $p < 0.05$  and  $0.1$ , respectively). The effect of temperature on gonad index was species dependent ( $p < 0.05$ ); GI increased with temperature in *M. galloprovincialis* but remained constant across all temperatures for *M. trossulus*. Table values represent mean  $\pm$  standard error of 16 samples.

Treatment	Condition Index ( $\text{g mm}^{-3} * 10^{-6}$ )		Gonad Index	
	<i>M. trossulus</i>	<i>M. galloprovincialis</i>	<i>M. trossulus</i>	<i>M. galloprovincialis</i>
11 °C	7.01 $\pm$ 0.33	5.09 $\pm$ 1.81	0.33 $\pm$ 0.01	0.20 $\pm$ 0.01
14 °C	6.77 $\pm$ 0.31	4.75 $\pm$ 1.69	0.32 $\pm$ 0.01	0.20 $\pm$ 0.01
18 °C	6.74 $\pm$ 0.33	4.53 $\pm$ 1.69	0.35 $\pm$ 0.01	0.22 $\pm$ 0.01
20 °C	6.66 $\pm$ 0.42	4.57 $\pm$ 1.72	0.35 $\pm$ 0.02	0.21 $\pm$ 0.01
22 °C	5.91 $\pm$ 0.51	4.59 $\pm$ 1.53	0.30 $\pm$ 0.02	0.23 $\pm$ 0.01
24.5 °C	6.01 $\pm$ 0.46	4.94 $\pm$ 1.55	0.32 $\pm$ 0.01	0.24 $\pm$ 0.01
<b>ANCOVA</b>				
T	F	4.6	0.9	
	df	1	1	
	p	<b>&lt; 0.05</b>	0.4	
S	F	72.1	208	
	df	1	1	
	p	<b>&lt; 0.001</b>	<b>&lt; 0.001</b>	
T*S	F	2.2	4.4	
	df	1	1	
	p	0.1	<b>&lt; 0.05</b>	

## **Chapter 3**

### **Seasonal weakening of *Mytilus trossulus* attachment strength can be predicted by high temperature, low pH, and low dissolved oxygen**

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### 3.1. Abstract

Mussel aquaculture is an economically important industry worldwide. In the most common farming practices, using rafts and long lines, mussels mold individual tethers, known as byssal threads, to form stretchy attachments to the rope or other mussels. These byssal threads are critical for keeping mussels attached to the lines. Weakened byssal threads increase the chance of “fall-off” or “sloughing” from aquaculture lines, reducing yields. Several studies on the mussel *Mytilus edulis* have found attachment strength varies seasonally. More recent laboratory studies identify two environmental conditions, low pH (below 7.6) and high temperature (above 18°C) weaken byssal threads in *M. trossulus*, but it is unknown whether these conditions are ever encountered in the field, or if they lead to weak mussel attachment. In this study, we measure concurrently mussel (*M. trossulus*) attachment strength (tenacity) and a suite of water conditions including temperature and pH. We find mussel tenacity is stronger in the winter than in the summer. Using GLM models, weak tenacity ( $< 2 \times 10^{-4} \text{ Nm}^{-2}$ ) is best predicted by high temperature ( $> 14^\circ\text{C}$ ), low pH ( $< 7.5$ ), and/or low dissolved oxygen ( $< 7 \text{ mg/L}$ ). These results suggest ocean warming, acidification, and hypoxia may threaten mussel aquaculture. Monitoring these conditions near farms can identify periods when attachment is expected to be weak and adapting alternative farming practices could produce higher yields.

### 3.2. Introduction

Mussels are economically and ecologically important molluscs, structuring communities and supporting large aquaculture operations in temperate ecosystems worldwide. Their presence and survival on coastal shores and aquaculture lines is made possible by their strong attachment (Carrington et al. 2009). In suspension cultures using rafts or long lines, each mussel is tethered to the rope or other mussels by byssal threads (Bell & Gosline 1996). These byssal threads are stretchy, collagen-like fibers mussels formed throughout their lifetime to maintain a secure attachment (Waite 1987). A reduction in byssus strength causes fall-off from aquaculture lines and rocky shores alike (Carrington et al. 2009, 2015). This both reduces both yields at mussel farms and opens up bare patches on rocky shores (Levin & Paine 1974, Carrington et al. 2009).

Fall-off is often driven by high waves and currents, but occurs most commonly when byssus strength is weakest (Carrington et al. 2009). Numerous studies have found strong seasonal patterns in the attachment strength of the mussels *Mytilus edulis*, *M. galloprovincialis* and *Perna perna* (Table 3.1, Figure S3.1). Whole mussel attachment strength can vary by a factor of two from one season to the next. These studies demonstrate seasonal weakening correlates with a variety of abiotic and biotic factors including high wind, temperature (both low and high), low food availability, seasonal spawning, and increased fouling and byssal thread decay (Price 1980, 1982, Carrington 2002, Zardi et al. 2007, Lachance et al. 2008, Carrington et al. 2009).

While these studies suggest environmental conditions alter byssal thread strength, they do not pinpoint the underlying mechanism by which abiotic conditions weaken byssus. Recent breakthrough studies have used controlled laboratory settings to test how isolated stressors can weaken the byssus of the mussel *M. trossulus*, which is closely related to the mytilids previously

studied. Specifically, these studies found low pH below 7.6 (O'Donnell et al. 2013), and elevated temperature above 18°C (Chapter 1, Chapter 2) weaken byssal threads. Moreover, the effects of these stressors are not additive; the stressor with the largest negative impact drives the response (Chapter 1). *Mytilus trossulus* also forms fewer byssal threads above 18°C exacerbating the weakening effect of elevated temperature on overall attachment strength (Chapter 2).

These laboratory studies suggest changes in water conditions, including temperature and pH, may drive seasonal changes in mussel attachment strength. Temperature variability in coastal environments is well known (Nishizaki and Carrington 2014, Murray et al. 2015). Recent studies find high pH variability in coastal environments similar to the ones where mussels grow (Feely et al., 2008, Hofmann et al. 2011, Murray et al. 2015, Kapsenberg et al. 2015). This variability is in part due to the activity of the rich biota common in these environments. Near the coasts of the northeast Pacific and Antarctica, for example, phytoplankton blooms increase O<sub>2</sub> and draw down CO<sub>2</sub> causing seasonal swings in pH up to 0.5 pH units (Murray et al. 2015, Kapsenberg et al. 2015). Coral reefs experience strong diel variation in pH as photosynthesis dominates during the day and respiration dominates at night (Hofmann et al. 2011). Photosynthesis and respiration also lead to large daily fluctuations of up to 0.8 pH units and seasonal variability of pH in kelp forests and seagrass beds up to 0.6 pH units (Hofmann et al. 2011, Frieder et al. 2012, Challener et al. 2015). Physical drivers also strongly influence ocean conditions. Thermal stratification, which occurs seasonally in many deep water bays and fjords, leads to hypoxia and hypercapnia below the thermocline. Upwelling can bring in cooler, lower pH water (Feely et al. 2008). River discharge events occur seasonally lowering the salinity and changing alkalinity, which can alter seawater pH (Murray et al. 2015).

Mussels growing in coastal inlets in the Salish Sea are also likely to experience seasonal and daily variability in seawater conditions. The Salish Sea is the home to numerous mussel aquaculture operations, including the nation's oldest and largest farm, Penn Cove Shellfish, LLC. This region experiences seasonal upwelling as well as river discharge from the Skagit River in the winter months. Penn Cove Shellfish farms *M. trossulus*, a species whose attachment strength weakens when pH is lower than 7.6 and temperature greater than 18°C under laboratory conditions (Chapter 1, Chapter 2, O'Donnell et al. 2013). The goal of this study is to determine whether mussels ever encounter these “stressful” in the field, and if so, whether they cause weak attachment.

In this study, we measure mussel tenacity over 24 months while simultaneously characterizing temperature, pH, dissolved oxygen, salinity, and chlorophyll. We find attachment strength in *M. trossulus* varies seasonally; mussels are 2-3 times more strongly attached in the winter than during the summer. These seasonal changes in attachment strength are due to changes in thread strength, not thread number. Weak tenacity correlates with high temperature, low pH, and low dissolved oxygen. We develop GLM models to predict mussel tenacity as a function of these three seawater parameters.

### **3.3. Methods**

#### *3.3.1. Study Site*

Penn Cove (48.22°N, 122.71°W) is a shallow (20 m depth) bay located on the eastern side of Whidbey Island, WA, USA within the Salish Sea. Wild populations of mussels lining the shores naturally seed the commercial aquaculture lines in the southwestern part of the bay. Here, Penn Cove Shellfish, LLC grows the bay mussel *M. trossulus* on vertical lines hanging from



floating rafts. The farm comprises 44 rafts encompassing an area of 0.2 km<sup>2</sup>. Mussels are grown on lines between the water depths of 1m and 7m. When mussels reach harvesting size (5 – 7cm), lines are brought up on a mechanical lift system for harvest. We used this system to access mussels growing at the top (1m) and bottom (7m) of the line.

### *3.3.2. Tenacity and Mussel Condition*

Monthly from August 2013 to July 2015, we removed large clumps (> 300 individuals) of mussels from the top (1m) and bottom (7m) of the aquaculture lines as they were being harvested. Because we relied on harvesting machinery to pull up mussels, the harvesters haphazardly determined the rafts from which we sampled mussels. Mussels from these clumps were then haphazardly selected for tenacity measurements following the procedure of Carrington (2002). Briefly, a 3 mm hole was drilled in the shell and a hook was inserted into the hole and attached to a 100N forge gauge (OMEGA, Stamford, CT, USA). We pulled a mussel normal to the substrate until it dislodged and recorded peak force as breaking force to the nearest 0.1 N. Force measurements were normalized to mussel planform area estimated as the area of an ellipse with axes of mussel height and mussel width. A total of 50 mussels were pulled from each of the two depths per month.

We collected a second group of 35 mussels from each depth to measure overall condition and reproductive state. Condition index, a measure of soft tissue (“meat”) per shell volume was measured following the procedure of Moeser et al. (2006), and gonad index, the proportion of soft tissue that is reproductive was measured following the procedure of Carrington (2002). Mussels at 7m were not assessed in May 2015, and for statistical purposes, this missing point was calculated from the closest two months by linear interpolation.

From August 2014 to June 2015, we examined if observed changes in mussel tenacity were due to a difference in thread strength or thread number. Mussels ( $n = 12$ ) from harvesting lines were relocated at each depth to “mussel hotels” (see Figure 3.1), which consisted of four vertically suspended 6 in<sup>2</sup> plexiglass plates. After 7 – 13 days, we removed these hotels from the water and counted the number of threads each mussel had made. Threads were stored dry on the plexiglass plates to which they were attached and rehydrated prior to testing. We measured the breaking force of individual threads by pulling threads in tension at a rate of 10 mm min<sup>-1</sup> with a 10N force gauge (OMEGA, Stamford, CT, USA) measured to the nearest 0.005 N.

### 3.3.3. *Water parameters*

Temperature was measured from August 2013 to July 2015 every 10 min using Hobo Pendant Temperature Loggers (UA-002-64, Onset Computer Corporation, Bourne, MA, USA) stationed at depths of 1m and 7m located on the most central raft of the farm.

Starting in August 2014, we installed YSI EXO2 data sondes (Yellow Springs, OH, USA) at depths of 1m and 7m to record temperature (to the nearest 0.01 °C), pH (NBS scale to the nearest 0.01), dissolved oxygen (to the nearest 0.01 mg/L), salinity (to the nearest 0.01 practical salinity units), and chlorophyll (to the nearest 0.01 µg/L). The sonde sensors were calibrated once a month for pH, dissolved oxygen, and chlorophyll, and once every three months for salinity. pH was calibrated on the NBS scale using NIST buffers of 7 and 10, chlorophyll optical sensors were calibrated with Rhodamine dye, dissolved oxygen was calibrated as 100% saturation in air, and salinity was calibrated with a standard conductivity solution.

The YSI EXO2 pH probe uses a glass bulb electrode that is documented to measure pH to an accuracy of  $\pm 0.2$  units (Johengen et al., 2015). We sampled water next to the probe twice per

month to convert pH from the NBS scale to the more commonly used total scale and verify pH accuracy. Samples were stored in PYREX® bottles (Corning, Corning, NY, USA), poisoned with 200 µL HgCl<sub>2</sub>, and later analyzed for pH spectrophotometrically with m-cresol purple dye according to the methods of Dickson et al. (2007). Spectrophotometric measurements were verified for accuracy with certified reference materials (CRM, Dickson Laboratory). Total alkalinity was measured using an endpoint titration method on a Mettler-Toledo DL15 titrator with a pH probe (Mettler-Toledo, Columbus, OH). Alkalinity measurements were calibrated using certified reference materials (CRM, Dickson Laboratory).

During the 11 months of data collection, sensors failed on occasion. The 1m conductivity readings failed from 6/9/15 to 7/6/15. We used a third backup YSI EXO2 outfitted with a conductivity sensor located next to the sonde for salinity readings during that period. Because the conversion of dissolved oxygen from 100% to mg/L is salinity dependent, these values were post corrected. At 7m, both salinity and temperature readings failed from 4/26/15 to 6/9/15. As with 1m, a backup YSI EXO2 was used for temperature and salinity recordings during this period and dissolved oxygen readings were corrected as above. pH is temperature dependent, thus when the 1m temperature sensor failed, we post-corrected pH values as follows. We can apply a temperature correction to pH if one other carbonate parameter (pCO<sub>2</sub>, total carbon, total alkalinity) is known. While we did not measure any of these parameters continuously, salinity can be used to predict the alkalinity provided a relationship has been quantified for a region. We developed this relationship using linear regression of data from our 32 bottle samples (alkalinity = 56.6\*salinity +394.2,  $r^2 = 0.974$ , Figure S2). We used this regression to adjust pH values for temperature and salinity in CO2Calc using the standards of K1 and K2 of Millero (2010) and KHSO<sub>4</sub> of Dickson (1990).

### 3.3.4. Statistical Analysis

We evaluated seasonal trends in our 24 month dataset using autocorrelation analyses on tenacity, temperature, gonad index, and condition index at each depth. Parameters that were autocorrelated were compared using cross-correlation analysis to determine lags between the data. We compared changes in tenacity, thread strength, and thread number between seasons using a two-way ANOVA with season and depth as fixed factors. Season was classified as Summer (May – Oct) and Winter (Nov – April).

We used general linear models to examine the effects of water parameters on mussel tenacity following the procedure of Ettinger et al. (2011). We did not measure all parameters for the same amount of time, therefore, models were run on the 11 month dataset (tenacity as a function of depth, temperature, salinity, chlorophyll, dissolved oxygen, and pH). The water parameters we measured (temperature, salinity, chlorophyll, dissolved oxygen, and pH) are often highly correlated (eg, see Table 3.2). Therefore for our model ranking selection process we did not consider models with parameters that were correlated with an  $r$  greater than 0.6. We evaluated 80 models for the 11 month dataset, including a null model. We first ranked parameters by their importance, that is, the number of times they appeared in all of the possible models. We next used these rankings to generate a predictive model for tenacity.

## 3.4. Results

### 3.4.1. Seasonal changes in mussel condition and tenacity

Mussel tenacity was not constant over the 24-month sampling period at both depths (Figure 3.2). At 1m depth, mussel tenacity followed a seasonal trend, whereby mussels were twice as strong in the winter months than in the summer months (autocorrelation = -0.451,

Figure 3.3A). Autocorrelation analysis did not detect any seasonal changes in tenacity at 7m on this monthly scale (Figure 3.3B). Mussel condition index at 1m also followed a seasonal pattern with greater mussel condition in the summer months than in the winter months (autocorrelation = -0.224, Figure 3.3A), with peaks preceding peak tenacity by 4 months (cross-correlation = 0.499, Figure 3.3C). No seasonal pattern was observed in gonad index (Figure 3.3A).

Mussel tenacity was greater in the winter than the summer, and this difference was most evident at 1 m depth (season:  $p < 0.001$ ; depth:  $p = 0.3$ ; season\*depth:  $p < 0.001$ , Figure 3.4A, Table 3.3). Mussels at both depths made threads that were 40% stronger in the winter months than the summer months (season:  $p < 0.01$ ; depth:  $p = 0.9$ ; season\*depth:  $p = 0.2$ , Figure 3.4B, Table 3.3). Mussels growing at 1m produced 60% more threads than mussels growing at 7m, regardless of season (two-way ANOVA, season:  $p = 0.8$ ; depth:  $p = < 0.001$ ; season\*depth:  $p = 0.4$ , Figure 3.4C, Table 3.3).

#### 3.4.2. *Water parameters*

Temperature varied seasonally at both depths, warmer in summer and cooler in winter (1m: autocorrelation = -0.675, 7m: autocorrelation = -0.554, Figure 3.2, Figure 3.3A, 3.3B). Mussels growing at 1m experienced a greater range of temperature variability; temperatures were as high as 20°C on warm days in the summer and as cool as 2°C on the coolest winter days (Figure 3.2). At 7m temperatures were more stable, ranging from 7°C to 16 °C (Figure 3.2). Cross-correlation analysis indicates a 6 month lag between peak temperature and peak tenacity (cross-correlation = -0.718, Figure 3.3C), implying the two parameters are negatively correlated.

pH, dissolved oxygen, chlorophyll, and salinity all varied over time and depth (Figure 3.5, Table 3.2). pH ranged from 7.09 – 8.72 at 1m and 6.97 – 8.53 at 7m. pH was lower at 7m

than at 1m for all months except from January - March. Dissolved oxygen fluctuated at both depths, ranging between 3 - 19 mg/L at 1m and 0.1 – 16 mg/L at 7m. Chlorophyll varied seasonally at both depths with very low concentrations in the winter (~0 µg/L) and high concentrations in the summer (~5 µg/L). Salinity showed some seasonal variation at 7m ranging from 13 - 30 with lower salinity in the winter. The range of variation was greater at 1m, salinity reached as low as 3 in the winter months as compared to a high of 30 in the summer months.

### *3.4.3. Factors related to tenacity*

We observed high correlation between salinity and temperature, pH and dissolved oxygen, and salinity and dissolved oxygen in the 11 month model (Table S3.1). Therefore, these parameters were not included with each other.

At 1m depth over 11 months, the monthly differences in tenacity are best explained by (in order of parameter importance) temperature, pH, salinity, depth, chlorophyll, and dissolved oxygen (Table 3.4). We did not observe the interaction between depth and any parameter to affect mussel tenacity significantly. Therefore, we evaluated both depths together.

Linear model fits find high temperature, low pH, high salinity, low chlorophyll and low dissolved oxygen correlate with weak tenacity. We believe the correlation between high salinity and weak tenacity is due to the correlation of salinity with temperature as there is no evidence in the literature to suggest marine mussels perform better under low salinity (Braby and Somero 2006). Likewise, we did not further explore the relationship between weak tenacity and high chlorophyll. We believe this is an artifact of the seasonal trend of low wintertime chlorophyll; we do not expect low chlorophyll (low food) to increase mussel attachment (Melzner et al. 2011). However, high temperature has been linked to a decrease in byssal thread strength in this species

(Chapter 2), as has low pH (O'Donnell et al. 2013). Dissolved oxygen and pH were highly correlated, and there is also evidence to suggest low dissolved oxygen could hinder mussel performance and thus weaken mussel attachment strength (Jakubowska and Normant 2015). None of these parameters interacted significantly with depth, therefore we considered both depths together. We there ran a model selection on both temperature and pH, and temperature and dissolved oxygen.

For the temperature and pH model, temperature, pH, and an interaction between temperature and pH best predict tenacity (Figure 3.6A, Table 3.5). This model predicts temperatures beyond 14°C and/or pH below 7.5 weaken mussel tenacity ( $< 2 \times 10^{-4} \text{Nm}^{-2}$ ). For the temperature and dissolved oxygen model, temperature and dissolved oxygen best predict tenacity (Figure 3.6B, Table 3.5). This model predicts temperature beyond 14°C ( $< 2 \times 10^{-4} \text{Nm}^{-2}$ ) and dissolved oxygen below 7 mg/L weakens mussel tenacity ( $< 4 \times 10^{-4} \text{Nm}^{-2}$ ).

To test the hypothesis that the effects of multiple stressors on byssal threads are independent of each other, we used the temperature and pH data to predict tenacity based on 1000 random temperature and pH combinations between the range of conditions observed in the field: temperature 6 - 16°C and pH 7.4 – 8.3. We then use ANOVA to compare tenacity under low temperature and high pH, high temperature, low pH, and high temperature and low pH. Average mussel tenacity was half as strong under high temperature, low pH, and high temperature and low pH (Figure 3.7, ANOVA,  $p < 0.001$ ). We find the combination of both stressors is no greater than the effects of each individual stressor alone (Figure 3.7, Tukey HSD,  $p = 0.8, 0.9$  respectively).

#### 3.4.4. Dissolved oxygen and pH accuracy

We verified the reported accuracy of the YSI EXO2 pH probes to  $\pm 0.2$  pH units using by-weekly bottle samples. Bottle samples from Aug 2014 to Dec 2014 exceed these bounds on one occasion (7m on 8/27/2014). However, three bottle samples from 1m from March to May 2015 fell well below this range. Since we cannot differentiate between pH sensor errors or errors in bottle sampling (strong currents mean we may not be sampling the same water as the sensor), we reconstructed pH from dissolved oxygen, chlorophyll, and temperature from the first 5 months when bottle samples showed no strong deviations. We used this relationship to predict pH for the whole dataset. This predicted pH at 1m only on one occasion goes beyond the 0.2 bounds (Figure S3.3). Therefore, we conclude our pH values are accurate to  $\pm 0.2$  pH units.

### 3.5. Discussion

Attachment strength (tenacity) of *M. trossulus* is not constant over time. At 1m depth, tenacity is 40% stronger in the winter months (as high as  $5.7 \times 10^{-4} \text{ Nm}^{-2}$ ) than in the summer months (as low as  $1.8 \times 10^{-4} \text{ Nm}^{-2}$ ). At 7m depth, tenacity fluctuated from  $2.2 \times 10^{-4} \text{ Nm}^{-2}$  to  $5.3 \times 10^{-4} \text{ Nm}^{-2}$  with the weakest tenacities recorded in the summer months. This is the first study to document seasonal differences in mussel tenacity for any species on the West Coast of North America. The water conditions (temperature, pH, dissolved oxygen, chlorophyll, and salinity) these mussels experienced also varied over time and depth. Notably, mussels growing at 1m experience warmer water in the summer, and mussels growing at 7m experience overall lower pH and dissolved oxygen. We find weak tenacity corresponded to periods of high temperature and low pH/dissolved oxygen, making these water parameters the best predictors of weak tenacity.



Seasonal differences in tenacity are due to changes in thread strength, not thread number. Although autocorrelation analysis did not detect a seasonal pattern at 7m, tenacity was on average greater in winter months, which was due to the production of stronger threads. Previous studies have attributed weak seasonal tenacity to weaker threads in *M. edulis* (Moeser et al. 2006, Lachance et al. 2008). However, Zardi et al. (2007) found seasonally weak byssus attachment was due to the production of fewer, not weaker threads in *P. perna* and *M. galloprovincialis*. Together, these studies suggest the thread properties that drive seasonal differences may be species dependent.

We found no significant difference in thread strength between depths, but mussels at 1m produced close to twice the number of threads than mussels at 7m. We observed much more mud and silt at 7m where flow was also lower (Newcomb, personal observation / data not shown). This increased sedimentation may decrease a mussel's ability to attach new threads to a hard surface. Although mussels produced fewer threads at 7m in comparison to 1m, there was no difference in tenacity between depths. This puzzling result may be explained by a greater rate of thread decay at 1m than 7m. While there may be more threads anchoring 1m mussels, the older threads may be weakened from decay and not contribute to overall strength.

In addition to seasonal changes in tenacity, we find water conditions in Penn Cove vary with depth and time. Both pH and dissolved oxygen were lower at 7m than at 1m. These parameters were also tightly linked, suggesting fluctuations in both are due to photosynthesis and respiration. Chlorophyll values were higher in the summer than the winter, presumably matching the time when phytoplankton is most abundant in the area. Salinity was lower in the winter than the summer at 1m. Runoff from the Skagit River rises in the winter from higher rainfall and

decreasing salinity (Trainer et al. 1998). Temperature variance was greater at 1m than 7m; temperature was highest at 1m in the summer and lowest at 1m in the winter.

By measuring mussel tenacity concurrently with water conditions, we were able to test our predictions from laboratory studies that high temperature (above 18°C) and low pH (below 7.6) weaken mussel tenacity (Chapter 2, O'Donnell et al. 2013). In the field, we observed weak tenacity when temperature > 14°C, and/or pH < 7.5. This temperature threshold is 2°C lower than that in the lab. While the reason for this discrepancy is not known, there are several plausible explanations. First, the laboratory study only grew mussels at their treatment temperatures for 3 days, while in the field the longer exposure to 14°C could be as stressful as the shorter exposure to temperatures >18°C. Further, the field threshold of 14°C was based on monthly mean temperature. In the warmest months, temperatures reached above 18°C and it is possible mussels are not responding to the mean temperature, but the maximum (Camacho et al. 2015). Chapter 2 predicts a range contraction of *M. trossulus* based on weakening past 18°C. The results of this study, that weakening occurs beyond 14°C, suggests this species may become restricted to cooler climates and environments over a shorter length of time than predicted in Chapter 2.

The observed threshold 7.5 for pH was very close to the threshold of 7.6 found in the laboratory study of O'Donnell et al. (2013) over 35 days. Interestingly, this threshold was not observed in Chapter 1: low pH (< 7.3) increased thread strength in Chapter 1, experiment 1 (Chapter 1). However, Chapter 1 experiment 2 finds a non-significant trend that long-term exposure (32 days) weakens threads while short-term exposure (3 days) strengthens threads. The field results here support the conclusion of O'Donnell et al. (2013) that long-term exposure to low pH weakens threads.

pH and dissolved oxygen were closely correlated, making it difficult to interpret their effects independently. While no studies have examined the effects of dissolved oxygen on byssus, dissolved oxygen at 20% saturation (~2 mg/L) compared to 100% saturation (~9 mg/L) lowered shell gaping activity in *M. trossulus* (Jakubowska and Normant 2015). Future work should examine how low dissolved oxygen could weaken byssus strength.

These results support the “weakest link” hypothesis put forth in Chapter 1. A byssal threads can be viewed as three regions, or links, that are connected in series: the plaque, distal and proximal regions. A thread can break in only one of three regions, the one that is weakest. Once a thread breaks in one region, the weakening effects of another stressor in another region are no longer relevant. For mussel byssus, different stressors target different regions of the thread. For example, elevated temperature weakens the proximal region (Chapter 1) and low pH weakens the plaque (O’Donnell et al. 2013). When combined, the stressor with the largest effect on its specific region determines the force needed to break the thread. In this manner, stressors act independent of each other and the greater stressor dominates. We observed tenacity under high temperature and low pH was no weaker than under high temperature or low pH alone. From a predictive standpoint, one that may be of interest to mussel growers, mussels are equally prone to low tenacity when either threshold is exceeded: temperature above 14°C, or pH below 7.5. The combination of these two stressors do not appear to impose additional harm.

Any predictive model carries the risk of false positives or false negatives. A false negative is the more worrisome scenario for mussel growers because they would be led to overestimate mussel attachment. The  $r^2$  for our two models range from 0.38 to 0.53, meaning 47-62% of the variation in mean monthly remains unexplained. Factors other than temperature, pH and dissolved oxygen likely contribute to variability in tenacity, and adding them into the model

will generate greater predictive capacity. A longer dataset would allow us to include more dependent variables, such as salinity and chlorophyll, and to tease apart how these multiple stressors interact to affect mussel attachment. It would be desirable to extend our measurements to include other parameters that could influence tenacity, such as sedimentation, water flow, and nutrients.

While these results provide field evidence that high temperature, low pH, and low dissolved oxygen weaken mussel attachment strength, the mechanism by which these parameters weaken byssal threads is not clear. One hypothesis is that different environmental conditions, such as high temperature and low pH, change the protein composition of the threads (Waite & Broomell 2012). Another hypothesis is that it is energetically costly to live in high temperature, low pH, and low dissolved oxygen waters leaving fewer resources available to produce byssus (Michaelidis et al. 2005, Ivanina et al. 2013). Previous work has demonstrated trade-offs between attachment and reproduction in *Mytilus* spp. (Carrington 2002, Zardi et al. 2007) that were not observed in this study. Gonad index did not vary seasonally or correlate inversely with mussel tenacity at either depth. It is possible mussels in our system prioritize resources to reproduction over byssus production and in those months where fewer resources are available due to adverse ocean conditions, tenacity suffers. Condition index (a ratio of meat per shell volume) in mussels growing at 1m, however, varied seasonally and peaks in condition lagged 4 months behind peaks in tenacity. This lag suggests a possible energetic trade-off because mussels do not maximize attachment strength and tissue mass at the same time.

In summary, this study is the first to show high temperature, low pH, and low dissolved oxygen weaken mussel tenacity in a field setting, corroborating the laboratory results of Chapters 1 and 2. This weakening is likely due to weakening individual byssal threads as opposed to

slowing thread production. Mussel growers have reported increases in fall-off in recent years (Carrington et al. 2015). The present study suggests temperature, pH, and dissolved oxygen can be monitored to a forecast of periods and locations when mussel tenacity is likely to be weak and fall-off most likely to occur. Ocean pH and temperature are projected to increase in the future (IPCC 2013), which could shift the baseline conditions to where critical thresholds are surpassed more frequently and severely, increasing the risk of fall-off for mussel farms. Growers may be able to mitigate these effects by adapting their farming practices to reduce fall-off during periods when attachment is predicted to be weak as well as siting new farms in areas where high temperature, low pH, and low dissolved oxygen do not occur as frequently.

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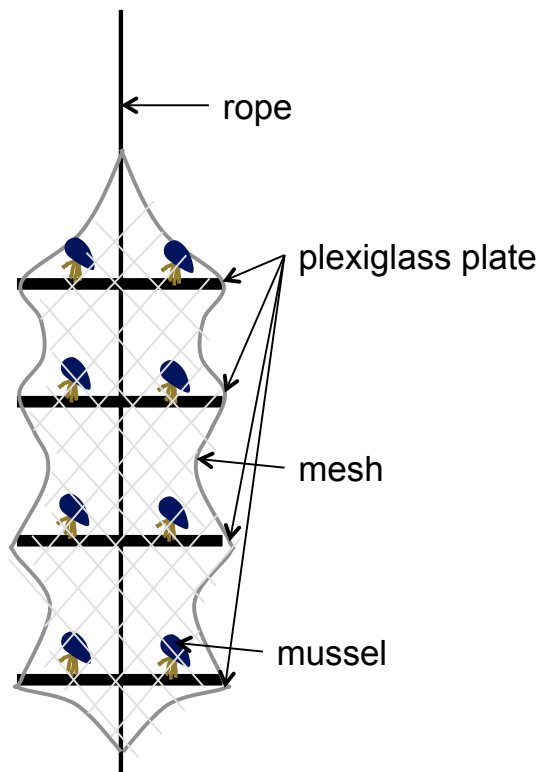
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### 3.7. Tables and Figures

**Table 3.1.** Summary of seasonal patterns in attachment strength in mussel species across the world.

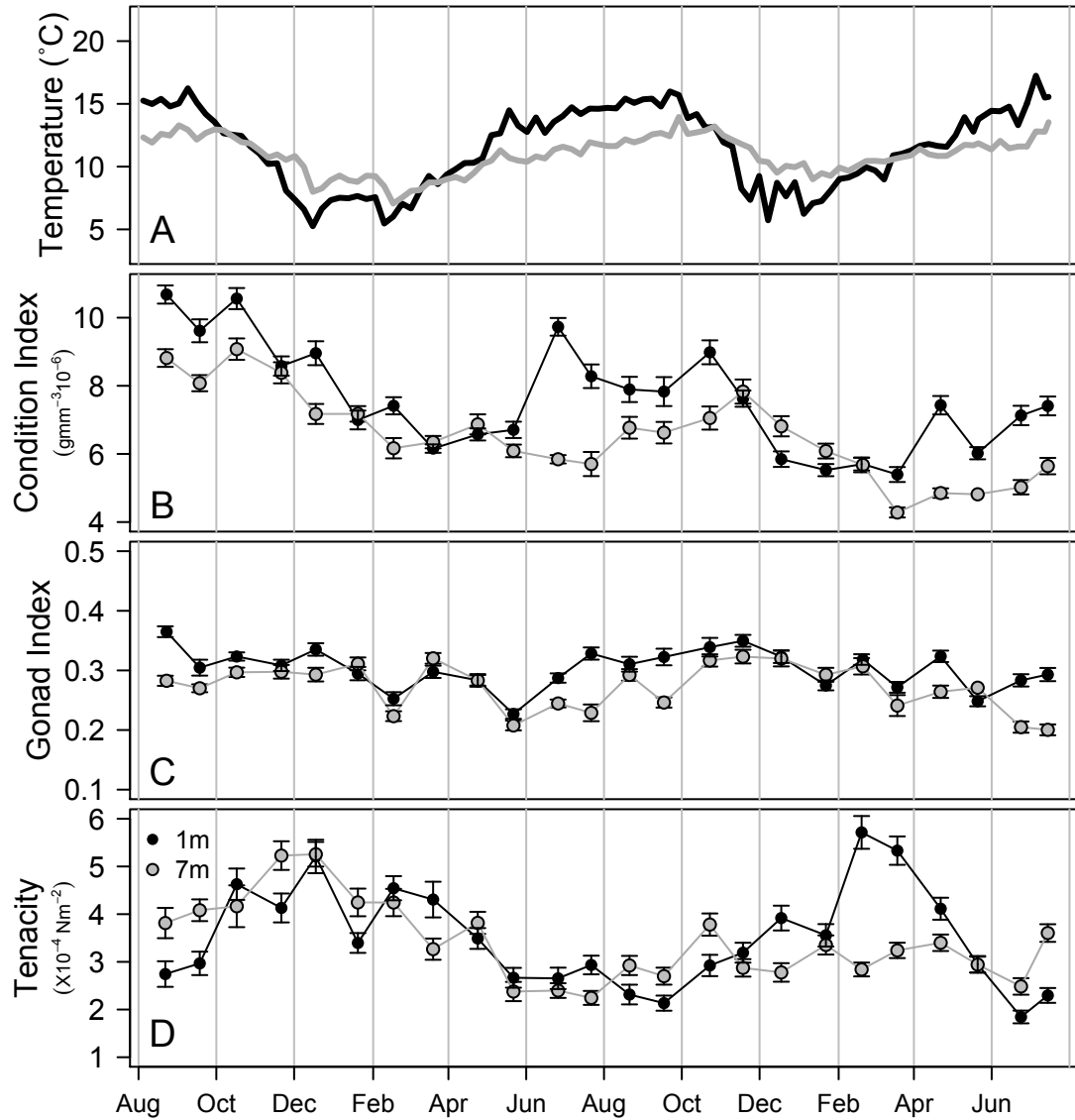
<b>Study</b>	<b>Species</b>	<b>Location</b>	<b>Seasonal Pattern</b>	<b>Responsible factors by correlation</b>
Price (1980) Price (1982)	<i>Mytilus edulis</i>	Northeast Atlantic	strongest in summer, weakest in winter	wind
Carrington (2002) Carrington et al. (2009)	<i>Mytilus edulis</i>	Northwest Atlantic	weakest in summer, strongest late winter/early spring	wave energy, gonad index, temperature, food supply
Lachance et al. (2008)	<i>Mytilus edulis</i>	Northwest Atlantic	weakest in summer, strongest fall/winter	temperature largest, then turbulence and gonad index
Zardi et al. (2007)	<i>Mytilus galloprovincialis</i>	Southeast Atlantic	stronger in summer, weaker in winter	temperature, wave energy, gonad index
Zardi et al. (2007)	<i>Perna perna</i>	Southeast Atlantic	stronger in summer, weaker in winter	temperature, wave energy, gonad index
Newcomb and Carrington (unpublished data)	<i>Mytilus californianus</i>	Northeast Pacific	weakest in summer, stronger in winter	NA
Present Study	<i>Mytilus trossulus</i>	Northeast Pacific	weakest in summer, stronger in winter	temperature, dissolved oxygen, pH



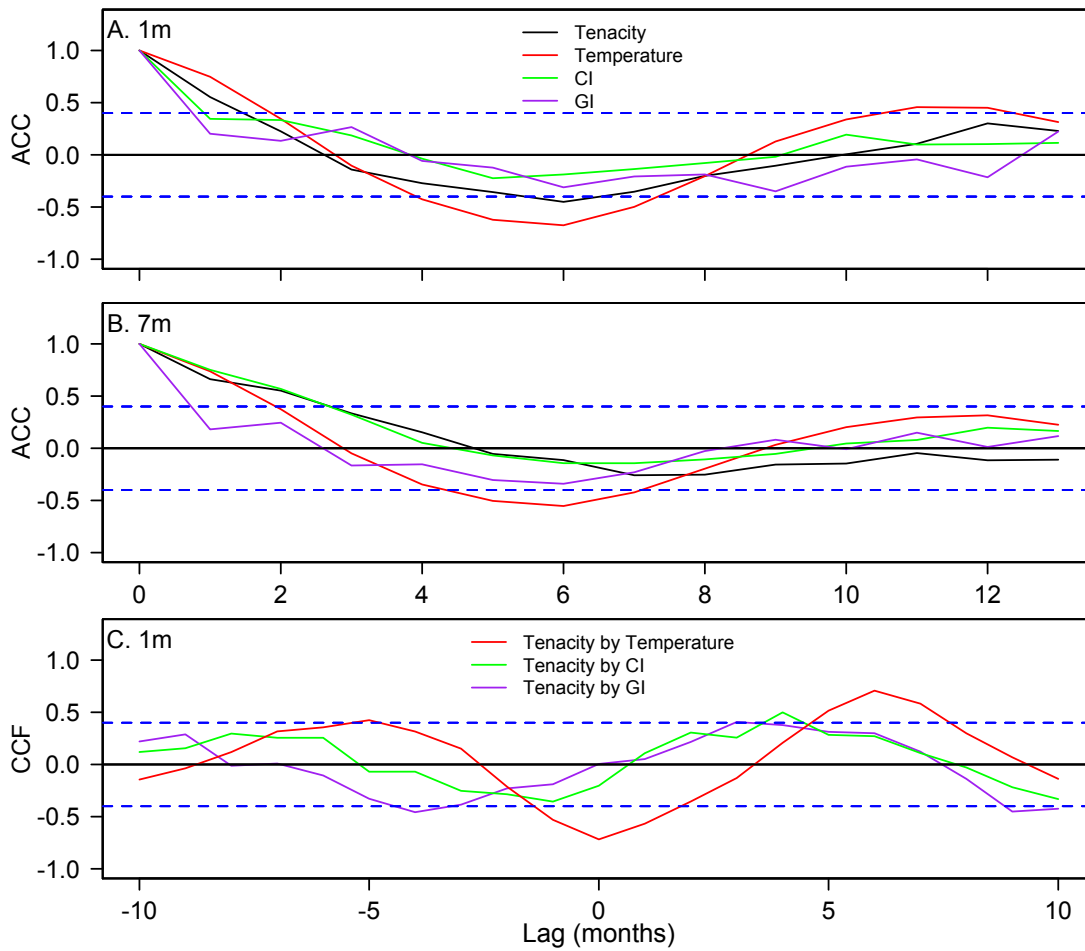
**Figure 3. 1.** Mussel hotels were constructed with rope, mesh, and plexiglass plates to assess individual mussel thread strength and production.

**Table 3.2.** ANOVA results of each water parameter as a function of depth and month. There was a significant interaction of depth and month for all factors. (DO = dissolved oxygen, Chl = chlorophyll).

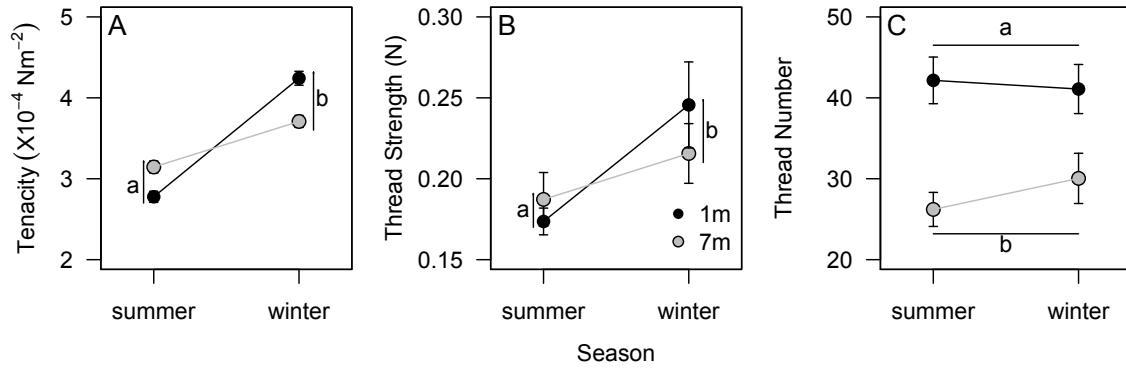
		pH	DO	Chl	Salinity	Temperature
depth	F	6539	11918	3.4	16164	102
	df	1	1	1	1	1
	p	<b>&lt; 0.001</b>	<b>&lt; 0.001</b>	0.06	<b>&lt; 0.001</b>	<b>&lt; 0.001</b>
month	F	392.5	141	32	1772	3614
	df	12	12	12	12	12
	p	<b>&lt; 0.001</b>	<b>&lt; 0.001</b>	<b>&lt; 0.001</b>	<b>&lt; 0.001</b>	<b>&lt; 0.001</b>
depth*month	F	271	55	63	517	1301
	df	10	10	10	10	10
	p	<b>&lt; 0.001</b>	<b>&lt; 0.001</b>	<b>&lt; 0.001</b>	<b>&lt; 0.001</b>	<b>&lt; 0.001</b>



**Figure 3.2.** Seasonal changes in weekly average temperature, monthly condition index, gonad index, and tenacity at depths of 1 and 7m in Penn Cove, Whidbey Island, WA from August 2013 to July 2015. Circles represent mean  $\pm$  SE from a sample of 35 (condition index and gonad index) and 50 (tenacity) mussels.



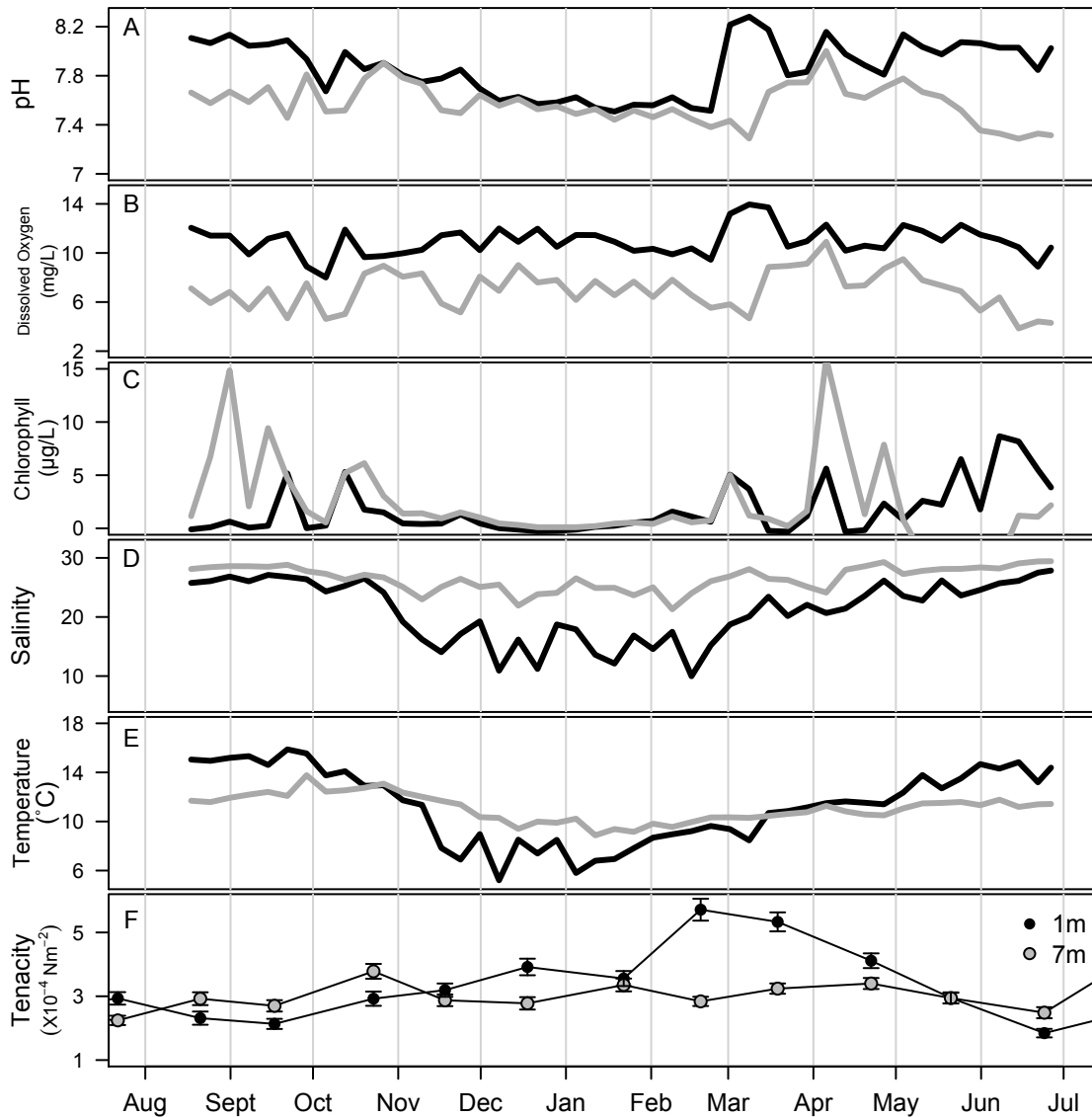
**Figure 3.3.** Auto correlation coefficients (ACC) of tenacity, temperature, condition index (CI) and gonad index (GI) at depths of (A) 1m and (B) 7m. (C) Cross correlation coefficients (CCC) of tenacity with temperature, CI, and GI at 1m depth. Solid lines represent correlation coefficients, dotted lines are 95% confidence levels for the significance of each correlation.



**Figure 3. 4.** (A) Mussel tenacity was 20% greater in the winter than the summer at both depths (season:  $p < 0.001$ ; depth:  $p = 0.3$ ; season\*depth:  $p < 0.001$ , Table 3.3). (B) Individual byssal threads were 40% stronger in the winter than the summer at both depths (season:  $p < 0.01$ ; depth:  $p = 0.9$ ; season\*depth:  $p = 0.2$ , Table 3.3). (C) Mussels growing at 1m produced 60% more threads than mussels at 7m in both seasons (season:  $p = 0.8$ ; season\*depth:  $p = 0.4$ , Table 3.3). There was no interactive effect of season on thread number ( $p = < 0.001$ , Table 3.3). Symbols represent mean  $\pm$  SE. Letters represent significantly different groups while bars indicate no difference between groups.

**Table 3.3.** Two-way ANOVA results of Tenacity, thread strength, and thread number as a function of seasons at both depths.

		Tenacity ( $\times 10^{-4} \text{ Nm}^{-2}$ )	Thread Strength (N)	Thread Number
season	F	179	8	0.05
	df	1	1	1
	p	<b>&lt; 0.001</b>	<b>&lt; 0.01</b>	0.8
depth	F	1	0	24
	df	1	1	1
	p	0.3	0.9	<b>&lt; 0.001</b>
season*depth	F	35	2	0.8
	df	1	1	1
	p	<b>&lt; 0.001</b>	0.2	0.4

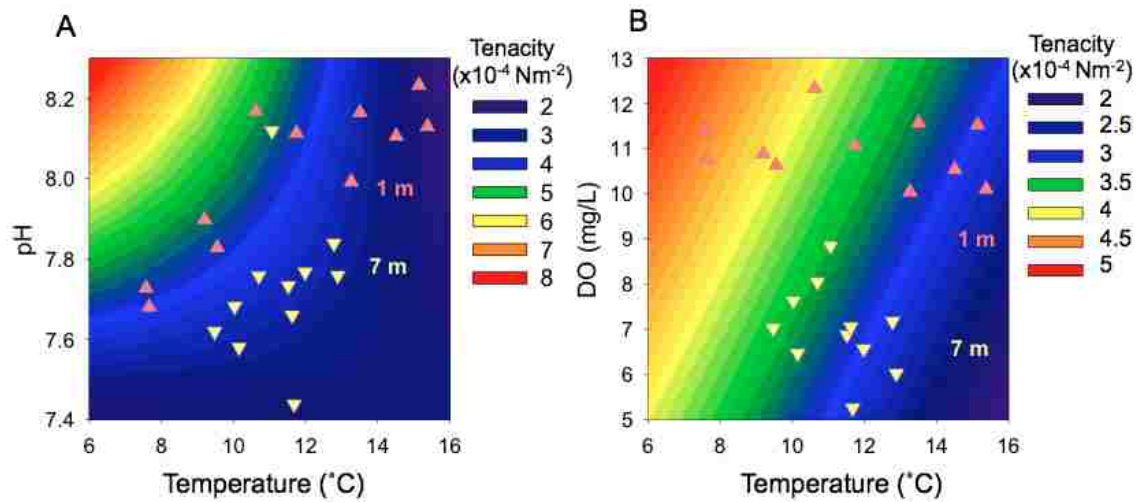


**Figure 3.5.** Water characteristics of Penn Cove, Whidbey Island, WA from August 2014 to June 2015. Black lines indicate conditions at 1m depth while gray lines indicate 7m depth. (A) Average weekly pH on the total scale, (B) average weekly dissolved oxygen, (C) average weekly chlorophyll as measured by fluorescence, (D) average weekly salinity, (E) average weekly temperature measured by YSI EXO2 data sondes. (F) Monthly tenacity of *M. trossulus* at 1m and 7m. Circles are mean  $\pm$  SE of 50 mussels.



**Table 3.4.** Water parameters listed by importance in all possible linear models. Full list of models are in Table S3.2.

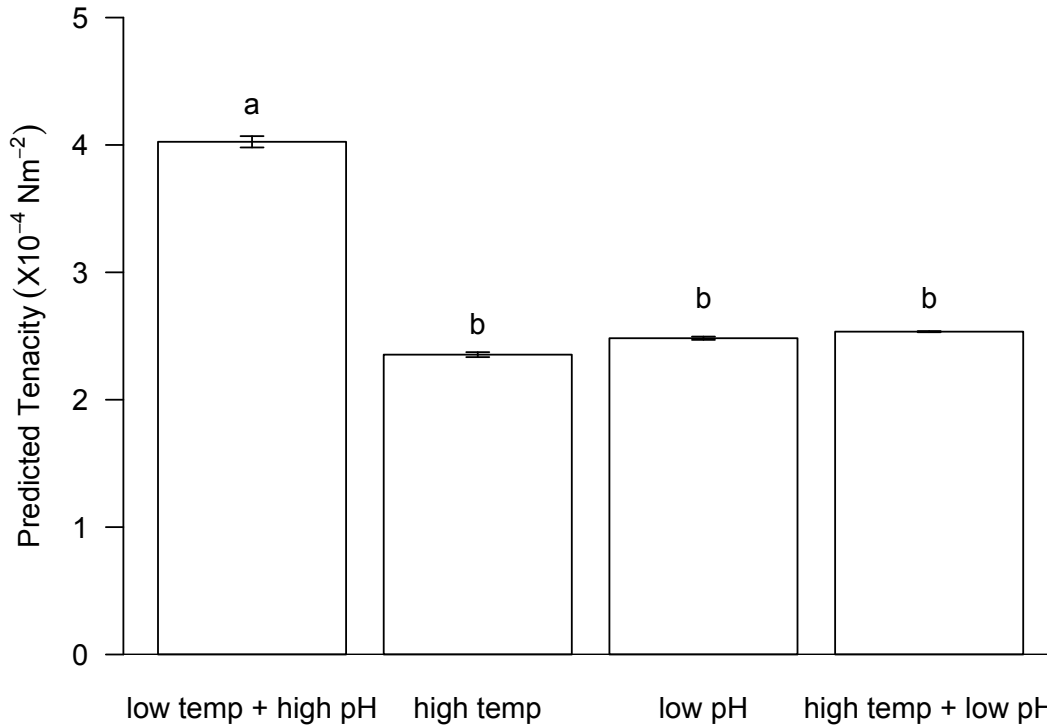
<b>Parameter</b>	<b>Parameter Importance</b>	<b>Number of Model containing parameter</b>
temperature	0.59	16
pH	0.54	14
salinity	0.41	12
depth	0.38	18
chlorophyll	0.14	9
dissolved oxygen	0.13	4
depth:pH	0.08	4
depth:salinity	0.08	3
depth:temperature	0.06	4



**Figure 3.6.** Contour plot of predicted tenacity based on GLM models. (A) Tenacity as a function of temperature and pH (tenacity= $6.003 \times \text{temperature} + 4.354 \times \text{pH} - 0.804 \times \text{temperature} \times \text{pH} - 84.212$ , Table 3.4). Tenacity decreases with increased temperature and decreased pH. Symbols represent each monthly mean temperature and pH conditions at 1m (pink upward triangle) and 7m (yellow downward triangle). (B) Tenacity as a function of temperature and dissolved oxygen (tenacity= $-0.235 \times \text{temperature} + 0.150 \times \text{dissolved oxygen} + 4.575$ , Table 3.4). Tenacity decreases with increased temperature and decreased dissolved oxygen.

**Table 3.5.** Generalized linear models to predict tenacity from temperature, pH, and dissolved oxygen (DO).

<b>Model Inputs</b>	<b>parameter</b>	<b>P</b>	<b>r<sup>2</sup></b>	<b>AIC</b>
temperature, pH	temperature	< 0.05	0.53	51.8
	pH	< 0.05		
	temperature*pH	< 0.05		
temperature, DO	temperature	< 0.001	0.37	56.1
	DO	0.05		

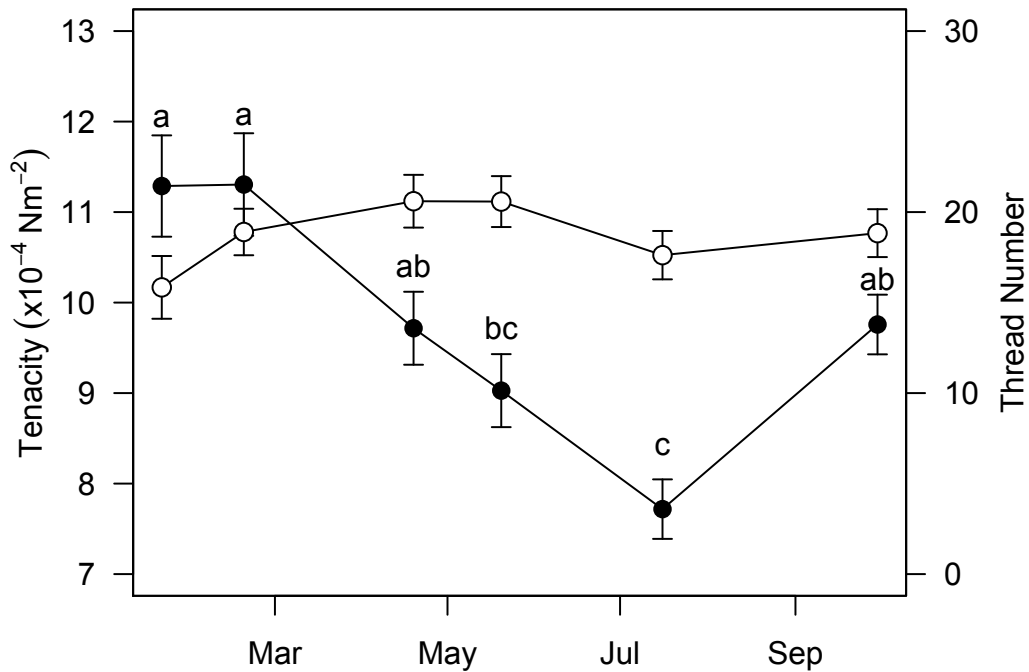


**Figure 3.7.** Low temperature and high pH act independently on mussel tenacity. Predicted tenacity from temperature and pH model run through 1000 simulations of random pH from 7.4 to 8.3 and random temperature from 6 to 16°C. Predicted tenacity under any stressor is 40% lower from non-stressful conditions (ANOVA,  $F_{3,996}=150$ ,  $p<0.001$ ). Tenacity when both temperature is high (>14°C) and pH is low (<7.5) is no different from tenacity when either temperature is high or pH is low (TukeyHSD,  $p = 0.8, 0.9$  respectively). Bars represent means, error bars are standard error, letters are significantly different treatments, N=743, 148, 82, 28.

### **3.9. Acknowledgements**

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### 3.8. Supplementary Tables and Figures



**Figure S3.1.** Tenacity (filled circles) varies seasonally in *Mytilus californianus* off of Sokol Point, WA, USA from January 2015 – September 2015 (ANOVA  $p < 0.001$ ). Attachment is 1.3 times greater in the winter than the summer. This pattern is likely explained by thread strength because thread number does not change across season (open circles, ANOVA  $p = 0.3$ ). Symbols are means  $\pm$  SE, letters denote significantly different treatments by Tukey Post-hoc test,  $N = 50$ .

**Table S3.1.** R values for parameter correlations at both depths. Parameters with  $r > 0.6$  (bolded) were not included together in any models for the model selections.

	temperature	salinity	chlorophyll	DO	pH
temperature		<b>0.70</b>	0.28	0.10	0.56
salinity			0.26	<b>0.60</b>	0.10
chlorophyll				0.06	0.30
DO					<b>0.73</b>
pH					

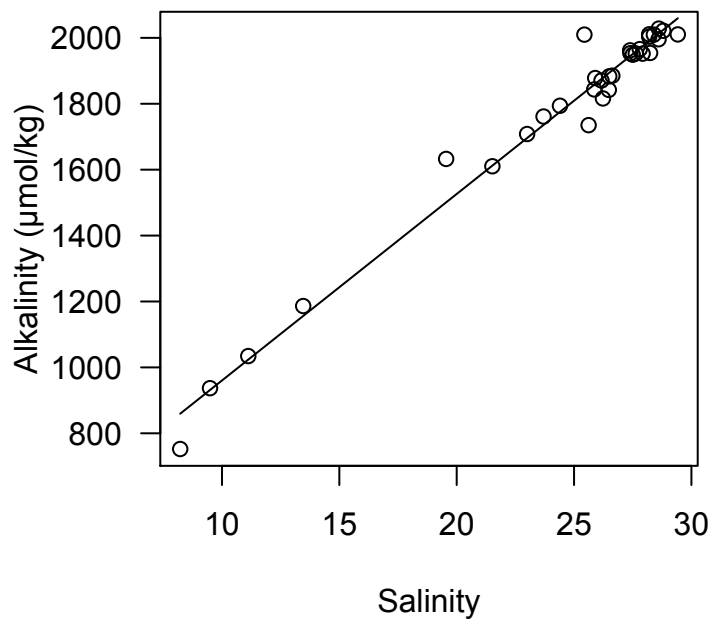
**Table S3.2.** All models included in parameter selection for 11 months. Chl= chlorophyll, DO = dissolved oxygen, sal=salinity, temp=temperature.

intercept	depth	chlorophyll	dissolved oxygen	pH	salinity	temperature	depth*chl	depth*DO	depth*pH	depth*sal	depth*temp	df	logLik	AICc	delta	weight
-9.08E+00				2.09		-0.36						4	-21.79	53.9	0	0.22
6.22E+00					-0.13							3	-23.86	55.1	1.12	0.13
4.58E+00			0.15			-0.23						4	-22.89	56.1	2.19	0.07
-8.33E+00	+			2.03	-0.21							5	-21.62	57	3.05	0.05
-4.43E+01	+			7.02	-0.40				+	+		7	-17.57	57.1	3.19	0.04
6.60E+00	+				-0.15							4	-23.45	57.2	3.31	0.04
-9.67E+00	+			2.16		-0.36						5	-21.79	57.3	3.39	0.04
-9.20E+00		-0.01		2.10		-0.36						5	-21.79	57.3	3.39	0.04
5.92E+00						-0.23						3	-25.11	57.5	3.61	0.04
6.31E+00	+					-0.25						4	-23.73	57.8	3.88	0.03
6.28E+00		0.03			-0.13							4	-23.76	57.9	3.94	0.03
4.18E+00				0.26	-0.13							4	-23.79	57.9	3.99	0.03
-1.06E+01	+			2.36		-0.41					+	6	-20.39	58.4	4.44	0.02
-1.13E+01	+			2.49	-0.24					+		6	-20.51	58.6	4.69	0.02
2.87E+00	+		0.29			-0.22						5	-22.57	58.9	4.95	0.02
4.63E+00		0.03	0.15			-0.25						5	-22.73	59.2	5.27	0.02
-2.39E+01	+			4.14	-0.27				+			6	-20.82	59.2	5.31	0.02
6.71E+00	+					-0.28					+	5	-22.90	59.6	5.62	0.01
2.57E+00	+		0.36			-0.26					+	6	-21.00	59.6	5.67	0.01
6.82E+00	+				-0.16					+		5	-23.18	60.1	6.17	0.01
-2.96E+01	+			4.95		-0.56			+		+	7	-19.06	60.1	6.19	0.01
6.68E+00	+	0.03			-0.15							5	-23.31	60.4	6.43	0.01
5.97E+00		0.02				-0.24						4	-25.04	60.4	6.5	0.01
-9.40E+00	+	-0.02		2.17	-0.21							6	-21.58	60.8	6.82	0.01
6.41E+00	+	0.04				-0.26						5	-23.54	60.8	6.89	0.01
-1.44E+01	+			2.80		-0.39			+			6	-21.69	61	7.04	0.01
-1.02E+01	+	-0.01		2.24		-0.36						6	-21.77	61.1	7.21	0.01
4.84E+00		0.02		0.18	-0.13							5	-23.73	61.2	7.27	0.01
7.44E-04	+		0.54			-0.21	+					6	-22.09	61.8	7.84	0.00
-4.46E+01	+	0.01		7.07	-0.40				+	+		8	-17.54	62.2	8.22	0.00
1.90E+00			0.15									3	-27.49	62.3	8.38	0.00
-1.28E+01	+	-0.04		2.65		-0.42					+	7	-20.19	62.4	8.45	0.00
-1.41E+01	+	-0.04		2.86	-0.25					+		7	-20.26	62.5	8.59	0.00
3.08E+00	+	0.02	0.28			-0.23						6	-22.48	62.5	8.61	0.00
3.24E+00												2	-28.98	62.6	8.65	0.00

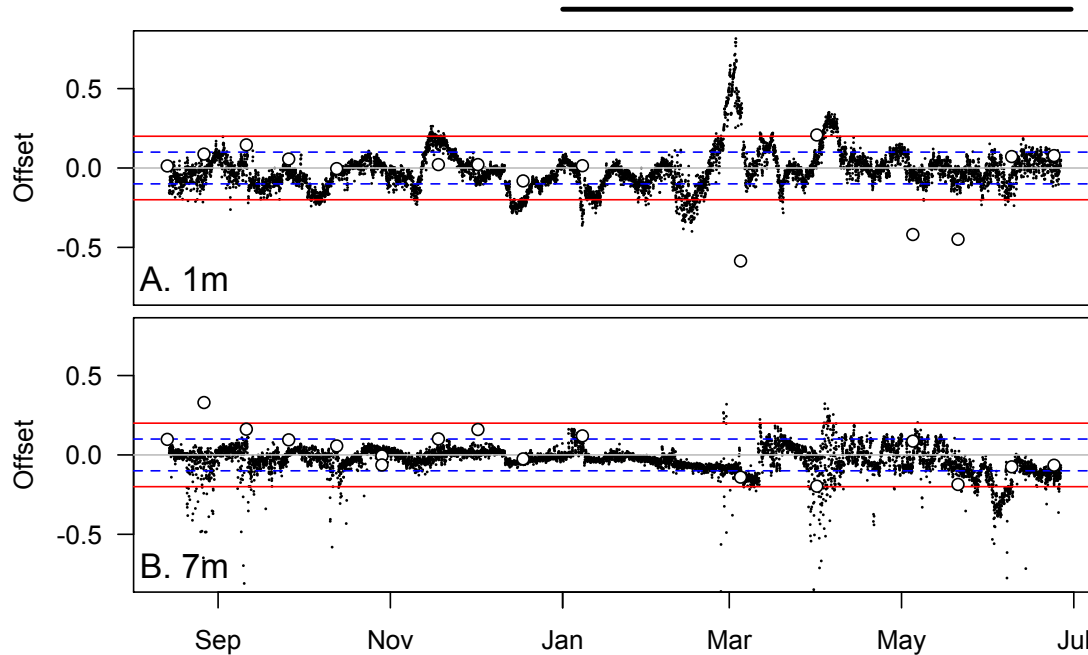


6.76E+00	+	0.02				-0.29				+	6	-22.82	63.2	9.3	0.00
-2.45E+01	+	0.02		4.24	-0.28			+			7	-20.78	63.6	9.63	0.00
8.61E-01	+		0.51			-0.25		+		+	7	-20.81	63.6	9.69	0.00
-1.08E+00	+		0.41								4	-26.66	63.7	9.75	0.00
6.88E+00	+	0.03			-0.16					+	6	-23.06	63.7	9.79	0.00
2.60E+00	+	0.00	0.36			-0.26				+	7	-21.00	64	10.07	0.00
3.45E+00	+										3	-28.34	64	10.08	0.00
6.61E+00	+	-0.01			-0.15		+				6	-23.25	64.1	10.16	0.00
6.35E+00	+	-0.02				-0.24	+				6	-23.40	64.4	10.47	0.00
3.30E+00		-0.03									3	-28.87	65.1	11.15	0.00
-9.64E+00	+	0.00		2.20	-0.21		+				7	-21.57	65.1	11.2	0.00
-2.96E+01	+	0.00		4.94		-0.56			+	+	8	-19.06	65.2	11.26	0.00
1.97E+00		-0.02	0.15								4	-27.42	65.2	11.27	0.00
1.59E+00				0.21							3	-28.95	65.2	11.29	0.00
-1.44E+01	+	0.00		2.80		-0.39			+		7	-21.69	65.4	11.44	0.00
-1.00E+01	+	-0.02		2.20		-0.36	+				7	-21.77	65.5	11.6	0.00
9.04E-03	+	0.03	0.55			-0.23		+			7	-21.90	65.8	11.87	0.00
-4.69E+00	+		0.74					+			5	-26.08	65.9	11.97	0.00
8.66E+00	+			0.65							4	-28.16	66.7	12.74	0.00
-1.11E+00	+	-0.03	0.42								5	-26.53	66.8	12.88	0.00
3.12E+00	+	-0.02	0.27			-0.22	+				7	-22.41	66.8	12.88	0.00
3.50E+00	+	-0.02									4	-28.28	66.9	12.98	0.00
-1.65E+01	+	0.04		3.22	-0.28		+			+	8	-19.95	67	13.05	0.00
2.13E+01	+			2.24					+		5	-26.70	67.1	13.21	0.00
-1.41E+01	+	0.02		2.83		-0.44	+			+	8	-20.05	67.2	13.23	0.00
-4.67E+01	+	0.09		7.38	-0.43		+		+	+	9	-17.18	67.4	13.43	0.00
6.72E+00	+	0.00				-0.28	+			+	7	-22.80	67.6	13.67	0.00
4.37E-01		-0.04		0.37							4	-28.79	67.9	14	0.00
6.83E+00	+	0.00			-0.16		+			+	7	-23.05	68.1	14.16	0.00
3.81E+00	+	-0.20					+				5	-27.29	68.3	14.4	0.00
-2.45E+01	+	0.01		4.23	-0.28		+		+		8	-20.78	68.6	14.7	0.00
8.33E-01	+	0.01	0.51			-0.26		+		+	8	-20.79	68.7	14.73	0.00
2.56E+00	+	0.02	0.36			-0.27	+			+	8	-20.98	69	15.11	0.00
-3.48E-01	+	-0.17	0.37				+				6	-25.86	69.3	15.39	0.00
-4.50E+00	+	-0.02	0.73					+			6	-26.02	69.6	15.71	0.00
8.36E+00	+	-0.01		0.61							5	-28.16	70.1	16.13	0.00
-1.46E+01	+	-0.02		2.83		-0.39	+		+		8	-21.66	70.4	16.46	0.00
1.99E+01	+	-0.06		2.04					+		6	-26.44	70.5	16.54	0.00
1.25E-01	+	0.00	0.54			-0.22	+	+			8	-21.87	70.8	16.87	0.00
-3.00E+01	+	0.03		5.00		-0.57	+		+	+	9	-18.98	71	17.02	0.00

8.04E+00	+	-0.18		- 0.53			+				6	-27.19	72	18.05	0.00
-3.39E+00	+	-0.15	0.65				+	+			7	-25.46	72.9	18.98	0.00
1.77E+01	+	-0.14		- 1.75			+		+		7	-26.20	74.4	20.46	0.00
7.79E-01	+	0.03	0.52			-0.26	+	+		+	9	-20.77	74.5	20.61	0.00



**Figure S3.2.** Salinity is an accurate predictor of alkalinity ( $r^2 = 0.943$ ,  $N=32$ , alkalinity =  $56.6 \times \text{salinity} + 394.2$ ). Alkalinity measurements were with a reported precision of  $\pm 2.6 \mu\text{mol/kg}$ .



**Figure S3.3.** pH offset from bottle samples (open circles) and prediction from predicted pH from temperature and oxygen relationship (black points) for (A) 1m and (B) 7m. Data points from August to December, where bottle samples did not deviate  $> \pm 0.2$  pH units from probe pH, were used to predict pH from Jan - June (indicated by solid black bar). Dotted blue lines indicate  $\pm 0.1$  unit deviation and red lines denote  $\pm 0.2$  unit deviation.