Effects of thermal stress and ocean acidification on the larval development of ascidian *Boltenia villosa*

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Abstract:

Climate change and ocean acidification significantly affect the development of many marine organisms, including the solitary ascidian, *Boltenia villosa*. This study investigates the effects of increased temperature and decreased pH levels on the development and survival rates of *B.villosa* embryos. It was hypothesised that embryos at higher temperatures would show faster development but lower survival rates while embryos at lower pH conditions would develop slower. In this study, the embryos were exposed to three different temperature conditions, low (9°C), mid (12°C), and high (16°C) and were also placed in seawater that was neutral (pH 7) and a control (pH 8) within each of these temperature settings. The time required for embryos to reach key development stages was observed, as was the survival rate to the tadpole stage. Our results show that embryos subjected to higher temperatures develop faster than those at lower temperatures but have lower survival rates. However, varying pH levels did not significantly affect embryonic development. These findings suggest that warmer temperatures expedite the rate of development but reduce the survivorship of the organism.

Introduction

Greenhouse emissions have seriously altered the physical and chemical properties of the ocean which have had profound impacts on marine species (Reid et al., 2009). The global ocean has absorbed 93% of the heat from these emissions, resulting in a 1°C increase in sea surface temperatures from the 1900s to 2020 (IPCC, 2023). Additionally, the oceans are absorbing more than a quarter of carbon dioxide from the atmosphere making them more acidic (Watson et al.,2020). Particularly, higher latitudes have a lower buffering capacity against pH change, leading to increased acidification along the U.S. coastal ecosystems (USGCRP, 2017). These changes pose significant challenges for marine species, necessitating a deeper understanding of their effects on individual organisms, particularly during critical developmental stages.

Among the diverse array of marine organisms, ascidians play a crucial role in marine ecosystems as efficient filter feeders. Earlier studies have observed that phytoplankton productivity in shallow fjords is controlled by tunicate populations (Peterson et al, 1992). These sessile organisms are vital components of marine food webs, serving as both a food source for molluscs, sharks, skates and other bottom-dwelling creatures as well as a suitable substrate for sponges and algae (Mesa Community College, n.d.).

Ascidians are hermaphroditic but are incapable of self-fertilization and reproduce by free-spawning (Swalla,2004). Tadpole larvae are motile and are invertebrate chordates, organisms that have a notochord at some point in their life history. Embryonic development has been well documented in many species of ascidians (Swalla, 2004). In solitary ascidians, cell fates, the specialized role each cell will play during embryogenesis, are fixed early on and they undergo 6 rounds of bilateral cleavage post-fertilization (Holland, 2016 and Swalla 2004). The tadpole stage is pivotal as it marks the transition from free swimming to seeking suitable environments for settlement and metamorphosis, a crucial phase for survival and dispersal (Davidson and Swalla, 2002; Karaiskou et al., 2015). Recruitment of tunicates, including ascidians, is influenced by environmental factors like temperature, acidity, swimming duration, and energy reserves at metamorphosis(Svane and Young, 1989). Despite their ecological importance, little is known about how ascidians, particularly the larval stages, respond to the dual stressors of ocean acidification and global warming.

Understanding the effects of these environmental stressors on ascidian development is essential for predicting their future survival and the health of marine ecosystems. By investigating the impacts of ocean acidification and global warming on the larval development of *B.villosa*, a common ascidian species in the North Pacific Ocean, we aim to

fill this knowledge gap. Specifically, we hypothesise that warmer temperatures will accelerate larval development but reduce survival rates, while increased acidity will slow developmental rates. Larval development will be measured by recording the time taken to reach key developmental stages. The actual data recorded will include time stamps for the occurrence of these events in each experimental group subjected to different temperature and acidity conditions. By comparing the timestamps across different pH and temperature conditions, we can assess the rate of larval development. Through this study, we seek to advance our understanding of how environmental changes affect ascidians and contribute to informed conservation and management strategies for marine ecosystems.

Methods

Sample collection: This study was conducted at the Friday Harbor Laboratories, University of Washington from April 13th to May 21st, 2024. Three adult ascidians, *B. villosa*, were collected from tires off docks located in the intertidal zone (48.54592°N, 123.01292°W) using a thick wooden stick to lift the tires. The tires provide a suitable substrate for *B.villosa* to grow in the crevices. Since ascidians are hermaphroditic but do not self-fertilize, three adults were collected to ensure an adequate supply of eggs and sperm, with sperm from two individuals available to fertilize the eggs of the third, guaranteeing a high percentage of fertilization. The three ascidians were placed in a sea table for 2 weeks under a lamp to prevent spawning and egg maturation.

Sample processing: To begin the study, the adult tunicates were placed in a dish filled with seawater. A straight cut was made between the siphons of a tunicate using a razor blade. The organism was spread apart to access the gonads, which were plucked using forceps and placed on a 200μm Nytex mesh. Filtered seawater was poured through the mesh to collect the gametes into a 250 mL beaker. This was done for all three tunicates with a separate beaker and Nytex mesh for each. Each beaker was labeled with the tunicate it came from as A, B, and C. Razor blades and forceps were thoroughly cleaned after cutting through each tunicate to prevent contamination. The beaker with gametes was left undisturbed for about 10 minutes, allowing the heavier eggs to settle at the bottom while the sperm remained mostly in the top half of the beaker.

The top portion of the beaker which mainly contained sperm was carefully transferred into a 15ml centrifuge tube, ensuring that no eggs were poured out. All tubes were labelled with respective tunicates from which the sperm came. Since tunicates are typically sexually active in the summer, and *B. villosa* were sexually immature during the experiment, 2-3 drops of pH 9 Tris HCl were added to each tube and mixed thoroughly to activate the sperm by inducing motility. A pH strip was used to ascertain the pH of 9 in each tube which were then set aside for 10-15 minutes. After this period, a slide was prepared with sperm from each tube to assess their motility.

The eggs in the bottom half of each beaker were washed with seawater until all the remaining sperm was removed. This was done by adding seawater to the beaker, allowing the eggs to settle to the bottom, and pouring out the water from the top which contains the remaining sperm until the water on top was not cloudy anymore (~3 rinses). Once clear, the solution was poured into a larger 500 mL beaker until 30-50 mL was left in each smaller beaker. To ensure successful fertilization, sperm from two tunicates were mixed with the eggs of a third tunicate, ensuring no self-fertilization. For example, sperm from tunicate A was added to eggs of tunicate B and C. The water in each beaker was swirled and the time of fertilization was noted. Each beaker with fertilized eggs was checked under a dissection microscope after 30 minutes to determine which had the highest number of fertilized eggs which was then chosen for the experiment.

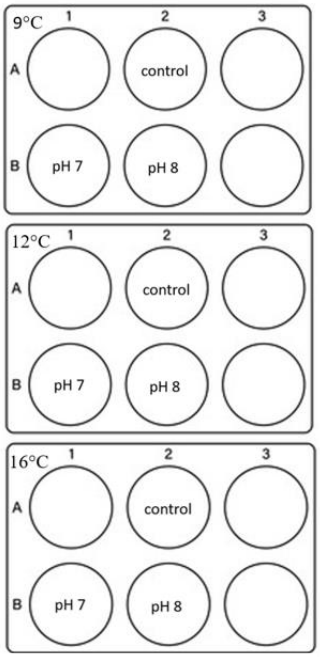

Figure 1. Experimental setup

Experimental setup: To set up the experiment, 3 mL of seawater was added to 3 wells in 3 different multi-well plates using a serological pipette, followed by 2 mL of fertilized eggs, resulting in a total volume of 5 mL per well. This volume allowed us to swirl the eggs in the wells without any spillage.

Three distinct pH treatments and three temperature treatments were established. Each multiwell plate contained three wells: one well was adjusted to pH 7 by adding a drop of pH 7 Tris-HCl, another to pH 8 using Tris-HCl of pH 8, and the third well was left as an unaltered control. The multiwell plates were then placed in temperature-controlled refrigerators set to 9°C, 12°C, and 16°C, respectively (Figure 1). Thermometers were used to ensure the accuracy of the temperature settings in each refrigerator.

Data collection: Data was collected by noting the time taken in minutes post-fertilization to reach the following important stages in embryonic development: 2-cell, 4-cell, 8-cell, 16-cell, 32-cell, gastrula, neurula, tail bud, and tadpole stages (Figure 2). Fertilization success was checked hourly and the time was only noted when 50% of the eggs had reached the same fertilization phase. After 24 hours, survival rates were calculated as the percentage of fertilized eggs that developed into a hatched tadpole. Conducting two trials to count embryos mitigated potential human error and ensured the validity of the data. This approach provided redundancy, safeguarding against inaccuracies by offering a second set of observations. Moreover, it helped ascertain that the observed times were not merely a result of chance, as consistency between the trials reinforced the reliability of the findings.

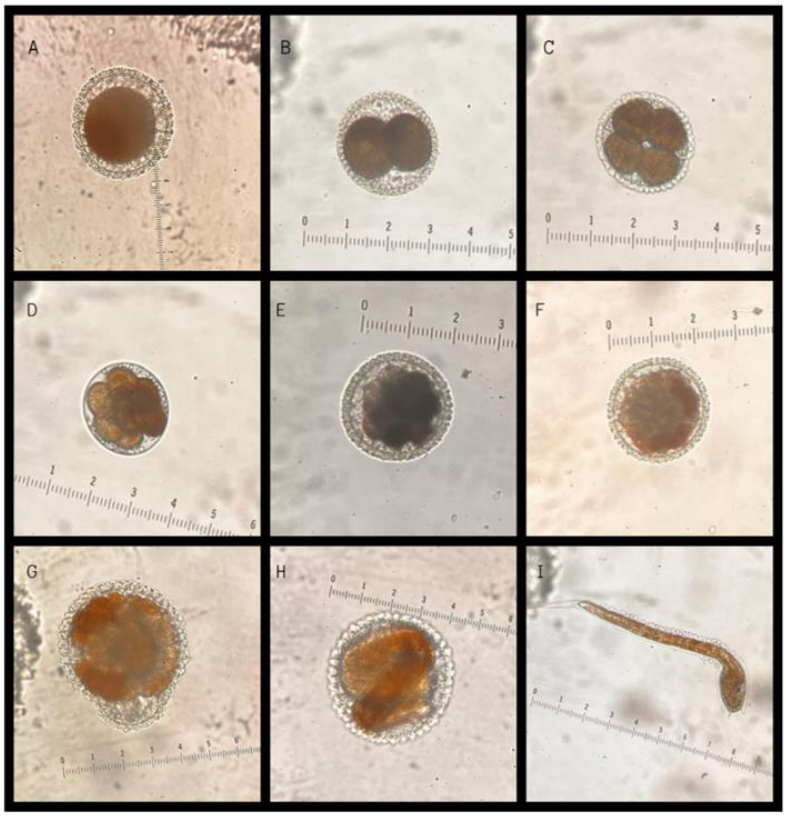

Figure 2. Developmental stages of *B.villosa*. A) start of fertilization B) 2-cell C) 4-cell D) 8-cell E)16-cell F) 64-cell G) tail-bud H) unhatched tadpole I) tadpole

Data analysis: Data was analysed using Python. Values for the eggs in the well with unaltered pH were selected, and a one-way ANOVA was performed for each developmental stage to assess the statistical significance of developmental times across different temperature settings. Subsequently, paired t-tests were conducted

using 9°C as a control and comparing it with 12°C and 16°C (Table 1).

To evaluate the effects of pH, paired t-tests were conducted for each developmental stage, comparing pH 7 and pH 8 while keeping the temperature constant (Table 2). Additionally, a one-way ANOVA was used to determine the significance of survival rates from fertilized eggs to hatched tadpoles across different temperature conditions at pH 8 (Figure 6). The pH 8 condition was used as the control, as it reflects typical seawater pH.

Results

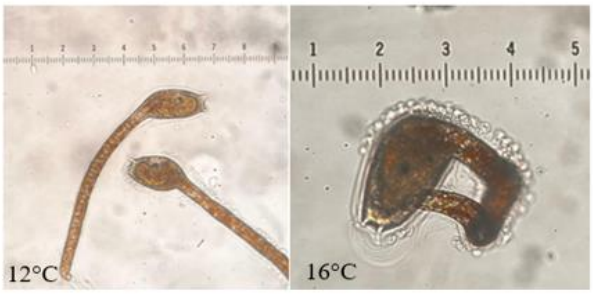

Figure 3. Morphological difference between tadpoles at ambient and warmer temperatures.

Morphologically, most tadpoles exposed to 16°C developed kinky, bent tails compared to tadpoles at 9°C and 12°C (Figure 3). The time taken by embryos to develop to all key stages was faster at 16°C followed by 12°C and then by 9°C (Figure 4).

The ANOVA tests revealed significant differences in development across various temperature settings for each key stage. Subsequently, the paired t-tests that were conducted to compare developmental times at 9°C with higher temperatures for each developmental stage differed significantly ($p<0.05$) between 9°C and 16°C embryos at every stage except the 2-cell stage. The developmental time between 9°C and 12°C was only significantly different at the 8-cell and tail-bud stages (Figure 5).

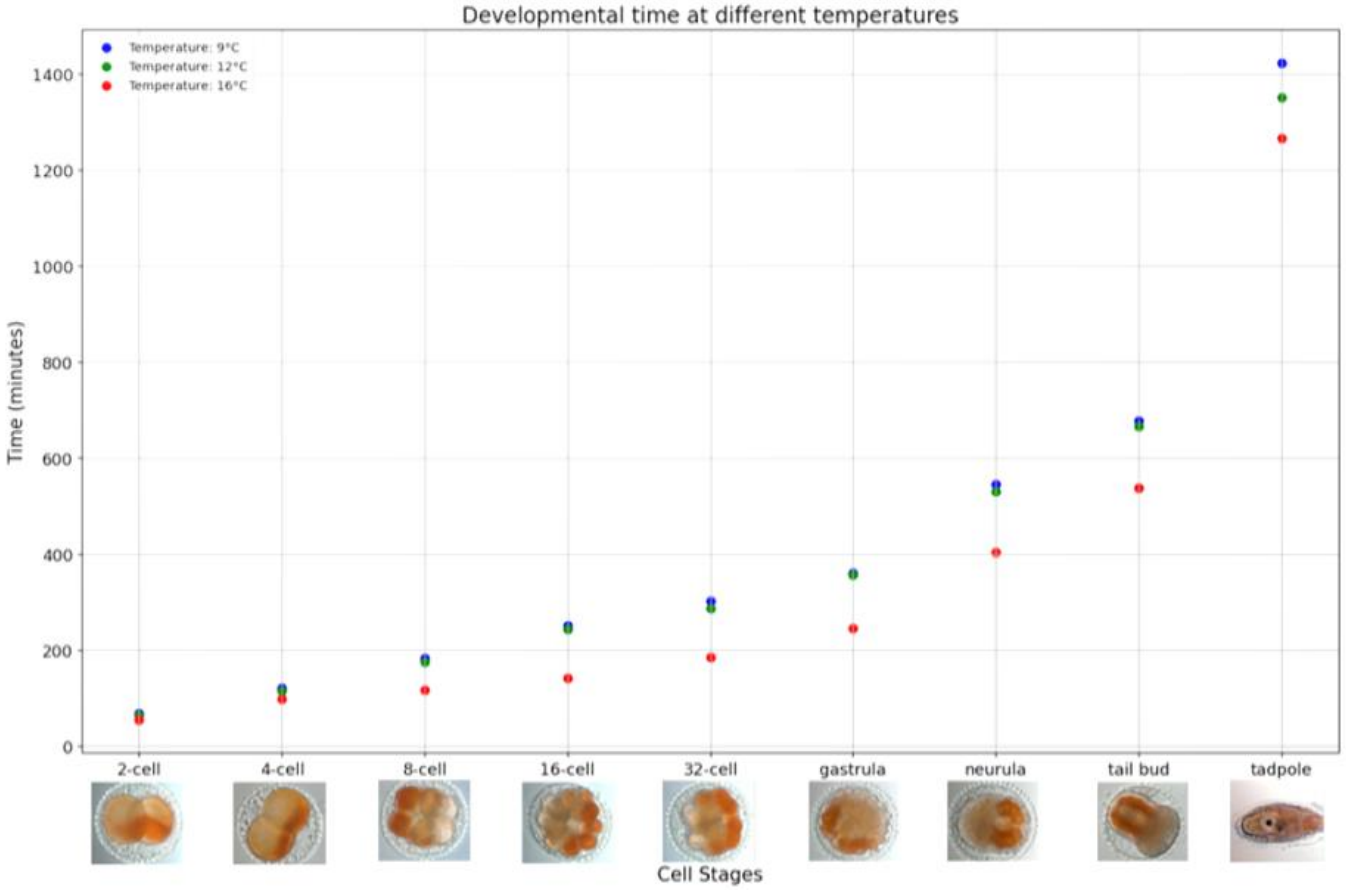

Figure 4. Time taken to reach key stages at different temperatures

Table 1. p-values for differences between 9°C and higher temperatures

Stage	9°C vs 12°C	9°C vs 16 °C
2-cell	0.577	0.622
4-cell	0.105	**0.032**
8-cell	**0.063**	**0.022**
16-cell	0.355	**0.037**
32-cell	0.319	**0.011**
Gastrula	0.563	**0.023**
Neurula	0.164	**0.018**
Tail-bud	**0.028**	**0.002**
Tadpole	0.069	**0.035**

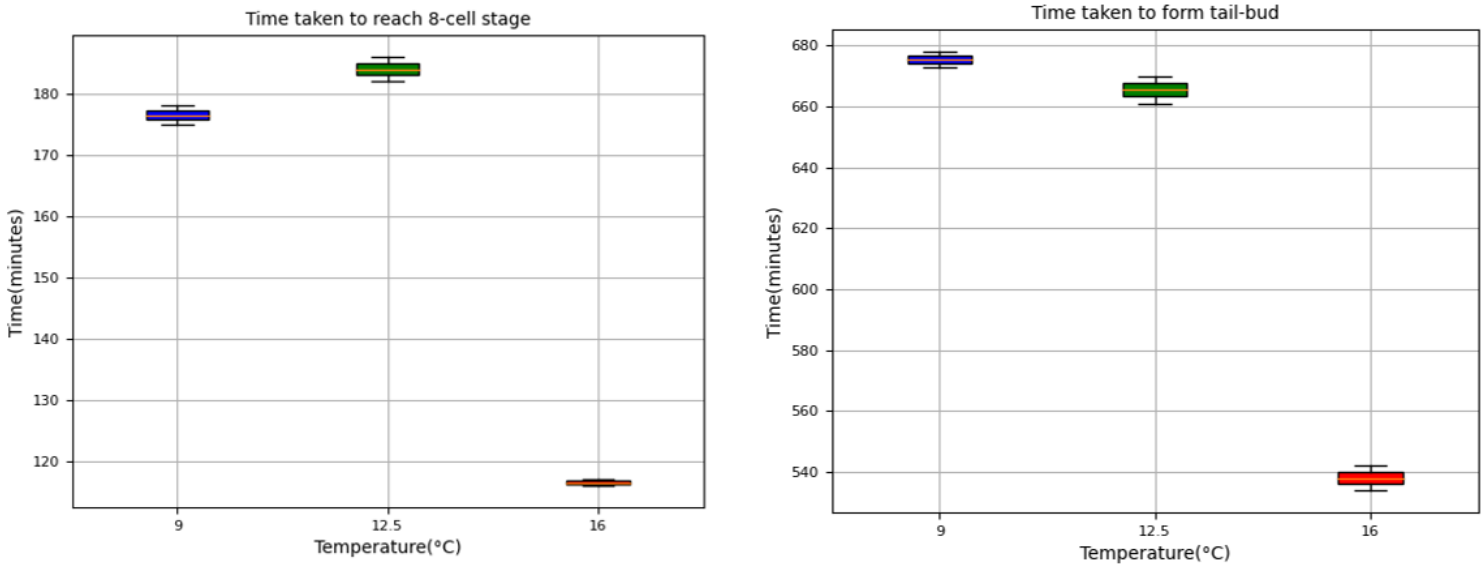

Figure 5. Stages that showed significant time differences post-fertilization in both temperature settings

Table 2. p-values between pH groups for each stage

Stage	9°C	12°C	16°C
2-cell	0.975	0.499	0.626
4-cell	0.897	0.677	0.452
8-cell	0.499	0.090	0.499
16-cell	0.912	0.499	0.895
32-cell	0.805	0.762	0.499
Gastrula	0.499	0.499	0.169
Neurula	0.675	0.330	0.917
Tail-bud	0.837	0.917	0.428
Tadpole	0.583	0.814	0.808

The developmental times at differing pH's were compared keeping temperature constant. The time taken to develop to key stages did not differ between different pH groups (Table 2).

The survival rates, the percent of fertilised eggs that hatched into a tadpole, decreased with increasing temperature. The average survival rate at 9°C, 12°C, and 16°C was 41%, 38.5% and 20% respectively. The difference in survival rate between 9°C and 12°C was not statistically significant (p=0.19) but the difference in survival rates was significant between 9°C and 16°C (p=0.019) and between 12°C and 16°C (p=0.018) (Figure 6).

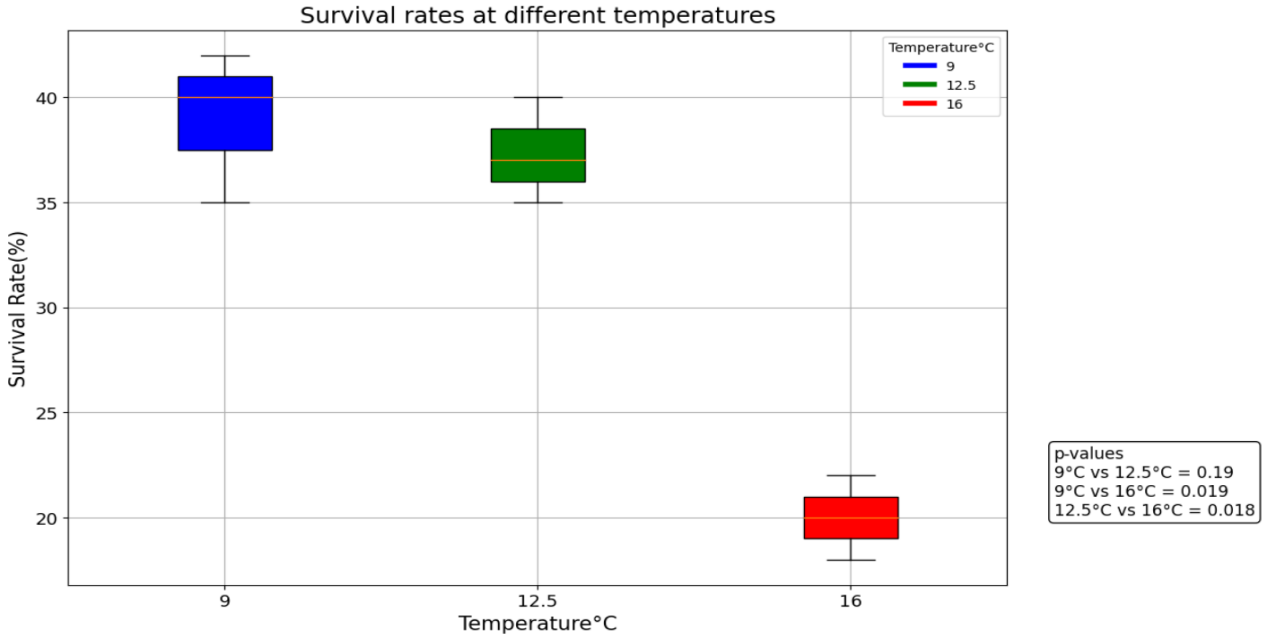

Figure 6. Percentage of fertilised eggs that hatched into a tadpole

Our results were consistent with our hypothesis that embryos at higher temperatures would develop faster but exhibit lower survival rates. However, our hypothesis that embryos at lower pH conditions would develop slower was not supported by the data.

Discussion:

Our study investigated the impact of temperature and pH on the development and survival of *B.villosa* embryos and larvae. The results supported our hypothesis regarding temperature: higher temperatures, specifically 16°C, accelerated development but reduced survival rates by 20% and induced morphological abnormalities compared to embryos kept at 9°C and 12°C. Additionally, at 16°C we observed most tadpoles having bent, kinky tails which were not observed at lower temperatures. Contrary to our expectations, our hypothesis that tunicates would develop more slowly under neutral conditions was not substantiated by the data. There was no significant difference observed in the developmental time to key stages between pH conditions of 7 and 8. This indicates that within the pH range tested, temperature is a more critical factor affecting development than pH. This finding diverges from studies conducted on other solitary tunicate species where tunicates showed slower development rates below a pH of 7.6 (Jones, 2023).

The Intergovernmental Panel on Climate Change (IPCC) predicts that climate change is expected to increase global temperatures worldwide by 1-3.7°C by the end of the century (IPCC 2013) and increase the occurrence and severity of marine heatwaves in a few decades (Meehl 2004; Perkins-Kirkpatrick and Gibson 2017). Higher summer temperatures have significantly impacted subtidal communities (Sorte et al. 2010; Smale et al. 2015). These observations suggest that as global warming proceeds, *B.villosa* populations may decrease drastically due to improper development and low survival rates. The experimental temperatures in our study, reaching up to 16°C, align with the temperature ranges predicted by the IPCC for the end of the century. This would potentially disrupt the ecosystem dynamics as it may lead to an increase in mussel and oyster populations due to reduced competition from tunicates (Rybovich et al., 2016).

Future studies should investigate the effects of pH independently from temperature, using a broader range from 6.5 to 8.2, as demonstrated in similar research (Jones, 2023). By analyzing multiple pH groups within this range, researchers can identify the critical pH level that tunicates can tolerate before their development is adversely affected. Furthermore, exploring the influence of salinity could offer a more holistic comprehension of external factors affecting the recruitment of solitary tunicates. This can be achieved through a parallel experimental design, substituting pH alterations with changes in salinity, ranging from the typical 35 ppt to 17 ppt (Shumway et al., 1978). As climate change alters the species composition of our oceans, attention must be paid to the competition between native and invasive species. Similar studies should be conducted for *Ciona savignyi*, *Styela clava*, and *Didenmun*, three invasive ascidians in the Pacific Northwest (Washington Invasive Species council, 2024). Comparing the heat, pH, and salinity tolerances of native and invasive species will help us understand whether invasive species possess a competitive advantage over native ones (Kenworthy et al., 2018). This information is crucial for informing effective conservation strategies in the Salish Sea.

In conclusion, the study of how temperature and pH influence the recruitment of *B. villosa* holds significant implications for conservation efforts, especially considering ongoing climate change. Ascidians play a crucial role in marine ecosystems by facilitating the downward flux of carbon and serving as environmental stress indicators (Pomeroy, 1980). By gaining a deeper understanding of the impacts of environmental factors on ascidian recruitment, we can take proactive steps to safeguard these organisms and preserve our marine ecosystems.

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