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Impact of fluctuating temperature and elevated CO2 on the growth, survival, and metabolic rate of the endangered pinto abalone (Haliotis kamtschatkana) in the Salish Sea

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Impact of fluctuating temperature and elevated CO_2 on the growth, survival, and metabolic rate of the endangered pinto abalone (*Haliotis kamtschatkana*) in the Salish Sea

By

Jaclyn Stapleton

Accepted in Partial Completion
of the Requirements for the Degree
Master of Science

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Master's Thesis

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Jaclyn Stapleton

May 16th 2023

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A Thesis
Presented to
The Faculty of
Western Washington University

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

by
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Abstract

Pinto abalone (*Haliotis kamtschatkana*) is the only abalone species found in the Salish Sea in the northeastern Pacific Ocean. They were recently declared as a State endangered species and human intervention is necessary to recover the species. Puget Sound Restoration Fund (PSRF) is one of the organizations trying to restore pinto abalone populations in the Salish Sea. Some of their outplant sites are less successful than others although they are physically similar. Currently, there is no research on how environmental variation affects juvenile pinto abalone survival, growth, and metabolic rate.

The goal of my thesis was to simulate summer sea water temperature fluctuations in the Salish Sea at different pCO_2 levels. I investigated how these treatments affected juvenile pinto abalone growth, survival, and metabolic rate. I also included abalone size as a factor in my growth and survival experiment. I hypothesized that (1) fluctuating temperature and elevated pCO_2 would negatively interact, resulting in less growth and survival, with larger abalone less affected. And (2) that fluctuating temperature and elevated pCO_2 would synergistically interact, resulting in abalone with an increased metabolic rate. Fluctuating temperature significantly decreased abalone survival. Growth was not impacted by initial size nor pCO_2 level. There was a significant interaction between temperature and pCO_2 on abalone metabolic rate.

In addition to the physical parameters used to determine outplant sites, PSRF can use my findings to choose outplant sites better suited for juvenile pinto abalone to increase the probability of their populations recovering in the Salish Sea.

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Thank you to my advisor, Dr. Deborah Donovan, who has mentored me from the moment I transferred to WWU for my undergraduate degree in 2017. You presented me with the opportunity to study abroad in 2018 and I am forever grateful for that experience. During my graduate degree you provided an environment that was supportive, encouraging, and fostered my growth as a scientist. Thank you to Elizabeth Diehl for being a wonderful lab partner and friend. We shared so many experiences, but some of my favorites include our lunch breaks outside during our research at Shannon Point Marine Center and travelling to California for the Western Society of Naturalists conference.

Thank you to my committee members, Dr. Brooke Love and Dr. Shawn Arellano, for their guidance and support. Our conversations led to my decision to use fluctuating temperature as a treatment, which improved my understanding of environmental fluctuation and the importance of this research. Thank you to Shannon Point Marine Center and all the staff/faculty there for providing lab space as well as housing accommodations during my experiments.

Thank you to the helpful staff at Puget Sound Restoration Fund for taking time to answer all my questions, provide food for the abalone and most importantly, for being willing to donate abalone for my research. Without your generosity, this project would not have been possible.

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Thank you to my parents, Bruce and Betty, for your support and encouragement throughout my college career. Finally, thank you to my incredible partner, Tyler. You have believed in me and my abilities every step of this journey and I am eternally grateful for your love and support.

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Introduction

Ocean Acidification

The world's oceans are undergoing shifts due to climate change. Multiple abiotic factors are included in these shifts, including temperature, pH, and salinity (Feely et al. 2009; Doney et al. 2009; Curry et al. 2013). These environmental factors can have individual effects on organisms' ability to grow, reproduce, and survive. When environmental factors are combined, however, there can be more of an impact on organisms (Bryne & Przeslawski 2013).

Since the Industrial Revolution, there has been an increase in anthropogenic carbon dioxide released into the atmosphere and the world's oceans have absorbed over 25% of those outputs (Friedlingstein et al. 2022). The absorption of CO_2 into the oceans may decrease the amount of global warming occurring but will directly affect the ocean's water chemistry (Fabry et al. 2008). As atmospheric CO_2 rises, more CO_2 is mixed into the surface waters of the ocean which shifts the carbonate chemistry, creating a more acidic environment (Doney et al. 2009).

$$H_2O + CO_2 \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \leftrightarrow 2H^+ + CO_3^{2-} \quad (1)$$

The increased carbon dioxide reacts with the surface water and leads to the formation of carbonic acid (H_2CO_3) which dissociates to create a hydrogen ion (H^+) and bicarbonate ion (HCO_3^-). H^+ can react with a carbonate ion CO_3^{2-} in the water to form HCO_3^- and H^+ (equation 1). Therefore, adding more carbon dioxide to seawater results in increased concentrations of H_2CO_3, H^+, and HCO_3^- and decreased concentrations of CO_3^{2-} (Fabry et al 2008). The acid-base balance in seawater is vital to the survival, growth, development, metabolism, and pH balance of marine

organisms (Melzner et al. 2011). Therefore, the increased acidity in seawater can create a harsh environment for marine organisms.

Ocean acidification decreases the concentration of carbonate ions in seawater, thus, animals that rely on calcification are more prone to their negative effects (Cai et al. 2020; Dupont et al. 2010; Kroeker et al. 2010). The decrease in available carbonate ions is especially detrimental to shell-forming species that rely on this compound to build and maintain their shells (Gazeau et al. 2013). The severity of the impact on these organisms depends on the calcium carbonate saturation state (Ω) of their environment (Fabry et al. 2008). The calcium carbonate saturation state is the product of Ca^{2+} and CO_3^{2-} concentrations divided by the solubility product of either calcite or aragonite, which are two types of calcium carbonate secreted by calcifying marine animals (equation 2).

$$\Omega = [Ca^{2+}][CO_3^{2-}]/K^*_{sp} \quad (2)$$

Thus, as more carbon dioxide is mixed with the oceans, CO_3^{2-} concentrations will decrease resulting in reduced Ω. A calcium carbonate saturation state of 1.0 for aragonite (Ω_{arag}) or calcite (Ω_{cal}) can favor the formation of shells and skeletons, however, the greater the Ω is, the less energy required for calcification to occur (Feely et al. 2009). The change in energy demands associated with Ω can create significant energy costs depending on the life stage which can result in negative outcomes, especially for early life stages. When Ω_{arag} or Ω_{cal} are low, shell dissolution can occur (Fabry et al. 2008; Libes 2009). Calcifying species such as molluscs, coralline algae, and echinoderms had reduced calcification when exposed to increased CO_2 treatments and low saturation states (Doney et al. 2009).

Rising and Fluctuating Temperature

The rise in atmospheric CO_2 has led to an increase in ocean temperature along with a decrease in ocean acidity (IPCC 2014). This increase in temperature can cause changes to ecosystems such as a shift in seasonal biological events (such as migrations or spawning events), local extinctions of populations, and shifts in population distributions, all of which can cause changes to food availability in an ecosystem (Pörtner & Farrell 2008). Temperature is one of the most influential environmental factors on invertebrates' developmental rate, growth, reproduction, and species distribution (O'Connor et al. 2007; Lawlor & Arellano 2020).

Marine organisms can also experience environmental temperature fluctuations due to location, seasonality, tidal patterns, and upwelling at different timescales (Evans et al. 2019). Thus, in addition to the gradual temperature increase over a long period of time associated with global warming, temperature can fluctuate in short time periods, such as over hours or days, that especially impact sessile or slow-moving marine organisms such as bivalves, snails, and corals (Micheli et al. 2012; Calderón-Liévanos et al. 2019). Short term extreme heat events, such as heat waves, have resulted in mass mortalities, recruitment failure, and increased susceptibility to disease in some molluscs (Boch et al. 2018; Morash & Alter 2015). The amount of time over which these temperature events occur can dictate the amount of damage done. Events that alter the environmental temperature but are very short, like tidal differences, are less likely to cause mass mortalities compared to longer events, sometimes lasting several days, like marine heat waves (Calderón-Liévanos et al. 2019). Thermal fluctuation can affect animals in different ways. Kang et al. (2019) found that ectotherms that experienced thermal fluctuations were more likely to have higher metabolic rates compared to ectotherms that were acclimated to higher stable

temperatures. However, daily temperature fluctuations can increase growth rates in some marine ectotherms (Dong et al. 2006).

Despite the fluctuations that occur in nature, most experiments are held at static conditions in global change biology research (Donham et al. 2022) and this likely does not give an ecologically meaningful representation of the animals response to temperature in a variable environment. For example, Jensen's inequality states that average performance in a variable environment is not the same as performance in the average environment (Jensen 1906, Denny 2017; Kroeker et al. 2020). Therefore, the results from these static condition experiments can lead to over- or under-estimating an organism's response to variable conditions in nature (Denny 2017). This phenomenon shows the importance of creating variable environmental conditions in experiments and can improve our predictions of how animals might respond to future climate conditions.

Multiple Stressors

Ocean acidification and warming are occurring simultaneously and can have antagonistic, additive, or synergistic effects when combined (Kroeker et al. 2013). Individual stressors have resulted in opposite outcomes for marine invertebrates, such as increased temperature increasing development rate and acidic conditions decreasing development rate (Bryne & Przeslawski 2013). For example, under ocean acidification, Ω decreases, requiring higher energy costs for calcification. However, when sea water temperature is increased, Ω increases, which lowers the energy cost of calcification and can increase calcification rates (Fabry et al. 2008) Thus, more studies are needed to understand how marine organisms respond to multiple stressors (Breitburg

2015). A meta-analysis by Harvey et al. (2013) compared the individual and combined effects of ocean acidification and warming on marine organisms that included corals, crustaceans, echinoderms, molluscs, phytoplankton, fish, seagrass, and calcifying and non-calcifying algae. The authors investigated the interaction between ocean acidification and warming effects on five biological processes (calcification, photosynthesis, reproduction, survival, and growth) across different life-history stages. They found that molluscs decreased in all responses (except photosynthesis), with reproduction and survival having the most negative impact by the combined effect of warming and ocean acidification. They also found that animals in different life-history stages differed in calcification and survival, with juveniles and larvae more negatively affected than adults (Harvey et al. 2013). Molluscs, such as bivalves and marine gastropods, were highly sensitive to both ocean acidification and warming. When reared in these conditions, larvae and juveniles lacked the ability to form shells and experienced higher rates of morphological abnormalities which resulted in lower growth and survival (Bryne & Przeslawski 2013). However, not all responses are negative when there are interactions. For example, the larvae of the Olympia oyster (*Ostrea lurida*) had increased tolerance to low salinities when paired with increased temperatures (Lawlor & Arellano 2020).

Metabolism

Changes in the marine environment, such as temperature, dissolved oxygen, or pH, can alter oxygen consumption rate and metabolism of organisms in the ocean. Because ectotherms do not use metabolic heat production to maintain their body temperature, their body temperature changes with the environment and can acclimatize to thermal fluctuations. Temperature controls the rate of most metabolic processes in ectotherms, with metabolism increasing as temperature

increases until thermal limits are reached (Morash & Alter 2015). In addition, some ectotherms are marine oxyregulators, which can maintain their metabolic rate even when dissolved oxygen is reduced, until a critical level is reached (Willmer et al. 2000). Finally, long term exposure to elevated CO_2 levels can depress metabolism and reduce growth in molluscs (Fabry et al. 2008).

Climate change is altering multiple environmental factors simultaneously. Studies have shown that these multiple stressors can increase metabolic rate and reduce the ability for ectotherms to recover from more extreme conditions (Knust & Portner 2007). This increase in physiological stress can cause an animal to divert energy needed for growth to energy for mechanisms that serve as protection from the environment. For calcifying organisms, this could mean diverting energy needed for calcification to other areas for survival (Bryne & Przeslawski 2013). Also, the increased ocean acidity associated with climate change decreases Ω, which increases the energy required for calcification (Feely et al. 2009). Environmental fluctuations can also be energetically costly. When frequently exposed to elevated thermal temperature fluctuations, some species maintain higher metabolic rates and have higher thermal limits (Donham et al. 2022). The increased demand for energy to be used for their metabolism can take away energy that could be used for production such as in somatic growth or for reproductive tissues (Willmer et al. 2000).

Energy budgets demonstrate how ingested energy (food and drink) is equal to the sum of energy directed to biological processes such as metabolism, growth, reproduction, and waste. The ingestion rate sets the limit for the energy to be used in the rest of the products. When an organism experiences environmental fluctuations, it can create an energy imbalance. As stated above, the metabolic rate of ectotherms will rise when they are exposed to elevated temperatures.

Economic and Cultural Implications

The combined effects of temperature, salinity, and acidification in the ocean will have negative consequences for marine organisms that ultimately affect human benefits including economic, cultural, and ecologic services (Narita et al. 2012). The decreased ability for calcification could decrease the availability of commercially important shellfish populations (clams, oysters, etc.) and ultimately impact the commercial shellfish industry (Cooley et al. 2009). Fisheries that provide millions of dollars in revenue each year, like the Dungeness crab fishery in Washington state, are at risk since increased ocean acidification could decrease catch sizes (Bednarsek et al. 2020; Barton et al. 2015). Many invertebrates, like abalone, have a deep connection with Native cultures. Native Americans have used abalone as a food source and as an item of trade; abalone shells were valuable due to the uniqueness of their shells which were often used for jewelry (Field 2008).

Pinto Abalone

Pinto abalone (*Haliotis kamtschatkana*) is the only abalone species found in the northeastern Pacific Ocean. Thus, pinto abalone are a symbol of cultural, economic, and ecological importance to both Indigenous and non-indigenous peoples of the Pacific Northwest (Neuman et al. 2018). Although pinto abalone inhabit the West Coast of North America, their population densities differ depending on location. In 2019, the decline of pinto abalone populations in Washington State caused the Washington Fish and Wildlife (WDFW) to list pinto abalone as a State endangered species (Carson & Ulrich 2019). Although the commercial and recreational fisheries have been closed since 1994, populations continue to decline, likely due to

low population densities, which prevent successful reproduction, and to environmental stressors (Crim et al. 2011).

Abalone are broadcast spawners and release their gametes into the water column for fertilization. This makes population density vital for abalone spawning in the wild (Babcock & Keesing 1999; Gascoigne et al. 2004). When population densities are low, abalone do not receive signals to spawn and/or they are not close enough for the males' broadcasted sperm to fertilize the distant females' broadcasted eggs in the water column (Gascoigne et al. 2004). Between 1992 and 2017, pinto abalone populations in the Salish Sea declined by 98% while shell length increased, indicating that the decline was due to low recruitment (Figure 1; Carson & Ulrich 2019). The increased size of abalone is significant because this means illegal poaching can be ruled out as a significant cause of population declines (Rothaus et al. 2008; Carson & Ulrich 2019). Low pinto abalone population densities coupled with multiple environmental stressors that impair larval development have made it increasingly important for restoration of pinto abalone populations (Read et al. 2012; Straus & Friedman 2009). After low population densities were observed in the Salish Sea, the need for human intervention was deemed necessary to recover the species (Bouma et al. 2012).

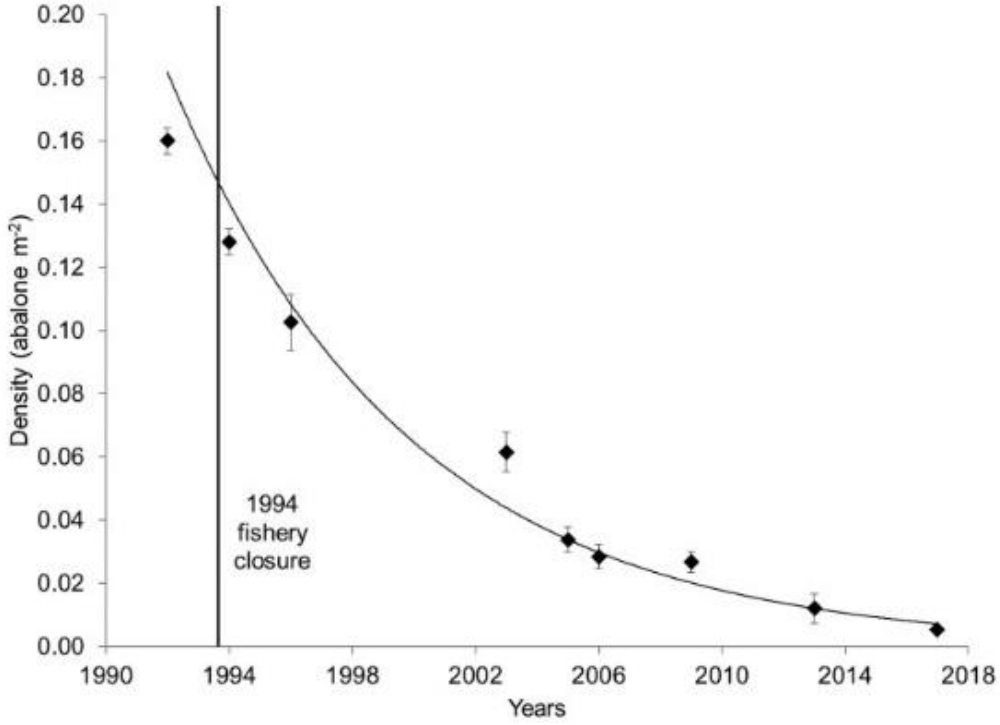

Figure 1. Average density of pinto abalone (abalone m^{-2}) at 10 survey sites in the Salish Sea from 1992-2017. Figure adopted from Carson & Ulrich (2019).

Organizations, such as Puget Sound Restoration Fund (PSRF), are working to restore pinto abalone populations through aquaculture and outplanting efforts in the San Juan Archipelago in the Salish Sea (Figure 2). The main goal of this PSRF project is to raise and outplant hatchery-reared pinto abalone to successfully recover the species and maintain a healthy rocky reef habitat in the Salish Sea (PSRF 2022). Puget Sound Restoration Fund uses 27 sites across the Salish Sea to outplant hatchery-reared abalone juveniles that are between 18 and 22 months of age. These sites are not disclosed to the public to prevent illegal poaching of outplanted abalone. Pinto abalone inhabit subtidal depths to 20 m mean lower low water (MLLW) and they outplant abalone from 0 to 10 MLLW (Bouma et al. 2012). Since 2009, PSRF have reared and released an estimated 45,000 juvenile abalone into the Salish Sea (PSRF 2022). Some outplant sites are less successful than others, even though sites are physically similar, but it is unclear why (Carson et al. 2019). Water chemistry varies greatly over time and space in the Salish Sea due to seasonality, location, and upwelling (Figure 3; Evans et al. 2019; Lowe et al. 2019), which could contribute to a site's success.

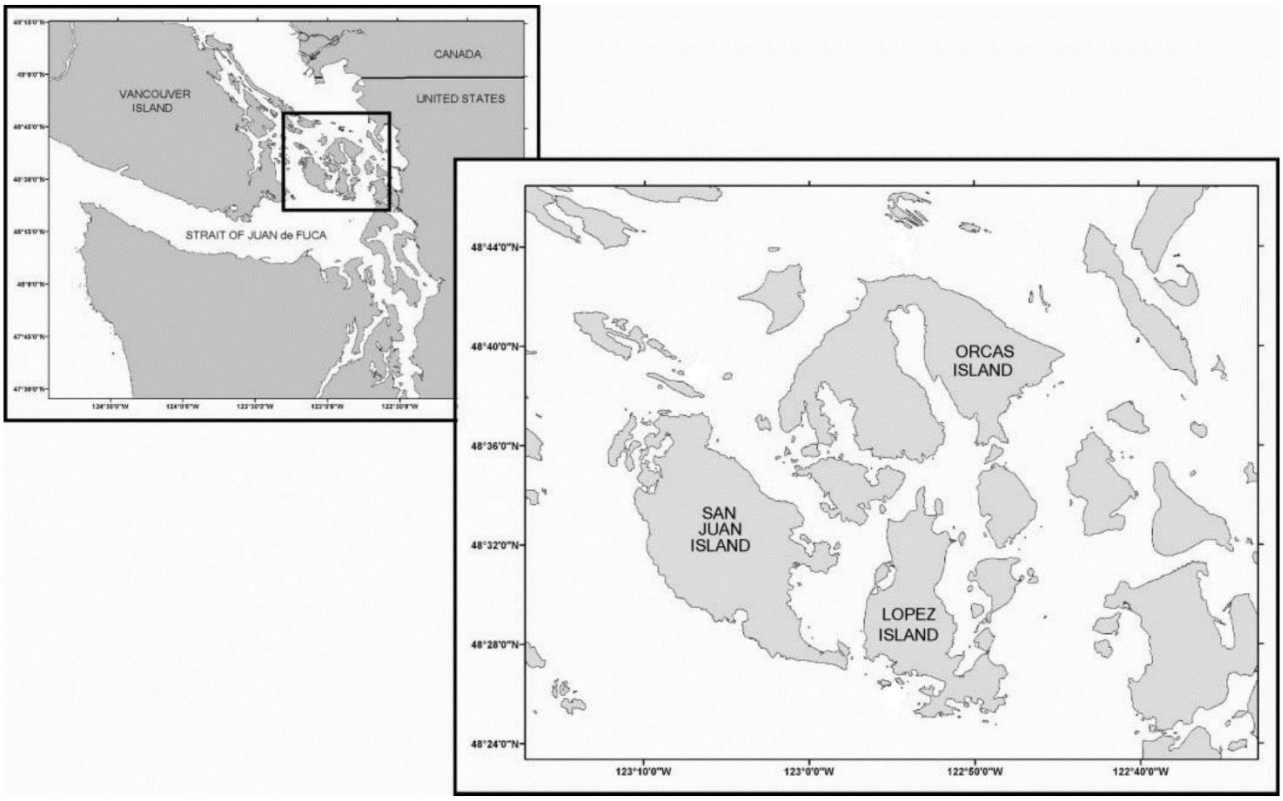

Figure 2. Map of San Juan Archipelago located in the Salish Sea. Image adopted from Bouma et al. (2012).

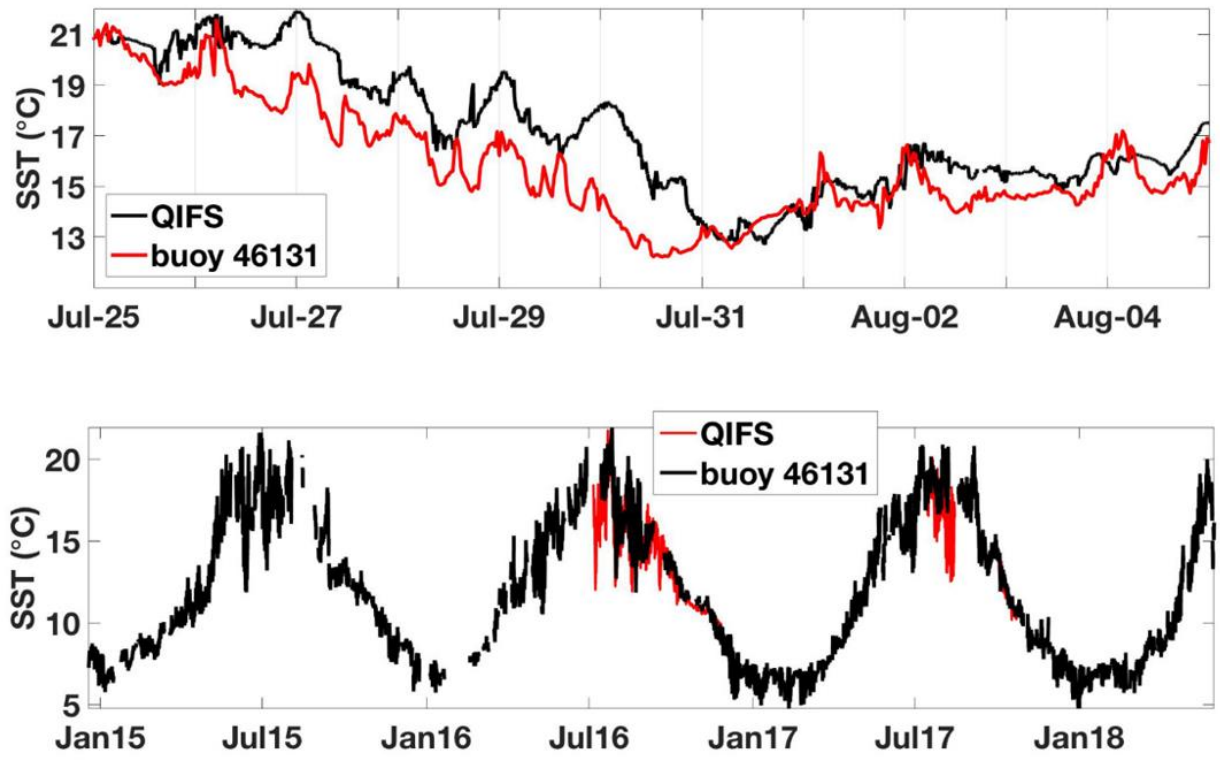

Figure 3. Weekly (top) and seasonal (bottom) sea surface temperature (SST) variation in the northern Salish Sea. Weekly SST data are from summer 2016 while seasonal SST data are from 2015-2018. Data were collected at Quadra Island Field Station (QIFS) and Environment Canada weather buoy 46131. Figure adopted from Evans et al. (2019).

Although there has been research showing the impact of ocean acidification on pinto abalone larval development and survival (Crim et al. 2011), there is currently no research on how ocean acidification affects juvenile pinto abalone. Also, there is no research on how temperature fluctuation in the environment affects juvenile pinto abalone survival, growth, and metabolic rate. Narrowing this gap in knowledge would allow PSRF to make more informed decisions on where to outplant juvenile abalone, which could save time, resources, money, and ultimately could help save an endangered pinto abalone species in Washington State.

I investigated the effects of fluctuating temperature and elevated pCO_2 levels on juvenile pinto abalone growth, survival, and metabolic rate. The purpose of this research was to provide PSRF and other abalone aquaculture programs with more information to better choose outplant sites for higher outplant success and to increase our understanding of the effects of multiple stressors on a mollusc species. My broad research questions were (1) how do temperature fluctuation and pCO_2 levels affect the survival and growth of juvenile abalone of different sizes? And (2) how do temperature fluctuation and pCO_2 levels affect the metabolic rate of juvenile abalone? My hypotheses were (1) that fluctuating temperature (10 → 14 → 10 °C) and elevated pCO_2 (1200 ppm) would negatively interact, resulting in less growth and survival in the juvenile abalone, with larger abalone less affected than smaller abalone and (2) that fluctuating temperature and elevated pCO_2 would have a synergistic interaction, resulting in juvenile abalone with an increased metabolic rate.

Methods

Experimental Organisms

Juvenile abalone from genetically distinct families and ~12 months old were obtained from the Puget Sound Restoration Fund (PSRF) hatchery within the Kenneth K. Chew Center for Shellfish Research and Restoration at NOAAs Manchester Research facility in Washington State. Diatoms (*Navicula incerta*, *Cylindrotheca closterium*, and *Amphora salina*), as well as the red alga *Palmaria palmata* (dulse) for feeding the abalone were also acquired. The abalone and food were transported to Shannon Point Marine Center (SPMC) in Anacortes, WA by car. Abalone were transported in a large container with seawater, which was placed on ice inside coolers and bubbled with air to provide oxygen to reduce mortality during transport.

Once at SPMC, the abalone were placed in one of two holding tanks on a sea table. Dulse was put in a separate sea table with a circulator to allow the dulse to tumble. The sea tables and holding tanks were supplied with a continuous flow of seawater from Guemes Channel. To create a diatom film in the holding tanks, the seawater flowing to the tanks was turned off and diatoms were added to each holding tank. After 20 minutes, the seawater was turned back on.

Experimental Design

To investigate the effects of constant and fluctuating temperature on growth and survival of juvenile abalone held at two different CO_2 levels, I used a fully crossed experimental design. To manipulate temperature, four water baths were placed inside the 10 °C walk-in environmental chamber at SPMC (Figure 4). Two of the water baths were held at the constant temperature (10 °C), while the other two water baths were equipped with aquarium heaters to allow for controlled

temperature fluctuation (10 → 14 → 10 °C). Temperature was adjusted by 1 °C each day to achieve fluctuation, so a full cycle lasted eight days. Constant temperature treatment is consistent with the PSRF hatchery (Josh Bouma, personal communications). Fluctuating temperature treatment is realistic summer sea water conditions pinto abalone likely experience in the Salish Sea (Evans et al. 2019; Josh Bouma, personal communications). All water baths contained a water circulator, iButtons (iButtonLink), and 10 treatment cups (475 mL, made of polypropelene plastic), each containing five juvenile abalone. Each cup had a clip on the edge to hold a tube used to bubble a CO_2/air mixture into the cup. Half the cups received elevated CO_2 levels (1200 ppm) and half received currently ambient CO_2 levels (400 ppm). Thus, the experimental unit was the cup.

To achieve different CO_2 levels for the treatments, the gas mixing system at SPMC was used to create specific and controlled levels of CO_2 mixed into air, which was then bubbled into the treatment cups. This system initially stripped CO_2 from ambient air then added controlled amounts of research grade CO_2 back to the stripped air to achieve experimental treatment levels (400 ppm and 1200 ppm). These mixed gases were bubbled into the cups holding the abalone in the water baths using 3 mm diameter aquarium airline tubing. The mixed gases were also bubbled into carboys in the walk-in environmental chamber holding water for water changes using the provided tubing with the gas system and air stones. One carboy was bubbled with 400 ppm CO_2 while the other was bubbled with 1200 ppm CO_2. This bubbled seawater was used to fill and refill the cups for the duration of the experiment. For a more complete description of the CO_2 gas mixing system, see Love et al. (2017).

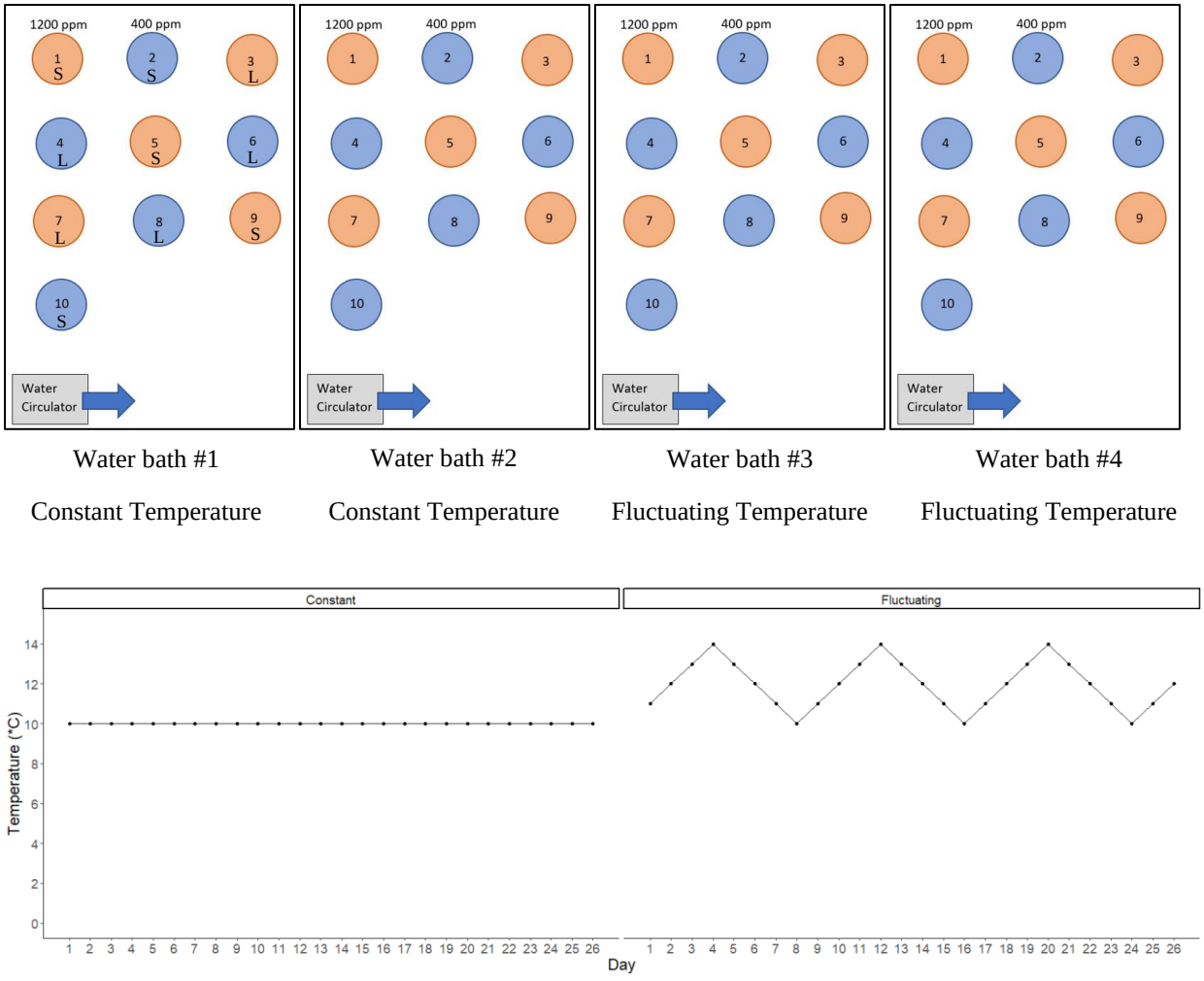

Figure 4. Experimental design for growth and survival experiment. Four water baths (top) – two with constant temperature and two with fluctuating temperature. Ten cups (circles) within each water bath – five cups bubbled with ambient CO_2 (400 ppm – blue circles) and five cups with elevated CO_2 (1200 ppm – orange circles). Abalone size is listed in each cup within water bath #1 (top left). "S" = small abalone, "L" = large abalone. Abalone size and cup is consistent for all water baths. Temperature treatments (below) for constant temperature water baths (10 °C) and fluctuating temperature water baths (10 → 14 → 10 °C).

Growth and Survival Experiment

Juvenile abalone were purposefully assigned to treatment cups based on size to include size as a factor in this experiment. Treatment cups were randomly assigned as "large" or "small" using a random number generator in Excel. Abalone were visually sorted into treatment cups – larger abalone (5.8 ± 0.2 mm) for large cups and smaller abalone (3.6 ± 0.1 mm) for small cups. All values are means ± standard error (SE), unless noted otherwise. During the sorting process, five abalone of similar size were photographed on a white background with a 1 cm reference line before the abalone were placed in their treatment cup. A Samsung Galaxy 10+ cellphone camera was used to take photos. The camera was in portrait mode and held over the white background so that the lateral sides of the background were aligned with the edge of the photo. Once sorted, cups were filled with ~300 mL of seawater bubbled with either 400 ppm or 1200 ppm CO_2 and placed in either a constant or fluctuating water bath. Diatoms and blended dulse were added to the cups, so food was not a limiting factor. Photographs were uploaded to ImageJ software to measure initial shell length which was used to calculate a mean initial shell length for each cup. Shell length was the longest distance from the anterior to the posterior ends of the shell. Thus, the treatments were fully crossed between temperature (constant or fluctuating), pCO_2 level (400 ppm or 1200 ppm), and abalone size (small or large). There were 10 cups per treatment, each containing five abalone.

The juvenile abalone were left in treatment for four weeks and monitored during water sampling that occurred every 4-6 days. Water sampling alternated between cups 1-3, cups 4-6, and cups 7-10 of all treatments and was performed to monitor water chemistry (see below). Each cup had a water change at least once a week. During water changes, abalone were observed to make sure they were alive. Movement of the foot, shell, and/or tentacles indicated the abalone

was alive. If no movement was observed, abalone were gently nudged with a spatula to see if they were actively attached to the treatment cup. If they did not budge, they were deemed alive. If they slid around easily, they would be removed from the cup and examined more closely to determine if they were alive. If any movement occurred, they were placed back in their treatment cup. However, if they did not show signs of life, they were determined dead and discarded. The number of live abalone in each cup was recorded during water changes to track survivorship over time. Survivors remained in cups, which were refilled with ~300 mL of pre-bubbled seawater and given some more dulse before being placed back in their water bath. Because the cups had a diatom film, diatoms were not added during water changes.

At the end of four weeks, survivors in each cup were counted and photographed using the same method described above to measure final shell length. Mean final shell length for abalone in each cup was determined. Surviving abalone were kept at SPMC until being transported back to the PSRF hatchery.

To analyze survival, I used a general linearized mixed model with binomial error function and modeled survival as the outcome variable, temperature treatment, pCO_2 level, and abalone size as fixed factors (including an interaction between temperature, pCO_2 level, and abalone size), and water bath as a random factor. Because salinity was high across all treatments (Table 1), mean salinity for each treatment was added as a fixed factor to see if abalone survival was impacted by salinity. I used a model selection procedure recommended by Zuur et al. (2009). Generally, I first fit the most complex model that contained only fixed factors. I then added the random factor and determined the most parsimonious model with and without the random factor. Using that most parsimonious model, I then removed the fixed factors in a backwards selection

process that yielded the most parsimonious model with fixed and random factors. I used the *lme4* package in RStudio (Bates et al. 2015) to perform these analyses.

Abalone growth could only be analyzed in different pCO_2 treatments in the constant temperature treatment due to extremely low survival in fluctuating temperatures (see Results). Final mean shell length was modeled as the outcome variable, initial mean shell length, pCO_2, and abalone size as fixed factors (including an interaction between pCO_2 and size), and water bath as a random factor. I then used the same backwards model selection described above to determine the most parsimonious model. I used the *lme4* package in RStudio (Bates et al. 2015) to perform these analyses.

Metabolic Rate Experiment

After the conclusion of the growth and survival experiment, another experiment was started to measure the effect of fluctuating temperature and elevated pCO_2 on pinto abalone metabolic rate. Size was not included in this experiment. Water baths were set up as in the growth and survival experiment, except each bath contained six cups rather than 10 (Figure 5). One abalone was placed randomly into each treatment cup and each cup received either 400 or 1200 ppm CO_2. Thus, there were six abalone per treatment in this experiment and abalone was the experimental unit. The cups were placed in their respective water bath (fluctuating or constant temperature) and the abalone were left to acclimate for one full temperature cycle (10 → 14 → 10 °C, adjusted by 1 °C each day).

Oxygen consumption was measured using optical oxygen sensors. Twenty 1 mL vials were prepped for metabolic rate measurements with oxygen sensor spots (PreSens Precision

Sensing GmbH, Regansburg, Germany) placed in the bottom. Vials were numbered so individual abalone were tested in the same vial on consecutive days. There were five blank vials to account for possible respiration of microorganisms in the seawater.

During this time, water sampling occurred every ~5 days. Water sampling followed the same procedure as the growth and survival experiment, except sampling alternated between cups 1-3 and cups 4-6.

To prepare for oxygen consumption measurements, each abalone was placed in a 1 mL vial with water from their treatment cups and the vials were set back in their respective cups. The abalone were left to adjust to the vials for 45 – 60 minutes. During this time, a PreSens Fibox with an optic cable (PreSens Precision Sensing GmbH, Regansburg, Germany) was calibrated for that day's atmospheric pressure, treatment temperature, and salinity. To calibrate the PreSens Fibox and optic cable, a vial containing seawater with 0% oxygen and a vial containing seawater with 100% oxygen saturation were created. The vial with 0% oxygen was made by dissolving 100 mg of sodium sulfite into 10 mL of autoclaved seawater with a magnetic plate and stir bar before putting the solution in a 1 mL vial. The vial with 100% oxygen was made by aerating autoclaved seawater for at least 30 minutes before being added to a 1 mL vial. The daily atmospheric pressure, treatment temperature, and salinity were recorded in the manual calibration setting in PreSens Measurement Studio 2. Atmospheric pressure was taken from the National Weather Service website for Anacortes, WA (forecast.weather.gov). The calibration continued with optic probe readings on the 0% and 100% oxygen vials. When the readings were at the appropriate level for each vial, the calibration was saved and measurements could start. PreSens Fibox optic probe calibrations were repeated every day that the treatment temperature was different than the previous day.

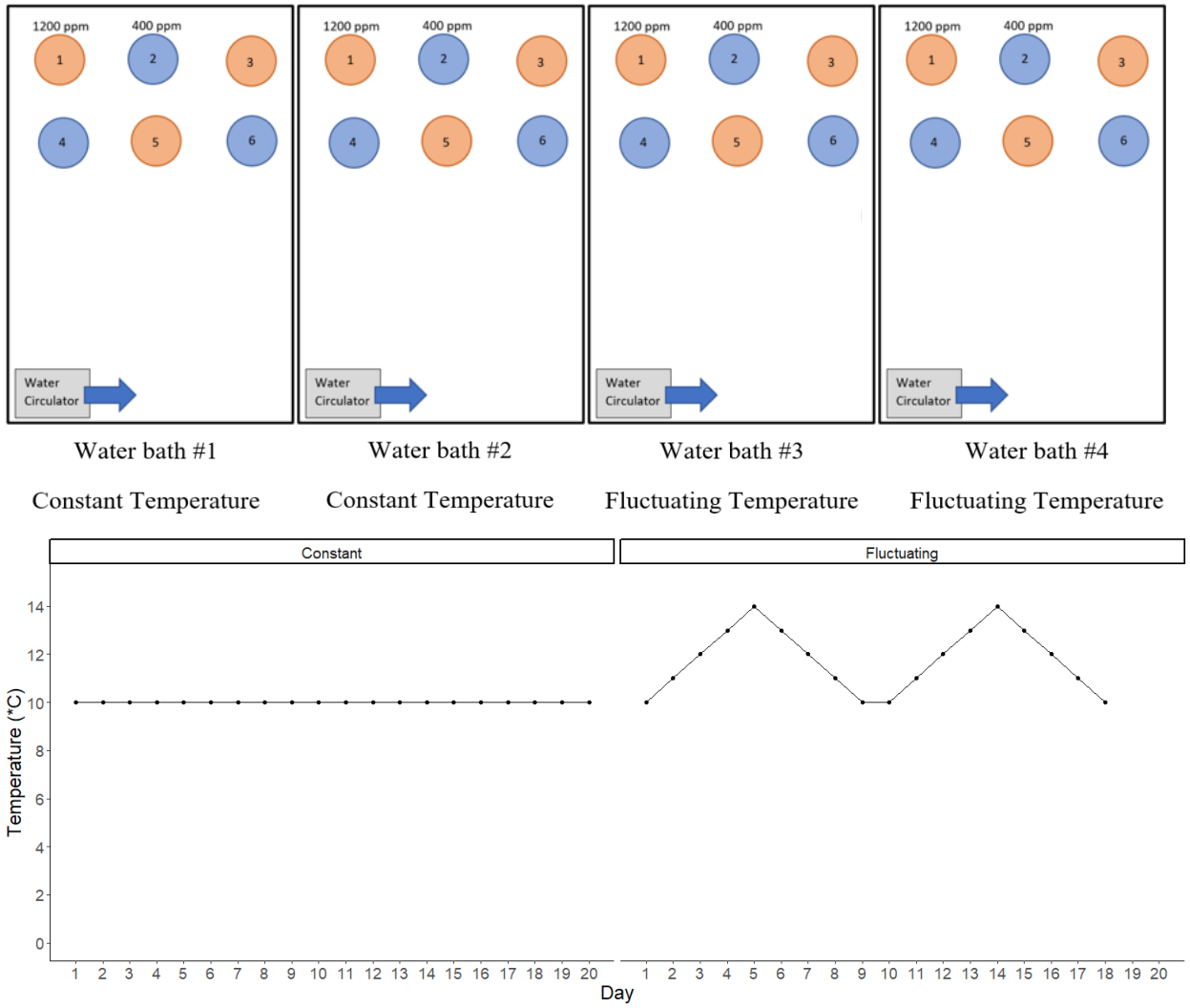

Figure 5. Experimental design for metabolic rate experiment. Four water baths (top) – two with constant temperature and two with fluctuating temperature. Six cups (circles) within each water bath – three cups bubbled with ambient CO_2 (400 ppm – blue circles) and three cups with elevated CO_2 (1200 ppm – orange circles). Temperature treatments (below) for constant temperature water baths (10 °C) and fluctuating temperature water baths (10 → 14 → 10 °C).

After the juvenile abalone had settled in their vials, the vials were removed from the treatment cups and the treatment water in the vials was replaced with autoclaved seawater that had been bubbled with the appropriate CO_2 level. The vials were filled slowly to minimize air bubbles. Blank vials were also filled with autoclaved seawater but did not have an abalone placed in them. After all 12 vials with abalone and five blank vials were filled with autoclaved seawater, they were covered in plastic wrap to prevent air bubbles before being capped. Blanks were capped first because they did not contain an organism, the other vials were capped in order of measurement (vial 1-12). Once capped, vials were placed upside down in a 24-well plate in numerical order. The 24-well plate was immediately placed in a Fisher Scientific Isotemp water bath set to the treatment temperature for that day.

The calibrated Fibox with an optic cable was connected to a laptop running the PreSens Measurement Studio 2 software. All lights were turned off in the lab to keep the room as dark as possible to minimize abalone movement. The 24-well plate containing the vials was removed from the water bath and gently inverted three times to prevent oxygen stratification. The O_2 optic sensor was placed on the bottom of the vial over the PreSens oxygen sensor spot for ~10 readings. Before moving onto the next vial, the O_2 optic sensor was placed on the edge of a vial, away from the oxygen sensor spots, for ~5 readings to show a break between vials in data collection. The vials were read in numerical order (vials 1-12) and then the blanks (vials B1-B5). Each round of measurements took ~5 minutes. After all vials were measured, the well plate was placed back in the water bath. After 10 minutes, measurements were repeated. This was repeated until the average percent air saturation (% a.s.) had decreased by ~20% for vials holding abalone, which took about 3 hours each day. This ensured that the abalone's oxygen consumption was not affected by declining oxygen levels since abalone are oxyregulators until about 80% a.s. (Morash

& Alter 2016). Once that day's measurements were complete, the abalone were removed from their vials and placed back in their treatment cups. This was repeated every day for the fluctuating treatment for a full temperature fluctuation cycle (10 → 14 → 10 °C, adjusted by 1 °C each day). After the last day of oxygen measurements, the live mass of each juvenile abalone was recorded before they were frozen. This procedure was then repeated for three consecutive days at 10 °C for abalone in the constant temperature treatment.

Abalone mass specific oxygen consumption was calculated from the decrease in percent air saturation in each vial. Treatment temperature, solubility of oxygen in water (Boutilier et al. 1984), vapor presser of water, and atmospheric pressure were used to find the concentration of oxygen in the water (μmol L^{-1}). This value was then multiplied by the slope of the change in % a.s. measured in each vial during the experiment to get the oxygen consumption (μmol O_2 L^{-1} min^{-1}). These values were used with the volume of the vial used in the measurement and mass of individual were used to calculate the mass specific metabolic rate for each individual (μg O_2 g^{-1} hr^{-1}).

To analyze juvenile abalone metabolic rate, I used a linear mixed effect model function (*lmer*) and modeled mass specific metabolic rate as the outcome variable, temperature treatment and pCO_2 level as fixed factors (including an interaction between temperature treatment and pCO_2), and vial (individual) and in-situ temperature as random factors to account for the repeated measurements. I used a model selection procedure recommended by Zuur et al. (2009) and used the same backwards model selection described above to determine the most parsimonious model. I used the *lme4* package in RStudio (Bates et al. 2015) to perform these analyses.

Water Chemistry

During each experiment, water in each experimental cup was changed on a weekly basis and some of this treatment water was preserved for water chemistry analysis. The following water chemistry methods are consistent with Lawlor and Arellano (2020). To prepare these water samples for pH and dissolved inorganic carbon (DIC) water chemistry, treatment water was first filtered into a 500 mL beaker through a 500 µm mesh filter. The filtered treatment water was then used to rinse 20 mL scintillation vials twice before filling a vial with the water sample. Vials were poisoned the same day with 10 µL mercuric chloride. Poisoned samples were stored for 2-3 weeks in a refrigerator at 2 °C until they were processed.

To determine the pH of the poisoned water samples, a S-UV-VIS flame spectrophotometer (Agilent 8453A) was used. First, the samples were moved from the fridge to a Fischer Scientific Isotemp water bath set to 25 °C. Measurements started when the water samples reached 25 °C. Once the poisoned samples reached the appropriate temperature, a 5 cm pathlength cuvette was filled with the sample. Three spectra were collected, a baseline and after the addition of a first and second aliquot (20 µL) of *m*-cresol indicator dye before moving onto the next sample. The spectrophotometer was read through the Ocean Optics software.

DIC was measured in preserved samples using an Apollo AS-C3 Analyzer (Apollo SciTech AS-C3). Room temperature and salinity were recorded for each sample. The analyzer extracted multiple sub-samples from the vial and ran them through the instrument which was calibrated to a standard certified reference material (CRM, Batch 149, Dickson, Scripps Institute of Oceanography). This was repeated for every water sample taken. The measured pH, DIC, and salinity, and in-situ temperature values were used in CO2SYS (Pelletier et al. 2012) to determine the pCO_2, pH (total scale), and aragonite saturation state ($\Omega_{aragonite}$) of each sample.

Results

Water Chemistry

The water chemistry deviated from expected values in the different experiments. Although the pCO_2 levels in the air bubbled into the cups were monitored at SPMC, the ambient pCO_2 (400 ppm) achieved in the treatment cups was higher than expected for the constant and fluctuating treatments for both experiments (Table 1; Table 2). This could be because the cups did not have lids, so there was more CO_2 mixed with the air in the walk-in environmental chamber. In addition to the bubbling air into cups, the increased CO_2 in the air could react with the water in the cups, creating higher pCO_2 levels than anticipated. The elevated pCO_2 (1200 ppm) was within 100 ppm of the target level in the growth and survival experiment but was much lower in the metabolic rate experiment. The pH corresponded with pCO_2 levels as expected; lower pCO_2 values resulted in higher pH values. Salinity was expected to be around 27-31 ppt, which is a normal range for the Salish Sea (Reum et al. 2014), however, salinity was higher than expected across all treatments in both experiments. Although elevated, the salinity levels were within salinity tolerances for abalone (Morash & Alter 2015). Salinity did not impact survival ($p>0.5$). The elevated salinity was likely due to the evaporation of seawater from bubbling pCO_2 into the cups. The temperature treatments were also lower than expected across all treatments in both experiments (Figure 6). This was likely due to the thermometer being incorrect in the environmental walk-in chamber, creating a colder environment than intended. The iButtons for the constant temperature treatment were incorrectly programmed for part of the metabolic rate experiment resulting in missing temperature data for the constant treatment which is shown by the gap in data (Figure 6). Aragonite ($\Omega_{Aragonite}$) was supersaturated across all treatments in both experiments (Table 1; Table 2).

Table 1. Water chemistry results for the growth and survival experiment. Ambient CO_2 treatments had an expected pCO_2 of 400 ppm and elevated CO_2 treatments had an expected pCO_2 of 1200 ppm. Constant temperature treatments had a targeted temperature of 10 °C and fluctuating temperature treatments had a targeted temperature fluctuation of 10 → 14 → 10 °C. Values are means ± SE.

Treatment	*n*	*Temp (°C)*	*Salinity (ppt)*	*pCO_2 (ppm)*	*DIC (µmol/kg)*	*pH (total scale)*	*Ω Aragonite*	*Ω Calcite*
Constant temperature								
Ambient pCO_2	30	9.0 ± 0.1	37.1 ± 0.5	603 ± 30	2376 ± 44	7.93 ± 0.02	1.89 ± 0.09	2.78 ± 0.13
Elevated pCO_2	30	9.0 ± 0.1	37.1 ± 0.6	1060 ± 49	2512 ± 45	7.71 ± 0.02	1.27 ± 0.07	1.75 ± 0.08
Fluctuating temperature								
Ambient pCO_2	12	9.6 ± 0.01	40 ± 0.5	1021 ± 295	3151 ± 83	7.89 ± 0.06	2.52 ± 0.23	3.96 ± 0.39
	8	9.8 ± 0.06	40 ± 2	659 ± 58	2952 ± 113	7.98 ± 0.05	2.85 ± 0.39	4.48 ± 0.62
	4	10.5 ± 0.03	37 ± 1.7	712 ± 31	2399 ± 57	7.86 ± 0.02	1.62 ± 0.12	2.53 ± 0.18
	2	12.0 ± 0.05	37 ± 0.5	639 ± 16	2284 ± 31	7.89 ± 0.01	2.7 ± 0.03	1.72 ± 0.02
	4	13.0 ± 0.0	36 ± 0.8	682 ± 55	2432 ± 62	7.89 ± 0.04	1.91 ± 0.21	2.99 ± 0.33
Elevated pCO_2	8	9.6 ± 0.01	43 ± 0.5	1193 ± 44	3926 ± 171	7.74 ± 0.03	2.81 ± 0.27	4.41 ± 0.43
	12	9.8 ± 0.04	42 ± 1	1317 ± 66	3446 ± 101	7.75 ± 0.02	1.95 ± 0.11	3.07 ± 1.14
	4	10.5 ± 0.03	37 ± 0.9	998 ± 42	2481 ± 46	7.73 ± 0.01	1.25 ± 0.04	1.96 ± 0.06
	4	12.0 ± 0.03	37 ± 0.3	1230 ± 68	2450 ± 38	7.64 ± 0.03	1.67 ± 0.12	1.07 ± 0.08
	2	12.7 ± 0.0	35 ± 1.3	1328 ±166	2605 ± 128	7.64 ± 0.03	1.12 ± 0.05	1.8 ± 0.08

Table 2. Water chemistry results for the metabolic rate experiment. Ambient CO_2 treatments had an expected pCO_2 of 400 ppm and elevated CO_2 treatments had an expected pCO_2 of 1200 ppm. Constant temperature treatments had a targeted temperature of 10 °C and fluctuating temperature treatments had a targeted temperature fluctuation of 10 → 14 → 10 °C. Values are means ± SE.

Treatment	*n*	*Temp (°C)*	*Salinity (ppt)*	*pCO_2 (ppm)*	*DIC (µmol/kg)*	*pH (total scale)*	*Ω Aragonite*	*Ω Calcite*
Constant Temperature								
Ambient pCO_2	18	9.4 ± 0.14	36.2 ± 0.4	549 ± 22	2289 ± 31	7.95 ± 0.01	1.79 ± 0.05	2.81 ± 0.08
Elevated pCO_2	18	9.6 ± 0.13	35.9 ± 0.4	812 ± 16	2293 ± 25	7.78 ± 0.01	1.23 ± 0.02	1.94 ± 0.04
Fluctuating Temperature								
Ambient pCO_2	4	9.5 ± 0.09	38.4 ± 0.7	608 ± 8	2594 ± 74	7.93 ± 0.02	2.12 ± 0.1	3.33 ± 0.23
	2	9.9 ± 0.05	34.5 ± 0.5	538 ± 6	2390 ± 36	7.98 ± 0.003	1.94 ± 0.03	3.05 ± 0.04
	4	10.7 ± 0.0	38.1 ± 0.7	561 ± 13	2461 ± 64	7.97 ± 0.02	2.17 ± 0.1	3.39 ± 0.2
	6	12.0 ± 0.0	36.2 ± 0.5	593 ± 28	2315 ± 28	7.93 ± 0.02	1.90 ± 0.1	2.97 ± 0.16
	2	12.5 ± 0.0	38.0 ± 0	515 ± 6	2431 ± 31	8.0 ± 0.01	2.45 ± 0.09	3.8 ± 0.13
Elevated pCO_2	2	9.5 ± 0.15	38.8 ± 0.3	1009 ± 26	2570 ± 49	7.73 ± 0.02	1.3 ± 0.1	2.04 ± 0.15
	4	9.9 ± 0.03	34.0 ± 0.9	862 ± 10	2454 ± 25	7.79 ± 0.0	1.3 ± 0.03	2.05 ± 0.04
	2	10.7 ± 0.0	37.5 ± 1.0	875 ± 59	2379 ± 5	7.77 ± 0.03	1.33 ± 0.07	2.08 ± 0.11
	6	12.03 ± 0.0	34.4 ± 0.4	844 ± 69	2257 ± 45	7.78 ± 0.04	1.30 ± 0.13	2.04 ± 0.35
	4	12.5 ± 0.0	36.8 ± 0.5	851 ± 15	2446 ± 27	7.8 ± 0.01	1.53 ± 0.04	2.40 ± 0.06

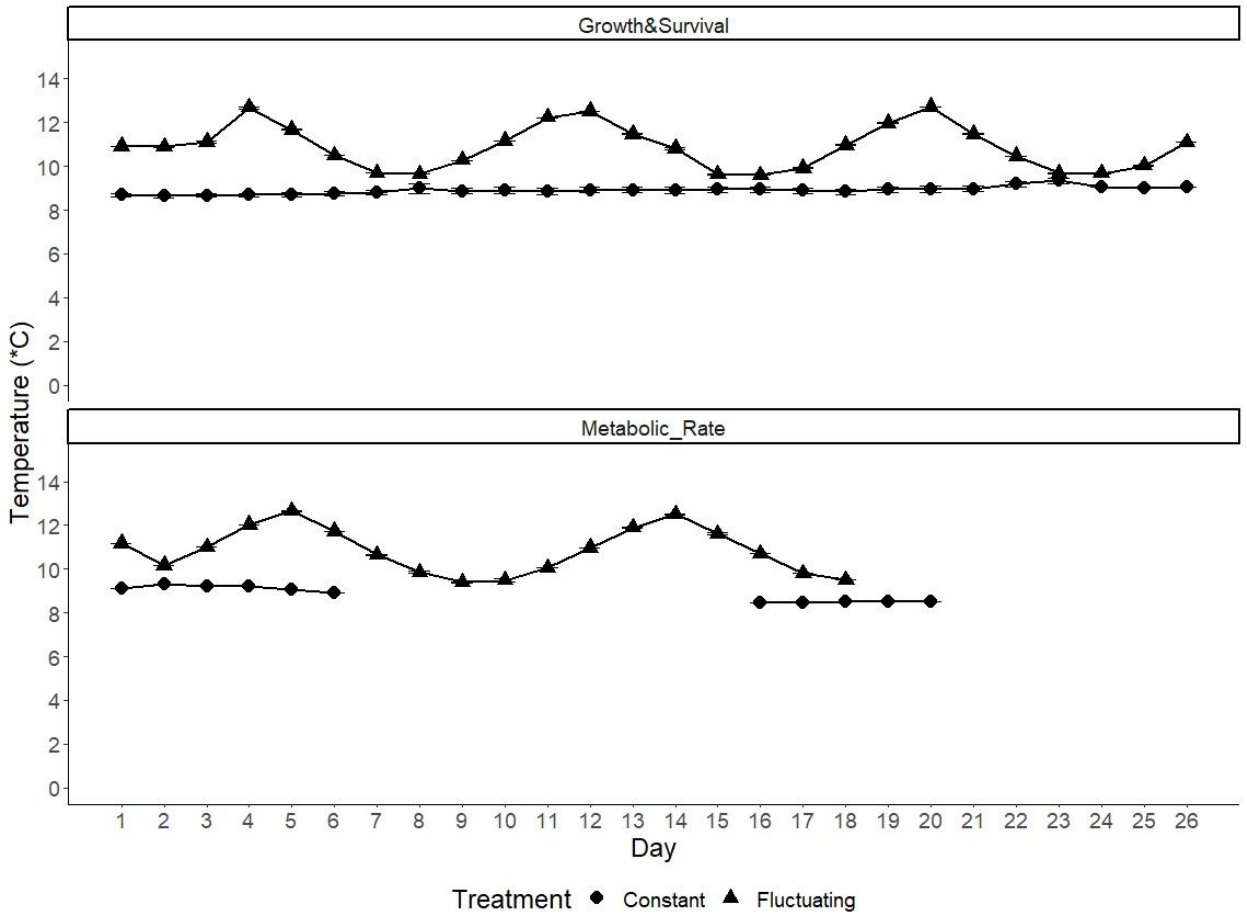

Figure 6. Measured temperature for constant and fluctuating temperature treatments over time in growth and survival experiment (top) and metabolic rate experiment (bottom). Gap in constant temperature treatment data in metabolic rate experiment due to human error. Values are means with SE error bars. Error bars smaller than points due to low SE.

Growth and Survival Experiment

There was no interaction between juvenile pinto abalone size, temperature, or pCO_2. Abalone size, salinity and pCO_2 were not retained in the best-fit model (Table 3). Fluctuating temperature had a significant negative effect on survival ($p<0.001$). Abalone survival was 75% in the constant temperature treatment with a mean of 3.8 ± 0.3 abalone survived per cup. However, only 16% of juveniles survived in the fluctuating temperature treatment with a mean of 0.8 ± 0.4 abalone survived per cup (Figure 7). In addition to having lower overall survival, abalone survivorship in the fluctuating temperature treatment decreased at a faster rate than in the constant temperature treatment regardless of pCO_2 treatment (Figure 8). Although insignificant, survivorship was higher for larger abalone compared to smaller abalone, with means of 2.75 ± 0.7 and 1.8 ± 0.5 abalone survived respectively.

Data from the fluctuating temperature treatments were not included in growth analyses due to extremely low survival in these treatments. There was no interaction between abalone size and pCO_2 level on juvenile abalone growth, and neither abalone size nor pCO_2 level were retained in the best-fit model (Table 3). Juveniles in the ambient (400 ppm) pCO_2 treatment had a mean growth of 0.53 ± 0.1 mm while juveniles in the elevated (1200 ppm) pCO_2 treatment had a mean growth of 0.45 ± 0.1 mm (Figure 9). Also, each treatment had one cup with "negative growth" likely from losing large individuals. Even excluding the negative values associated with two cups of large juveniles, the small juvenile abalone experienced more growth on average (0.63 ± 0.1 mm) compared to the large juveniles (0.48 ± 0.1 mm).

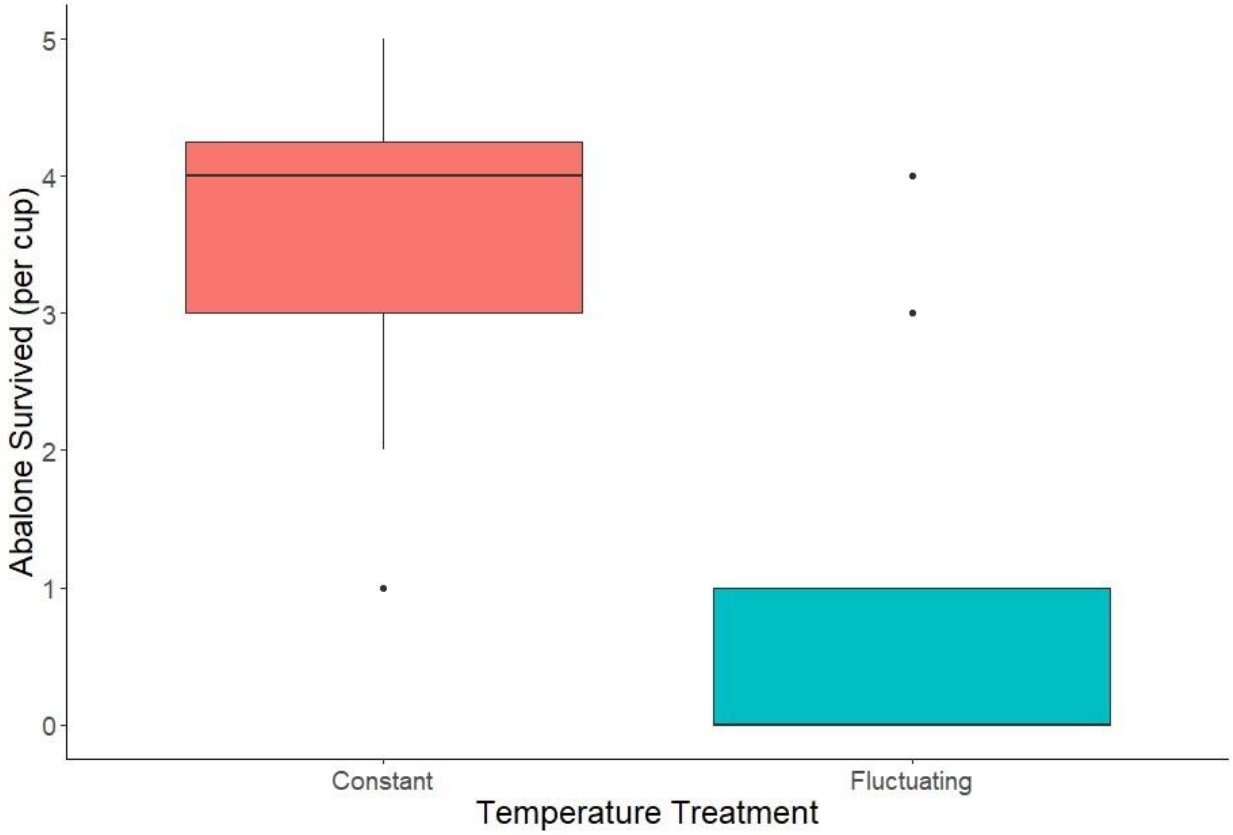

Figure 7. Juvenile pinto abalone survival in constant and fluctuating temperature treatments. All cups started with 5 juvenile abalone and remained in treatment for four weeks before final survivors were counted. The fluctuating temperature treatment had a significant negative impact on juvenile abalone survival ($p<0.001$).

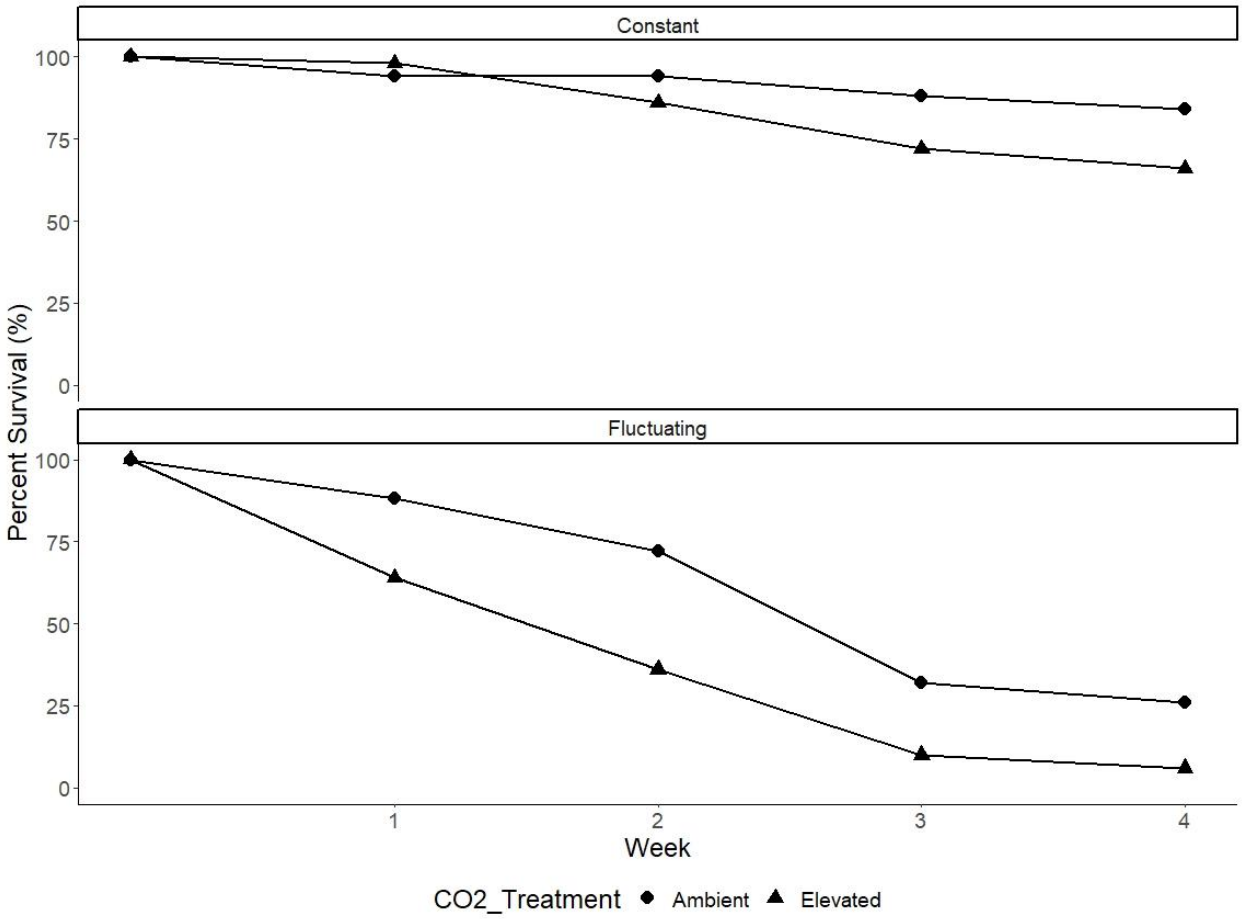

Figure 8. Percent survival over time for growth and survival experiment. Constant temperature treatment (top) and fluctuating temperature treatment (bottom) with percent survival for elevated pCO_2 (1200 ppm – triangle) and ambient pCO_2 (400 ppm – circle). Values are total survived each week per treatment divided by total from start of experiment x 100%.

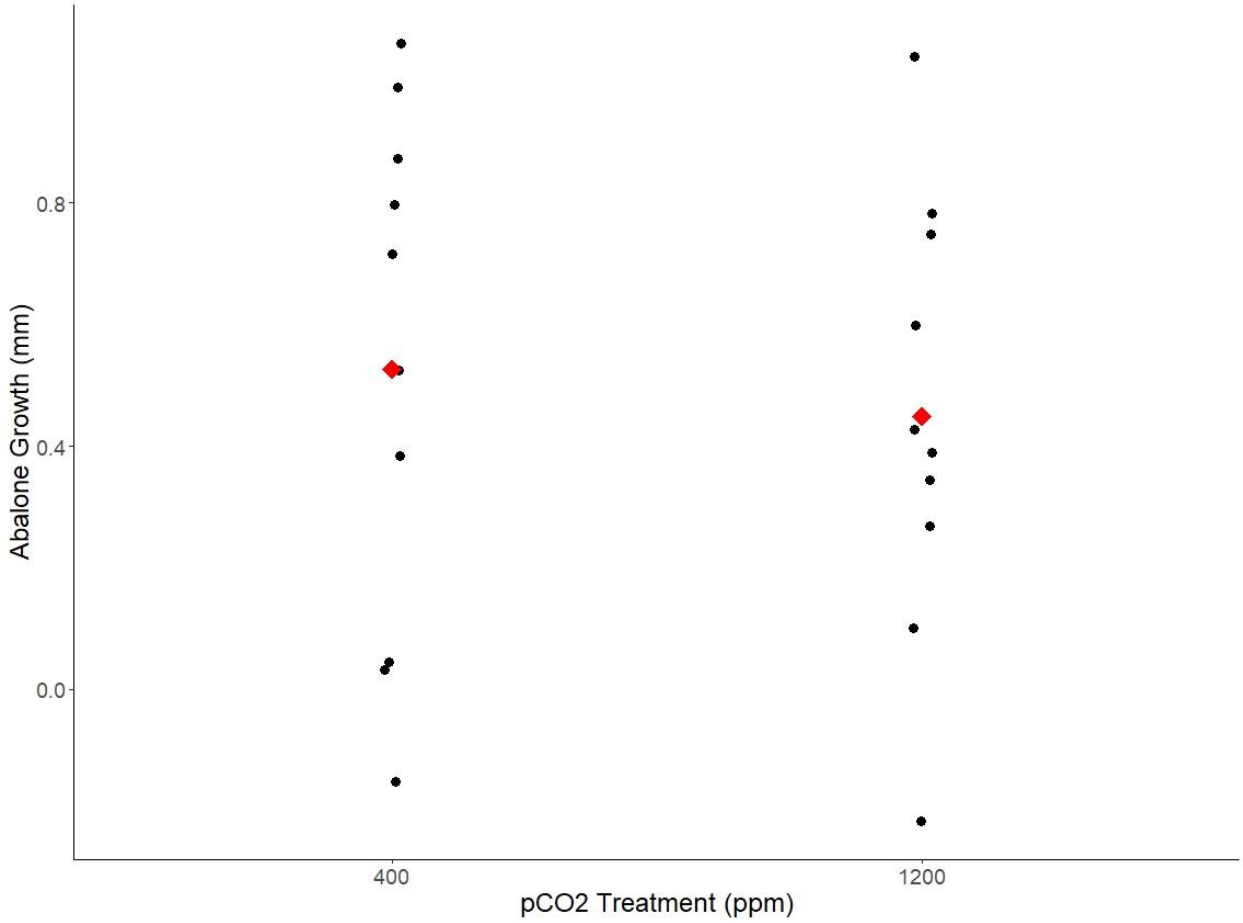

Figure 9. The effect of ambient (400 ppm) and elevated (1200 ppm) pCO_2 treatments on juvenile abalone growth (mm) after 4 weeks in a constant 10 °C temperature treatment. Red points represent treatment means. Juvenile pinto abalone shell length growth was not significantly affected by pCO_2 ($p>0.05$).

Metabolic Rate Experiment

Both temperature treatment and pCO_2 level had significant effects on abalone metabolism, and there was a significant interaction between temperature treatment and pCO_2 ($t = 2.264$; Table 3). In-situ temperature and individual were also retained in the final model as random factors. Abalone in the constant temperature treatment at ambient (400 ppm) pCO_2 had a mean mass specific oxygen consumption of 19 ± 2.8 µg O_2 g^{-1} hr^{-1}, which was the lowest of the treatments (Figure 10). Both fluctuating temperature and elevated pCO_2 increased abalone oxygen consumption, but in nonadditive ways as indicated by the significant interaction. The mass specific oxygen consumption of the abalone in the constant temperature treatments were relatively stable compared to the mass specific oxygen consumption rates of the abalone in the fluctuating temperature treatment (Figure 11). The oxygen consumption rates increased as the temperature increased, as expected. However, the oxygen consumption rates were lower for the abalone after the "peak" temperature of 14 °C when compared to the same temperature before the peak (Figure 11).

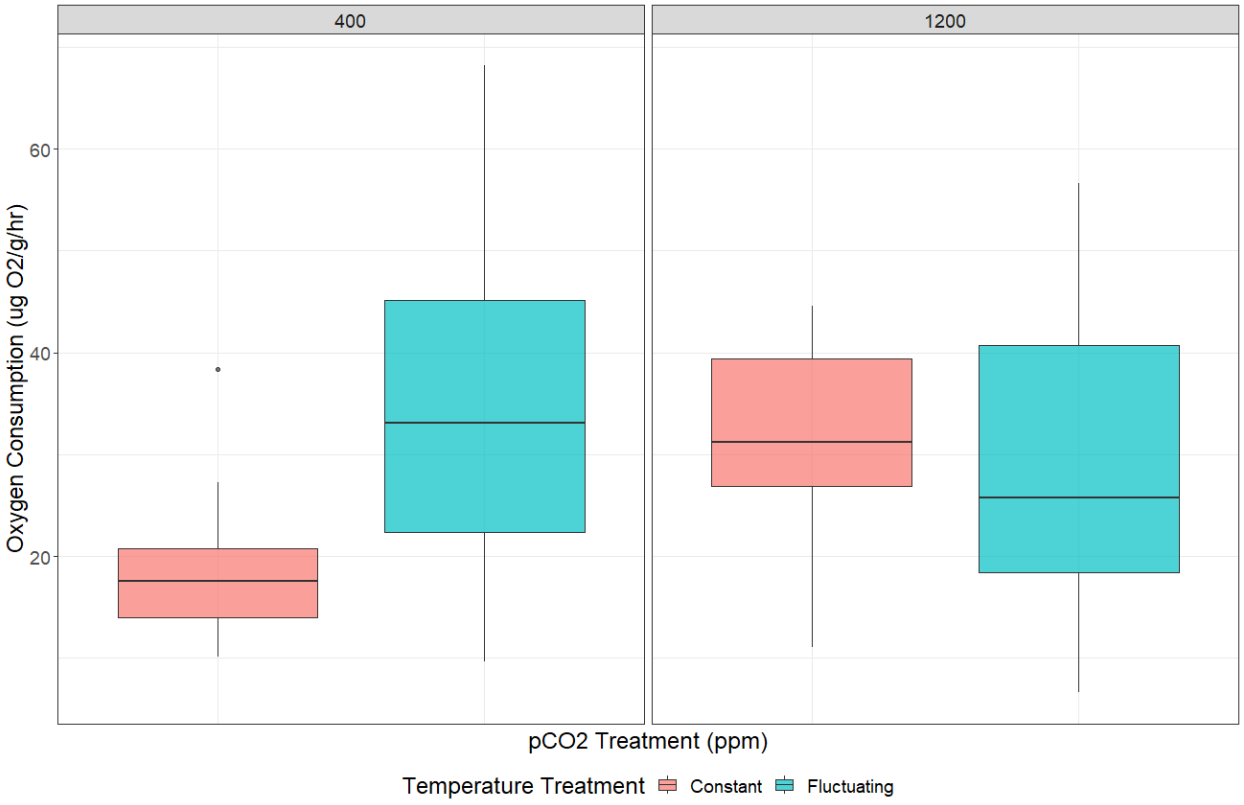

Figure 10. Boxplot of juvenile pinto abalone mass specific oxygen consumption (µg O_2 g^{-1} hr^{-1}) across different temperature and pCO_2 treatments. The interaction of temperature and pCO_2 had a significant effect on juvenile pinto abalone metabolic rate ($t>2.0$).

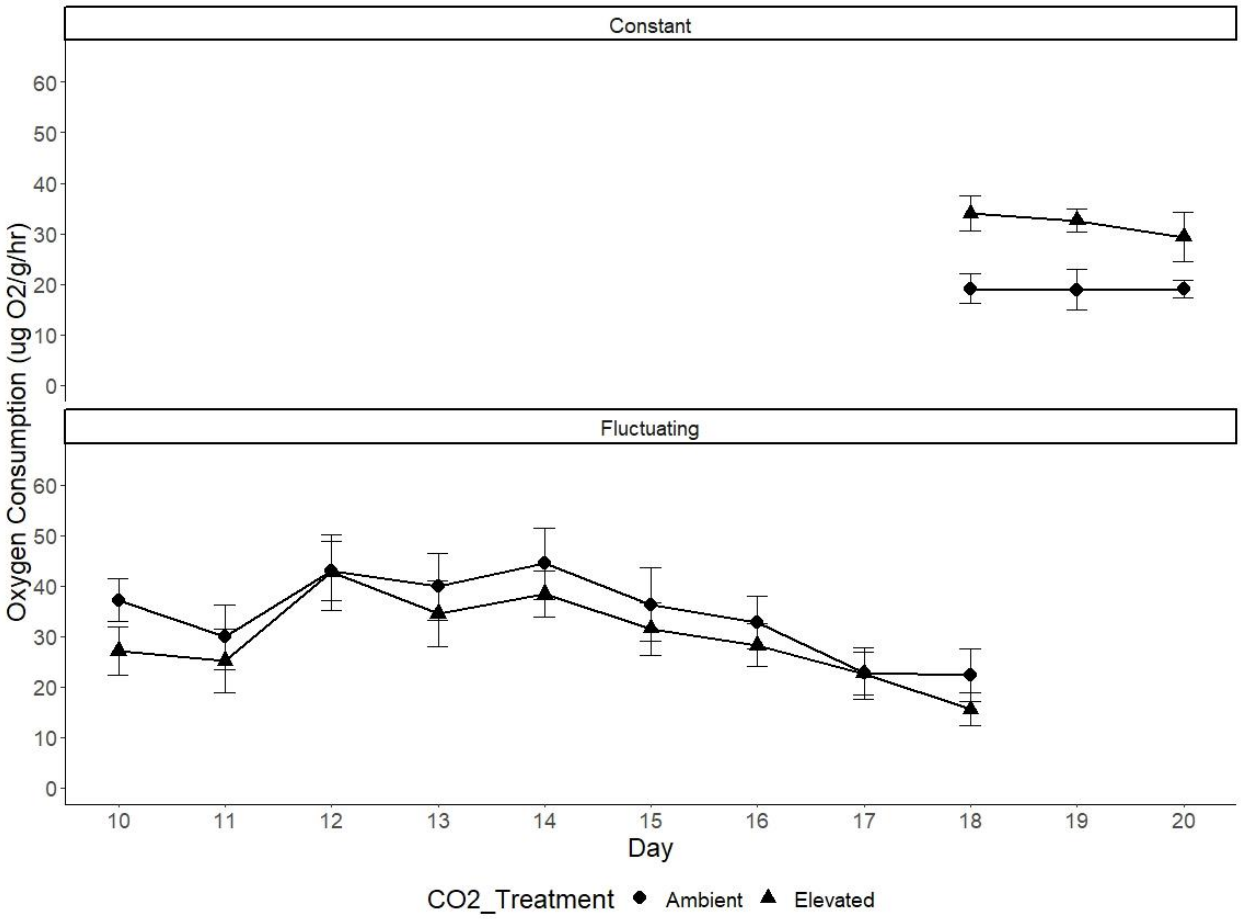

Figure 11. Juvenile pinto abalone mass specific oxygen consumption (µg O_2 g^{-1} hr^{-1}) over duration of metabolic rate experiment. Constant temperature treatment (top) and fluctuating temperature treatment (bottom) with metabolic rate for elevated pCO_2 (1200 ppm – triangle) and ambient pCO_2 (400 ppm – circle). Values are means with SE error bars.

Table 3. Outputs from best-fit models for juvenile pinto abalone shell length growth, survival, and metabolic rate analyses. The *glm* package in RStudio was used for the survival analyses and generated *z* values while the *lm* and *lmer* packages used for the growth and metabolic rate analyses, respectively, generated t values. The *lmer* package does not include p values in the output, however, a *t* value >1.96 is considered significant.

	Estimate	SE	*t* or *z* value	p value
Growth and Survival Experiment				
Growth				
SLf ~ SLi				
Intercept	1.06	0.37	2.87	0.01
Initial Shell length	0.88	0.07	11.87	<0.001
Survival				
(NumAbsf, NumAbsi) ~ Temp Treatment				
Intercept	-0.09	0.19	0.49	0.63
Fluctuating temperature	-1.55	0.31	4.98	<0.001
Metabolic Rate Experiment				
Metabolic Rate				
*Mass Specific Metabolic Rate ~ Temp Treatment*pCO2 + (1|in-situ temp) + (1|individual)*				
Intercept	25.71	5.38	4.78	
Fluctuating Temperature	9.39	5.76	1.63	
High pCO_2 (1200 ppm)	12.99	5.80	2.24	
Fluctuating Temp*high pCO_2	-17.72	7.83	2.26	

Discussion

In this study, I investigated the effect of fluctuating temperature and elevated pCO_2 on juvenile pinto abalone growth, survival, and metabolic rate. I hypothesized that temperature fluctuations and elevated pCO_2 levels would result in less growth and decreased survival for abalone and that their metabolic rates would increase under these conditions. I did find that juvenile pinto abalone survival was negatively affected by fluctuating temperature, but the hypothesis regarding a negative effect of high pCO_2 on survival was not supported. Abalone growth was not affected by pCO_2 level (temperature could not be analyzed due to very poor survival in the fluctuating temperature treatment). Finally, there was an interaction between fluctuating temperature and pCO_2 level on abalone metabolic rate. Abalone in the constant temperature treatment had higher metabolic rates when exposed to elevated pCO_2, however that pattern was not observed in the fluctuating temperature treatment when exposed to elevated pCO_2.

Growth and Survival Experiment

Juvenile pinto abalone survival was negatively impacted by temperature fluctuation, however pCO2 level did not significantly affect abalone survival as I expected. My finding that fluctuating temperature decreased survival compared to constant temperature could be explained in different ways. First, the maximum temperature in the fluctuating temperature treatment (14 °C) was higher than the constant temperature treatment (10 °C), so it is possible that abalone mortality was due to peak temperature. Pinto abalone thermal tolerance, however, is well above 14 °C. Paul and Paul (1998) estimated the CT_{max} of *H. kamtschatkana* at 24-27 °C. In addition,

Hosikawa et al. (1998) determined that the optimal temperature for growth was 15 °C. These findings suggest that abalone mortality in my study was not due to the peak temperature in the fluctuating temperature treatment. Second, the mean temperature in the fluctuating treatment (12 °C) was higher than the mean temperature of the constant temperature treatment (10 °C). Again, temperature tolerance and optimal growth temperature for *H. kamtschatkana* suggest that the difference in mean temperature between the two treatments was not the cause of mortality. Thus, it seems likely that it was the fluctuation in temperature, and not the actual temperatures, that caused increased mortality.

Most research on abalone mortality due to temperature change is on temperatures close to the thermal limit of the species. For example, Kang et al. (2019) investigated the response of caged Pacific abalone (*Haliotis discus hannai*) to diurnal temperature fluctuation in a shallow bay in different seasons. They found that caged abalone experiencing temperatures that fluctuated from a high temperature to temperatures below a thermal maximum experienced lower mortality compared to abalone held in constant high temperatures. Interestingly, the sites that had lower average temperature fluctuations (~1 – 3.5 °C day^{-1}) had higher abalone mortality rates than the site that had the highest average temperature fluctuation (~2 – 7.5 °C day^{-1}), although the higher fluctuation resulted in abalone experiencing cooler temperature. These fluctuations occurred in August when the water temperature in Wando Bay is around 28 °C, which is the thermal limit of Pacific abalone (Morash & Alter 2015). Boch et al. (2018) found conflicting results when exposing green abalone (*Haliotis fulgens*) to fluctuating temperatures in the field and in the lab. The green abalone were outplanted at multiple sites over two years near Isla Natividad, Baja California, Mexico. Survivorship varied by outplant location and by year. In the first year, one site had nearly 0% survivorship while the rest of the sites had ~70%

survivorship. However, in the second year, there was more variation in the temperature and dissolved oxygen at the outplant locations, which decreased survivorship to ~21% across all locations. When these field temperature conditions were replicated in the lab, however, survivorship was not impacted by temperature fluctuation (Boch et al. 2018). Although the temperature fluctuations in the lab did not impact survivorship, the authors concluded that other environmental factors, such as hypoxia can have a negative effect on growth and survival (Boch et al. 2018). Their mixed results indicate the importance of conducting multi-stressor experiments in lab settings.

In the case of my experiment, mortality was associated with temperature fluctuation well below the thermal limits of *H. kamschatkana*. There are some possible explanations for this result. Fluctuating temperature can increase metabolic demand for ectotherms, which can lead to difficulty maintaining linked physiological processes (Kang et al. 2019). Fluctuating temperature might also lead abalone to rely on anaerobic metabolism, which can't be maintained over long periods of time in abalone (Morash and Alter 2016). Finally, pinto abalone are less resilient to temperature change than other abalone species due to their purely subtidal habitat. In this case, Dahlhoff and Somero (1993) found that the mitochondria of *H. kamtschatkana* did not acclimate to increased temperature compared to other abalone species from intertidal habitats. This could indicate that pinto abalone might be more susceptible to temperature fluctuation than other abalone species. Low survival in fluctuating temperature is consistent with observations in the PRSF hatchery where they try to maintain constant water temperature in the tanks to prevent abalone mortality (PSRF hatchery manager, Josh Bouma, personal communication).

In addition to abalone, other ectotherms, such as amphibian tadpoles, have shown that survival is greatly impacted by temperature fluctuations in the environment. When three species

of Australian frog tadpoles (*Limnodynastes peronii*, *Limnodynastes tasmanienses*, and *Platyplectrum ornatum*) were exposed to large daily temperature fluctuations (18-38 °C), two of the three species had zero survivors at the end of the study period (Kern et al. 2015). *P. ornatum* was the only species that survived the largest temperature fluctuations. It was also the only species that came from a habitat that regularly experienced large temperature fluctuations, indicating that regular exposure to environmental variation may increase plasticity in some ectotherms (Kern et al. 2015). However, ectotherm survival does not always decrease with fluctuating temperatures. Dong and Dong (2006) investigated the effect of fluctuating temperature on juvenile sea cucumber (*Apostichopus japonicus*) growth. The treatments included constant temperature treatments (15 and 18 °C) and fluctuating treatments that fluctuated around the constant temperatures with different amplitudes (15 ± 2, 15 ± 4, 15 ± 6 °C and 18 ± 2, 18 ± 4, 18 ± 6 °C). The sea cucumbers were placed in one of the eight temperature treatments for 40 days and the survival rates in all treatments were 100% (Dong & Dong 2006).

Optimal and critical temperatures vary depending on the species and the climates where they are found. Thus, abalone found in warmer climates have higher optimal temperatures than those found in cooler climates. For example, Diaz et al. (2006) found that the green abalone and pink abalone from the Pacific coast of Baja California, Mexico have optimal temperatures of 25.4 and 25.0 °C, respectively. Pinto abalone, which are found in cooler climates in the NE Pacific Ocean, have an optimal temperature of 15 °C (Morash & Alter 2015; Hoshikawa et al. 1998). Blacklip and greenlip abalone, which are endemic to Australia, have optimal temperatures ranging from 17-19 °C (Diaz et al. 2000; Gilroy & Edwards 1998). Exposure to elevated temperatures can be lethal for abalone. Like optimal temperatures, critical temperatures also vary by species and the climate in which they are found. The critical temperatures listed below were

determined by CT_{50}, unless otherwise noted. Because green and pink abalone are found in warmer water, they have critical temperatures of 33.6 and 32 °C, respectively (Diaz et al. 2006), whereas pinto abalone, which are found in cooler waters of the NE Pacific Ocean, have a critical temperature ranging from 24-27 °C (Morash & Alter 2015; Paul & Paul 1998). The blacklip and greenlip abalone of Australia have critical maximum temperatures of 26.9 and 27.5 °C, respectively (Gilroy & Edwards 1998). To determine the blacklip and greenlip abalone critical thermal maximum, Gilroy & Edwards (1998) increased temperature until all abalone had detached from a vertical substrate. Ocean temperatures and temperature fluctuations associated with climate change are expected to increase (IPCC 2014) and this could result in favorable conditions for some species while being detrimental to others (Kern et al. 2015).

There was no significant effect of pCO_2 on abalone growth, contrary to my hypothesis that growth would be negatively affected by elevated pCO_2. These results contradict previous literature on how abalone growth rates respond to elevated pCO_2 levels. Harris et al. (1999) found that juvenile blacklip and greenlip abalone decreased growth rates for mass and length when exposed to highly acidic conditions ($pH < 7.75$). My growth results may not align with these for several reasons. First, the pH values in my study were relatively close to or greater than 7.75 for the treatments used in my growth analyses (Table 1). Second, these results could be because my ambient (control) pCO_2 level was higher than anticipated in the growth and survival experiment (Table 1), creating less of a difference between the two pCO_2 treatments. Third, the constant temperature was lower than anticipated (Figure 6). Higher mean temperatures are often associated with higher growth rates because of higher feeding rates at high temperatures (Dong & Dong, 2006). Therefore, having a lower mean temperature could have negatively impacted the growth results for this experiment. Finally, the aragonite was supersaturated across all treatments

(Table 1), indicating that concentrations were likely high enough to prevent shell dissolution and promote shell building, even in acidic conditions (Fabry et al. 2008).

Some molluscs do not have a negative response to acidic conditions when exposed to other stressors such as food scarcity and changes in temperature and salinity. Lawlor and Arellano (2020) concluded that Olympia oyster (*Ostrea lurida*) larvae are more affected by temperature and salinity than by ocean acidification. When the larvae in their experiment were exposed to lower salinity and higher temperature the larvae increased their developmental rate, indicating that Olympia oyster larvae would likely benefit from future conditions. When blue mussels (*Mytilus edulis*) were grown in acidic conditions but with different levels of available food, the mussels with lower food availability had significantly less growth than those with high food availability (Melzner et al. 2011). Although shell length was still negatively affected by elevated pCO_2 levels, Melzner et al. (2011) concluded that the combination of food scarcity and elevated pCO_2 had a larger effect on shell length in blue mussels than seawater with elevated pCO_2 levels alone.

The life stage of the mollusc may also determine the impact that acidification or fluctuating temperature has on the individual. Larval pinto abalone reared in different pCO_2 treatments (400, 800, and 1800 ppm) had significant differences in shell development and shell length (Crim et al. 2011). Most of the pinto abalone larvae in the ambient pCO_2 treatment (400 ppm) developed normal shells while the middle pCO_2 treatment (800 ppm) had significantly fewer abalone with normal shells, which were also shorter in length than the abalone in the ambient treatment. The high pCO_2 treatment (1800 ppm) contained zero abalone with normal shell development and no shell lengths recorded (Crim et al. 2011). A meta-analysis conducted by Kroeker et al (2013) found that growth differs for molluscs at different life stages in response

to acidification. Mollusc growth in larval or juvenile life stages have a significant negative response to near-future ocean acidification compared to adult life stages which show no significant change (Kroeker et al. 2013).

Metabolic Rate Experiment

The interaction between fluctuating temperature and pCO_2 level resulted in abalone exhibiting a lower metabolic rate than expected in the high pCO_2 – fluctuating temperature treatment. The effect of increased metabolic rate with high pCO_2 is only present in the constant temperature treatment, and not in the fluctuating temperature treatment. This was contrary to my hypothesis that juvenile abalone in this treatment would have the highest metabolic rate out of all the treatments.

Fluctuating temperature affects abalone metabolic rate. When Pacific abalone (*Haliotis discus hannai)* were exposed to fluctuating temperatures (20 – 26 °C) over a six-day period, their metabolic rates were lower than abalone exposed to the same temperatures under stable conditions (Kang et al. 2019). The pinto abalone in my fluctuating treatment were acclimated to the fluctuating treatment that they were going to be exposed to during their oxygen consumption measurements. However, Kang et al. (2019) acclimated the Pacific abalone to 26 °C, which was the highest temperature in their fluctuating treatment. Acclimating the abalone to a higher temperature and then exposing them to a fluctuating treatment that includes lower temperatures is likely why they found a decrease in metabolic rate, and I did not. Calderón-Liévanos et al. (2019) also found a decrease in green abalone (*Haliotis fulgens*) respiration rate when they were exposed to short-term events with multiple stressors. The green abalone had an increased

respiration rate when exposed to only elevated temperature, however when exposed to elevated temperature and decreased dissolved oxygen, their respiration rate decreased significantly (Calderón-Liévanos et al. 2019). They also used modelling to determine how the length of exposure to stresses (elevated temperature, decreased dissolved oxygen, and the combination of both) changes the effect on green abalone mortality. They found that at 24 hours, elevated temperature had the greatest impact on mortality in green abalone and the combined treatment had the next largest impact. However, after 48 hours, the combined treatment increased to a greater effect size than the elevated temperature treatment. After 96 hours, the elevated temperature treatment had even less of an effect on abalone mortality, but the combined treatment remained high. This model indicates that abalone may be more sensitive to stress in the long-term which may be more impactful than stress in the short-term (Calderón-Liévanos et al. 2019).

In another study on ectotherms, Kern et al. (2015) investigated the effects of fluctuating temperature on the metabolic rate of tadpoles reared in fluctuating or constant temperature treatments. They had two different fluctuating temperature treatments, a small (20-30 °C) and a large (18-38 °C) daily temperature fluctuation, and a constant temperature treatment (24 °C). Although two of the three species of tadpoles did not survive the large daily temperature fluctuation, the metabolic rate of the species that survived the large fluctuation (*Platyplectrum ornatum*) was not significantly different when compared to tadpoles of that species in the smaller daily temperature fluctuation or constant temperature treatment. Dong & Dong (2006) investigated the effects of fluctuating temperature on oxygen consumption on sea cucumbers (*Apostichopus japonicus*). When the sea cucumbers were exposed to temperature fluctuations with various amplitudes, sea cucumber oxygen consumption rates decreased five of the six

fluctuating temperature treatments when compared to sea cucumbers in the constant temperature treatment. Interestingly, the treatments at 18 ± 2 and 18 ± 4 °C were significantly lower than the constant 18 °C treatment while the 18 ± 6 °C fluctuating treatment was the same as the constant 18 °C (Dong & Dong, 2006). The individuals in the treatments with lower oxygen consumption rates were the same individuals with higher specific growth rates (Dong & Dong, 2006). The higher growth rate in the individuals in the less stressful conditions (smaller temperature fluctuations) indicate that more energy can be allocated to growth when less energy is needed for metabolic rate in more stressful conditions (larger temperature fluctuations).

Abalone metabolism is also affected by pCO_2 levels. Studies focused on the effect of pCO_2 on metabolic rate found that juvenile greenlip abalone depress their metabolism as a survival tactic when exposed to elevated pCO_2 levels (Harris et al. 1999). This may be due to a reverse Bohr effect found in many abalone species, such that oxygen is bound more tightly to hemocyanin at lower pH (Wells et al. 1998) and a heavy reliance on anaerobic metabolism during environmental and functional hypoxia (Donovan et al. 1999). Depressed metabolic rates under elevated pCO_2 levels have also been observed in other marine invertebrate species including scallops, periwinkles, and mussels (Michaelidis et al. 2005; Bibby et al. 2007; Liu & He 2012). In contrast, some species of oysters have shown no significant change to their metabolic rate (the pearl oyster, *Pinctada fucata*) or an increase in metabolic rate (the Sydney rock oyster, *Saccostrea glomerata*) when exposed to these conditions (Liu & He 2012; Parker et al. 2012).

Limitations and Future Studies

Although there were several limitations to this study, the main limitation was difficulty in controlling some of my water chemistry parameters. The temperature, salinity, and pCO_2 levels were not at the levels I planned for this research. To improve the temperature, I would use additional thermometers in the environmental walk-in chamber and adjust the temperature as needed to have treatment temperatures closer to the intended temperatures. I would also include multiple iButtons in each water bath, instead of one. This would prevent the gap in temperature data that I have in my metabolic rate experiment. To decrease the salinity of the seawater in all treatments in both experiments, I would measure the salinity of the seawater being bubbled with CO_2 in the carboys before conducting water changes. This would allow for me to add more filtered seawater to the carboy to decrease the salinity if needed. To make the pCO_2 treatments closer to their intended levels, I would conduct water chemistry tests when the number of cups were changed between experiments to improve the pCO_2 levels in the metabolic rate experiment. I would be able to increase the gas flow into the cups to create pCO_2 treatments closer to what I planned. I would also use lids on my treatment cups to prevent the escape of CO_2 into the air in the environmental walk-in chamber and to prevent the air in the environmental walk-in to mix with the seawater in my treatment cups.

If I were to conduct these experiments again, I would make a few changes to my methods. To improve the growth and survival experiment, I would measure shell length of the dead abalone as they were removed from their treatments. This would enable me to include fluctuating temperature in my growth analysis in addition to the pCO_2 treatment, which may have influenced growth. To improve the metabolic rate experiment, I would measure the oxygen consumption over more fluctuating temperature cycles to see if the results I found were

consistent over longer time periods or if longer exposure to the combined stress would change its effect on pinto abalone oxygen consumption.

Some limitations to this study also included the inability to replicate more of the environmental variables in the lab. In nature, abalone experience fluctuations in temperature and pCO_2, however they also experience changes in salinity and dissolved oxygen. Controlling a couple variables proved to be hard considering the treatment levels were not where they were intended. The addition of more variables in the lab setting may make the conditions more realistic to what abalone experience in nature, however, they would make the experimental design and data interpretation more challenging.

Conclusion

Future temperature conditions in the Salish Sea may decrease survival for juvenile pinto abalone. Global warming is predicted to increase the temperature of seawater; however, it is also predicted to increase marine heat waves in both number and intensity (Calderón-Liévanos et al. 2019; IPCC 2014). Pinto abalone have a critical temperature ranging from 24-27 °C (Morash & Alter 2015; Paul & Paul 1998), which is much higher than the maximum temperature (14 °C) and the highest mean temperature (12 °C) reached in this experiment. Therefore, is not necessarily the increase in temperature that is detrimental, but the temperature fluctuation that might create the harsh environment. Based on my results, if environmental temperature fluctuations increased in intensity or number of events occurring annually in the Salish Sea, unfortunately, the likelihood of juvenile pinto abalone surviving in the Salish Sea would decrease.

The increasing acidity of the Salish Sea that is predicted may not have as large of an effect on these calcifying molluscs as temperature fluctuation does. According to my results, growth and survival were not significantly affected by elevated pCO_2 levels. However, aragonite was supersaturated in all treatments. This could have mitigated shell dissolution in the elevated pCO_2 treatment. Therefore, the saturation state in the Salish Sea would also need to be supersaturated, with higher saturation states requiring less energy for abalone to calcify. The interaction between fluctuating temperature treatment and elevated pCO_2 decreased the juvenile pinto abalone oxygen consumption which could benefit them in nature. Assuming food intake was constant, the decreased metabolic rate could allow for more energy to be allocated to somatic or reproductive growth (Willmer et al. 2000).

Although PSRF cannot control the impacts of climate change on the Salish Sea, they can control some aspects that may improve their success, such as supporting research and where they outplant abalone. Continuing research in fluctuating temperature treatments will help PSRF and others in global change biology understand the effect that fluctuating temperatures have on abalone and other marine animals. PSRF is currently collecting abiotic data for their outplant sites (Eileen Bates, personal communication). When this is complete, they can apply my results to choosing a better suited environment that promotes survival of the juvenile abalone that they outplant. Sites that experience higher survivorship will have a better probability of population densities being high enough to induce spawning, which will lead to higher pinto abalone populations in the Salish Sea.

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