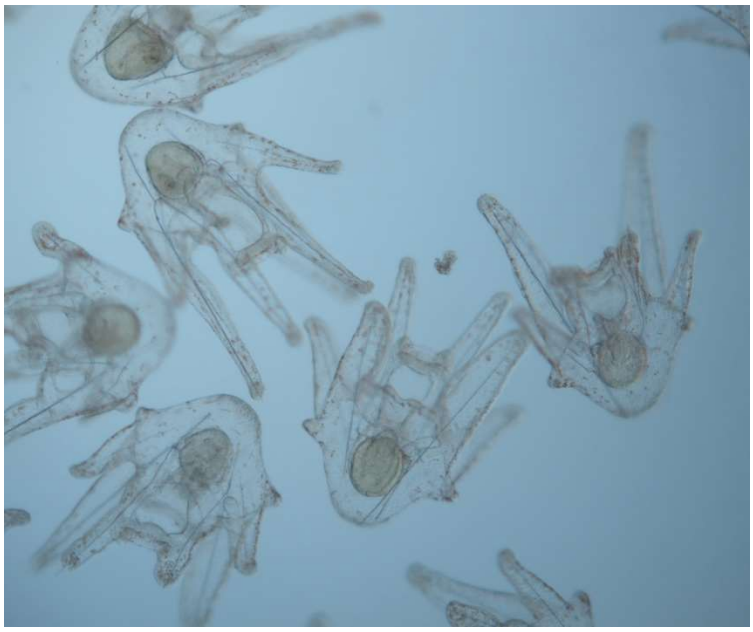
UNIVERSITY OF GOTHENBURG

Department of Biological and Environmental Sciences

Fluctuating pH simulating natural variability modulates larval growth for *Strongylocentrotus droebachiensis*



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Front page photo: Bryn Anderson, *Strongylocentrotus droebachiensis* larvae, Kristineberg Marine Research Center, April 14, 2022

Abstract

Ocean acidification (OA) is the downward trend of ocean pH mainly resulting from the absorption of carbon dioxide (CO_2) emissions from anthropogenic sources. pH of the open ocean is expected to drop to 7.7 by the end of the century. Previous experiments investigating biological response to OA often use this open ocean prediction to assess response and neglect the potential modulating effects of a dynamic, fluctuating coastal ecosystem. pH in the Gullmar Fjord on the west coast of Sweden experiences natural fluctuations of pH as a result of biological processes that exceed the end-of-the century predictions, and as such these natural fluctuations need to be considered. The aim of this study was to investigate which part of the natural variability cycle, minimum pH experienced or duration of exposure under fluctuating conditions, drives the biological response of green sea urchin larvae (*Strongylocentrotus droebachiensis*), which are residents in the Gullmar Fjord. It was hypothesized that both intensity and duration of different pH exposures contributes to the stress experienced by an organism and further that (1) the level of stress is dependent on both intensity and duration of exposure in a cumulative manner (intensity*time); (2) for a given intensity, the negative effect on sea urchin larvae will increase with the duration of exposure; and (3) for a given duration of exposure, the negative effect on sea urchin larvae will increase with the intensity. The main results were that overall fluctuating conditions were beneficial to growth relative to constant conditions, intensity*time predicted stress response for body length growth, ignoring the role of modulating effects on pH can overestimate biological response to OA, and an overall change in shape was observed under fluctuating conditions. Future studies should further investigate this change in shape and also assess biological response in the context of natural fluctuations combined with other global change stressors.

Key words: ocean acidification, global change, sea urchin, duration, intensity, biological response

Popular Science Summary

Ocean acidification (OA) is just one of the many global environmental changes impacting Earth's ecosystems as a result of anthropogenic activities. Carbon dioxide (CO_2) emissions are the main driver of OA and have increased dramatically since the industrial revolution, primarily from the burning of fossil fuels. While some of the CO_2 that is released from these human activities stays in the atmosphere, much of it settles into the ocean. The ocean absorbs roughly 25% of the CO_2 that it is emitted, making it one of the most effective carbon sinks on the planet (IPCC WG2, 2022). While this helps slow the rate of other environmental challenges, such as global warming, it leads to an acidifying ocean, which has catastrophic consequences for many marine organisms and ecosystems. Calcifying organisms, those which build skeletons out of calcium carbonate, have become a primary target for OA research since they are known to be sensitive to the resulting changes in seawater carbonate chemistry (Dupont and Thorndyke, 2013). Since the beginning of the industrial revolution, the global average pH of the open ocean has dropped from 8.2 to 8.1, marking a severely rapid pace of pH decline (Calderia and Wickett, 2003). Global projections under a business-as-usual scenario for CO_2 emissions predict that the average pH of the open ocean will be 7.7 by 2100 (IPCC WG2, 2022). The pH of the open ocean doesn't change much on shorter timescales; while pH trends downward, it is relatively stable over long periods of time. However, coastal environments (tidal pools, coral reefs, fjords, etc.) have different pH regimes than the open ocean. Coastal pH can fluctuate as a result of biological activity on a daily, monthly, or annual scale, oftentimes exceeding pH values not expected for the open ocean until the end of the century (Hoffmann et al., 2011).

Previous experiments investigating the biological response of organisms to OA often use open ocean scenarios even if they are studying species that reside in coastal ecosystems. When coastal pH naturally fluctuates between a high and low pH, it offers small bits of time where organisms are not constantly exposed to the lowest pH, but rather a more moderate one (Schulte, 2014). The variability cycle that is inherent in coastal ecosystems means that organisms experience different combinations of minimum pH and duration of exposure to that minimum pH. Ignoring the potential effects of this fluctuation provides an unrealistic view of organismal response.

Echinoderms are a calcifying phylum of marine invertebrates whose larvae are known to have a general negative response to OA (Dupont and Thorndyke, 2013). They reside in coastal ecosystems and therefore experience fluctuating pH on varying timescales (Dorey et al., 2013). Urchins are a group of echinoderms, and within this group, the green sea urchin *(Strongylocentrotus droebachiensis)* has become a primary target for OA research. They are residents in the Gullmar Fjord, on the west coast of Sweden, where this experiment took place. Larvae were exposed to different combinations of intensity of pH and durations of exposure in an attempt to identify which part of the natural variability cycle triggers the stress that drives the biological response to OA. It was hypothesized that both intensity and duration of different pH exposures contribute to the stress experienced by an organism. This initial hypothesis is followed up by three subsequent hypotheses: (1) the level of stress is dependent on both intensity and duration of exposure in an cumulative manner (intensity *time); (2) for a given intensity, the negative effect on sea urchin larvae will increase with the duration of

exposure; and (3) for a given duration of exposure, the negative effect on sea urchin larvae will increase with the intensity.

Five parts of the urchin larval body were measured and analyzed to determine growth. It was found that: (1) intensity and duration significantly influenced growth for two parameters, (2) intensity alone did not significantly influence any parameters while duration alone significantly influenced two parameters, (3) stress was negatively correlated with intensity*time for body length growth, introducing a novel method for predicting stress, (4) there was a change in overall shape for larvae raised in fluctuating conditions; specifically, larvae had longer arms relative to body length, and (5) overall, larvae raised in fluctuating conditions performed better than those raised in constant conditions.

Considering these results, it can be confirmed that ignoring the fluctuations in pH that occur in coastal ecosystems when designing laboratory experiments presents a false representation of reality. Moreover, larvae actually performed better in fluctuating conditions, indicating that to fully understand the biological response to OA, a complete understanding of the modulating effects of pH fluctuations must first be understood. A change in the shape of larvae might have further consequences for other parts of urchin development, and there might be negative consequences as a result of this enhanced growth (i.e. more energy needed to grow), or further modulations with other parameters such as food availability. Future experiments should focus on natural pH fluctuations and further investigate the changed shape of larvae. Additionally, all organisms reside in complex ecosystems experiencing many different stressors at once. Creating and designing experiments that can fully incorporate all of the environmental challenges that species will face in the future will provide the most accurate account of biological response to global change and inspire the best possible conservation initiatives.

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1. Introduction

Over the past two decades, global environmental changes and their biological effects have become a dominant area for scientific research. Ocean acidification (OA), the downward trend of oceanic pH, is a component of environmental change that is severely altering the carbonate chemistry of the ocean. OA can be attributed to the continued burning of fossil fuels and the resultant carbon dioxide (CO_2) emissions, 30% of which are absorbed by the world's oceans (Caldeira and Wickett, 2003). The ocean represents one of Earth's largest carbon sinks, and the excess CO_2 humans are putting into the atmosphere will continue to make its way to the ocean and have profound effects on marine ecosystems and species. Since the beginning of the industrial revolution, the average pH of the open ocean has dropped from 8.2 to 8.1 and is expected to drop to 7.7 by the year 2100 (IPCC WG2, 2022).

Previous research on species response to OA has often used this open ocean OA scenario to assess impacts. Mean pH in the open ocean is very stable and does not fluctuate significantly day-to-day or month-to-month. Because of its stability, the open ocean behaves in a very predictable way, adhering to the global models projecting that surface CO_2 concentrations will be in equilibrium with atmospheric pCO_2 (Hofmann et al., 2011). The air-gas exchange that occurs between the ocean and the atmosphere means that as atmospheric CO_2 increases, so does the concentration in the ocean, thereby inducing OA. Several chemical reactions occur when atmospheric CO_2 dissolves into the water. These are displayed in Equations 1-4 (Zeebe and Wolfe-Gladrow, 2001).

$$CO_2\ (g) \leftrightarrow CO_2\ (aq) \qquad (1)$$

$$CO_2\ (aq) + H_2O \leftrightarrow H_2CO_3 \qquad (2)$$

$$H_2CO_3 \leftrightarrow H^+ + HCO^{3-} \qquad (3)$$

$$HCO^{3-} \leftrightarrow H^+ + CO_3^{2-} \qquad (4)$$

When gaseous (g) atmospheric CO_2 dissolves in water (aq), it reacts to form carbonic acid (H_2CO_3). This carbonic acid is deprotonated to form bicarbonate (HCO^{3-}) and eventually carbonate (CO_3^{2-}), releasing H^+ ions into the water, thereby reducing pH. CO_3^{2-} acts as a buffer for the ocean, absorbing an H^+ ion, which aids in keeping the pH as constant as possible. However, there is still one H^+ ion that is released, so the result of increased CO_2 entering the water is not only an increase in H^+ ions and a decrease in pH, but also a reduced availability of CO_3^{2-} and thus a decrease in the buffering capacity of the ocean, also known as alkalinity.

While increased atmospheric CO_2 concentrations will drive OA in the open ocean, all other marine environments will also experience OA. In coastal environments, the chemical changes associated with OA are not only driven by increased atmospheric CO_2, but also other drivers such as currents or biological activity. This leads to natural variations in pH that

oftentimes exceed the projected open ocean values for 2100 (Hofmann et al., 2011). Natural variations in pH can occur on multiple time scales for a multitude of reasons. Diurnally, photosynthesis, calcification, and respiration of organisms residing in coastal ecosystems can cause significant fluctuations up to 1 pH unit (Wootton et al., 2008, Dorey et al., 2013, Challener et al., 2015). On a longer time scale, pH can fluctuate monthly or annually due to biotic parameters influenced by changing temperatures and growth seasons (Challener et al., 2015, Torres et al., 2021).

In previous laboratory experiments, researchers have often designed experiments that use open ocean OA scenarios regardless of whether or not they are researching the effects of OA in a coastal ecosystem (Hofmann et al., 2011), and therefore would not consider geographical or temporal variability (McElhany and Busch, 2012). The modulating pH regimes that are present in coastal ecosystems mean that the use of these open ocean scenarios and models can give a false representation of future response to OA (Vargas et al., 2017, 2022) as the tested scenarios are within the present range and thus not driving a true stress. It is important to consider how these modulating effects affect species in their ability to cope with current and future OA (Hofmann et al., 2011). With future OA expected to cause a Δ-0.4 units (IPCC WG 2, 2022) in the overall mean pH of seawater, there will also be a Δ-0.4 shift in the natural variability (Dorey et al., 2013). Furthermore, species that dwell in ecosystems with modulating pH regimes also respond differently to fluctuations in pH and have different adaptive capabilities than species dwelling in stable conditions (Comeau et al., 2014, Kapsenberg et al., 2017, Johnson et al., 2019). While it is still relatively unknown if a species residing in variable conditions is better able to cope with future OA, it is known that they behave and adapt differently, and therefore this condition needs to be taken into account when designing OA experiments. Resolving the modulating role of variability in species response, and in particular what part of the variability is driving the observed effect, is critical for projection of future impacts on species and ecosystems.

Across all phyla, responses to OA appear to be very species-specific (Doney et al., 2009). Not all species are necessarily negatively affected by OA, but calcifying organisms have proven to be one of the most negatively affected group of organisms (Dupont and Pörtner, 2013). Marine invertebrates are an abundant group of calcifying organisms in the ocean, and it is known that pH is an important environmental factor that contributes to determining the physiology, morphology, and behavior of such organisms (Foo et al., 2020). Coastal ecosystems are home to a diverse range of species, including marine invertebrates, which are often well adapted to thrive in heterogenous environments because of the natural variability that they experience (Dorey et al., 2013).

Echinoderms are a phylum of marine invertebrates that have become a primary target for OA research, with known negative consequences for certain developmental parameters (Dupont and Pörtner 2013, Bednaršek et al., 2021, Shetye et al., 2021). Echinoderms are found from the arctic to the tropics, demonstrating their ability to survive in a wide variety of environmental conditions. Sea urchins are a group of echinoderms which often fill the role of a keystone species in many habitats (Dupont et al., 2010). Future OA will affect their ability to thrive in coastal ecosystems, potentially disrupting the function of entire ecosystems that they inhabit (Dupont et al., 2010, Dorey et al., 2013). Within urchin species, green sea urchins (*Strongylocentrotus droebachiensis*) have become one of the dominant study species

in the field of OA. Larvae in particular are an ideal study organism as they are sensitive to change and are a reliable species for assessing the effects of global change. Additionally, *S. droebachiensis* are widely distributed among coastal boreal ecosystems where they play a key role in the ecosystem and are of economic importance (Jager et al., 2016).

The Gullmar Fjord on the west coast of Sweden is characterized by strong fluctuations of pH, creating variable conditions for its resident organisms (Figure 1). pH fluctuates between 8.6 and 7.6 annually, meaning organisms that live in this ecosystem experience low pH that exceeds the open ocean OA end of the century scenario (7.7) (Dorey et al., 2013).

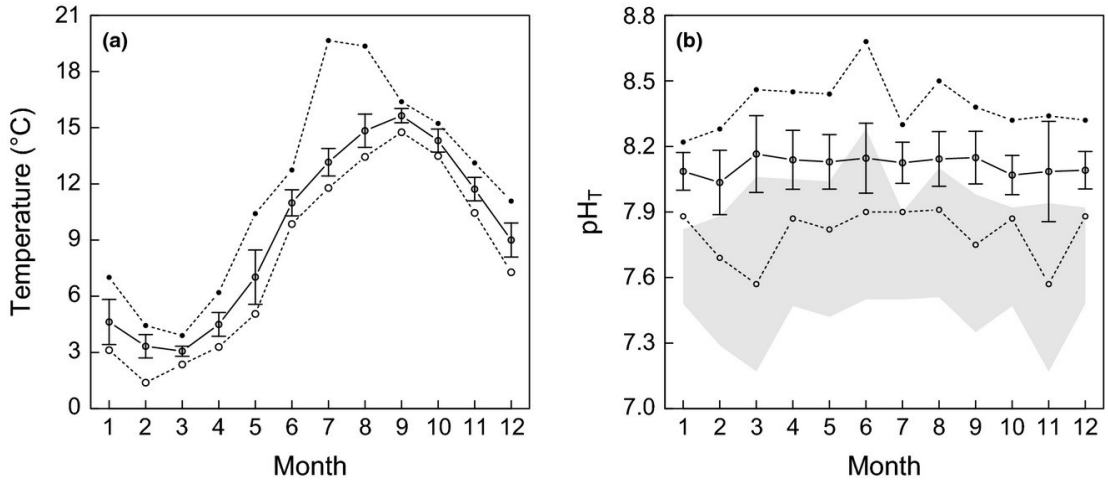

Figure 1. Monthly temperature (a) and pH$_T$ (b) variability at the Gullmar Fjord on the west coast of Sweden. The dotted lines represent the maximum and minimum values, and the middle line represents the mean. Monthly pH variation ranged from 0.34 to 0.89 pH units, with the lowest value of 7.58 and the highest of 8.68. The grey area represents the expected shift in pH, including variability, under future OA (Δ-0.4). pHT values are expected to range from 7.17 to 8.28 pH units, with a mean annual value of 7.71. pH data were recorded monthly (Släggö station: 58°15'5N–11°26'0E; depth = 0–40 m) by the SMHI (Database Svensk Havsarkiv) from 1921 to 1987. From Dorey et al. (2013).

A biological response to OA is currently observed in many species in multiple ecosystems around the globe, and the intensity of the biological response is expected to increase as OA increases (IPCC WG2, 2022). For *S. droebachiensis*, effects of OA have been observed for various parameters. Dupont et al. (2013) found negative effects of low pH on female fecundity in the reproductive conditioning period, likely reflecting the increased energy larval and juvenile urchins need to survive in a new and challenging environment. However, this effect disappeared after a longer exposure time demonstrating the potential of adults to acclimate to these conditions. Dorey et al. (2013) identified a developmental tipping point for *S. droebachiensis* larvae at pH$_T$ 7.3; however, more recent findings identify pH$_T$ 7.5 as the developmental tipping point (Jaeger et al., 2016).

S. droebachiensis larvae will be affected by OA, but the biological responses of these organisms also need to be assessed in the context of the natural variability that is present in their native ecosystems, such as the Gullmar Fjord. The episodic nature of OA in coastal environments, i.e. the constant ups and downs in pH, leads to the idea that intensity and

duration of an OA event can result in varying responses. This has been addressed in the context of low aragonite saturation states and it was found that a longer exposure to, or a shorter recovery time between aragonite undersaturation events, may decrease the time to recover from such a stressful event, and one or both of these factors drives the biological response (Hauri et al., 2013). For practical reasons, most published experiments considered constant conditions and the contribution of natural variability remains to be resolved.

Low pH relevant in the context of OA can drive a stress response. Stressful environments are ones that cause the fitness performance of an individual to decrease from a specified level. The effect on fitness of a stressful environment on an individual can be both a function of the length of time (or duration of exposure to the stressor) and the intensity of the stressor, depending on the circumstance (Schulte, 2014). The timing of stress-inducing events and the fluctuations in magnitude that are experienced by organisms are critical to understanding how an organism will respond to the event (Gunderson et al., 2016), and the natural variability in an ecosystem will inherently involve changes in duration and intensity of the stress (Boyd et al., 2016).

Natural variability in an ecosystem is a cycle; as pH fluctuates, an organism will experience different pHs for different amounts of time. The aim of this study is to identify which part of this natural variability cycle drives the biological response of an organism, i.e., the minimum pH experienced (intensity), the duration an organism is held at a certain pH, or both. It is known that *S. droebachiensis* larvae exhibit a biological response to low pH, and, that over their lifetime, they are exposed to a natural pH variability cycle (Dorey et al., 2013). Since organisms living in coastal ecosystems experience such variation in the pH that they encounter daily or annually (Figure 1), the duration and/or intensity of the low pH exposure is driving the biological response. The fjord represents an incredibly dynamic ecosystem that experiences natural fluctuations in conjunction with OA (Andersson et al., 2008), and as such, it is important to understand how the natural variability cycle plays a role in determining the response of *S. droebachiensis* to further OA.

The main hypothesis of this study is that both intensity and duration of different pH exposures contribute to the stress experienced by an organism. This initial hypothesis is followed up by three subsequent hypotheses: (1) the level of stress is dependent on both intensity and duration of exposure in a cumulative manner (intensity*time); 2) for a given intensity, the negative effect on sea urchin larvae will increase with the duration of exposure; and (3) for a given duration of exposure, the negative effect on sea urchin larvae will increase with the intensity.

Stress as a function of intensity* time is based on the concept of degree-days, first introduced as a method to predict the effect of temperature on biological processes (Baskerville and Emin, 1968) and has been used as a method optimizing efficiency in agriculture (Wilson and Barnett, 1983). For development in organisms, degree-days are the total amount of heat required for an organism to develop from one stage in its lifecycle to another (UC IPM, 2016); it is a function of temperature vs. time and represents the metabolically relevant thermal energy experienced by an individual over time (Honsey et al., 2018). The degree-day unit is the area under the curve of that function, or the integral (UC IPM, 2016). Degree-days have also been used as a method to explain variation in fish growth and development and to quantify the amount of energy that was experienced over a given

amount of time (Chezic et al., 2013). Additionally, degree days in fish development has provided a valid physiological understanding of how growth responds to temperature (Honsey et al., 2018). This same idea can be applied to pH as a function of time. The area under the curve, or integral of that function, will be the amount of stress produced in a stage of development of a biological organism. The concept of degree-days can be used to assess if different combinations of intensity and duration of low pH can produce an equal amount of stress. This leads to the development of a stress index. To date, this is a novel method for use in projecting the effect low pH as a function of pH intensity and duration.

The hypotheses were tested by exposing *S. droebachiensis* larvae to different combinations of intensity and duration of low pH exposures. There were three pH values used: 8.0 (present average pH), 7.7 (future average pH and extreme of the present natural variability), and 7.4 (outside of the present range of current natural variability). In addition to exposing larvae to these constant pH values, larvae were also exposed to different levels of variability (6h and 12h exposure to low pH) (Figure 2), for a total of six treatments. The three treatments with different time and intensity combinations were designed to simulate natural variability scenarios. The stress (expressed in pH units per day) for the fluctuating scenarios was calculated by multiplying the change in pH from the constant 8.0 treatment by the percentage of each day that the treatment was applied for. For example, the 12h 7.7 treatment stress unit was calculated by multiplying 0.3*0.5=0.15 (Δ 0.3 from pH 8.0*1/2 of a 24-hour day spent at the treatment pH). This is based on the concept of degree-days and the stress unit represents the area under the curve of pH vs. time, or the integral of that function.

Several endpoints were considered, including mortality, larval growth rates, and morphometrics via allometries. Allometries are recorded changes in a body part measurement in relation to body length (BL) (Brown et al., 2000).

Treatment	Exposure (h)	Intensity (min pH)	Stress	Treatment #
Constant 8.0	0	8.0	0	1
Constant 7.7	0	7.7	0.3	2
Constant 7.4	0	7.4	0.6	3
12h 7.7	12	7.7	0.15	4
12h 7.4	12	7.4	0.30	5
6h 7.4	6	7.4	0.15	6

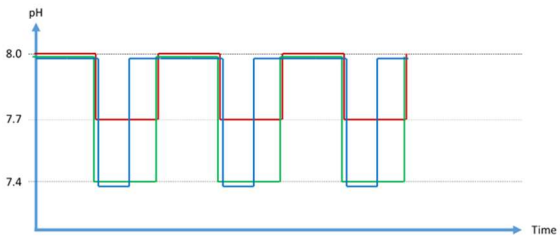

Figure 2. Summary of the six treatments that were applied to larvae over a 16-day period. Three of the six treatments were designed to simulate natural variability scenarios. Grey lines correspond to constant treatments, and the colors highlighted in the table correspond to the treatments shown in the schematic to the right. Time is represented in hours.

2. Methods

Location and duration

The experiment took place at the Kristineberg Marine Research Station of the University of Gothenburg in Fiskebäckskil, on the west coast of Sweden from March 22, 2022- April 14, 2022.

Animal collection and larval culture

Adults of the green sea urchin *S. droebachiensis* were collected by divers in Tromsö, Norway in February 2021, shipped to the Kristineberg Marine Research Station, and kept in natural filtered seawater (FSW) following natural fluctuations. Adults were fed with *Saccharina latissima* collected from the Kristineberg shoreline.

Spawning was triggered on March 24, 2022 by injection of 1mL of 0.5M KCl in FSW across the peristomal membrane. Sperm was collected dry, transferred into Eppendorf tubes and kept on ice for ~1 hour until use. Eggs were collected in filtered sea water (FSW). For fertilization, gametes from one male and one female were pooled in FSW to a final concentration of ≈40 µl of sperm /1 liter of eggs solution in FSW. Fertilization took place for ~15 minutes in FSW with pH_T ≈8.0 at 10°C. Embryos were left to develop at 10°C until the two-cell stage.

After the first cleavage, embryos were adjusted to a final concentration of 10 embryos ml^{-1} FSW and transferred into three 5 L glass bottles and kept until Day 5. At this time, larvae had a well-developed calcium carbonate skeleton and were ready to eat. At Day 6, the larvae in the three larger bottles were concentrated and transferred into 18 ≈610 ml bottles (Figure 1a) and filled to the top with FSW at the target pH (see below). The larvae were kept in a thermo-constant room at 10°C for the duration of the experiment (21 days). Once they began to develop a stomach (Day 5), the were fed 100 µL of a solution of the microalgae *Rhodomonas sp.* to a final concentration of ≈3000 cells per mL. On Day 8, the amount of food given twice daily was increased to ≈6000 cells per mL.

Experimental design and seawater carbonate chemistry

To assess which part of the natural pH variability cycle is driving the biological response in *S. droebachiensis*, larvae were raised in 18 bottles representing six different treatments with 3 replicates per treatment. After transfer on Day 6, the experiment was continued until Day 21. The six treatments were: (1) constant (CST) pH 8.0, (2) CST pH 7.7, (3) CST pH 7.4, (4) 12 hour fluctuating 7.7 pH→8.0 pH, (5) 12 hour fluctuating 7.4 pH → 8.0 pH, and (6) 6 hour fluctuating 7.4 pH → 8.0 pH (n=3 bottles for all treatments, 18 total bottles) (Figure 1a). To simulate the natural variability cycle in the fluctuating treatments, the water in each bottle for treatments 4-6 was changed to the experimental value at 08:00. For treatment 6, water was changed back to pH 8.0 after 6h, at 14:00. For treatments 4 and 5, water was changed back to pH 8.0 after 12h, at 20:00 (Figure 2). The pH of the seawater used for the transfer was maintained in three separate 50 L buckets (Figure 2a). For the 8.0 pH seawater, the water was continuously aerated and mixed through air bubbling to reach equilibrium with the air which had a CO_2 concentration of approximately 440 ppm. Pure CO_2

was bubbled into the other two buckets to reach their respective pH values, 7.7 and 7.4, and controlled by a pH-stat system. Air was also continually aerated and mixed through bubbling in these two buckets. Temperature in the thermo-constant room was held at 10°C.

Seawater pH, alkalinity, and temperature were monitored twice a week following the recommendations of Dickson et al., 2007. mV was measured after calibration using TRIS and was then converted to pH on the total scale (pH_T). Total alkalinity (TA) was assessed on samples with a titration system following recommendations by Dickson et al., 2007. Carbonate system parameters (pCO_2, Ω_c, Ω_a) were calculated from pH_T, TA, temperature, and salinity using CO_2sys with the dissociation constants from Mehrbach et al. (1973) as refitted by Dickson & Millero (1987), following Dorey et al. (2013).

Biological measurements

Mortality and developmental abnormality. Two subsamples of 10 ml from each larval culture were sampled daily in the morning before the water change. Individuals were immediately fixed with a drop of paraformaldehyde solution (4% PFA in FSW). For each culture, 50 larvae were placed on a slide and looked at under a microscope, and the number of individuals that appeared abnormal were counted and recorded (usually out of 50 individuals, but sometimes fewer if fewer individuals were found in the two subsamples). For each culture, relative density was calculated each day as the number of live larvae divided by the maximum number of larvae ever counted during the experiment and corrected for the 20 ml of replaced FSW every day. Mortality rate (day^{-1}) was calculated as the coefficient of the significant linear relationship between relative density and time (day). An example of the mortality calculation can be seen in Figure 3.

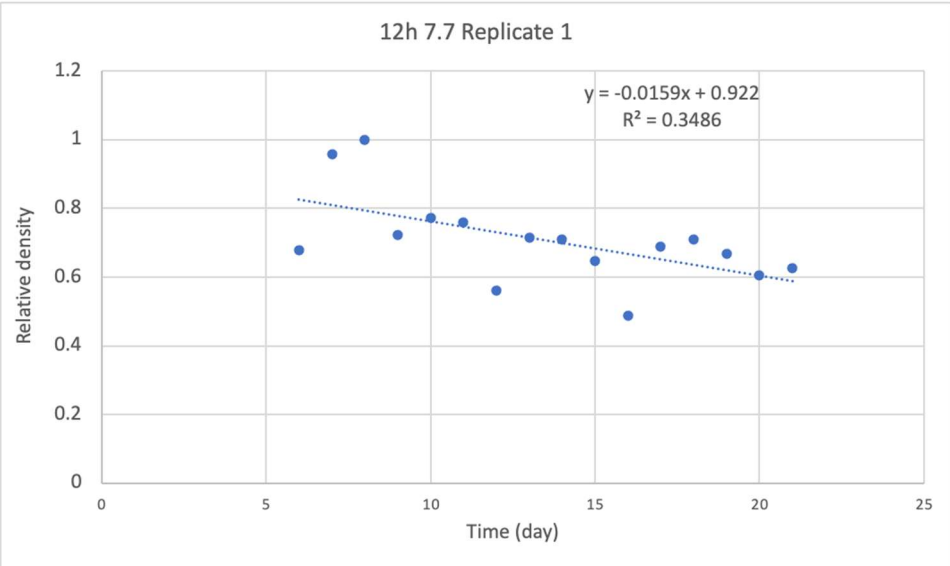

Figure 3. Example of mortality rate (day^{-1}) calculation from Replicate 1 of the 12h 7.7 treatment. In this example, relative mortality is calculated as the coefficient (-0.0159 day^{-1}) from the significant linear regression between relative density and time (day). Relative density has no unit as it is between 0 and1, 1= no mortality as the observed density equals the maximum number of larvae observed over the course of the experiment.

Body length and morphometry. To measure larval size and other morphometric parameters, photographs were taken every day of the experiment (Day 0-21). Ten photos were taken of each of the replicates. All pictures were measured using ImageJ; 2878 larvae were photographed and measured by the conclusion of the experiment.

Body length (BL) along with four other morphological parameters were measured, depending on larval stage: body rod (BR), post-oral rod (POR), posterolateral rod (PLR), and stomach diameter (S) (Figure 4).

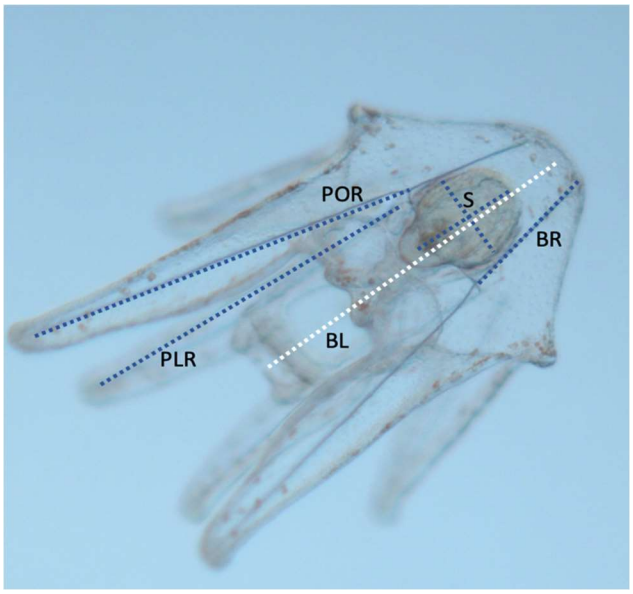

Figure 4. A total of five morphological parameters were measured for S. droebachiensis larvae ; body length (BL) body rod (BR), post-oral rod (POR), posterolateral rod (PRL), and stomach (vertical and horizontal diameter, S).

Growth rates (GR in μm logday^{-1}) for BL were calculated as the coefficient of the significant logarithmic relationship between body length (μm) and time (day), BL = GR x ln(time) + Intercept. For the other parameters (body rod length, posterolateral rod length, post-oral rod length), allometries (μm μm^{-1}) were calculated as the coefficient of the significant linear relationship between the longest arm for each parameter (μm) and the body length (μm). Stomach volume (SV) was calculated as $SV = \frac{4}{3}\pi \times [\frac{S1+S2}{4}]^3$, where S1 and S2 are the two measured diameters of the larval stomach. All rates that were used for analysis can be found in Appendix 1, Table 1a. Only images taken after Day 5 (Day 6-21) were used to analyze all parameters other than BL since there was no calcium carbonate shell or fully formed stomach prior to Day 6.

Statistical analysis

General linear models (GLM) were run on the SAS statistical software, and graphs were made in Microsoft Excel. The level of significance for all statistical analyses was 5%. The relationships between parameters and the two variables (time and intensity) were tested to assess growth rates and allometries. An additional GLM was run to test the correlation of

each parameter with the proposed stress index. Finally, one last GLM was run to test the relationship between each allometry and BL growth rate. A summary of all statistics can be found in Tables 2, 3, and 4. For the parameters of the chemistry, effects of target pH were also tested with a GLM followed by a post-hoc Scheffe's test.

3. Results

Seawater chemistry

Carbonate chemistry of each of the 50L buckets, each with a different target pH, is summarized in Table 1. There was a significant difference in measured pH between the 3 target pHs (GLM, $F_{2,17}$=78.63, p<0.0001). There was no significant difference between the alkalinity measurements (GLM, $F_{2,17}$=2.28, p=0.1366). There was a significant difference between CO_2 (GLM, $F_{2,17}$=25.51, p<0.0001), calcite saturation state (Ω_c) (GLM, $F_{2,17}$=150.07, p<0.001), and aragonite saturation state (Ω_a) (GLM, $F_{2,17}$=149.54, p<0.001) among target pHs. In summary, there was a significant effect of target pH for all parameters except for alkalinity, which was expected. Seawater was undersaturated both with respect to calcite and aragonite at pH_T 7.4. All tested parameters were followed by a post-hoc Scheffe's test to ensure that the treatments were different from each other.

Table 1. Seawater carbonate chemistry parameters presented as Mean±SE; pH and alkalinity were measured a total of 6 times over the course of the experiment. pH is presented on the total scale (pH_T) and this along with alkalinity was used to calculate CO_2 partial pressure (pCO_2; µatm), as well as calcite and aragonite saturation states (Ω_c and Ω_a respectively), for a salinity of 32 and a temperature of 10°C.

	Measured		Calculated		
Target pH	pH_T	Alkalinity (µmol/kgSW)	CO_2 (µatm)	Ω_c	Ω_a
8.0	8.10±.07	2384±23	368±12	3.53±0.11	2.23±.07
7.7	7.72±.03	2434±20	986±77	1.68±0.11	1.07±.07
7.4	7.40±.05	2449±24	2214±313	0.87±0.11	0.55±.07

Biological measurements

For all biological measurements, GLMs were used to test the effect of the two tested variables and their interaction: time, or duration of exposure (0 hours, 6 hours, and 12 hours), and intensity (8.0, 7.7, 7.4).

Morphology and mortality. The effect of intensity, time and their interaction on morphological parameters was tested using GLM models. All statistics are summarized in Table 2. Both time and intensity had a significant effect on body length growth rate (BL) and body rod allometries (BR) but no interaction was observed (see below for a description of the effects on the two parameters using the stress index). For the post-oral rod (POR) and the posterolateral rod (PLR), only time had a significant effect. Exposure to 6h and 12h variability had a positive effect on PRL and POR allometries as compared to exposure constant conditions (Figure 5). No significant effect of time, intensity or their interaction was observed for the stomach volume (SV) and the mortality rate. When no interaction between

the tested variables occurs, it means that the variables are acting independently from one another.

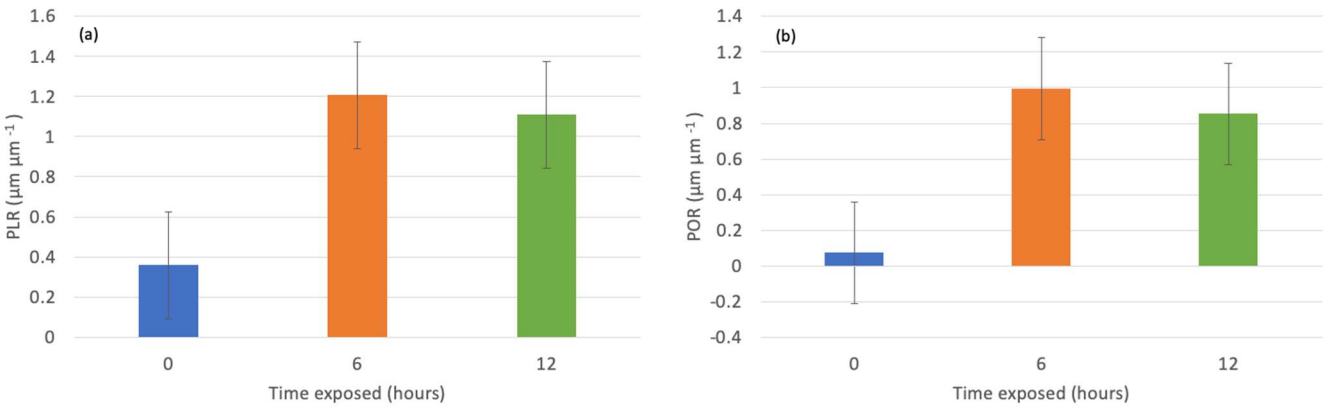

Figure 5. Duration of exposure is the only factor contributing to different growth rates for (a) posterolateral rod length (PLR) and (b) post-oral rod length (POR). Constant exposure (0 hours) has a much smaller growth rate than either of the two fluctuating durations (6 hours or 12 hours). The average is presented along with the standard error (SE) as error bars.

Table 2. Summary of statistics for growth rates and allometries. The model was a general linear model (GLM). Time is duration of exposure, intensity is the target pH value that larvae were exposed to, and interaction is the interaction of both variables. Results of the GLM are given (F-value, P-value). ***Bold*** *parameters indicate significance (p<0.05).*

	Model		*Time*		*Intensity*		*Interaction*	
	$F_{5,17}$	p	F_2	p	F_2	p	F_1	p
BL rate (mm log day^{-1}	**9.03**	**0.0009**	**20.62**	**<0.0001**	**8.42**	**0.0052**	0.17	0.69
BR allometry (µm µm $^{-1}$)	**29.19**	**<0.0001**	**8.37**	**0.0053**	**33.97**	**<0.0001**	2.90	0.1144
POR allometry (µm µm $^{-1}$)	**38.57**	**<0.0001**	**58.17**	**<0.0001**	3.28	0.0731	0.05	0.8235
PLR allometry (µm µm $^{-1}$)	**26.77**	**<0.0001**	**43.07**	**<0.0001**	1.46	0.2706	0.47	0.5053
SV allometry(µm µm $^{-1}$)	**5.07**	**0.0099**	2.87	0.0957	5.95	0.0160	0.92	0.3559
Mortality (day^{-1})	0.98	0.4673	0.16	0.8528	1.55	0.2510	0.59	0.4570

Stress index. For each measured parameter, the relationship was tested between the stress index and the parameter using linear regression. The stress index measures the cumulative effect of intensity*time. All statistics are summarized in Table 3. The regression between BL and the stress index was tested with two different sets of data, one which included the CST 8.0 treatment and one which did not. Both regressions were significant, but the regression without the CST 8.0 treatment had a higher R^2. This was done for comparison purposes. As the stress index increased, BL growth rate decreased (Figure 6). No significant linear relationship with the stress index was observed for the other allometries or mortality (Figure 7).

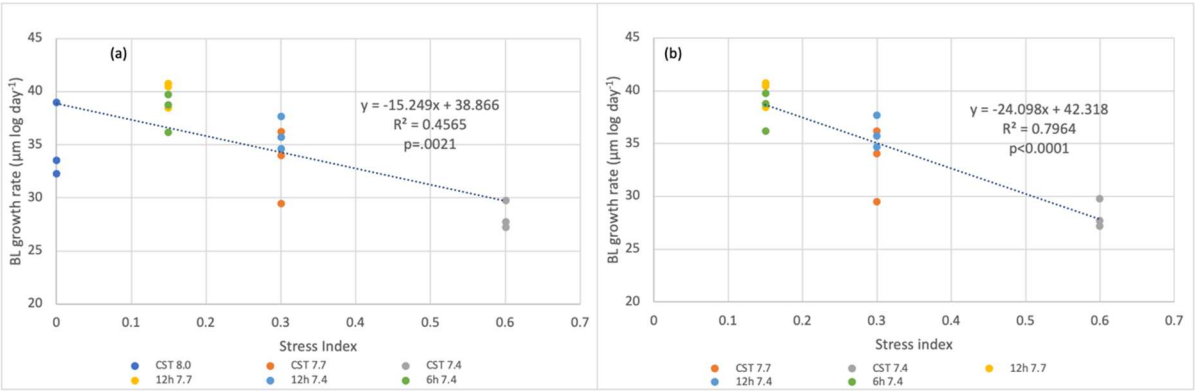

Figure 6. Body length (BL) growth rate expressed in µm log day^{-1} plotted against the stress index. Both graphs represent the same parameter (BL) but graph (a) includes all treatments whereas graph (b) excludes treatment CST 8.0 because it does not fit the trend of all other treatments. Both results are significant, however, in graph (b) the correlation is much stronger. R^2=0.4565 in (a) and R^2= 0.7964 in (b). Each dot represents the regression coefficient extracted from the logarithmic relationship of BL and time.

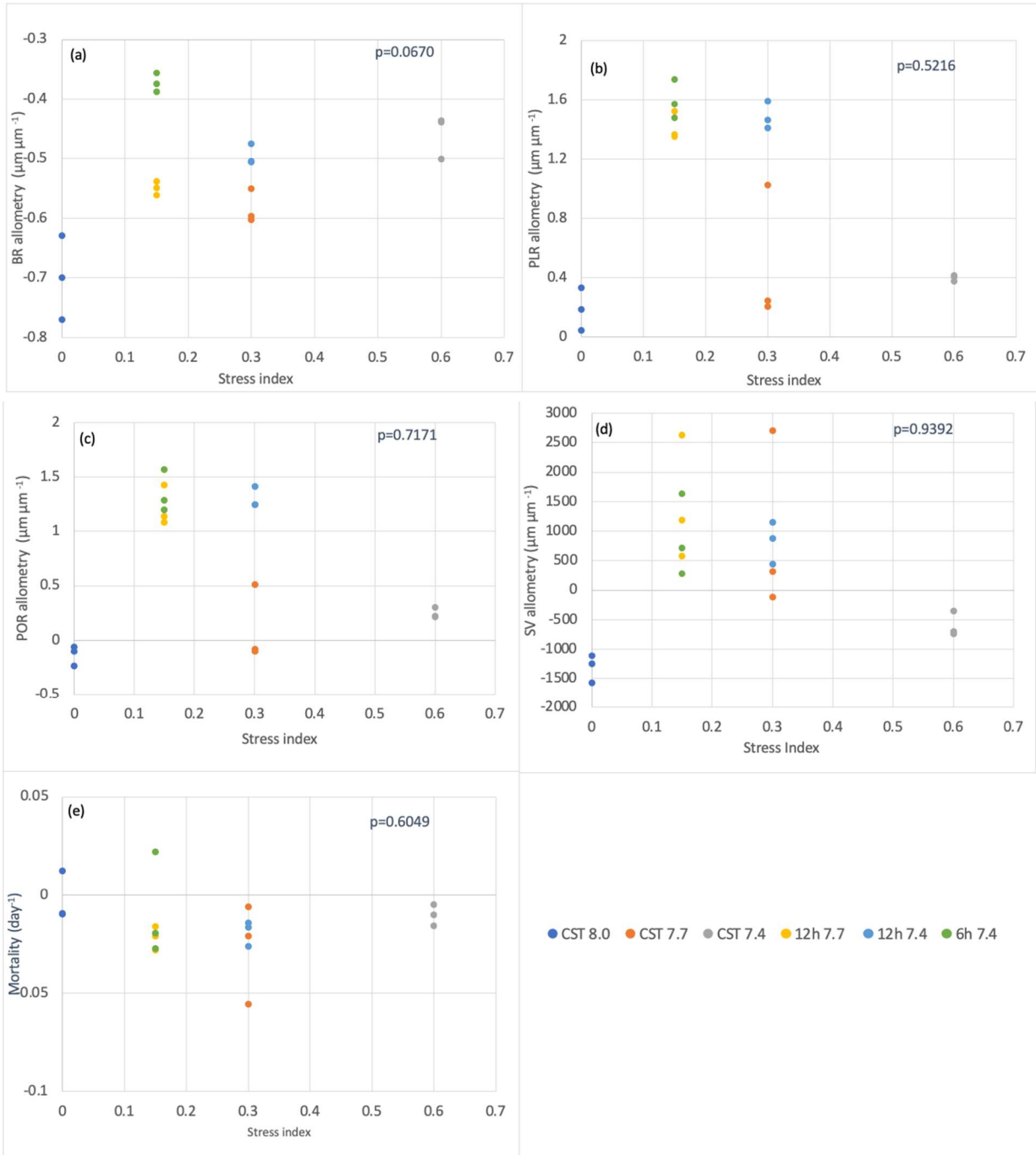

Figure 7. BR allometry (a), PLR allometry (b), POR allometry (c), SV allometry (d), and mortality (e) plotted against the stress index. Each dot represents the regression coefficient extracted from the logarithmic relationship of BL and time. These allometries were not significant in the context of the stress index.

*Table 3. A summary of statistics for the regression to test if the stress index predicted growth in the different parameters. Results of the regression are given (F-value, P-value, and R^2). **Bold** data indicate significance ($p<0.05$).*

		Model		
	$F_{1,14}$	$F_{1,17}$	p	R^2
BL rate w/8.0 (µm log day^{-1})	--	**13.44**	**0.0021**	**0.456474**
BL rate (µm log day^{-1})	**50.84**	--	**<0.0001**	**0.796381**
BR allometry (µm µm $^{-1}$)	--	3.86	0.0670	0.194461
POR allometry (µm µm $^{-1}$)	--	0.14	0.7171	0.008428
PLR allometry(µm µm $^{-1}$)	--	0.43	0.5216	0.026134
SV allometry (µm µm $^{-1}$)	--	0.01	0.9392	0.000375
Mortality (day^{-1})	--	0.28	0.6049	0.017112

Allometries vs. BL growth rate

The linear relationship between all allometries and BL growth rate were tested. All statistics are summarized in Table 4. These relationships were significant for POR and PRL. POR and PRL increased as BL growth increased (Figure 8). Additionally, it can be seen from Figure 8 that all fluctuating treatments have higher values on the graph than constant treatments. There were no other significant results for the other tested parameters (Figure 9).

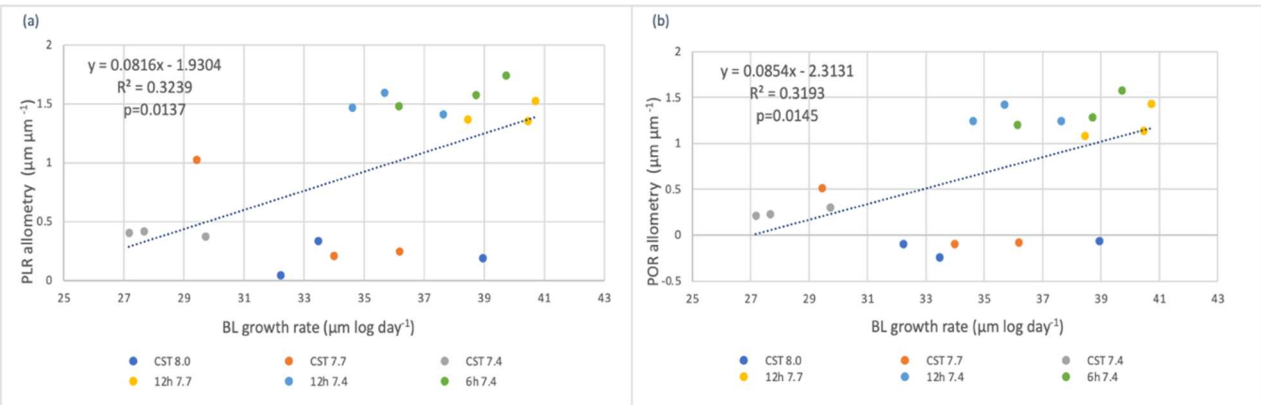

Figure 8. Relationship between PLR (a) and POR (b) allometries and BL growth rate. PLR and POR are the two out of the five measured allometries that were significant when plotted against BL growth rate. This shows that as BL increases over time, PLR and POR also increase. Each dot represents the regression coefficient extracted from the linear relationship between the allometry and BL growth rate.

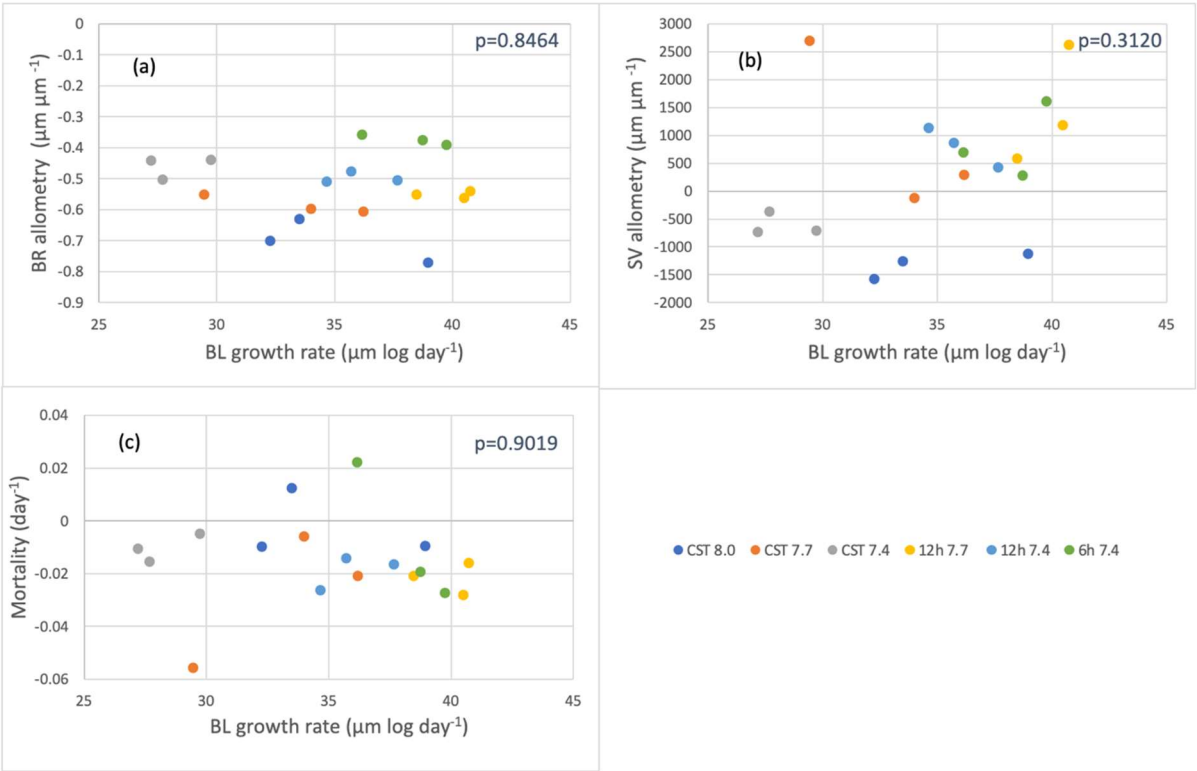

Figure 9. Relationship between BR allometry (a), SV allometry (b), mortality (c) and BL growth rate. None of these relationships showed significant results. Each dot represents the regression coefficient extracted from the linear relationship between the allometry and BL growth rate.

*Table 4. A summary of statistics for the regression to test if the BL growth rate was correlated with increased growth in another allometry. Results of the regression are given (F-value, P-value, and R^2) **Bold** data indicate significance ($p<0.05$).*

	Model		
	$F_{1,17}$	p	R^2
BR (µm µm $^{-1}$)	0.04	0.8464	0.002416
POR (µm µm $^{-1}$)	**7.51**	**0.0145**	**0.319317**
PLR (µm µm $^{-1}$)	**7.67**	**0.0137**	**0.323942**
SV (µm µm $^{-1}$)	2.52	0.3120	0.136055
Mortality (day^{-1})	0.02	0.9019	0.000978

4. Discussion

Intensity, duration, or both?

The initial hypothesis that both intensity and duration of pH exposures contribute to the overall stress experienced by an organism and subsequent biological response can be assessed in several ways. While stress was not directly measured or quantified in this experiment, it was evaluated through various indicators (larval growth, allometries, and mortality). No differences between treatments were observed for mortality, which is consistent with the results of Dorey et al. (2013), who identified the tipping point for *S.*

droebachiensis larvae mortality to be around pH_T 7.0. The experimental treatments that larvae were exposed to in this study never dropped below that tipping point; therefore, the fact that no significant effect on mortality was observed is consistent with what was expected. Of the four allometries (BR, POR, PLR, SV) and one growth rate (BL) that were measured, two showed that both intensity and duration contribute to the biological response (Table 2), BL growth rate and BR allometry. As previously mentioned, OA research on biological response is often solely focused on intensity, and it is known that a low pH can have effects on morphology. As displayed in Dorey et al. (2013), *S. droebachiensis* larvae exposed to decreased pH between 7.0 and 8.0 experienced lowered growth rates. Yu et al. (2013) found very similar results in an arctic urchin species, *Sterechinus neumayeri;* at the highest pCO_2 concentrations, 730 µatm which is somewhat similar to the pH_T 7.7 treatment of this experiment (986 µatm), significant reduction in arm length was observed. Additionally, Matson et al. (2012) found that larval growth rate in *Strongylocentrotus purpuratus* was reduced at higher pCO_2 concentrations, corresponding to lower pH despite similar energy usage. While there is a suite of articles investigating the impacts of low pH on sea urchin larvae, these are just a few that are mentioned repeatedly in the literature. A review of the overall negative effects of low pH on sea urchin larvae growth and development are summarized in Dupont and Thorndyke (2013).

However, for two of the allometries, POR and PLR, time (duration of exposure) was the only significant variable contributing to the observed differences (Figure 5, Table 2). Interestingly, these are the two arm allometries, meaning that arms grew faster in fluctuating conditions even though they were at some points in time reaching a lower pH than the high constant treatment (pH_T 8.0), as they were designed to. It is known that previous research on biological responses to OA often neglects duration of exposure and pH fluctuation as a factor, so the fact that it is the sole contributor to the observed differences in allometries for two parameters indicates a gap in the knowledge of OA response for organisms residing in environments that experience various durations of exposure as a result of natural variability. This implies that ignoring the effects of duration of exposure would falsely identify a low pH as the driver of a biological response when in fact fluctuations causing different durations of exposure drives the biological response for some allometries. Not only does duration of exposure modulate biological response, but the biological response actually seems to increase growth in fluctuating conditions relative to constant conditions, contrary to what most of the literature has found in the past, that low pH conditions negatively impact larval development. While our results still suggest that constant low pH exposure negatively affects growth rate, low pH exposure does not negatively affect growth rates when exposure happens periodically in fluctuating conditions. Larvae that grew in constant conditions had a more negative response (e.g. lower growth rates) even if they grew in the relatively high pH that is at the high end of the natural fluctuation they experience (pH_T 8.0) and which is considered "present-day conditions." Fitness could therefore be expected to decrease when analyzing biological response under constant conditions when, in reality, fitness may actually increase as a result of increased growth when analyzed under the proper fluctuating conditions.

Chan and Tong (2020) found that the tropical species *H. crassispina* had a faster arm growth rate in fluctuating conditions rather than in constant low pH conditions, demonstrating that the stress response was not a result of the minimum pH experienced,

much like is observed here. The results of Chan and Tong (2020) combined with our results could indicate that what is more important than the lowest pH experienced by an organism is the overall mean the organism experiences over time, including the highs and lows of the fluctuation. Of course when the high pH values of fluctuation are included in the overall level of pH exposure, the average increases.

For a given intensity, it was hypothesized that the negative effect on sea urchin larvae will increase with duration of exposure. For most allometries (all except SV), duration of exposure had a significant effect on growth (Table 2). Moreover, it can be seen from the results that nearly all of the fluctuating treatments had higher growth allometries when compared to constant treatments (Figure 8), meaning that the negative effect on sea urchin larvae does in fact increase with duration of exposure (6 and 12 hour data appear higher on graphs than constant treatments). This is consistent with what is known about coastal environments and what conditions *S. droebachiensis* are adapted to. Conditions are far from constant in coastal environments (Figure 1), so allowing larvae to develop in constant pH is a condition that they do not experience daily, monthly, or annually, and it makes sense that this would not be beneficial as they are not adapted to such an environment. Moreover, there were changes in overall ratios between different aspects of the larval body. These results are discussed further below (*A changed shape)*.

Garcia et al. (2018) found similar results when exposing the urchin *Paracentrotus lividus* to a 12h fluctuating pH regime (8.1→ 7.7) and a constant pH (8.1). Larvae in the fluctuating conditions developed better than those in the constant conditions, meaning they had an increased growth rate. They also found a delay in development in the constant treatment vs. the fluctuating treatment. Considering the results of Garcia et al. (2018) and the results of this experiment, which showed an increase in growth and allometries, would suggest that in a fluctuating environment, sea urchin larvae are not limited in their growth, but their growth is rather enhanced, perhaps because they receive a small respite from the fluctuating conditions. This is true even when pH drops to below what is experienced in today's natural variation. When larvae spent 12 hours at 7.4 and were then returned to the CST 8.0 treatment, overall growth and allometric relationships were still higher as compared to those left to continually develop in constant conditions.

To understand how certain allometries and growth rates affect sea urchin larval development, considering the two primary roles urchin larvae serve is important; successful larvae that continue into the successive life stages (1) consume food to grow and (2) find a suitable substrate to settle and metamorphose into a juvenile (Hinergardner, 1969). When food is abundant and growth is faster, larvae may settle sooner to begin their metamorphis into a juvenile (Meidel et al., 1999); they leave the pelagic water column and settle into their benthic habitat. This early settlement could benefit fitness parameters indirectly. The longer a larvae is in the pelagic environment, the longer it is exposed to potential predators, and subsequent increased mortality followed by an obvious decrease in fitness (Dupont et al., 2010). Since fluctuating conditions followed a similar pattern in enhancing growth as does increased food availability (Meidel et al., 1999), it could be said that larvae raised in fluctuating conditions could metamorphose into juveniles more quickly, reducing their chance of mortality as a result of predation in the water column.

It was also hypothesized that for a given duration of exposure, the negative effect on sea urchin larvae would increase with each increase in intensity. This hypothesis could not be fully assessed because not every intensity had all three durations of exposure (constant, 6 hours, and 12 hours). However, it can be seen from the stress index that stress does increase when intensity increases for a given duration, at least for BL growth rate (Figure 6).

Stress index

The development of the stress index was a novel method for quantifying stress as a function of pH intensity and time. If the level of stress is dependent on intensity*time, with each increase in negative effect, there would be an increase in the stress index (see Figure 2). The results showed that stress is a function of intensity*time for BL growth rate, but not for allometries. Additionally, the correlation between stress and increased intensity*time gets much stronger when the CST 8.0 treatment is removed. The removal of the CST 8.0 treatment was for comparative purposes only. Given that this is a novel method for stress assessment, there are no previous studies to directly compare to. However, if compared to the thermal index described in the introduction, results are consistent with what was observed in growth development for fish in response to a change in temperature. Fish growth as a function of temperature variation showed a linear relationship (Honsey et al., 2018), much like the linear relationship observed here for BL growth (Figure 6). The stress index, therefore, could be a useful predictor of stress in response to changing pH in the future, particularly for BL growth rates.

Additionally, since the stress index was accurately able to assess stress for BL growth rate, combined with the previously mentioned result that larvae had increased growth rates for arms in fluctuating conditions (Figure 5), we can conclude that a little bit of stress (i.e. fluctuating environments) is actually helpful for larval development. Interestingly, the stress index says there is 0 stress at CST 8.0; there is 0 change from present conditions and larvae should respond well to this treatment, but we know this isn't necessarily true since larval growth was enhanced under fluctuating conditions, particularly arm growth. This is most likely because larvae in the field grow under fluctuating conditions and are also under variable conditions in their spawning period, much like how larvae are adapted to live in variable conditions as previously mentioned. Research has shown that when spawning in sea urchins occurs in the variable conditions that a species is adapted to, the negative effects of the frequent low pH exposure is mitigated as compared to a narrower variability regime (i.e. constant pH) (Kapsenberg et al., 2017). A little bit of stress as predicted by the stress index is therefore helpful for larval growth because it is the condition they are adapted to in the field and the conditions they most readily spawn in.

A changed shape

Arm rods are essential for many functions of sea urchin larvae, including feeding, swimming, and protection from predation (Strathmann et al., 1992). Longer arms can enhance feeding efficiency, but there is also the potential for morphological trade-offs, such as reduced stability in the water column as a result of longer arms (Strathmann and Grünbaum, 2006). Arm length is often a function of food availability, and arm to BL ratios change when there is a change in the abundance of food (Soars et al., 2009). Overall food

availability can influence not only overall BL growth but also the relationships between BL and arm lengths. Food availability was not a variable that was investigated in this experiment, but results indicating a change in ratios were found.

Specifically, we observed a statistically significant change in the ratio between arm length and BL growth; as BL growth rate increased, both sets of arms also increased in length. Additionally, for all of the fluctuating treatments, regardless of how long of a fluctuation or intensity of pH that was reached during the fluctuation, there were observed higher values than those of the constant treatments (Figure 8). In other words, fluctuating pH induces longer arms relative to body length, creating a different overall shape when compared to larvae from the constant conditions. Interestingly, a similar morphology change was observed in Carrier et al. (2019) when *S. droebachiensis* larvae from Norway were fed *ad libitum* vs. a restricted diet; longer arm to body length ratio was correlated with a non-restricted diet. This was a reversal from the usual trend that was observed on the east and west coasts of the United States where a restricted diet induced a larger arm to body length ratio. Additionally, previous research has also found that arm length increases relative to body length to acquire food particles when food is scarce, and larvae grow shorter arms when there is an abundance of food (Pedrotti and Fenaux, 1992), making the reversed results identified in Carrier et al. (2019) more confounding. This is also interesting because food availability is imperative for growth (Meidel et al., 1999), but there is also a relationship between different types of growth and food availability and resulting ratios between measured parameters. Perhaps the fluctuating conditions introduce a new modulating effect when combined with food availability, inducing longer arm growth relative to body size when the opposite effect was expected. When conditions were fluctuating, presumably less energy was needed to acquire food and therefore the arms were able to grow longer. In our experiment, maybe this could be due to the fact that food was mixed more often as a result of the water change, making food more available for certain periods of time but less available for others.

A changed shape for larval sea urchins could have profound effects on population dynamics (Chan et al., 2015). Larval sea urchins grow and swim in order to find a good location to settle and begin a metamorphosis into a juvenile, and a change in shape can affect this settlement. Chan et al. (2015) found that an overall change in shape to a shorter larvae for *S. droebachiensis* was observed when exposed to constant low pH conditions, without an overall change in swimming speed, suggesting adaptive potential and phenotypic plasticity associated with the changed shape. However, Foo et al. (2020) found almost a reverse effect for the urchin species *Arbacia lixula*; in field experiments, they found that low fluctuating pH combined with high food levels induced longer arms relative to body length, a phenotype which was not expected based on laboratory experiments. They hypothesized that this could be due to an inhibition of the sensory biology disrupted by pH fluctuations. Overall, shorter armed larvae, expected in high food conditions for most populations, are expected to settle earlier and begin metamorphis sooner due to the saved energy they accrue from not having to grow longer arms to capture scarce food (Pedrotti and Fenaux, 1992). However, Carrier et al. (2019) explained that in food abundant conditions, populations of *S. droebachiensis* had longer arms. Since pH fluctuations induced a change in shape, the effects this might have on life stages of sea urchin larvae and the time they spend in the pelagic environment should be

fully understood in the context of food availability. A change in shape could have consequences for the amount of time a larvae will spend in the pelagic environment before settling, greatly impacting overall fitness for individuals, further implying the necessity of fully understanding the modulating effects of pH fluctuations.

Consequences and next steps

It is apparent from the results of this study that ignoring the modulating effects of fluctuating pH in coastal environments will not provide an accurate representation of biological response to OA. Future experiments should take into account this role so that a better understanding of response in a changing environment can be assessed. If the modulating role of fluctuating pH is ignored, the effects of OA on larval development might be overestimated, considering that fluctuation enhanced larval growth compared to constant conditions. From these results, it can be observed that overall low pH is still bad for growth when compared to fluctuating conditions, but completely ignoring the role of the fluctuations does not offer the best overall view of the ecosystem.

This experiment could have provided more information on what effect a specific duration of exposure has on biological response if each intensity was able to have all three durations of exposure (constant, 6h, 12h). However, for practical reasons, this was not possible, but a future design could incorporate such fluctuations on different time scales so that more information regarding specific time fluctuations can be assessed. Additionally, further statistical analysis could be performed to identify more specific differences among treatments.

A changed shape as a result of fluctuating conditions is an interesting result that deserves a more detailed investigation. Population dynamics could be more accurately modeled if there is a better understanding of how overall time spent in the water column and the predicted mortality due to the continual exposure to predation is affected by fluctuation and the correlated increase in arm length. Given that food availability also altered larval shape in a similar population, it could be interesting to test to see if the result of the two very different treatments investigating the role of two very different variables actually produce the same biological response. Designing an experiment to directly test the response of changed food availability and fluctuation would provide insight into how organisms in the field change and alter their shape to enhance their growth or their ability to capture food. In the field, food supply is not necessarily constant, and with known pH fluctuations, the two variables combined could also have modulating effects. There is potential for different adaptation techniques under predictable and nonpredictable food availability for some marine larvae (Hu et al., 2017) and investigating such adaptations for *S. droebachiensis* larvae could provide insight into adaptations under fluctuating conditions to better acquire food or to understand what is causing the changed shape if it is not food availability. In addition, the energy spent to grow longer arms should also be assessed to understand if there is increased energy going towards growing longer arms that might delay metamorphosis into a juvenile.

Additionally, there are other physical conditions, such as temperature and salinity, that also vary on a spatial and temporal scale that should be taken into account when assessing the biological response of organisms to global changes. Climate change is causing a shift in the natural variation of the range of temperatures present in coastal marine

environments. An interesting approach could be to combine the modulating effects of pH fluctuation with the modulating effects of temperature fluctuation into a multiple-stressor experiment. This would provide a more accurate representation of what happens in the field. Organisms do not feel the effect of just one stressor, but all of them combined, and the combination could induce an entirely different response than the one seen here. Just as OA will continue to alter marine environments, so will all of the other global changes that are the result of anthropogenically-induced climate change. Exploring the consequences of multiple global environmental changes would be challenging but could ultimately provide great insight into better understanding how natural variability cycles of multiple stressors influence biological response.

5. Conclusion

Ocean acidification is a constant threat to marine environments. From the open ocean to coastal ecosystems, OA has and will continue to have profound effects on marine ecosystems (IPCC WG2, 2022). Global CO_2 emissions are the main driver of the continuous change in carbonate chemistry globally (Calderia and Wickett, 2003). Open ocean models predict that by the end of the century, pH will drop from its current 8.1 average to a global average of 7.7. Previous research on OA often uses open ocean scenarios to assess biological response, regardless of whether or not the aim of the research is to investigate responses in a dynamic environment. pH in coastal ecosystems can fluctuate significantly day-to-day or month-to-month, exposing resident organisms to a much lower pH than that which is expected for the open ocean by 2100 (Hoffman et al., 2011). Ignoring the modulating effects of pH fluctuation and duration of exposure can potentially overestimate the effects of the low pH threshold driving negative effects in a marine organism.

The Gullmar Fjord on the west coast of Sweden is characterized by strong variations in seawater pH annually (Dorey et al., 2013). *S. droebachiensis* reside in the fjord and were used as the study species for this experiment. In this experiment *S. droebachiensis* larvae were exposed to different combinations of intensity of pH (8.0, 7.7, 7.4) and durations of exposure (constant, 6h, 12h) to attempt to identify which part of the natural variability cycle drives the biological response to OA. The main hypothesis for this experiment was that that both intensity and duration of different pH exposures contribute to the stress experienced by an organism. This initial hypothesis is followed up by three subsequent hypotheses: (1) the level of stress is dependent on both intensity and duration of exposure in a cumulative manner (intensity *time); (2) for a given intensity, the negative effect on sea urchin larvae will increase with the duration of exposure; and (3) for a given duration of exposure, the negative effect on sea urchin larvae will increase with the intensity.

After analysis of five morphological parameters, results showed that both intensity of pH and duration of exposure affected growth in *S. droebachiensis* for two parameters, and only duration of exposure affected growth for two other parameters. Additionally, the stress response index negatively correlated with BL growth, but not the other parameters. An unexpected result of this experiment was a change in shape of the larvae under fluctuating conditions. Larvae grew longer arms relative to body size under fluctuating conditions which is the same response observed when food was increased in another experiment (Carrier et al.,

2019). Ultimately this result should be further explored and confirming whether or not food availability has the same effects of fluctuating pH could have profound impacts on the understanding of the biological response to OA and population dynamics.

This experiment showed that when natural fluctuations of pH occur in ecosystems, it is important to consider the modulating effects of those fluctuations on species response to OA. Only focusing on the minimum pH that an organism experiences will not provide an overview of the actual biological response and can overestimate the effects of an OA scenario on organisms. If a more comprehensive review of the role of natural variability of pH on the biological response of organisms can be developed, it will ultimately lead to a better overall understanding of stress responses of organisms to decreasing pH in waters. This experiment provided valuable insight into biological responses of an organism residing in a highly dynamic environment. More studies should focus not only on minimum pH experienced, but also duration of exposure so that a more accurate representation of biological response can be presented. Further assessment of a wider species in other dynamic environments would be useful in advancing the understanding of the natural pH variability cycle. CO_2 emissions will continue to rise, and marine ecosystems will continue to be affected. An integrated assessment of all parts of the natural variability cycle will allow for a stronger predictive power for assessing community and population dynamics in a constantly changing world. In turn, there will be better opportunities for creating innovative and productive management and conservation programs that best account for natural conditions.

6. Acknowledgments

I learned an incredible amount about my topic and overall scientific investigation throughout the process of writing my thesis. My supervisor, Sam Dupont, was the biggest part of that; he always provided constructive feedback that allowed me to further my knowledge and was eager to make sure I understood the process. It was an amazing learning experience to work with a leader in the field of ocean acidification, and I truly appreciate the opportunity. I could not have completed this project without the help and support of Ida Vartia, a bachelor's student working on a similar thesis at the same time at the Kristineberg Marine Research Center. She assisted in so many ways, but I am most thankful for her support on long days in the lab and dedicating so much time to assisting me with my project. I feel fortunate that I was able to complete this work at the Kristineberg Marine Research Center and am so thankful to everyone there who supported me throughout this process. I am also thankful to my classmates who made long days in the library engaging rather than draining.

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8. Appendix 1

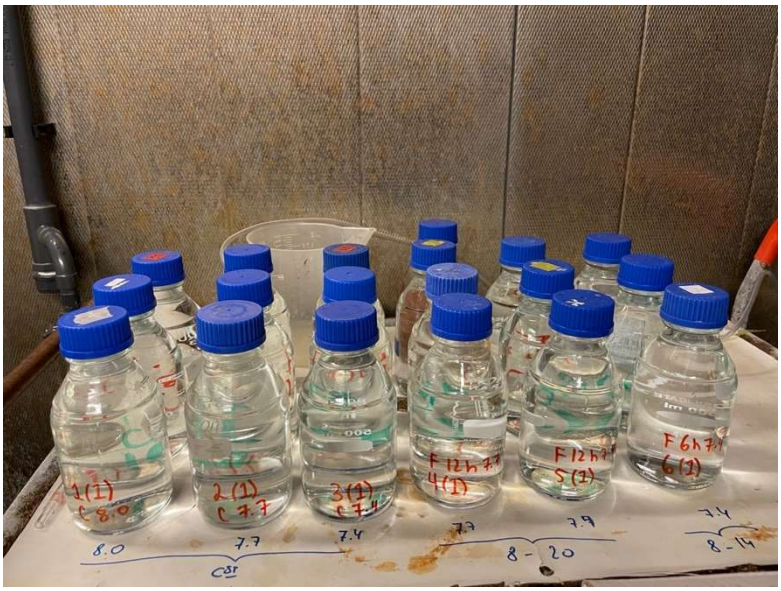

Figure 1a. Experimental set-up in the thermo-constant room. 6 treatments, n=3; from left to right the 6 treatments are labeled as CST 8.0, CST 7.7, CST 7.4, 12h F 7.7, 12h F 7.4, 6h F 7.4.

Figure 2a. Three 50 L buckets that maintained the 3 different target pH seawater needed to perform water changes. The farthest left bucket maintained 8.0 pH water, the middle 7.7 pH water, and the farthest right 7.4 pH water. The 8.0 bucket was in equilibrium with the CO_2 concentration in the air (~440 ppm) while the 7.7 and 7.4 water was bubbled with pure CO_2 and maintained with a pH stat system. All buckets were continuously aerated.

Table 1a. Rates for BL, calculated from the logarithmic relationship between BL and developmental time, the 4 other allometries measured (BR, POR, PLR, SV), calculated from the relationship between BL and each parameter, and mortality calculated form the relationship between relative density and developmental time. Rates come from the equation of the regression line: y=ax+b, where x is the rate. The rates are what were used for statistical analysis.

	CST 8.0			***CST 7.7***			***CST 7.4***		
	1	***2***	***3***	***1***	***2***	***3***	***1***	***2***	***3***
BL	32.235	38.945	33.473	29.436	36.174	33.993	29.722	27.677	27.173
BR	-0.6992	0.7696	-0.629	-0.5507	-0.6029	-0.5963	-0.4361	-0.5004	-0.4385
POR	-0.1013	-0.0656	-0.2404	0.5112	-0.0795	-0.1014	0.3041	0.2239	0.2119
PLR	0.045	0.1845	0.3328	1.0249	0.244	0.2037	0.3726	0.4159	0.4032
SV	-1567.1	-1117.6	-1247.9	2700.2	311.1	-113.31	-698.44	-355.54	-735.56
Mortality	-0.0099	-0.0095	0.0124	-0.0556	-0.0209	-0.0059	-0.0048	-0.0156	-0.0103

	12h 7.7			***12h 7.4***			***6h 7.4***		
	1	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
BL	40.712	40.459	38.448	35.686	37.636	34.616	36.148	39.727	38.715
BR	-0.5378	-0.5612	-0.5495	-0.4754	-0.5045	-0.5057	-0.3566	-0.3877	-0.3741
POR	1.4271	1.1371	1.0811	1.4173	1.2423	1.2459	1.1982	1.5726	1.2832
PLR	1.5247	1.3495	1.3643	1.5913	1.4081	1.4639	1.4784	1.7384	1.572
SV	2628.1	1190.9	583.45	880.37	439.23	1144.6	709.93	1628.8	284.32
Mortality	-0.0159	-0.028	-0.0208	-0.0141	-0.0165	-0.0262	0.0221	-0.0273	-0.0194