A disaster for the *Pisaster*? Temperature effects on Ochre sea star larval development

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FHL 470 Research in Marine Biology

Metamorphosis in the Ocean and across Kingdoms

Spring 2019

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Keywords: larva, development, cloning, temperature, Ochre sea star, *Pisaster ochraceus*

Abstract

Marine ecosystems are threatened by climate change. Sea surface temperatures are rising as a result of global warming, which may influence the larval development and survival rates of many species. Decreasing the quantity of successful larvae, especially for keystone species such as *Pisaster ochraceus* (Ochre sea star), has the potential to disrupt the predator-prey balance in a variety of habitats. The sea star wasting disease that passed through the west coast of the United States in 2013 greatly impacted populations of a variety of sea stars, notably that of the Ochre sea star, and emphasized the importance of sea star presence in intertidal ecosystems. By rearing ten day old *P. ochraceus* larvae in three different temperatures for a week, this study examines the growth, survival, and cloning rate of Echinoderm larvae in response to elevated sea surface temperatures. Larvae residing in temperatures of 20.15℃ +/- 2.65℃ experienced the highest mortality rates and fastest growth, while larvae in local San Juan Island, WA, temperatures (9.13℃ +/- 0.47℃) exhibited the lowest mortality rates and slowest growth. A temperature range of 15.58℃ +/- 1.48℃ produced intermediary growth and mortality rates, although the success of the latter is comparable to that of local temperatures. Medium temperatures induced the highest frequency of asexual reproduction. There was no difference between the cloning rate of high temperature and low temperature larval cultures. This study concludes that due to the exhibited larval sensitivity to temperature changes, it is possible *P. ochraceus* may experience population shifts or geographic redistribution as a result of sea surface temperature rise.

Introduction

Over the last century, sea surface temperatures have been on the rise as a result of climate change (Houghton et al. 1995, Intergovernmental Panel on Climate Change 2007, Intergovernmental Panel on Climate Change 2013). Having already increased globally by 0.11°C per decade since 1995, climate models predict that sea surface temperatures may increase by as much as 1.1°C to 6.4°C by 2100 (Intergovernmental Panel on Climate Change 2007, Intergovernmental Panel on Climate Change 2013). The University of Washington's Climate Impact Group found the Pacific Northwest's Puget Sound region warmed significantly faster than global averages throughout the 20th century, increasing by 1.3°C (Snover 2005, Mote et al. 2010). A rise in sea surface temperatures may impose drastic challenges to organisms residing in the Pacific Northwest. Dead zones arising from hypoxia events and changes in plankton populations may likewise alter the foundation of food webs and amplify throughout trophic levels (Glick et al. 2007). Aside from influencing the composition of the Pacific Northwest's food chain, sea surface temperatures impact larvae development and physiological growth rates (Orton 1920, Thorson 1950, Pechenik 1987). As global warming continues to pressure the stability of marine environments, predator populations may decline as a result of decreased larval success (Belkin 2009).

Pisaster ochraceus (Ochre sea star) is a keystone predator of intertidal ecosystems throughout the Pacific Northwest, that consumes bivalves, chitons, limpets, snails, and sea

urchins (Feder 1959). In 2013-2015, *Pisaster ochraceus* experienced intense density and biomass declines as a result of sea star wasting disease, which causes adults and juveniles of numerous species to lose arms or fully disintegrate (Menge et al. 2016). Due to decreased predation on mussels, biodiversity declined and intertidal community structure shifted (Menge et al. 2016). In the San Juan Islands, WA, an increase in sea surface temperature apparently drove the onset of sea star wasting disease (Eisenlord 2016). For years after the virus subdued, surrounding habitats experienced a biodiversity imbalance as a result of less predation by *Pisaster ochraceus* and other predatory sea stars. To gain a holistic understanding of *Pisaster ochraceus* population dynamics, and how the species may respond to environmental changes in the future, it is advantageous to examine what may be the most vulnerable of life stages: larvae and their metamorphosis.

Morphological responses to salinity and food concentration in *Pisaster ochraceus* larvae has been examined (Vickery et al. 2000, Pia et al. 2012), both of which are expected to vary with respect to climate change. *P. ochraceus* are distributed from Prince William Sound, Alaska, to Baja California, Mexico (Lamb et al. 2006). These areas often have sea surface temperatures ranging from -1℃ to 22℃, so Ochre sea star larvae should have the capability to withstand a large range in sea surface temperatures (National Oceanographic Data Center, National Oceanic and Atmospheric Association). Sea surface temperature rise possesses the capability to decrease Ochre sea star populations, or force the species to shift geographic distributions northward, which may offset the ecological balance of intertidal ecosystems. The average Friday Harbor, San Juan Island, WA, spring sea surface temperature is 10.5°C (Strathmann 1978); establishing control temperatures within a close proximity of local habitats, introducing larvae to increasing increments of temperature may yield results as to whether the species can survive in warmer ecosystems. This study examines the effects of rearing *Pisaster ochraceus* larvae at three various temperatures: 9.13℃ +/- 0.47℃ as a control, 15.58℃ +/- 1.48℃, and 20.15℃ +/- 2.65℃.

It is expected *P. ochraceus* larvae will undergo normal larval development with a few instances of cloning and experience the highest survival rates at local temperatures (10.5℃), as the species has been known to be successful in the San Juan Islands (Lamb et al. 2006). Echinoderm larvae have shown faster developmental rates under higher temperatures (Stanwell-Smith et al. 1998), yet may not be as viable. The alternative hypothesis for this study states that *Pisaster ochraceus* larvae raised at the highest temperatures will exhibit the lowest survival rate, highest cloning rate, and fastest development (determined by the normalized straight length of the right anterior enterocoel against the individual larva's maximum length). Larvae residing in lower, local temperatures may develop more slowly but more will survive. Medium temperatures may fall somewhere intermediate for cloning, survival, and developmental rates. The null hypothesis states that various temperature values have no influence on larval development rates, survival, or cloning frequency.

Methods

Fertilization

Three *Pisaster ochraceus* were collected from the University of Washington's Friday Harbor Laboratories docks and point; one male and one female from 48°32'45.9"N 123°00'27.3"W and one female from 48°32'44.2"N 123°00'45.8"W. Male gametes were collected by siphoning sperm from the aboral surface of the male Ochre sea star using a pipette, and transferring the solution to an Embryo-safe, sealable vial, with as little sea water as possible. It was not necessary to inject the male Ochre with a spawning inducer, as he began spawning immediately after collection. Sperm was stored in the fridge until fertilization was initiated. To induce spawning in the other stars, the volume of each Ochre sea sear was determined by measuring the amount of water displaced in a 5000mL beaker. 1mL of 1-methyladenine for every 100mL of sea star volume was then injected with a 16 gauge needle inside two opposite arms, with the total solution distributed equally among them (Strathmann 1987). After gonads began to contract (approximately one hour), each sea star exhibited a lifting behavior, and then was placed ventral side up into a beaker to collect eggs (approximately 30 minutes). Excess water was decanted so the beaker was full of concentrated eggs. Eggs are dark-pink or red in color, while sperm is white. Fertilization was achieved by combining 250 µL of eggs and a pipette with a minimal amount of sperm, in an effort to prevent multiple fertilization events on one egg. For the first three days post fertilization, the top layer of water was decanted to limit bacterial contamination due to dead sperm. After three days, larvae were swimming, and fed 2500 cells/mL of *Rhodomonas sp.* and 3000 cells/mL of *Dunaliella tertiolecta* every two days. Larvae were kept in the same culture jar post-fertilization, and exposure to various temperatures began ten days post fertilization (10 dpf).

Experimental design

Six 400mL and three 1000mL Embryo-safe identical beakers were obtained. Ideally, the experiment would take place in nine identical jars, but due to a shortage in the availability of Embryo-safe glassware, the experimental design had to be adjusted. Nine identical lids – also Embryo-safe – were obtained; it is crucial for the lids to protect from water evaporation, yet still allow adequate oxygen flow, so they should be covering the top of the beakers but not be completely sealed. Three 1000mL mason jars, also Embryo-safe, were labeled as "Water Change Stock High/Mid/Low Temp," with the respective condition. The purpose of these jars is to minimize larval temperature shock during a water change by providing a thermally equilibrated source, as neglecting to do so may be fatal. Each of the experimental beakers were labeled with numbers one through nine. Jars one through three were designated as high temperature cultures, while four through six were medium, and seven through nine served as the controls. The mason jars, lids, and beakers were cleaned with reverse osmosis filtered (RO) water, and rinsed with 0.45 micron-filtered sea water sourced from the Salish Sea. Experimental beakers were then filled with one hundred fifty milliliters of 10.5℃ of 0.45 micron-filtered sea water.

Using a glass pipette and dissecting scope (out of focus to limit potential bias), nine hundred ten day old *P. ochraceus* larvae were hand selected from a mass culture and evenly distributed among the jars. Using a calibrated compound microscope at 100x view, measurements of ten larvae within each beaker were taken. The maximum length, width, and stomach diameter were recorded and averaged to obtain a starting data point (Figure 1). One larva from the original culture was removed and placed on a microscope slide with a raised cover slip. Using a compound scope, QImaging camera and software, a photograph of the specimen was obtained at 100x view to establish a starting developmental stage (Figure 1).

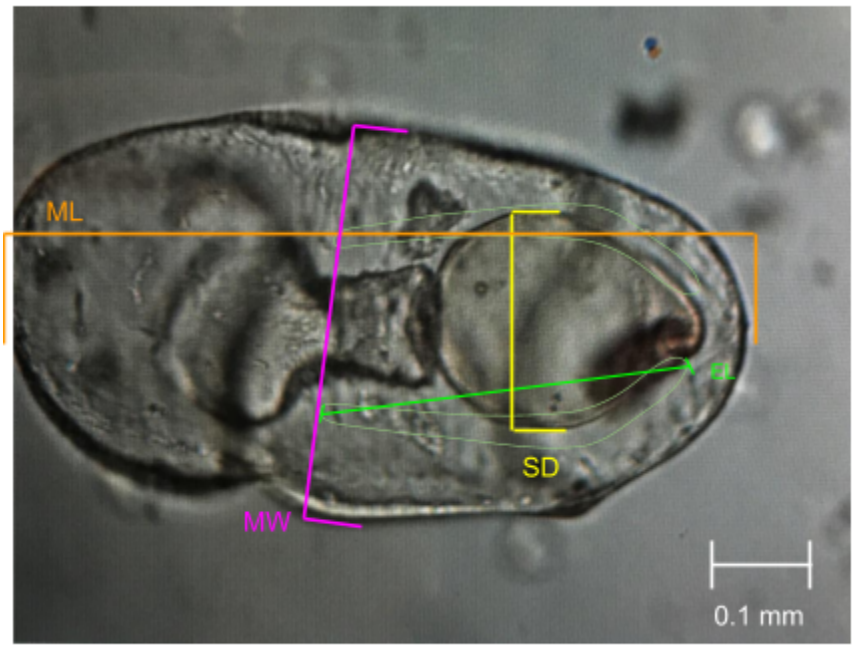

Figure 1. *Pisaster ochraceus* x *Pisaster ochraceus* larvae at ten days post fertilization, immediately prior to initiating the experiment. Three measurements were taken: "ML" denotes maximum length, "MW" maximum width, and "SD" stomach diameter. Seven days after exposure to various temperatures (seventeen days post fertilization), the straight length of the right anterior enterocoel (designated as "EL") was measured as well. The right and left anterior enterocoel is drawn in light green, as larvae at ten days post fertilization are too young to exhibit this structure. View at 100x, including ocular magnification. Photo taken with a Nikon Eclipse E600, QImaging MicroPublisher 5.0 RTV, and QCapture Suite PLUS (v. 3.1.3.10).

In a random fashion, each beaker was then distributed amongst three water baths, which were already equilibrated to the appropriate temperature and residing in a culture room kept at 10.5℃. Treatment jars were kept in water baths for seven days (Figure 2). Every day, the locations of each beaker was rotated, in an effort to eliminate any external influences on larval growth as a result of beaker location. Every other day, each beaker underwent a temperature recording, water change, and feeding.

Figure 2. Experimental setup, involving three water bath apparatuses in a cold culture room kept at 10.5°C. The highest temperature water bath resides on the left, and lowest temperature (control) resides on the right. Images taken with an iPhone 7, on the first day of treatment.

Temperatures were recorded using the same thermometer (NSF Taylor 9842) in Celsius; although the experimental design deploys controlled water bath apparatuses, it is valuable to recognize that temperature fluctuates in controlled settings and in local environments. Water changes were performed with a 28 μm filter apparatus, by siphoning up used seawater with a syringe, and refilling the beaker to 150 mL using the equilibrated micro filtered sea water kept in the "Water Change Stock" mason jars. After each water change the filter is visibly checked and thoroughly washed with R.O. water to prevent larval transfer between beakers. Similarly to Pia et al. 2012, larvae were fed a mixture of *Rhodomonas sp.* and *Dunaliella tertiolecta.* Food concentrations were counted using a hemocytometer to obtain 2500 cells/mL and 3000 cells/mL, of *Rhodomonas sp.* and *Dunaliella tertiolecta,* respectively. Food cultures were then spun down in a centrifuge for ten minutes per fifty milliliters to concentrate viable algal cells, and excess culture medium was decanted and then resuspended in filtered sea water. *Rhodomonas sp.* and

Dunaliella tertiolecta was then combined, and equally distributed to each treatment beaker immediately following a water change.

Data collection

Halfway through and at the conclusion of the experiment – four days and seven days after placement into water baths – five larvae were selected (using the naked eye to minimize selection bias). Each was removed from the beaker and placed onto a microscope slide with a raised cover slip. With the same microscope, imaging camera, and imaging software as the initial starting point photograph, images of these fifteen larvae were obtained to examine the differences of coelomic development between the three treatments. For the photographs taken at four days, larvae were then returned to the respective treatment jar in an effort to maintain a 100 larvae/150mL density.

At the conclusion of the experiment (seven days) each beaker was removed from water baths at the same time. Ten larvae were selected from each jar, according to the selection method mentioned previously. Measurements of larval maximum width, maximum length, stomach diameter, and straight length of the right anterior enterocoel were recorded using a calibrated compound microscope at 100x view. When the larva's mouth is facing the cover slip, with it's mouth on top and stomach on the bottom, the right anterior enterocoel is located on the right (Figure 1). Inversely, when the larva's stomach is facing the cover slip, the right anterior enterocoel is located on the left (Wessel et al. 2014). Larval viability was calculated by individually separating and counting the number of alive, active, swimming larvae divided by the total number of swimming larvae and deceased larval carcasses. Subtracting one hundred (starting number of larvae) from the total number of swimming larvae and deceased larval carcasses counted from each beaker yields the approximate number of clones. Up until two weeks post-budding, clones are visually identifiable, noting their smaller and deformed shape (Vickery et al. 2000). Possible clones were counted out separately, to determine the number of viable larvae from the original culture. Although approximate, dividing the number of viable larvae that do not appear to be clones by one hundred yields the percent survival.

Statistical Analysis

Data regarding larval size measurements, viability, and survival percentages were compiled, averaged, and graphed. To determine whether viability and survival percentages were significantly different, a two sample, two-tailed t-test assuming unequal variances was used. Using Microsoft Excel (v. 16.25), a two sample t-test assuming unequal variances was also performed against each treatment on the slopes and y-intercepts of maximum length, and enterocoel measurements, in order to analyze whether individual data points are significantly variable between each replicate. Under the circumstance where variance is at a minimum ($p>0.25$), justification to compile the replicates is provided (Underwood 1997), and average linear length of the right anterior enterocoel may be plotted linearly against the respective larva's

maximum length. The strength of linear regression among the three treatments is comparable via slope intensity and correlation strength, in order to determine whether temperature had any significant influence on larval development (in terms of normalized right anterior enterocoel size via maximum length).

Results

The initial measurements of larval size may be found in Table 1. High temperature treatments experienced substantially lower survival and viability rates when compared to medium and lower temperatures (Figure 3). Graphing average survival besides average viability shows little variance between the two. The t-test showed the difference between viability and estimated survival percentages is not statistically significant ($p=0.916$). The cloning frequency peaked at medium temperatures, and was observed most frequently in jar 5 (Figure 4). Averaging each larvae measurement across each treatment shows larval development is relatively comparable across each of the three treatments (Figure 5). No outstanding visual trend is available, although high temperature cultures exhibited the smallest sizes for each of the measurements – with the exception of maximum width, which was greater than the medium temperature beakers by 3.52μm.

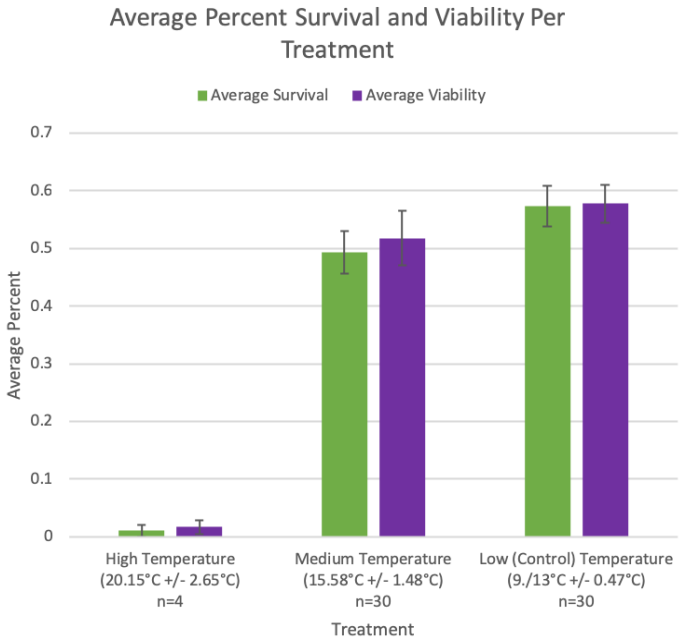

Figure 3. The average percent of viable larvae (purple) is determined by individually counting the number of alive (suspended and actively moving) larvae divided by the total number present in the jar. Survival percentage (green) calculated by individually counting the number of larvae that appear to have undergone normal development (i.e. not abnormally-shaped clones) divided by the initial number of larvae (one hundred). Standard error bars are calculated by dividing the standard deviation by the square root of appropriate measurements that make up the mean, and thus are respective to each data range.

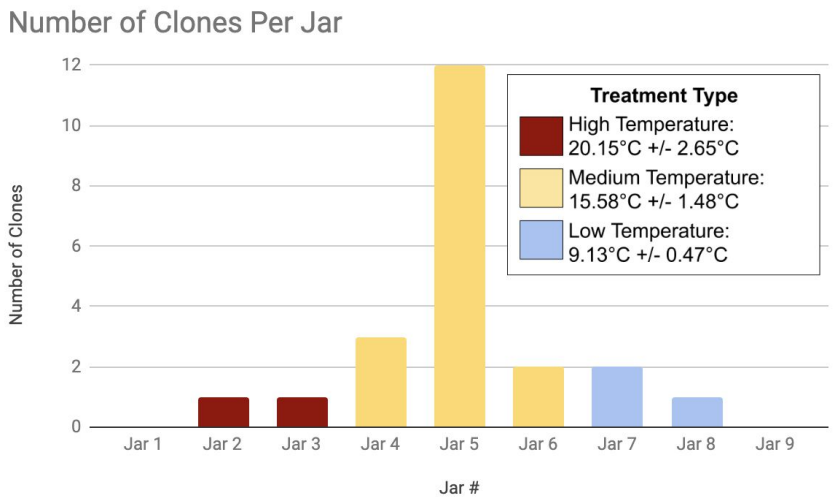

Figure 4. Cloning frequency for each jar determined by counting the number of larvae present (alive or deceased) and subtracting the original quantity of larvae (one hundred).

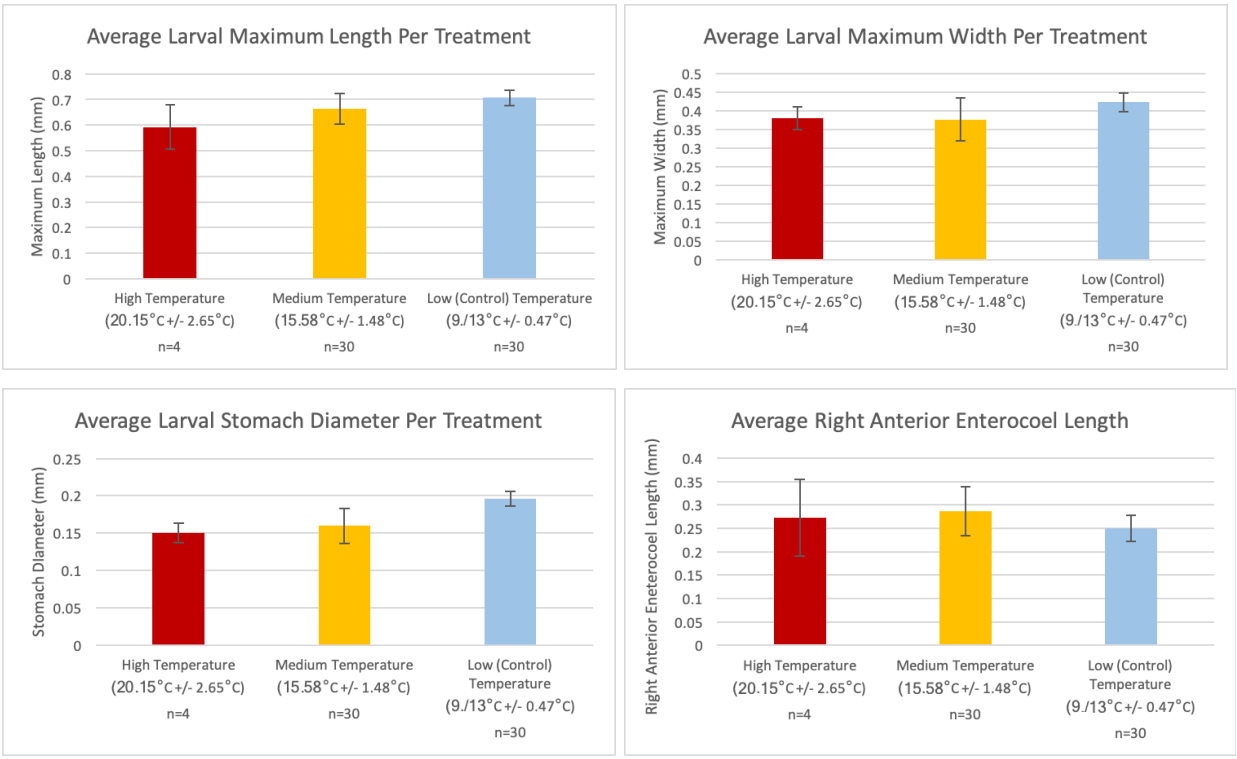

Figure 5. Final average larval measurements for each jar in terms of maximum length, maximum width, stomach diameter, and right anterior enterocoel, in millimeters. Sample size (n) represents the number of larvae measured; ten larvae were chosen at random from each experimental replicate (three per treatment). Of the three hundred larvae residing in high temperatures, only four were found alive. Data compiled on a calibrated compound microscope at 100x, seven days after introduction to various temperatures (17 dpf). Standard error bars are calculated by dividing the standard deviation by the square root of appropriate measurements that make up the mean, and thus are respective to each data range.

The slopes and y-intercept of normalized right anterior enterocoel length via maximum length were calculated for each treatment. Performing a two-tailed t-test assuming unequal variances for each treatment proved the variance within each replicate is not statistically significant ($p>0.25$, Table 2). The calculated p-values provide justification for combining the replicates and performing a linear regression of normalized enterocoel length to compare treatments following Underwood's (1997) accepted $p>0.25$. The linear regression reveals a clear trend between the relationship of larval growth and culture temperature (Figure 6). Higher temperatures yielded the highest R^2 value (0.953) while lower temperatures showed little correlation ($R^2 = 0.4384$). Therefore, larval cultures residing in temperatures of 20.15℃ +/- 2.65℃ show a strong relationship between maximum and enterocoel length, and display a greater rate of development and metamorphosis. The slowest growing larvae were found in control temperatures 9.13℃ +/- 0.47℃.

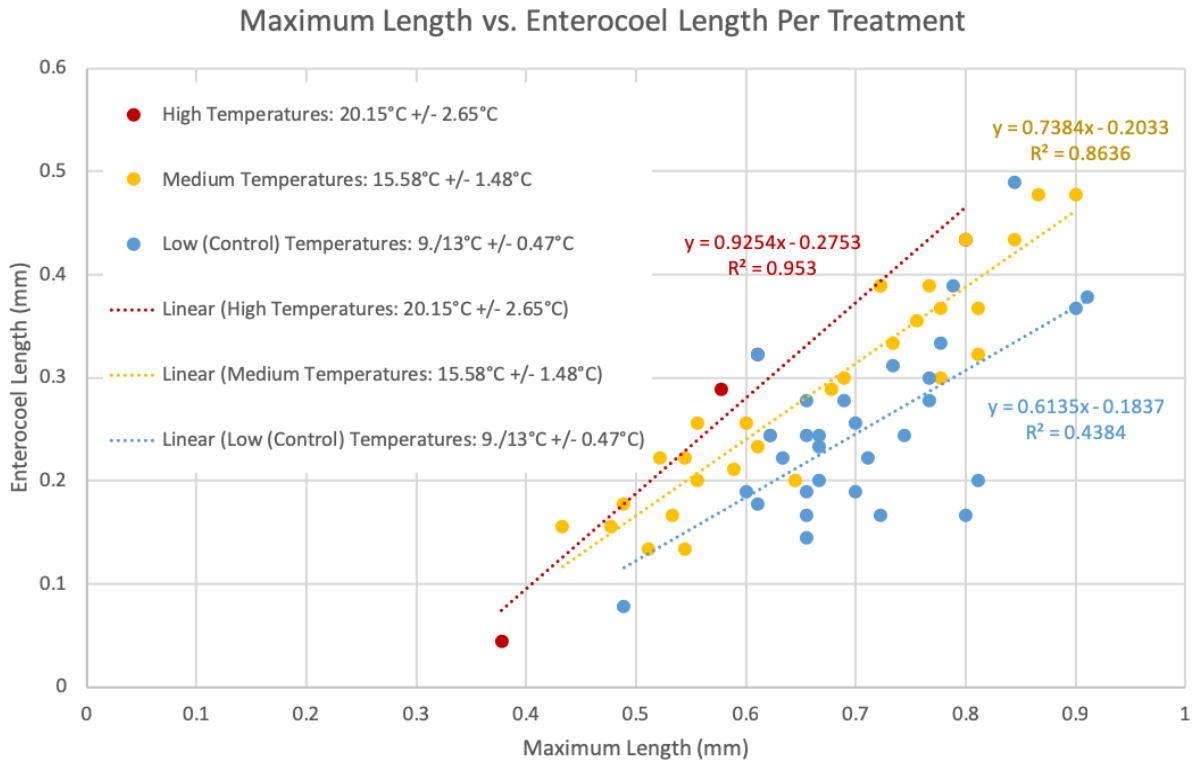

Figure 6. Linear regression of the normalized enterocoel length via maximum length for each individual larvae in each treatment.

Discussion

This data exhibits a correlation between high temperature and increased growth rate (represented by normalized enterocoel length against maximum length), and a lower chance at survival or viability. Survival and viability percentages between low and medium temperatures were similar, yet there was a substantial difference between high temperature cultures. It was

expected cloning rates would be most frequent at high temperatures (20.15℃ +/- 2.65℃), when in fact more instances were recorded at medium (15.58℃ +/- 1.48℃) temperatures.

Considering the cloning process is energetically demanding, it is likely that only larvae under ideal conditions may invest in asexual reproduction (Vickery et al. 2000). Temperature and food availability have been known to influence cloning frequency (Levitan 1995, Morgan 1995); considering food availability is held constant among each treatment while temperature is variable, it is appropriate to state that temperature in itself may affect the rate of asexual reproduction. Comparing survival and viability percentages, it is crucial to note that survival percentage is subjective, seeing as the larvae were assumed clones based off visual examination. However, a t-test ($p=0.916$) provides evidence that it is relatively reasonable to distinguish clones based off larval structure up until two weeks post-budding, following suit of Vickery et al.'s (2000) findings.

It is curious that averaging each larval measurement across the treatments did not provide a clear difference between each condition. However, when normalizing the measurements (i.e. graphing enterocoel length with respect to maximum length), a substantial trend is notable. Higher temperature environments result in greater relative development of coeloms to larval structures, which may indicate a greater rate of metamorphosis. Inversely, low temperature environments experience slower relative development of coeloms with respect to larval length, yet may have a greater chance of survival. Granting a larvae chosen at random does not represent the population as a whole, it is possible to observe the difference in larval structure between the treatments in the photographs taken at four and seven days post temperature exposure (Figure 7).

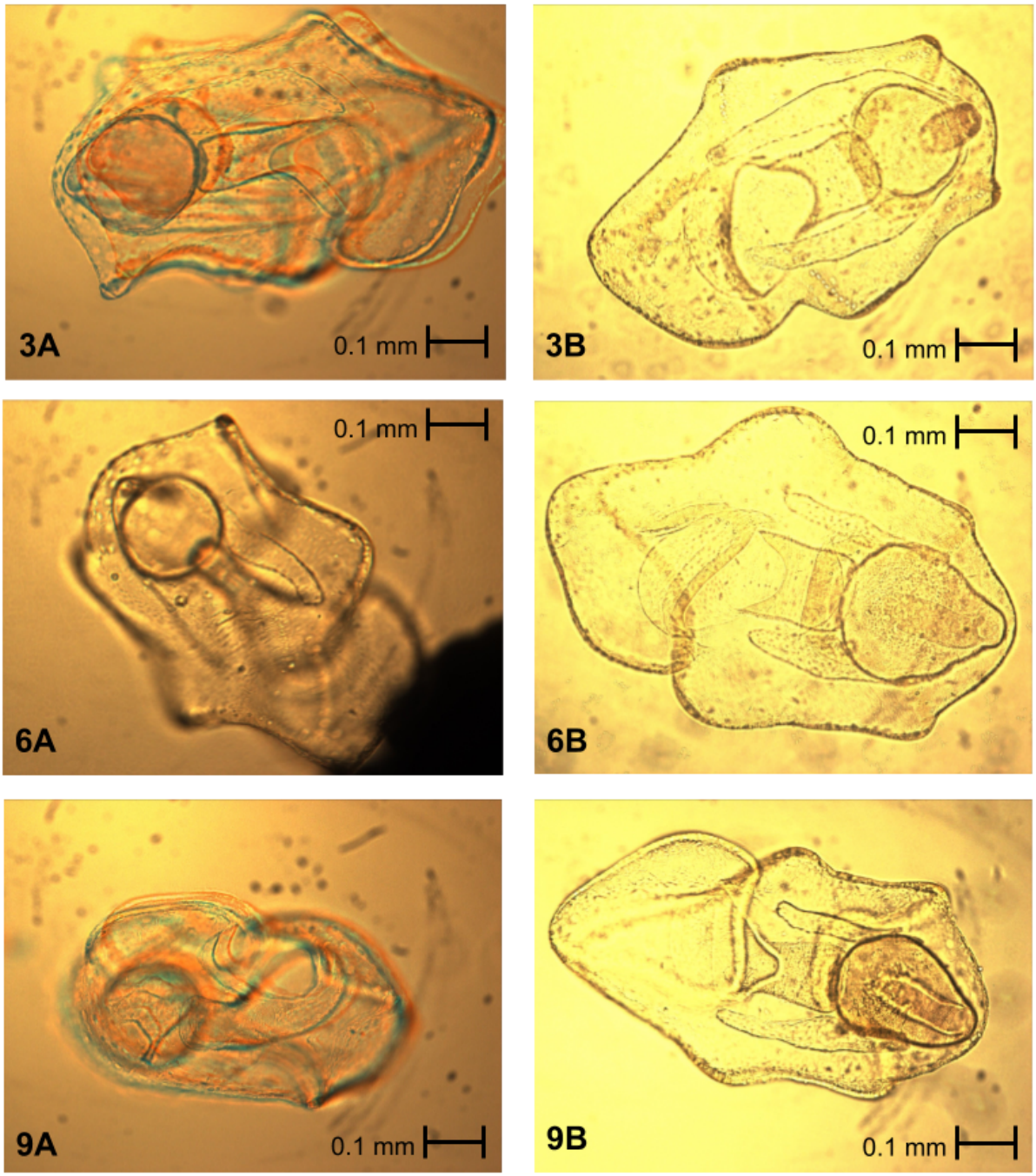

Figure 7. *Pisaster ochraceus* x *Pisaster ochraceus* larvae from treatment beakers 3 (high temperature), 6 (medium temperature), and 9 (low temperature). To determine which replicate of each treatment to photograph, a random number generator was used, and the same treatment beakers were selected for the second round of images. Larvae were chosen at random, using an unfocused dissecting scope. View at 100x, including ocular magnification. "A" figures represent images taken when larvae were fourteen days post fertilization (14 dpf), and had been exposed to experimental conditions for four days. "B" figures represent images taken when larvae had been exposed to experimental conditions for seven days (17 dpf). Photos taken with a Nikon Eclipse E600, QImaging MicroPublisher 5.0 RTV, and QCapture Suite PLUS (v. 3.1.3.10).

Temperature has a substantial effect on larval survival and viability; those in temperatures of 20.15℃ +/- 2.65℃ endure in increased risk of fatality, while those in temperatures of 9.13℃ +/- 0.47℃ or 15.58℃ +/- 1.48℃ are far more likely to succeed. *P. ochraceus* reside in habitats that can reach 22℃, and with respect to the Intergovernmental Panel on Climate Change 2007's climate models, it seems that rises in temperature will pose an increasing threat to the species (National Oceanic and Atmospheric Association). Evidence suggests *Pisaster ochraceus* spawn in the fall, as long day lengths – like in the summer and spring – initiate gametogenesis (Pearse et al. 1982). Baja California, Mexico, is the southernmost location in which *P. ochraceus* are found, and in fall seasons the ocean averages at approximately 20.5°C (National Oceanic and Atmospheric Association). Therefore, *Pisaster ochraceus* larvae already reside in areas where they may experience high mortality rates, and survival may become increasingly difficult.

If this experiment were to be repeated, it would be interesting to observe the difference in larval development between a greater range of temperatures, and for a longer period of time. Observing larvae through metamorphosis to juvenile stages, which occurs after settlement around 50 dpf, may yield results of greater significance or stronger trends (Vickery et al. 2000). Additionally, it may be advantageous to perform population counts of larvae multiple times throughout the study; larval carcasses may disintegrate or be discarded after a water change, which may misrepresent the calculated instances of asexual reproduction. Because ten larvae were selected at random to represent the growth of the one hundred total larvae in their respective beaker, it is possible cloning may distort the data. Beakers with a higher instances of asexual reproduction endure a higher likelihood of a clone becoming randomly selected to undergo measurement. This would cause beakers with more clones to exhibit a lesser growth rate. In this study, the treatments with the highest frequency of cloning (medium temperatures) proved an intermediary growth rate, as is expected considering the boundaries of high and low temperature findings, so it does not appear to be a major cause of concern. In order to avoid this situation with the repetition of this experiment, the ten larvae selected at random could undergo a visual analysis prior to recording measurements, and if the selected individual appears to be a clone, one could recollect another larva. However, this may introduce a selection bias, and it would be difficult to visually determine which individuals are clones if the study lasted longer than two weeks (Vickery et al. 2000). It is ideal to measure every single larvae in the study. It would also be interesting to analyze the metaphorical process of *Pisaster ochraceus* larvae sourced from parent individuals residing in higher temperatures, such as in Baja California, Mexico. *Pisaster ochraceus* that may withstand extreme temperatures could potentially be subject to identification as a subspecies.

The broader implications of this experiment refer to the balance of habitats which may have to adjust to the loss of a keystone predator, if temperatures rise at the rates that are expected. *Pisaster ochraceus* may impact habitats by consumptive or non-consumptive pressures, and thus may be referred to as a keystone predator or keystone intimidator,

simultaneously (Gosnell et al. 2012). Ochre sea star presence has been shown to affect the diet, growth, reproduction, and behavior of whelks, *Nucella emarginata* (Gosnell et al. 2012). *Pisaster ochraceus* also composes 0.6% of a sea otter's diet (*Enhydra lutris kenyoni*) in coastal Washington (Laidre et al. 2006). Ecosystems without *Pisaster ochraceus* – which Baja California, Mexico, may have to adapt to in the near future – may encounter increased presence of bivalves, chitons, limpets, snails, and sea urchins, a decrease in biodiversity, and a shift in the structure of the intertidal community (Feder 1959, Menge et al. 2016). Intense environmental changes along this scale may pose drastic consequences towards the success of the habitat, as well as any anthropogenic economic revenue deriving from it or higher trophic levels. Inversely, rising sea surface temperatures along the north end of the *Pisaster ochraceus* geographic range may allow the species to expand further into Alaska, which also has the potential to disrupt the stability of those environments by introducing a new predator dynamic. Sea surface temperature rise in the upcoming decades may encourage a geographical shift for *Pisaster ochraceus*, as well as manipulate the composition of habitats along the southern and northernmost ranges.

Tables

Jar #	Average ML (µm)	ML Std Dev (µm)	Average MW (µm)	MW Std dev (µm)	Average SD (µm)	SD Std dev (µm)
1	488.8888889	40.23252034	288.8888889	43.50866713	155.5555556	18.14436847
2	400	41.90262407	211.1111111	24.00274333	133.3333333	18.14436847
3	511.1111111	26.70778723	244.4444444	29.16299213	155.5555556	17.37191022
4	470	30.5639719	222.2222222	23.42427896	133.3333333	20.95131204
5	467.7777778	24.25855244	277.7777778	41.57397096	155.5555556	39.89010968
6	466.6666667	25.66001196	255.5555556	20.28602065	155.5555556	11.71213948
7	477.7777778	38.84477215	244.4444444	29.16299213	155.5555556	18.14436847
8	466.6666667	25.66001196	255.5555556	20.28602065	133.3333333	20.95131204
9	455.5555556	17.37191022	255.5555556	20.28602065	155.5555556	11.71213948
Average across all jars	467.1604938	29.9387807	250.617284	24.28680935	148.1481481	11.11111111

Table 1. Average maximum length, maximum width, and stomach diameter measurements from larvae ten days post fertilization, directly prior of exposure to various temperatures. “Std dev” describes the appropriate standard deviation.

	Slope Mean	Slope Standard Error	P-value (two-tail)	Intercept Mean	Intercept Standard Error	P-value (two-tail)
Jars 4, 5, 6	0.5825	0.02323252	0.8002499128	-0.1095	0.00509683	0.5037437284
Jars 7, 8, 9	0.55543333	0.005553903	0.8002499128	-0.1456	0.00171412	0.5037437284

Table 2. Two sample t-tests assuming unequal variances compared the slopes and y-intercepts of jars 4, 5, and 6 (medium temperatures) and jars 7, 8, and 9 (low temperatures). Slopes and y-intercepts were obtained using linear regression. None of the larvae in jar 1 survived, and only one viable, possible clone, was present in jar 3. Therefore, a t-test is not possible for the high temperature data. These p-values provide justification for compiling replicates of each treatment together for linear regression analysis.

Acknowledgements

I would like to thank Billie Swalla, director of Friday Harbor Laboratories, for providing access to campus equipment and laboratory space. Jason Hodin, the instructor of FHL 470, provided unparalleled insight for larval development, procedures, resources, experimental design, statistical analysis, and the editorial process. Karly Cohen, the teaching assistant for FHL 470, served as a mentor in completing the research process successfully, and provided advice on statistical analysis and preliminary deliberation on experimental organisms and design. Katie Hearther conducted a similar experiment on *Pisaster ochraceus* larvae development in response to various food availability, and collaborated on appropriate data collection, larvae care, and editorial support. Ashley Cullen and Jason Hodin are responsible for the fertilization of parent organisms, larval spawning, and initial post-fertilization care. Ashley Cullen, Justin Harshman, and Marissa Leatherman aided in the transfer and set up of the water bath apparatuses. Thank you to Megan Dethier and Tom Mumford for managing yet another successful Zoology-Botany program. Tom Mumford also provided access to a thermometer and stage micrometer. Mo Turner graciously attempted to collect organisms for a similar experiment, but weather conditions unfortunately prevented successful scientific dives. Morgan Eisenlord provided advice on FHL's Ocean Acidification Laboratory use, and volunteered personal time to assist in experimental set up. Peggy Combs contributed in the accumulation of Embryo-safe glassware, as well as temperature bath apparatuses, and other materials necessary for the success of this experiment.

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