Effects of temperature and salinity on *Pisaster ochraceus* and *Pycnopodia helianthoides* larvae

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Abstract

Increasing global temperatures are resulting in marine heating events which can increase temperature in these environments up to 22°C. These marine heating events are becoming more frequent and intense, increasing the physiological stress on marine invertebrates and their larval forms in the water column. In the Salish Sea, *Pisaster ochraceus* and *Pycnopodia helianthoides* are important ecosystem predators which maintain the structure of the systems they dominate through predation. A decrease in the abundance of these species attributed to lessened juvenile recruitment from heat-induced morphological variation, could result in cascading effects for the balance of their ecosystems. This study implemented conditions of decreased seawater salinity and increased water temperatures to determine the effects on sea star larval growth over time. *Pisaster ochraceus* larvae were exposed to 4 treatments with 3 replicate jars per treatment: Three jars at low temperature and low salinity (LTLS), 3 jars at low temperature and high salinity (LTHS), 3 jars at high temperature and low salinity (HTLS), and three jars at high temperature and high salinity (HTHS). *Pycnopodia helianthoides* were kept under two temperature treatments: A high temperature treatment between 15 and 18°C and a low temperature treatment between 9 and 14°C. Total larval length and width were measured for 12, 24 and 32 day-old *Pisaster* bipinnaria and early brachiolaria larvae, and for 60 and 63 day-old *Pycnopodia* brachiolaria larvae. Our results indicate that temperature had a significant effect on larval length and width for both species and that the variation in these variables decreases as larvae age. These findings suggest that the timing of marine heat waves may be critical for successful larval development to metamorphosis of these species.

Introduction

In recent years, global-scale heating has become a topic of increasing concern, due to the projected increase of stressors in the natural environment. Marine environments are expected to undergo an increase in the intensity and duration of marine heatwave events, resulting in the degradation of habitat structures and functions (Cooley et al., 2022). Marine rocky-shore environments are highly susceptible to the impacts of these heating events. Rocky shore ecosystems, such as those found on the Northwest coast of the United States, are notably composed of biotic communities with complex ecological interactions. These ecosystems rely on the stability of top-predator populations, as they play a key role in maintaining the functions within these system (Hermosillo-Núñez, 2020; Paine, 1969; Power et al.,1996). Rocky shores are inhabited by a variety of invertebrates including sea stars like *Pisaster ochraceus* and *Pycnopodia helianthoides*, which function as top predators within their environments. These sea stars have a significant contribution to the structure of their environment and maintain population control over their prey, such as mussels and sea urchins (Harvell et al., 2019). Sea star populations are experiencing increasing strain due to global heating, and faced mass mortality from sea star wasting disease, which became prevalent among wild sea star populations in 2012 (Montecino-Latorre et al., 2016).

These heating events also result in an influx of freshwater into marine systems, which result in additional environmental stress for these sea stars and increases their risk of mortality, as they lack the ability to osmoregulate (Held and Harley, 2009). Locally, the Fraser River (B.C Canada) accounts for a significant portion of the freshwater that reaches the Salish Sea (Khangaonkar et al., 2017) and the seasonal influxes of this freshwater into the Salish Sea may increase as global heating persists.

The larval life phase of these marine invertebrates is not entirely understood, as a result our comprehension of larval response to environmental stressors such as heating, and salinity is not definitively known. Understanding the effects this changing abiotic environment has on larvae is essential to understanding the possible morphological and physiological changes that are occurring among developing larvae under these conditions, and how these early stressors may affect their juvenile and adult stages.

This study focused on determining the effects of temperature, as well as salinity on larval size, specifically total larval length, and larval width. We based our predictions on the physiological responses of echinoderms to variations in environmental conditions. An increase in environmental temperatures will typically result in an increase in metabolic rate, and an increase in bodily growth, assuming food is non-limiting (Carey et al., 2014; Marsh et al., 1999). Additionally, larval echinoderms have exhibited salinity-dependent growth, where low-salinity often results in a decrease in developmental rates, compared to high-salinity conditions (Roller and Stickle, 1985). We predict that *Pisaster* larvae in the high temperature and high salinity treatment will show the highest size difference from the other treatments, resulting in significantly larger larvae in this treatment. We predict that *Pycnopodia* larvae will also be larger in length and width in the high temperature, compared to the low temperature treatment.

Methods

Collection, spawning and fertilization

On April 15, 2023, 5 adult *Pisaster ochraceus* sea stars were collected from Friday Harbor Washington (48°32’45” N, 123°0’47” W) and placed in laboratory holding tanks equipped with a flow-through seawater system. On April 17, 2023, the adult seas stars were injected with 4 ml of 100µmol 1-Methyladenine and within a few hours, a single female spawned followed by a male. As spawning occurred, gametes were collected from the water surrounding the spawning individual and placed into small beakers. The sperm from one male was used to fertilize the eggs produced. Fertilization was 99% successful. The embryos were then distributed into several jars and placed into low temperature seawater tanks.

On March 21, 2023, adult *Pycnopodia helianthoides* spawned naturally in outdoor holding tanks. The eggs were collected, fertilized, and the embryos were added to stock jars and maintained in a low temperature seawater tank. On May 16, 2023, *Pycnopodia* larvae were divided into jars and placed in low and high temperature seawater tanks.

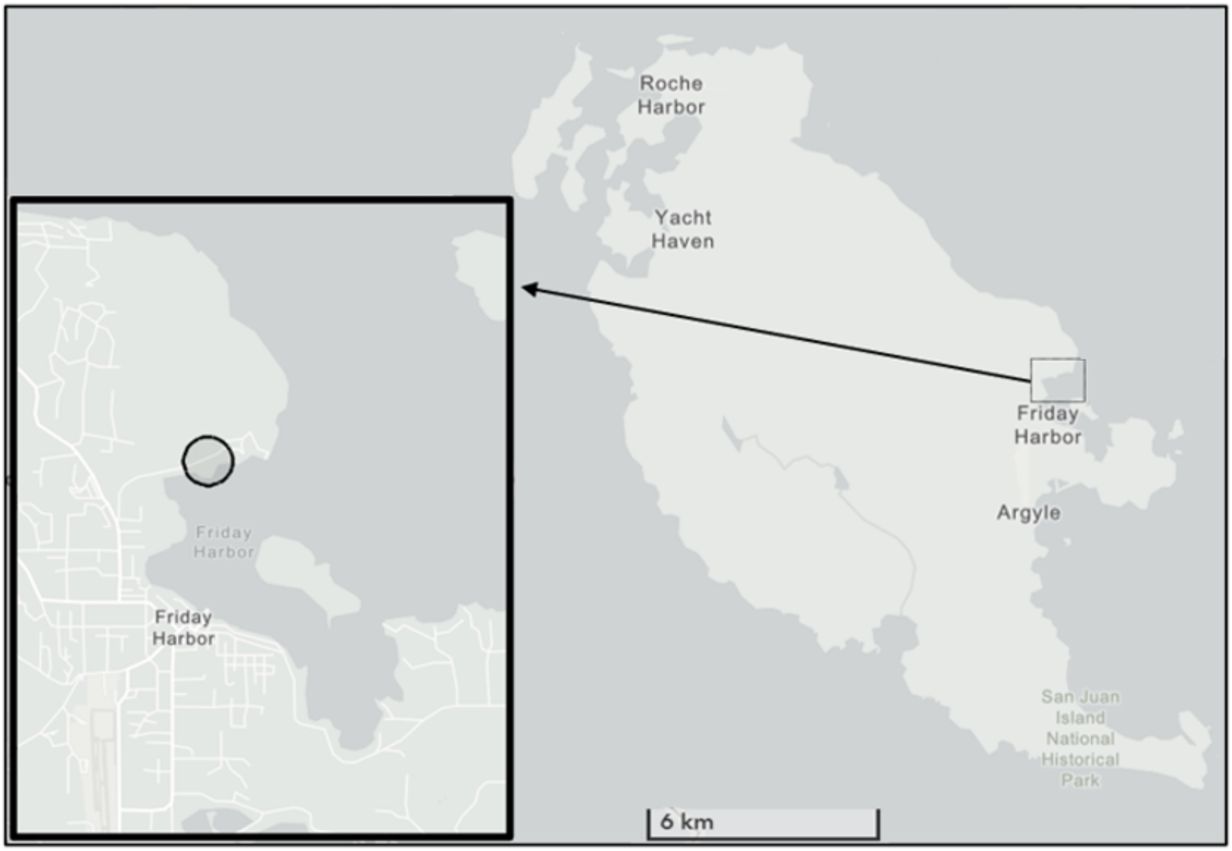


Fig. 1. Map of study location in Friday Harbor, San Juan Island. Laboratory location and collection area is indicated by encircled area (Map: National Geographic, Public Domain).

Experimental Design

To determine the effects of salinity and heating events on larval echinoderms, laboratory experiments were conducted on *Pisaster ochraceus* and *Pycnopodia helianthoides.* Three water-filled sea tables (2 large, and 1 small) were used throughout the experiments. The large sea tables measured 104.5 cm x 104.5 cm x 14.5 cm, one was kept at high-temperature and the other at low-temperature. The smaller sea table was kept at low temperature and used to hold *Pycnopodia* larvae. Each sea table had an incurrent and excurrent flow of seawater pumped in from the Harbor.

Each sea table was equipped with a system of swinging paddles, powered by an electric motor (Strathmann, 1987). The paddles were suspended into the jars and each jar was mixed by a single paddle. This mixing system allowed for food and larvae to remain suspended within the seawater of each jar. The high-temperature tank was equipped with up to three 200-watt submersible heaters (Hygger HG-802) and their respective temperature controllers (Inkbird ITC-308) were initially set to 22°C. To maintain a temperature of 22°C, the incurrent flow of seawater into the high temperature tank was reduced to a lower rate. The low-temperature tanks had a normal flow rate of incurrent seawater, and the temperature was maintained at ambient incurrent seawater temperatures (typically between 9 and 14°C). A small submersible water pump was placed in each tank to provide further circulation and consistency of temperature.

Within the temperature treatments, *P. ochraceus* received treatments of varying seawater salinities; high salinity (HS, 33‰) and low salinity (LS, 22‰) 0.45 µm filtered seawater. For the duration of the experiment, *P. ochraceus* larvae were kept among 4 treatments with 3 jars per treatment: Three jars at low temperature and low salinity (LTLS), 3 jars at low temperature and high salinity (LTHS), 3 jars at high temperature and low salinity (HTLS), and three jars at high temperature and high salinity (HTHS). The experiment began with a total of 12 jars. Each jar had a total volume of 2000ml of 0.45 µm filtered seawater and approximately 2000 larvae. To create the low salinity treatment, filtered seawater of approximately 33‰ salinity was diluted by combining with reverse osmosis (RO) water, until 22‰ salinity seawater was achieved.

P. Helianthoides larvae were kept in 2 treatments with 3 jars per treatment: Three jars in the low temperature (LT) and three jars in the high temperature (HT). Each jar was filled with 2400ml of 0.45 µm filtered seawater and approximately 400 *Pycnopodia* larvae.

All larvae were fed an algal monoculture diet consisting of *Rhodomonas* sp. and *Dunaliella tertiolecta* every 48 hours. All 12 of the *P. ochraceus* larval jars were provided with the same quantity of food. Over the course of the experiment the concentration of algae gradually increased as the larvae of both species developed. The amount of algae provided to the jars varied from 3ml to 10ml, this amount was equivalent to 2,500 to 5,000 cells per ml of *D. tertiolecta* and *Rhodomonas* sp. respectively. *P. helianthoides* larvae were fed at a slightly higher rate and were provided up to 6000 cells/ml of *Rhodomonas* sp. every 48 hours. The jars were cleaned once a week using established methods (Strathmann, 2014). Jars were cleaned by siphoning out up to 70% of the seawater, then each of the jars were manually scrubbed and rinsed, larvae were then returned into their jars and replenished with fresh 0.45 µm filtered seawater.

Data collection

As the jars were siphoned during cleaning, a 40ml sample containing larvae was removed from each jar and set aside. Using a Pasteur pipette, larvae were placed onto a glass slide. A glass cover slip was gently placed on top of the *Pisaster* larvae, a sufficient layer of water between the glass slide and coverslip ensured larvae were not compressed, to achieve accurate measurements. *Pisaster* larvae were observed under a trinocular compound microscope (Nikon Eclipse Ci) using a 4x objective lens and images were captured with an attached video camera (HAYEAR 34MP) using HAYEAR computer software. A coverslip was not used for *Pycnopodia* larvae. Instead, a small amount of water was removed from the slide using a pipette, to reduce their movement. *Pycnopodia* larvae were observed using a trinocular microscope (Olympus BH-2) with a 4x or 2x objective lens based on larval size. A digital camera was attached to this microscope (AmScope MU1000B 10MP) and AmScope computer software was used for image capture. Images of *Pisaster* larvae were captured for 3 age groups (12, 24, and 32 day old larvae). For each age group, 12-15 larvae were randomly selected from each jar, placed under the microscope, and photographed. Images of *Pycnopodia* larvae were captured for two age groups (60 and 63 days after fertilization). For each age group, 11-15 larvae were selected from each jar placed under the microscope and photographed. Photos were saved and later measured using Fiji (ImageJ) Software. Approximately, 426 *Pisaster* and 75 *Pycnopodia* larvae were measured. Before measuring larvae, a scale was set to convert image pixel units to microns and all final measurements were taken in micrometers (µm). Total body length (TL) was measured from the posterior to anterior of the larval body (Fig. 2B). Larval width (W) was measured at the widest point across the anterior section of the body (Fig. 2A).

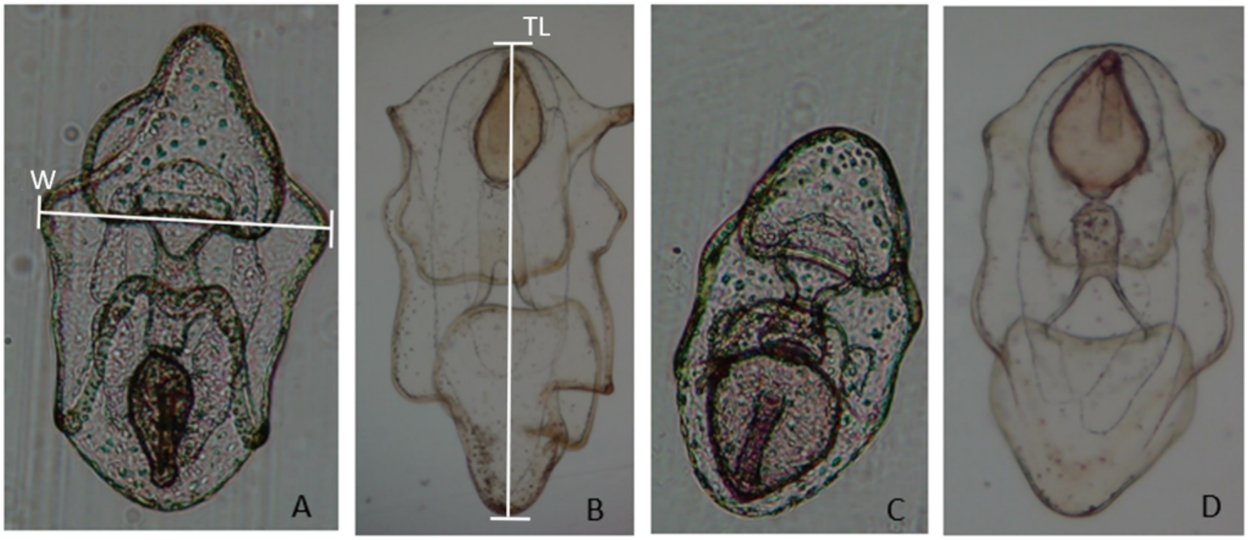


Fig. 2. Early and late bipinnaria larvae of *Pisaster ochraceus*. (A) 12 day old *Pisaster* larvae in high temperature and low salinity with white horizontal line indicating larval width (W), (B) 32 day old *Pisaster* larvae in high temperature and low salinity with white vertical line indicating total Length (TL), (C) 12 day old *Pisaster* in low temperature and low salinity, (D) 32 day old *Pisaster* larvae in low temperature and low salinity.

Data analyses

The packages lme4 and LmerTest in the software RStudio were used to perform all statistical analyses. To determine whether temperature or salinity affected *Pisaster ochraceus* larval growth, a model 3 nested ANOVA with a block design was used. The temperature and salinity were fixed factors and jar was a random factor. To determine whether temperature affected *Pycnopodia helianthoides* larval growth, a linear mixed effects model was used.

Results

Pisaster ochraceus larvae

For this experiment there were 4 treatments: 3 jars at low temperature and low salinity (LTLS), 3 jars at low temperature and high salinity (LTHS), 3 jars at high temperature and low salinity (HTLS), and three jars at high temperature and high salinity (HTHS).

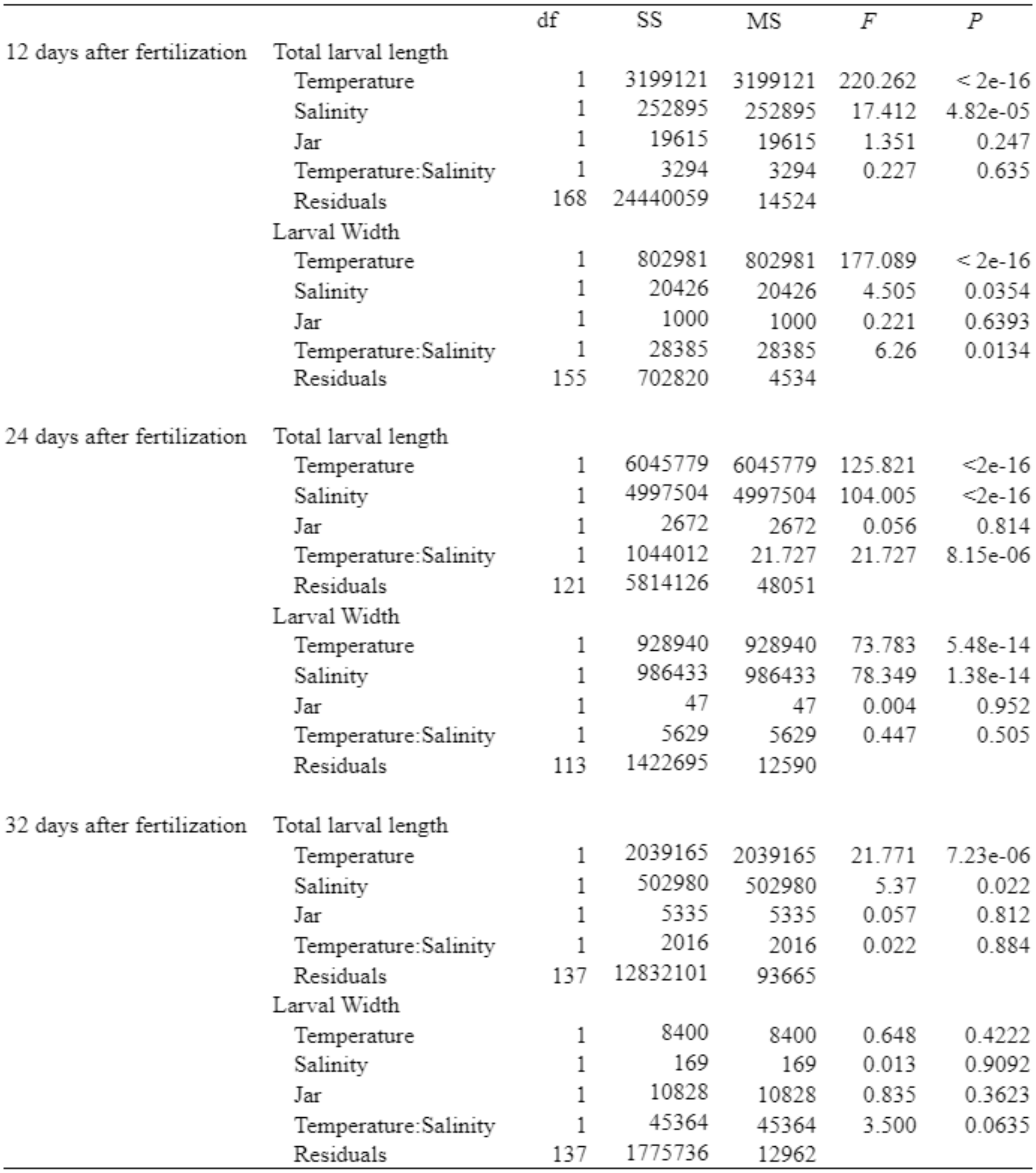
At twelve days old, all larvae in the high temperature treatment were significantly bigger (both longer and wider) than those in the low temperature treatments (Table 1, Fig.3; 12 day old; Total length: F = 220.3, p = 2e-16, and F=17.4, p = 4.82e-05 respectively, n=173; Total width: Fig. 4; F = 177.1, p = 2e-16, and F = 4.5, p = 0.03 respectively, n=160). Larvae in high salinity were generally larger; they were longer and wider than larvae in low salinity (Fig.3, Fig.4). One exception to this was between HTLS and HTHS, larval widths were not significantly different (Fig. 4).

In the twenty-four day old larvae, length and width were significantly affected by temperature and salinity (Table 1, Larval length: F=125.8, p < 2e-16 and F=104.0, p < 2e-16 respectively, n=126; Larval width: F=73.783, p = 5.48e-14, and F=78.349, p = 1.38e-14 respectively, n=118). Twenty-four day old larvae in HTHS were longer and wider than those in the HTLS and larvae in the LTHS were longer and wider than those in the LTLS (Fig.3, Fig.4.). The larvae in the high temperature treatments were bigger than those in the low temperature, when comparing across salinities.

Thirty-two day old larvae lengths were also significantly affected by temperature and salinity (Table 1, Fig. 3; Age 32, F=21.7, p=7.23e-06, and F=5.4, p=0.022 respectively, n=142). Thirty-two day-old larvae widths were not significantly affected by temperature or by salinity (Table 1, Fig. 4, F=0.648, p=0.4222, and F=0.013, p=0.9092 respectively, n=142). Thirty-two day old Pisaster larvae in the HTHS treatment were longer than those in the HTLS treatment, and larvae in the LTHS treatment were also longer than those in the LTLS treatment (Fig. 3; Age 32). Additionally, visual assessment of the plot (Age 32, Fig.3) shows a wider distribution of data points around the median of each treatment, indicating that the difference in larval length among the treatments is gradually decreasing.

The interaction between salinity and temperature was only significant for larval width for 12-day old *Pisaster* larvae (Table 1, Fig.4, F=6.260, p=0.0134) and larval length for 24-day old larvae (Table 1, Fig.3, F=21.727, p=8.15e-06). Twelve-day old larvae in the LTHS treatment were wider than those in the LTLS but larvae in the HTHS and HTLS did not differ in larval width. The difference in larval length between temperatures for 24-day old larvae was greater for larvae in the high salinity treatments than in the low salinity treatments.

Table 1. Model 3 nested ANOVA with a block design for length and width of 12, 24 and 32 day old Pisaster *larvae kept at high(22°C) and low temperatures (9-14°C) and two salinity treatments (22 and 33‰).*



		df	SS	MS	*F*	*P*
12 days after fertilization	Total larval length					
	Temperature	1	3199121	3199121	220.262	< 2e-16
	Salinity	1	252895	252895	17.412	4.82e-05
	Jar	1	19615	19615	1.351	0.247
	Temperature:Salinity	1	3294	3294	0.227	0.635
	Residuals	168	24440059	14524		
	Larval Width					
	Temperature	1	802981	802981	177.089	< 2e-16
	Salinity	1	20426	20426	4.505	0.0354
	Jar	1	1000	1000	0.221	0.6393
	Temperature:Salinity	1	28385	28385	6.26	0.0134
	Residuals	155	702820	4534		
24 days after fertilization	Total larval length					
	Temperature	1	6045779	6045779	125.821	<2e-16
	Salinity	1	4997504	4997504	104.005	<2e-16
	Jar	1	2672	2672	0.056	0.814
	Temperature:Salinity	1	1044012	21.727	21.727	8.15e-06
	Residuals	121	5814126	48051		
	Larval Width					
	Temperature	1	928940	928940	73.783	5.48e-14
	Salinity	1	986433	986433	78.349	1.38e-14
	Jar	1	47	47	0.004	0.952
	Temperature:Salinity	1	5629	5629	0.447	0.505
	Residuals	113	1422695	12590		
32 days after fertilization	Total larval length					
	Temperature	1	2039165	2039165	21.771	7.23e-06
	Salinity	1	502980	502980	5.37	0.022
	Jar	1	5335	5335	0.057	0.812
	Temperature:Salinity	1	2016	2016	0.022	0.884
	Residuals	137	12832101	93665		
	Larval Width					
	Temperature	1	8400	8400	0.648	0.4222
	Salinity	1	169	169	0.013	0.9092
	Jar	1	10828	10828	0.835	0.3623
	Temperature:Salinity	1	45364	45364	3.500	0.0635
	Residuals	137	1775736	12962		

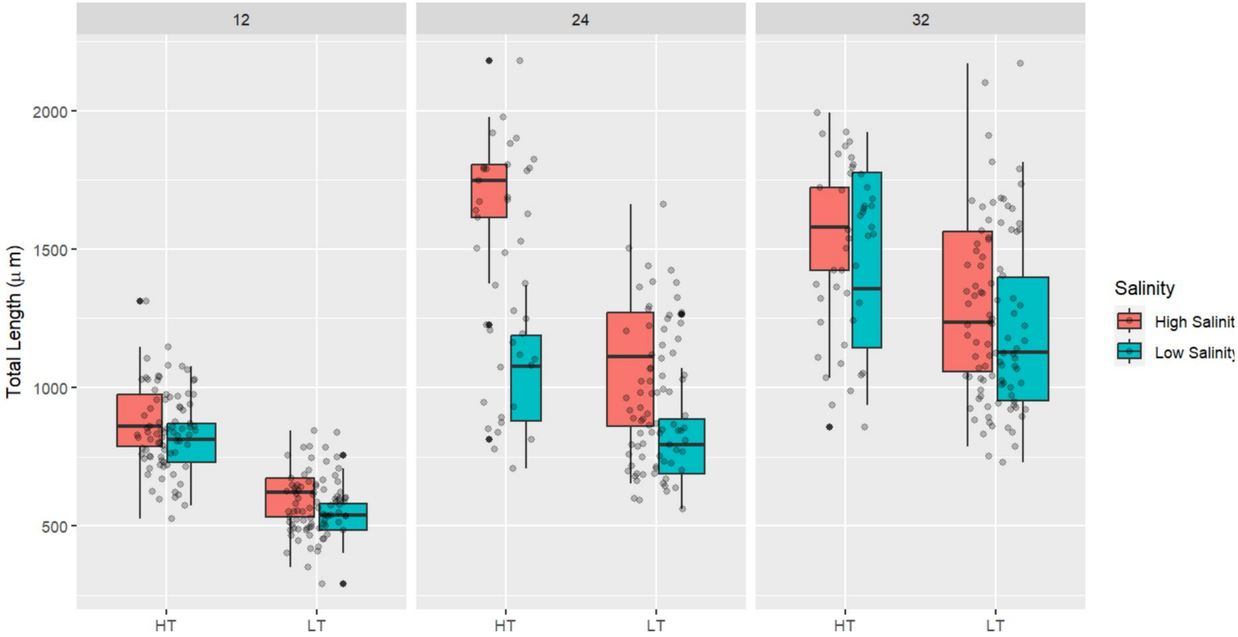


Fig. 3. Boxplots of total larval length (µm) for *Pisaster ochraceus* larvae under high temperature (HT), low temperature (LT) and at two different salinity treatments. The age of larvae measured is specified at the top of each plot (12, 24, and 32 days after fertilization). The median is represented by a bold black line in each box. Each point represents a single larval measurement.

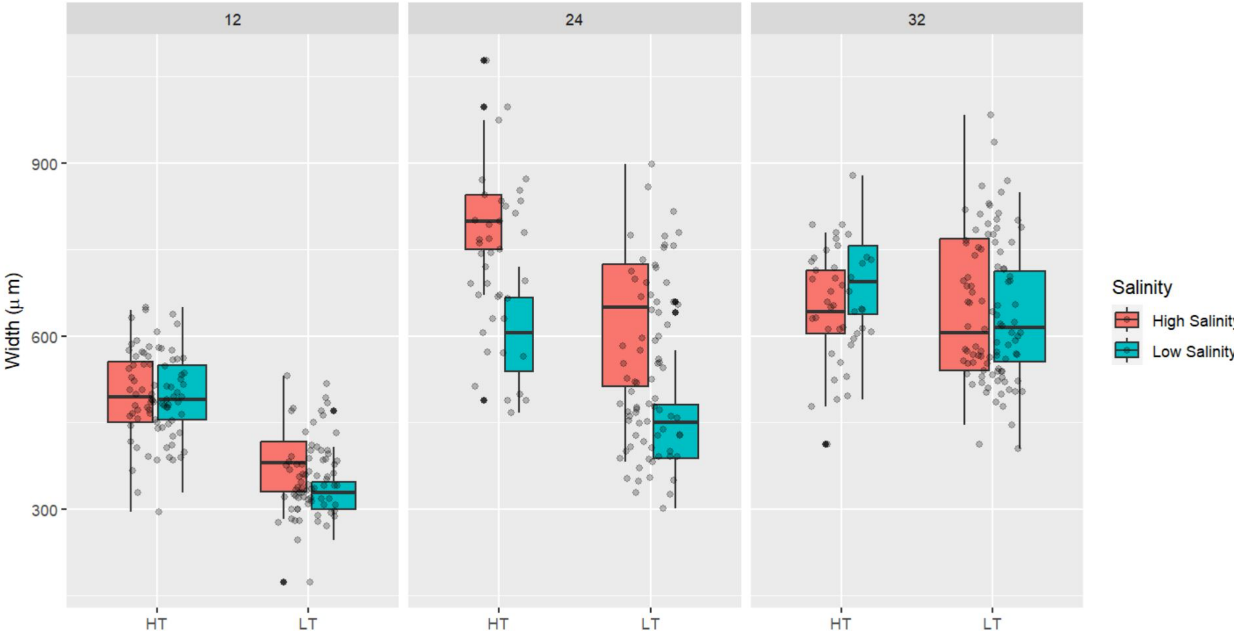
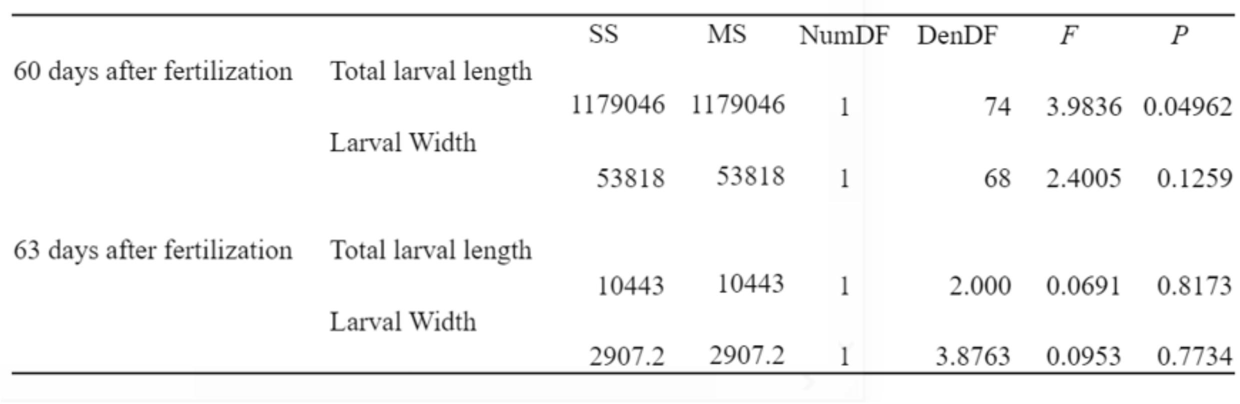


Fig. 4. Boxplots of total larval width (µm) for *Pisaster ochraceus* larvae under high temperature (HT), low temperature (LT) and at two different salinity treatments. The age of the larvae that were measured is specified at the top of each plot at 12, 24, and 32 days after fertilization. The median is represented by a bold black line in each box. Each point represents a single larval width measurement.

Pycnopodia helianthoides larvae

Temperature had a significant effect on total length of 60 day-old *P. helianthoides* larvae (Table 2, Fig. 5, F=3.9836, p=0.0496, n=76). The total larval length for 60 day old larvae in the low temperature treatment was significantly higher than for those in the high temperature treatment (Fig. 5). Temperature had no effect on the width of 60-day old larvae (Table 2, Fig. 6, F=2.4, p=0.1259, n=70). Likewise, temperature had no effect on larval length and width of 63-day old larvae (Table 2, Fig. 5, F=0.0691, p=0.8173, n=69, and F= 0.0953, p=0.7734, n=59, Fig. 6, respectively).

Table 2. Analysis of variance to determine the effect of high and low temperature on the total length and width for 60- and 63-day old *Pycnopodia helianthoides* larvae.



		SS	MS	NumDF	DenDF	*F*	*P*
60 days after fertilization	Total larval length	1179046	1179046	1	74	3.9836	0.04962
	Larval Width	53818	53818	1	68	2.4005	0.1259
63 days after fertilization	Total larval length	10443	10443	1	2.000	0.0691	0.8173
	Larval Width	2907.2	2907.2	1	3.8763	0.0953	0.7734

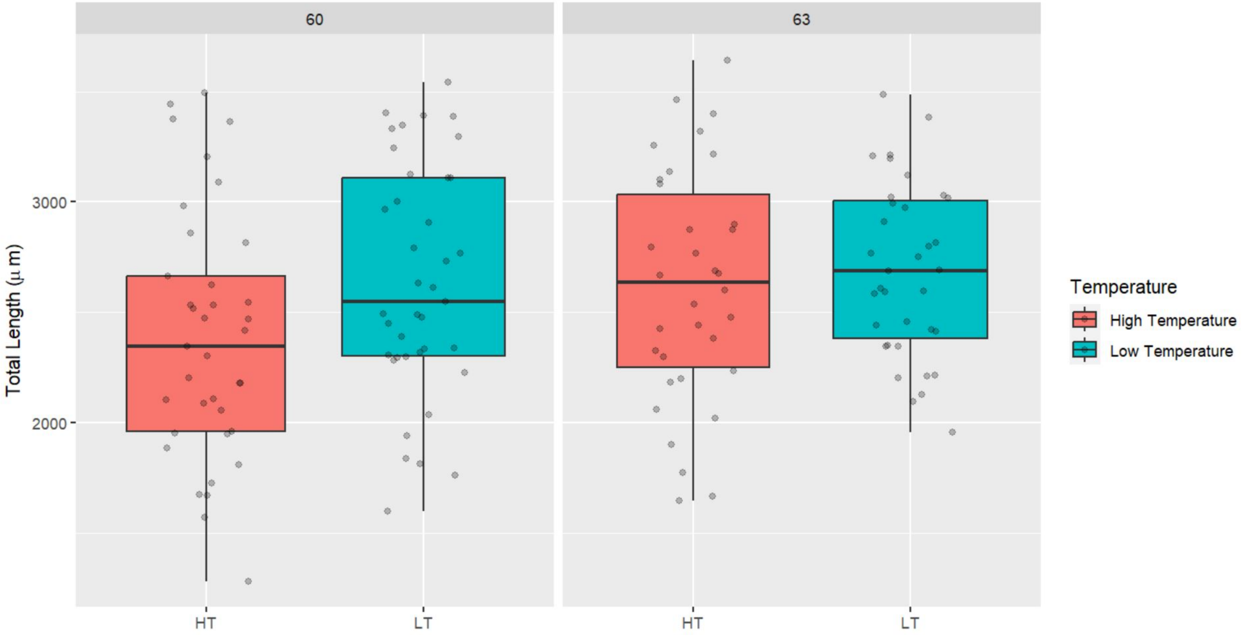


Fig. 5. Box plots of total length for *Pycnopodia helianthoides* larvae under high temperature (HT) and low temperature (LT) treatments. The age of the larvae that were measured is specified at the top of each plot at 60 and 63 days after fertilization. The median is represented by a bold black line in each box. Each point represents a single larval total length measurement.

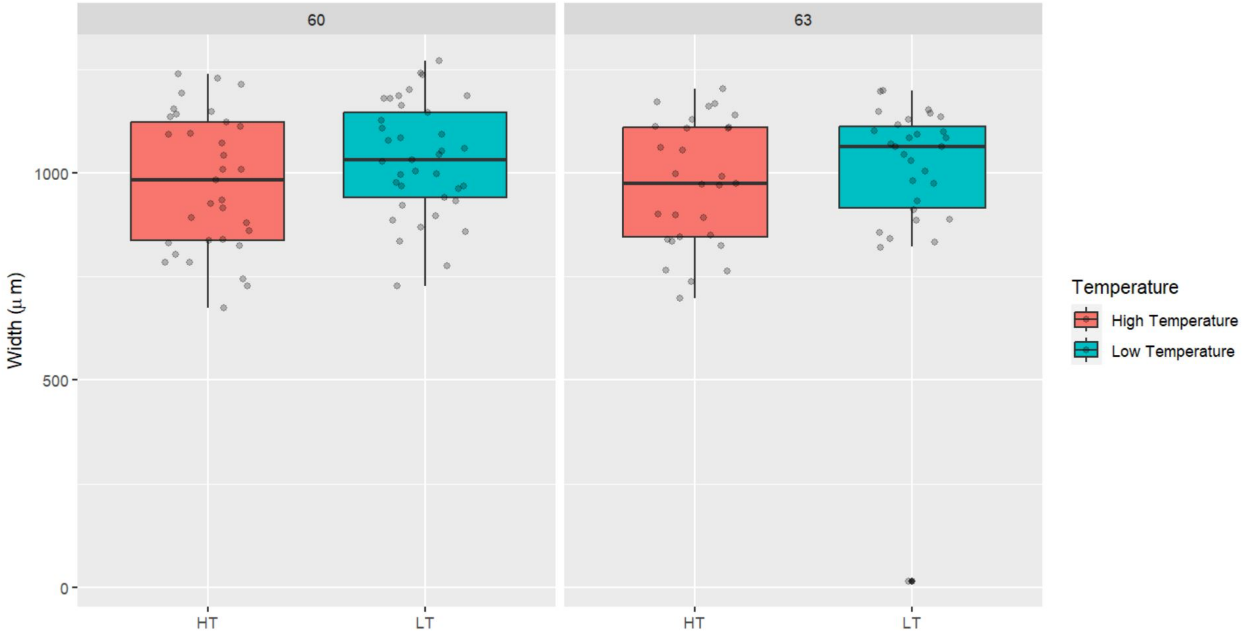


Fig. 6. Boxplots of total larval width (µm) *Pycnopodia helianthoides* under high temperature (HT) and low temperature (LT) treatments. The age of the larvae that were measured is specified at the top of each plot at 60 and 63 days after fertilization. The median is represented by a bold black line in each box. Each point represents a single larval width measure.

Discussion

The results indicate that there are significant differences among the treatments for both species. Our initial hypothesis for these experiments was that the high temperature and high salinity (HTHS) conditions would result in *Pisaster* larvae that were larger in body length and width, this hypothesis was supported by the results from the study. *Pisaster* larvae reared at high temperature and high salinity were significantly larger and wider than the other treated larvae, these findings are also supported by past studies that demonstrate similar larval growth trends occur under such conditions (Watts et al., 1982). Differences in total larval width persisted throughout development but these differences became less evident among the 32-day old *Pisaster* larvae in the various treatments.

The resultant effect of temperature on the size of 60-day old *Pycnopodia* larvae was different from our hypothesized outcome. The only significant difference in size variation of *Pycnopodia* was observed at day 60 and the significantly larger length appeared from the low temperature treatment, rather than the high temperature treatment. After 63 days there was no significant difference between the sizes of larvae from either treatment.

The variation in morphometric measurements over the course of development in *P. ochraceus* and *P. helianthoides* indicates that there may be a trend for body size to be less influenced by the abiotic environment as larvae grow. As larval *P. ochraceus* aged, variation in sizes among the treatments decreased, until the larvae shared a similar width distribution and showed much less variation in length across treatments by day 32. The decrease in size variation with age was also apparent in *P. Helianthoides*, as the differences in length between the high and low temperatures was only present at 60 days and ceased at day 63. As the larvae of both species aged and approached their settlement phase, their sizes varied much less within the experimental treatments.

As larvae age and increase in size, they become increasingly capable of ingesting particles from the water column, because of an increase in ciliated band length (George et al. 2021). It is possible that the larvae in these treatments reached a favorable size that allowed for optimal food intake and their growth rate plateaued after that point. All jars were provided with the same quantities of food in each of the experiments, and this may explain why larval sizes from the various treatments began to converge as they aged. But it is likely that confounding factors among jars could have occurred, such as differential mortality.

The tendency of larvae morphometrics to converge around a similar size range may have positive implications as global heating shows no signs of slowing. If larvae tend to result in a similar size distribution after experiencing the environmental effects of these marine heatwaves, this may indicate greater resilience to these events that we had previously thought. From a morphological development perspective, we observed that temperature and salinity changed the rate of development in larvae up until a certain age. In the context of marine heatwaves, larvae that grow at a faster rate due to increase sea surface temperatures may encounter shortages of food if the seasonal variation of plankton has not yet peaked, to feed the growing demand. A lack of food could then increase competition among larvae for resources and possibly result in mass mortality of these young larvae. Past studies have indicated that larval echinoderms can experience morphological variation due to salinity changes (Pia et al., 2012), this was also observed at various stages along the development of *P. ochraceus* during this experiment, further supporting our need to continue improving our understanding of the abiotic marine effect on larval development. To do this, future studies could be performed. These future studies may require longer durations of experimental observation where the larval development is recorded from fertilization to settlement and measured at close intervals throughout the growth period. This would provide us with a clearer timeline to view growth rate variation among all the developmental stages, possibly offering a more conclusive assessment on the observed growth trends between different treatments over time.

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