12-1-2017

Effects of Ocean Warming and Acidification on Fertilization Success and Early Larval Development in the Green Sea Urchin, Lytechinus variegatus

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Thesis of
Brittney L. Lenz

Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science

M.S. Marine Biology

Nova Southeastern University
Halmos College of Natural Sciences and Oceanography

December 2017

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HALMOS COLLEGE OF NATURAL SCIENCES AND OCEANOGRAPHY

EFFECTS OF OCEAN WARMING AND ACIDIFICATION ON FERTILIZATION SUCCESS AND EARLY LARVAL DEVELOPMENT IN THE GREEN SEA URCHIN, *LYTECHINUS VARIEGATUS*

By:

Brittney Lenz

Submitted to the Faculty of

Halmos College of Natural Sciences and Oceanography

in partial fulfillment of the requirements for

the degree of Master of Science with a specialty in:

Marine Biology

Nova Southeastern University

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Abstract

      Climate change is predicted to affect the larval stages of many marine organisms. Ocean warming can reduce larval survival and hasten larval development, whereas ocean acidification can delay larval development. Ocean acidification is especially concerning for marine organisms that develop and grow calcified shells or skeletons in an environment undersaturated with calcium carbonate minerals. This study assessed the effects of ocean warming and acidification on the fertilization and larval development of the green sea urchin, *Lytechinus variegatus*, a tropical species common in Florida and the Caribbean. After spawning, gametes were fertilized and embryos/larvae were reared at: 1) 28°C and pH 8.1 (control), 2) 28°C and pH 7.8 (ocean acidification scenario), 3) 31°C and pH 8.1 (ocean warming scenario), and 4) 31°C and pH 7.8 (ocean warming and acidification scenario). Exposure to acidified conditions had no effect on fertilization, but delayed larval development, stunted growth and increased asymmetry. Exposure to warm conditions decreased fertilization success at a high sperm to egg ratio (1,847:1), accelerated larval development, but had no significant effect on growth. Under exposure to both stressors (ocean warming and acidification), larval development was accelerated, but larvae were smaller and more asymmetric. These results indicate that climate change will have a serious impact on the larval development and growth of the green sea urchin, *L. variegatus,* and may negatively affect its persistence.

1. Introduction:

Increased levels of carbon dioxide and other greenhouse gases in the atmosphere are causing changes to global climate (IPCC 2014). Burning of fossil fuels is the main source of carbon dioxide (Houghton et al. 2001). Global atmospheric carbon dioxide levels have increased exponentially since the Industrial Revolution, surpassing 400 ppm in 2013, and are projected to continue to rise (IPCC 2014). Since carbon dioxide is a greenhouse gas, higher levels of carbon dioxide trap more heat in the atmosphere. As a result, global temperatures have increased at an average rate of 0.17°C per decade and are expected to increase further (IPCC 2014). The ocean absorbs both heat and carbon dioxide from the atmosphere, resulting in ocean warming, acidification (OAW) (Revelle and Suess 1957, Takahashi et al. 1997), and the reduction of carbonate ion concentrations (Zeebe and Wolf-Gladrow 2001, Caldeira and Wickett 2003, Orr et al. 2005, Meehl et al. 2007, Feely et al. 2009). Specifically, dissolved carbon dioxide reacts with water to form carbonic acid ($CO_2+H_2O \rightarrow H_2CO_3$). Carbonic acid then dissociates into hydrogen ions and bicarbonate ($CO_2 + H_2O \rightarrow H^+ + HCO_3^-$). The increase in hydrogen ion concentration reduces seawater pH, thus acidifying the ocean. In addition, the hydrogen ions bind to carbonate ions to create more bicarbonate ($H^+ + CO_3^{2-} \rightarrow HCO_3$). As carbonate ions become depleted, seawater becomes undersaturated in aragonite and calcite, which are calcium carbonate minerals that many marine organisms use to build their shells and skeletons (Raven et al. 2005, Dupont et al. 2010, Hofmann et al. 2010, Barton et al. 2012, Kroeker et al. 2013).

Ocean warming and acidification affect numerous marine organisms, including species that are vital to the balance and persistence of marine ecosystems. Climate models project that global ocean temperatures will rise 2-4°C over the next century (IPCC 2014). Such an increase alters survival and growth of key ecosystem species such as reef-building corals (Cantin et al. 2010, Hughes et al. 2017); estuarine clams, which control pollution by removing trace chemicals and hydrocarbon pollutants, and reduce turbidity (Kennedy et al. 1971); abalone, which are an important food source for many marine organisms (Vilchis et al. 2005), and sea urchins, which control algal growth from tropical coral reefs to kelp forests (Hughes et al. 1987, Clemente et al. 2014).

Additionally, the 0.1 to 0.4 drop in pH, projected to occur by 2100 (i.e., a greater than 30% increase in acidity, Orr et al. 2005, Meehl et al. 2007, IPCC 2014) has been shown to reduce growth and survival in key ecosystem species with calcifying structures (Orr et al. 2005), e.g., temperate sea urchins that help control algae growth on reefs (Dupont et al. 2010); reef-building corals and calcareous algae (Hoegh-Guldberg et al. 2017), and reef-inhabiting sea stars that regulate the diversity, distribution and abundance of their prey (Dupont et al. 2010). Also, increased CO_2 levels impair behaviors in predator-prey interactions in tropical gastropods (Watson et al. 2014, Watson et al. 2017), coral reef fishes (Munday et al. 2009b, Dixson et al. 2010), and tropical squid (Spady et al. 2014).

Ocean warming and acidification can affect all life stages of a species, including critical stages for recruitment and replenishment of populations, such as fertilization success and larval development. Both stressors can negatively impact the fertilization success of marine organisms with external fertilization, diminishing larval production, chances of dispersal and consequent gene flow between populations. Existent studies suggest that the success of fertilization under ocean warming and acidification conditions projected for 2100 varies considerably between species (Byrne et al. 2010, Gibson et al. 2011). For example, the tropical sea urchin *Echinometra lucunter* successfully fertilized over a wide temperature range (12-37°C, Sewell et al. 1999), while corals (Albright and Mason 2013) were less successful under warmer conditions, 30°C, versus ambient, 27°C. Reduced pH decreased fertilization success in several species of sea urchins (Havenhand et al. 2008, Moulin et al. 2011, Kapsenberg et al. 2017), oysters (Barton et al. 2012) and coral (Albright and Mason 2013).

Temperature and pH variations beyond the tolerance range of a species can have drastic effects on larval survival and development (Pechenik 1987, Hofmann et al. 2010). Both may alter larval duration and dispersal patterns, and jeopardize recruitment, replenishment, and, therefore, population persistence (Munday et al. 2008, Munday et al. 2009a). Increases in ocean temperatures can have positive or negative impacts on larval development and growth of marine species. Gibson et al. (2011) reported slower development and reduced larval growth when a temperature increase exceeded +3-4°C; however, most studies reported that warmer temperatures hasten larval development and

growth rates (O'Connor et al. 2007, Brennand et al. 2010, Byrne et al. 2013a, Figueiredo et al. 2014). The differences in susceptibility to warming likely depends on the magnitude of temperature increase tested relative to the natural thermal range of the species (Tewksbury et al. 2008). Ocean acidification also dramatically affects development and growth of larvae that have calcifying structures, including oysters (Kurihara 2008, Watson et al. 2009, Barton et al. 2012), sea urchins (Kurihara 2008, Brennand et al. 2010, Byrne et al. 2013a, Byrne et al. 2013b), brittle stars (Dupont et al. 2008), spider crabs (Walther et al. 2010), and mussels (Kurihara 2008). Larvae without calcifying structures, such as coral planula and some sea star lecithotrophic larvae, respond much more robustly to predicted near future-ocean acidification conditions during larval development (Kurihara 2008, Gibson et al. 2011, Nguyen et al. 2012, Chua et al. 2014).

Although many studies have assessed the impacts of climate change on marine species, they typically evaluate the effects of ocean warming and acidification separately, instead of in combination, as they will occur in nature. It is important to examine the combined effects of ocean warming and acidification, because they may have antagonistic effects, the effect of one stressor may prevail over the other, or their effects may be additive or even synergistic. Those studies that have assessed the combined effects of ocean warming and acidification indicate that the two stressors interact, with most species being more vulnerable to the effects of ocean acidification when the water was warmest. For example, acidified seawater marginally reduced primary polyp growth in the stony coral *Porites panamensis*, but the combination of increased temperature and lowered pH reduced the primary polyp growth by nearly one third (Anlauf et al. 2011). Rodolfo-Metalpa et al. (2011) found that acidification significantly affected the stony coral *Cladocora caespitosa* but not the coral, *Balanophyllia europaea*, or the molluscs, *Mytilus galloprovincialis* and *Patella caerulea*. However, all four species experienced an increase in mortality rates and a significant decrease in gross calcification when exposed to both acidification and warming (Rodolfo-Metalpa et al. 2011). In the Sydney rock oyster, *Saccostrea glomerata*, increased temperature and decreased pH caused decreased fertilization and embryonic development when applied separately. However, both stressors combined generated a greater decrease in fertilization and embryonic development relative to the individual effects, leading to lower survival, higher rates and

magnitude of abnormalities, and smaller sizes (Parker et al. 2009). Thus, to better predict the impacts of climate change in marine ecosystems, it is critical to assess the combined effects of ocean acidification and warming on species with key ecosystem functions (e.g., herbivory or habitat construction), particularly those in which all life stages are expected to be affected, such as sea urchins.

The study of ocean warming and acidification on sea urchins is important because these organisms control algal growth on macroalgal beds, kelp forests (Elner and Vadas 1990), and coral reefs (Hughes et al. 1987). Sea urchins are entirely marine, found around the world from the intertidal zone to the deep sea, and occur on both hard substrates and sediment bottoms (Andrew et al. 2003). Shallow-water species are most diverse in warmer environments (Schultz 2006). The sea urchin life cycle is divisible into six distinct phases: (1) fertilized egg; (2) development through blastula and gastrula to pluteus larva (when the egg nutrients are usually consumed); (3) growth and development of the planktotrophic pluteus into a mature larva; (4) development of the urchin rudiment inside the growing larva, (5) metamorphosis, and (6) growth of the juvenile urchin into a reproductive adult (Hinegardner 1969). Sea urchins are gonochoric broadcast spawners that release eggs and sperm into the water column (James and Siikavuopio 2011), usually once a year. Spawning is generally cued by seasonal and lunar cycles, temperature and other environmental factors that promote optimal larval growth and development, such as food abundance, e.g., algal blooms (Reuter and Levitan 2010, James and Siikavuopio 2011). After spawning, fertilization occurs, where the sperm penetrates the egg's membrane. The sperm and egg then fuse, and the sperm nucleus enters the egg cytoplasm, which triggers the rise of the fertilization envelope indicating successful fertilization.

Fertilization success in sea urchins, however, is dependent on many different factors such as sperm concentration, egg density, pH and paternity. Sperm concentration is a very influential factor because limiting sperm can reduce fertilization success due to lower chances of gamete encounter, whereas abundant sperm can result in polyspermy, which occurs when an egg is fertilized by more than one sperm (Levitan 2004, Levitan and Ferrell 2006). Egg density also affects fertilization because with abundant eggs, egg

competition occurs and only the larger eggs will be fertilized because they are easier for sperm to locate. With a low egg density, the eggs will again be at risk of polyspermy because there will be sperm competition over the low amount of eggs (Levitan 1993, 2004). Activation of sperm occurs by intracellular ion concentration, where the ATPase activity of sperm depends upon the pH of the medium (Christen et al. 1982, 1983). As pH increases, ATPase activity increases, however, dynein ATPase is inactive below 7.3, thus inhibiting sperm activation (Christen et al. 1982, 1983). Paternity can also impact fertilization success in that, some males may have higher quality sperm than others. Studies have shown that differences in sperm performance between individual male sea urchins can lead to variation in fertilization rates (Palumbi 1999, Levitan and Ferrell 2006, Evans et al. 2007).

After successful fertilization, cell division and cleavage of the embryo occur until the echinopluteus larval stages are reached. The echinopluteus larva is similar to the ophiopluteus larva of brittlestars (Ophiuroidea). Echinoplutei have long paired ciliated arms supported by slender calcified rods. Once the digestive tract develops, larvae become facultative feeders, which reduces the risk of starvation (Byrne et al. 2008). However, when larvae deplete their egg energetic reserves, they become obligate planktotrophs and require exogenous nutrients for further development (McEdward et al. 1997, Reitzel et al. 2005, Byrne et al. 2008). Ciliated bands along the arms function in swimming and feeding on phytoplankton and other small, suspended particles in the water column (Strathmann 1975). In the laboratory, sea urchin larvae can be reared using formulated feeds (George et al. 2004, Liu et al. 2007). With proper nutrition, the rudiment develops and, given suitable food and good environmental conditions, the larva eventually ceases swimming and undergoes metamorphosis (Burke 1980).

Sea urchins are one of the key ecosystem species that are expected to be drastically affected by ocean acidification and warming (OAW), however, only a few studies have assessed the combined effects of ocean warming and ocean acidification on their larvae. Independently, higher temperatures stimulated larval growth and development in *Tripneustes gratilla,* while increased acidity reduced larval growth and larval calcification (Brennand et al. 2010). However, when exposed simultaneously to

OAW, the negative effects of acidification outweighed the positive effects of warmer temperatures and thus larval growth rates were reduced (Brennand et al. 2010). Similar results were found for *Heliocidaris tuberculata* under conditions projected for 2100: acidification [pH 7.8] at the ambient temperature [20°C] strongly reduced larval growth and development. However, an increased temperature [24°C] facilitated larval growth at both current and future pH conditions [pH 8.1 and 7.8, respectively] (Byrne et al. 2013a).

Most studies of the effects of ocean warming and acidification on sea urchins have concentrated on temperate species, whereas tropical sea urchins are still poorly studied. As herbivores, sea urchins have a key role in the health of seagrass beds and of tropical coral reefs, where they regulate algal abundance and thus facilitate coral settlement, survival and growth (Hughes et al. 1987, Jones and Andrew 1990, Coyer et al. 1993). Tropical species are expected to be less tolerant to changes in climate, because they evolved in more stable environments than those at temperate latitudes (Pörtner and Knust 2007, Tewksbury et al. 2008). Determining the effects of OAW on the fertilization success and larval development of tropical reef-dwelling sea urchins will help predict future impacts on these ecosystems. This study evaluated the combined effects of ocean acidification and warming on the fertilization success, larval growth and development of the sea urchin, *Lytechinus variegatus*, a common species on the reefs and seagrass beds of South Florida and the Caribbean Sea. The larvae of *Lytechinus variegatus* have previously been cultured through metamorphosis fed either the algae *Dunaliella tertiolecta* or an artificial feed (George et al. 2000, George et al. 2004). Despite undergoing metamorphosis at the same time, larvae fed the artificial diet (which is easier, as it does not require culturing phytoplankton) initially had slower growth rates than with the algae. Thus, this study compared larval growth and development using the same artificial feed (EZ Larva) with a previously untested and commercially available algal paste (*Nannochloropsis* sp.), which also does not require culturing phytoplankton.

2. Objectives:

This study investigated the combined effects of ocean acidification and warming (OAW) on the fertilization success and larval development, growth and survival of *Lytechinus variegatus*. Specifically, by:

- evaluating fertilization success under control, acidification and warming conditions;
- determining the effect of OAW on larval growth and morphology (total body length, body width, arm rod length, and asymmetry between rods);
- assessing larval development under OAW (i.e., duration of each developmental stage);
- and measuring larval respiration under warm conditions.

In an attempt to improve the larval rearing methodology of the species, larval growth and development of *L. variegatus* using an artificial feed (EZ Larva) was also compared with a previously untested, commercially available algal paste (*Nannochloropsis* sp.).

3. Methods:

3.1 Species Description and Specimen collection

The green sea urchin, *Lytechinus variegatus* (Figure 1), is common throughout the Western Atlantic and Caribbean (Watts et al., 2001). It is found in shallow waters, typically on seagrass beds, rocky substrates or reefs, and feeds on algae and seagrasses (Watts et al., 2001, Schultz, 2006). Urchins are sexual mature at 40 mm test diameter, but adults can reach 110 mm across (Moore et al., 1963). Both fertilization and larval development of *Lytechinus variegatus* have not been previously studied under combined OAW conditions.
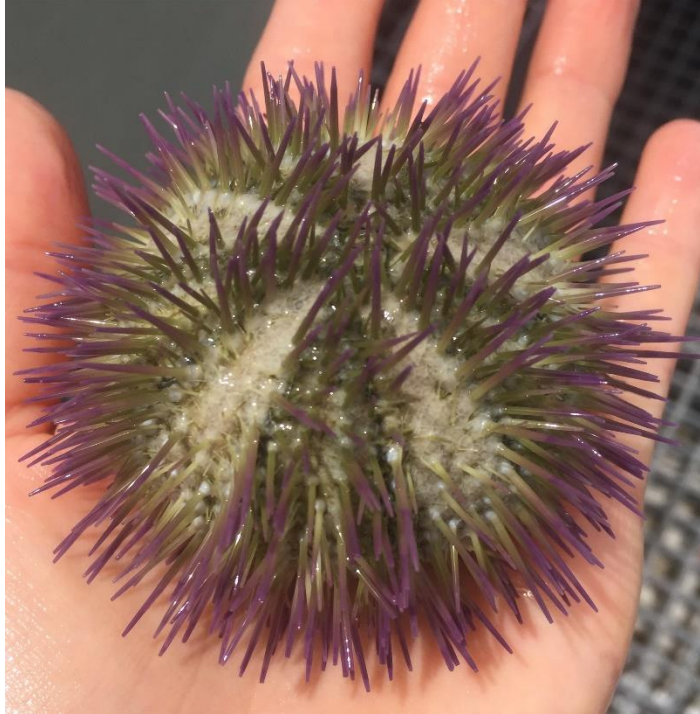
Figure 1: Green sea urchin, *Lytechinus variegatus*

Specimens were collected during high tide at Blue Heron Bridge in Riviera Beach, Florida (26°46'58.3"N, 80°02'37.0"W), during shore dives using SCUBA. For each trial, 50 individuals were collected to ensure that enough males and females were available for successful fertilization (under Permit SAL-17-1902-SRP). Only sea urchins >40 mm across were collected to maximize the likelihood of selecting reproductive individuals. Upon collection, individual sea urchins were placed in mesh bags underwater. Once on shore, the mesh bags containing sea urchins were placed into a cooler with seawater to be transported back to Nova Southeastern University's Guy Harvey Oceanographic Center (NSU GHOC). Temperature, salinity, and pH were measured onsite at Blue Heron Bridge. At NSU GHOC, the sea urchins were placed into a 379-L recirculating tank and maintained at conditions that mimicked the natural environment (28°C, 35 ppt). Specimens were fed macroalgae (*Caulerpa* sp. and turf

algae) while in captivity. After spawning, adults were returned to the collection site.

Figure 2: Map of collection site (red star) at Blue Heron Bridge.

3.2 Experiment 1: Effect of ocean warming and acidification on Fertilization

Spawning and fertilization followed methods in Levitan et al. (1991, 1992) and Levitan (2000). Individuals were removed from the tank and injected with 0.5 mL of 0.55M KCl on each side of the mouth. The K^+ ions in the KCl solution depolarized the muscles surrounding the gonads causing them to contract and release their gametes (Levitan et al. 1992). After injection, the sea urchins were gently rocked back and forth to mix the KCl solution. Then, they were placed mouth-side down onto a petri dish. After five minutes, the gametes of some sea urchins started to extrude from the gonopores on the aboral surface, and the sex of the individual was determined. Sperm was collected with a pipette without seawater and placed on a watch glass dish over ice to maximize viability. Eggs were collected by placing a sea urchin aboral side down over a petri dish with seawater so that the eggs would fall into the seawater and settle on the bottom. Released eggs were then poured into a large beaker to create an egg stock solution. Sperm was rinsed off the watch glass with sea water and into a jar collectively to create a

sperm stock solution. Three samples from the egg (1 mL) and sperm (0.1 µL) stock were collected and the number of gametes counted to estimate gamete density in each stock solution, and the final sperm:egg ratio. The egg and sperm stocks were then divided evenly among twelve 500-mL mason jars, three per treatment. Each mason jar contained 100 mL of filtered seawater with pH and temperature adjusted to represent each experimental treatment: 1) 28°C and pH 8.1 (control-ambient), 2) 28°C and pH 7.8 (ocean acidification treatment - OA), 3) 31°C and pH 8.1 (ocean warming treatment - OW), and 4) 31°C and pH 7.8 (ocean warming and acidification treatment - OAW). All non-control treatments represent conditions projected for 2100 (IPCC 2014). Mason jars were placed in a water bath equipped with a heater and controller that maintained experimental temperatures. The pH was maintained by bubbling CO_2 into the mason jars using a pH controller. There was no evidence that bubbling CO_2 had any effect on sperm and egg contact.

After an hour of exposure, two 1-mL samples of eggs per replicate jar were observed under a microscope to check for the presence of the fertilization envelope, and the ratio of fertilized eggs to total number of eggs was recorded. This experiment was repeated following the same methodology, but including an extra temperature (33°C) in an attempt to observe a further trend in the effects of elevated temperature on fertilization success (Trial 2). Again, the temperatures of 28°C and 31°C were crossed with pH of 8.1 and 7.8 with 3 replicates each. The additional temperature of 33°C was only tested for ambient pH (8.1), but also had 3 replicates. The larvae resulting from Trial 1 and 2 of fertilization were used in the various subsequent experiments as indicated in Table 1.

Table 1: Indicates the experiments in which the larvae from each Fertilization Trial were utilized.

Fertilization:	Trial 1	Trial 2
Larvae used in Experiment:	Experiment 2: Larval Growth and Development	Experiment 4: Respiration
Experiment 3 - Diet:	EZ Larva	*Nannochloropsis* Instant Algae

3.3 Experiment 2: Effect of ocean warming and acidification on larval growth and development

The embryos from the first fertilization trial were used to test the effect of warming and acidification on larval development and growth. Specifically, embryos from each fertilization experimental treatment were moved to a larval culture tank with the same pH and temperature conditions. Embryos were therefore reared throughout their early larval development stages in an indoor system of 11.35-L plastic cylindrical tanks at: 1) 28°C and pH 8.1 (control-ambient), 2) 28°C and pH 7.8 (OA), 3) 31°C and pH 8.1 (OW), and 4) 31°C and pH 7.8 (OAW). Each treatment was replicated in three tanks (Figure 3). Each tank was subject to constant moderate aeration using a 120-V Danner air pump to maintain a constant water flow.
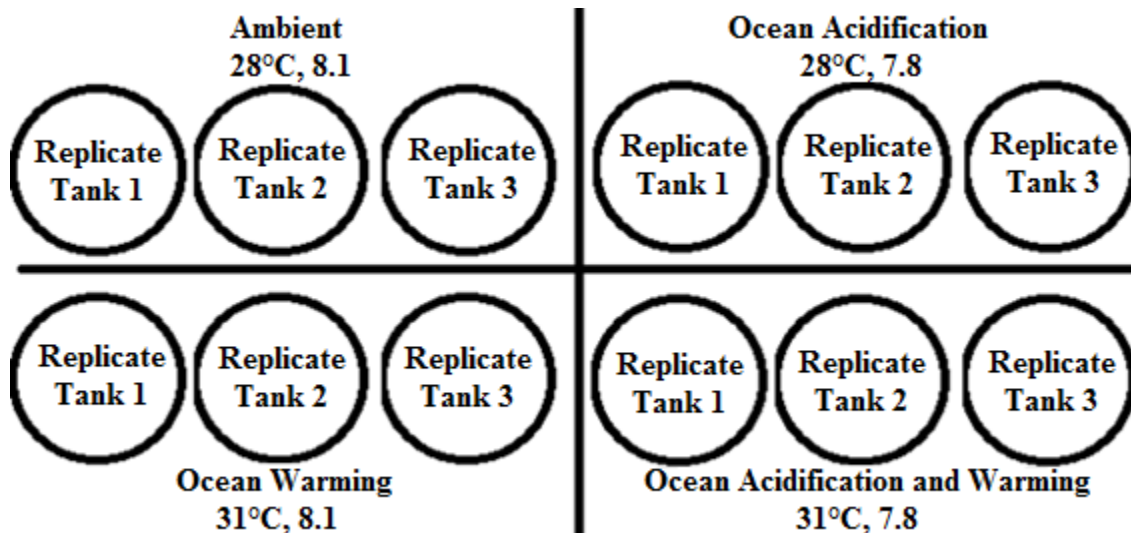
Figure 3: Experimental setup showing placement of replicates (tanks) and the conditions of each treatment.

The temperature of each tank was maintained within 1°C of the desired temperature using heaters connected to temperature controllers. Room temperature was controlled by air conditioning set below the tested temperatures. All tanks were supplied with 1-µm filtered and sterilized seawater. Tanks subject to reduced pH (7.8) had a pH probe connected to an American Marine Pinpoint pH controller and a CO_2 tank. The pH in the tanks was maintained using a pH controller and kept within 0.05 units of the desired pH. Temperature, pH and salinity from each tank were measured daily to ensure

accurate conditions. Salinity was maintained at 35 ppt by adding reverse osmosis water as needed. Ammonia was checked every day. Elevated levels of ammonia (>0.10 ppm) indicating poor water quality were recorded every other day, thus, water changes were performed on day 3 and every other day thereafter by gentle filtration using a 25-μm mesh filter. There was no evidence of damage to larvae due to filtration. The larvae were fed Zeigler Feeds EZ Larva daily beginning day 2 post-fertilization (George et al. 2004).

Larvae were sampled daily from day 1 post-fertilization until day 5, and then every other day until no larvae remained. Subsamples of 10 larvae were randomly selected from each replicate of each treatment and were used to measure development and growth. The tanks were homogeneous due to bubbling air creating constant water flow and samples were taken from various depths of the tank. Each subsample was placed onto a Sedgewick-Rafter counting cell microscope slide (1 mL) and development stages of all larvae were recorded. A minimum of ten individuals from each treatment were randomly selected and photographed under the microscope using a LC20 Olympus Camera. The photos were analyzed in the program CellSens to determine the developmental stage (Mazur et al. 1971, McEdward and Herrera 1999) and measure total body length, rod length, and body width (Figure 4, following Brennand et al. 2010, Stumpp et al. 2011, Byrne et al. 2013). Total body length was measured from the base of the body to a line drawn between the rod tips so that the intersection was perpendicular. Length of both lateral rods was measured from the rod tip to the center of the bottom of the larva. Body width was measured across the body immediately above the gut (Dorey et al. 2013). Asymmetry between the two rod lengths of each larva was determined as (modified from Lamare et al. 2017):

$$\frac{|\text{ rod length 1 (mm)} - \text{rod length 2 (mm)}|}{\text{maximum rod length (mm)}}$$

Figure 4: Echinopluteus larval growth measurements: total body length, length of each rod and total body width.

3.4 Experiment 3: Effect of diet on larval development and growth

To determine the effect of diet on larval development and growth, development of larvae (from the first fertilization trial) reared in ambient conditions and fed with EZ Larva feed (particles 10-50 µm) was compared with larvae from the second fertilization trial reared also at ambient conditions, but fed Instant Algae® (*Nannochloropsis* sp.). Specifically, embryos from the second trial were fertilized in ambient conditions, placed into three replicate tanks and reared at 28°C and pH of 8.1. In the EZ Larva (10-50 µm)

diet treatment, larvae were fed a 10-fold seawater-diluted solution. Feeding began day 2 post-fertilization when feeding appendages were apparent. Larvae were fed 1.5 mL of feed from days 2 to 7. From day 8 until no larvae remained, larvae were fed 3 mL of feed daily. In the Instant Algae® (*Nannochloropsis* sp.) diet treatment, the larvae were fed 1 mL of the paste from days 2 to 4, and 0.5 mL from days 5 to 11, due to mass mortality and poor water quality, which began on day 3. Larval growth and developmental stages were assessed as described for Experiment 2.

3.5 Experiment 4: Effect of ocean warming on Larval respiration

Evaluation of respiration under the different experimental temperatures (28° and 31°C, 6 replicates per treatment) followed methods adapted from Edmunds et al. (2011). Specifically, two days after fertilization, two 200-mL samples were taken from the larval culture tanks kept at ambient conditions (28°C, pH 8.1). These samples were placed in glass jars with one heated to 28°C and the other to 31°C, using a water bath equipped with heaters and controllers. To minimize potential measurements errors, including oxygen consumption by the oxygen probe, two glass jars containing only seawater were also kept in the 28 and 31°C water baths. All jars were aerated in order to saturate the samples with oxygen. All samples were given 2 h to acclimate to the temperature of the water bath. After the acclimation period, three 1-mL subsamples from each jar containing larvae, and one 1-mL sample from each jar containing only saltwater (blank), were put into Wheaton vials, each with an oxygen sensor spot (SP-PSt3-NAU, PreSens) glued to its inside wall prior to the start of the experiment. Each vial was topped off with saltwater and sealed with Parafilm™ to ensure that no air was contained within.

To measure initial oxygen concentration, a polymer optical cable (POF, PreSens) was used to scan the oxygen sensor spot in the vial. The POF measures oxygen saturation (% O_2) by transferring a light from the cable to the sensor and back to the Fibox 4® meter (PreSens). The oxygen concentration of each vial was then recorded, and each vial was returned to the water bath at the designated temperatures. The vials were swirled every 5 min to keep larvae from settling and to ensure a homogenous sample. After 1 h, all vials were removed to re-measure oxygen concentration. Each sample containing

larvae was then placed onto a Sedgewick-Rafter slide (1mL) and larvae were counted under a dissecting microscope and counts recorded.

Oxygen consumption was calculated by subtracting the final from the initial oxygen concentration. These measurements were adjusted by subtracting the oxygen consumption in the blank sample, thereby accounting for the amount of oxygen lost to the oxygen probe or diffusion of oxygen within the vial. Then, oxygen consumed was divided by the number of larvae per vial and experimental duration to determine how much oxygen was consumed per larvae per minute.

3.6 Data Analysis

To determine the effect of temperature and pH (fixed factors) on fertilization success, a factorial analysis of variance (ANOVA) was used. To assess the effect of time, temperature and pH (fixed factors) on larval growth (total body length, rod length, body width, and the asymmetry between rods), factorial ANOVAs were used. To describe larval development and how it differed among treatments, descriptive statistics of the proportion of larvae in each development stage in each treatment over time were applied. To assess the effect of time and larval diet (fixed factors) on larval growth (total body length, rod length, body width, and the asymmetry between rods), factorial ANOVAs were used. To assess the effect of temperature (fixed factor) on respiration rate, a two independent samples t-test was used. The statistical software R was used to conduct all statistical analysis.

4. Results

4.1 Experiment 1: Fertilization Success

Trial 1: Of 50 sea urchins, nine males and nine females spawned. Egg and sperm stock concentrations were 6,441 eggs mL^{-1} and 11.791 x 10^6 cells mL^{-1}, respectively, with a ratio of 1,847 sperm cells:1 egg. Fertilization success was significantly affected by temperature (p = 3.35x10^{-5}) but was not significantly affected by pH (p = 0.11). The interaction between temperature and pH among treatments was not significant (p = 0.12). Fertilization success was much lower at the higher temperature (31°C, OW and OAW treatments) than at current temperature (28°C, ambient and OA treatments, Figure 5):

88.4±1.4 %, 88.4±1.4 %, 72.4±5.5 %, 81.5±1.7 %, respectively for ambient, OA, OW and OAW treatments.

Trial 2: Of 50 sea urchins, 13 males and nine females spawned. Egg and sperm stock concentrations were 3,821 eggs mL^{-1} and 29.031 x 10^6 cells mL^{-1}, respectively, with a ratio of 7,896 sperm cells:1 egg, a high sperm to egg ratio. Fertilization success in this trial was not significantly affected either by temperature (p = 0.84), pH (p = 0.49), or the interaction between the two (p=0.29). However, Trial 2 had higher fertilization success rates than in Trial 1 (Figure 5): 97.8±0.3 %, 95.4±1.7 %, 97.9±0.7 %, 97.0±1.8 %, respectively for ambient, OA, OW and OAW treatments.
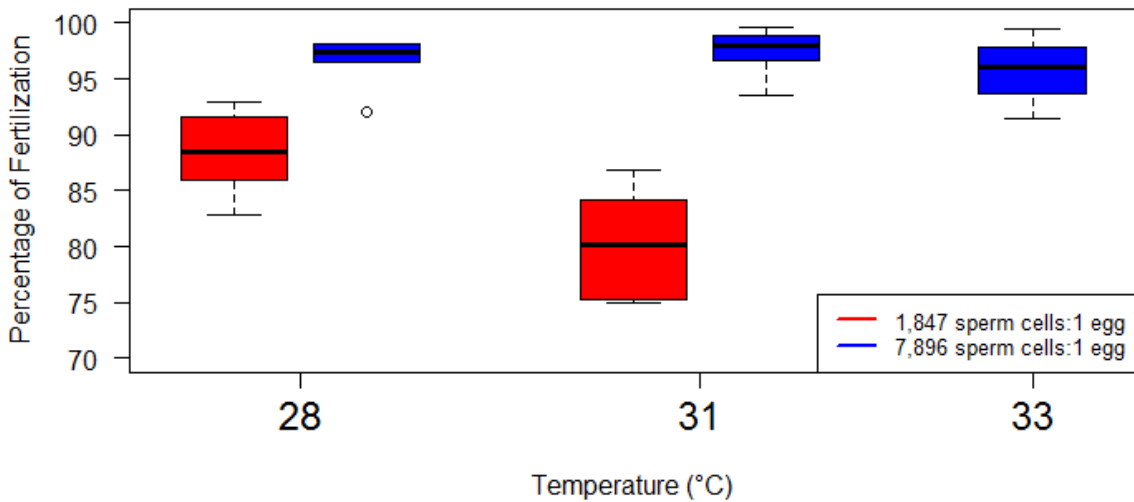

Figure 5: Fertilization success of Trial 1 (red) and 2 (blue) for each temperature (note: since pH did not have a significant effect on fertilization success, the data was pooled).

4.2 Experiment 2: Larval growth and development

Data for larval growth and development was collected up to day 13. The larvae in the ambient and OA treatments survived past day 13. However, samples collected on day 15 included no living larvae. Prior to day 13, all larvae in the OW and OAW treatments had died, as well as the larger and more developed larvae in the ambient and OA treatments; only smaller and less developed larvae were still alive on day 13.

Total body length of larvae differed significantly over time (p< 2.2x10^{-16}) and with pH (p= 4.42x10^{-16}), but not with temperature (p= 0.10). All interactions between

time, temperature and pH were not significant (p= 0.71, p=0.34, p=0.91, p=0.05, respectively, for Time x Temperature, Time x pH, Temperature x pH and Time x Temperature x pH). Under all treatments, total body length of larvae increased over time (Figure 6). Larvae reared under lower pH conditions (OA and OAW treatments) had smaller total body length than larvae reared under current pH conditions (ambient and OW treatments) (Figure 6).
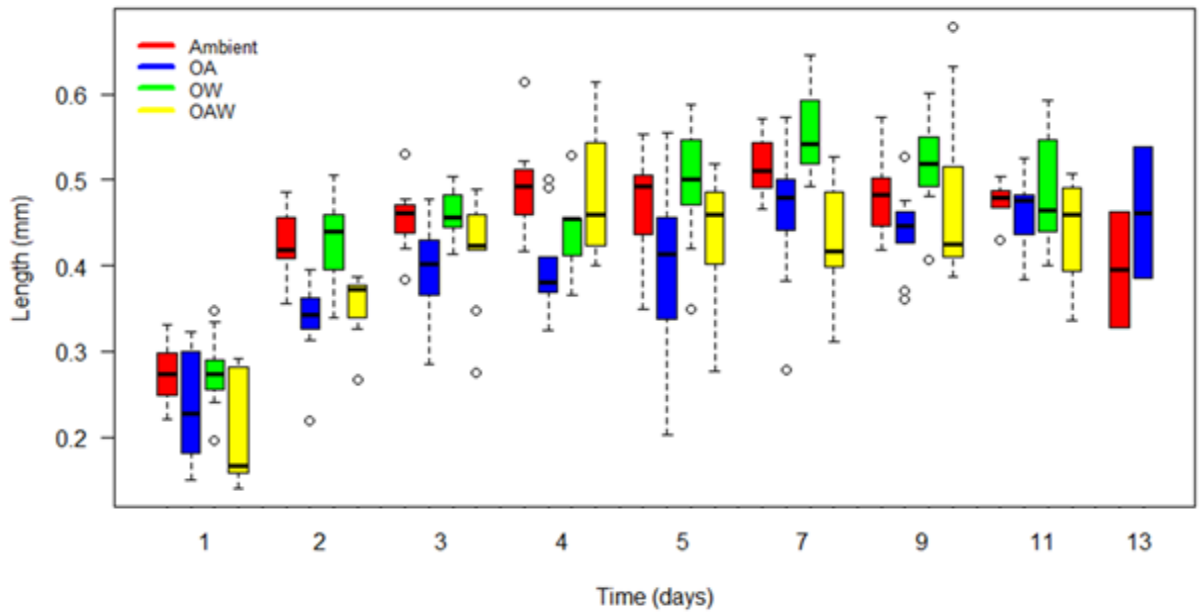

Figure 6: Total body length (mm) for each treatment over time (days).

Average rod length of larvae changed significantly over time (p<2x10^{-16}) and with decreased pH (p<2x10^{-16}), but not with temperature (p= 0.37). All interactions between time, temperature and pH were not significant (p= 0.81, p=0.79, p=0.89, p=0.20, respectively for Time x Temperature, Time x pH, Temperature x pH and Time x Temperature x pH). Rod length increased over time in all treatments (Figure 7). However, average rod length was small in larvae reared under decreased pH conditions (OA and OAW) than in those reared under current pH conditions (ambient and OW) (Figure 7).
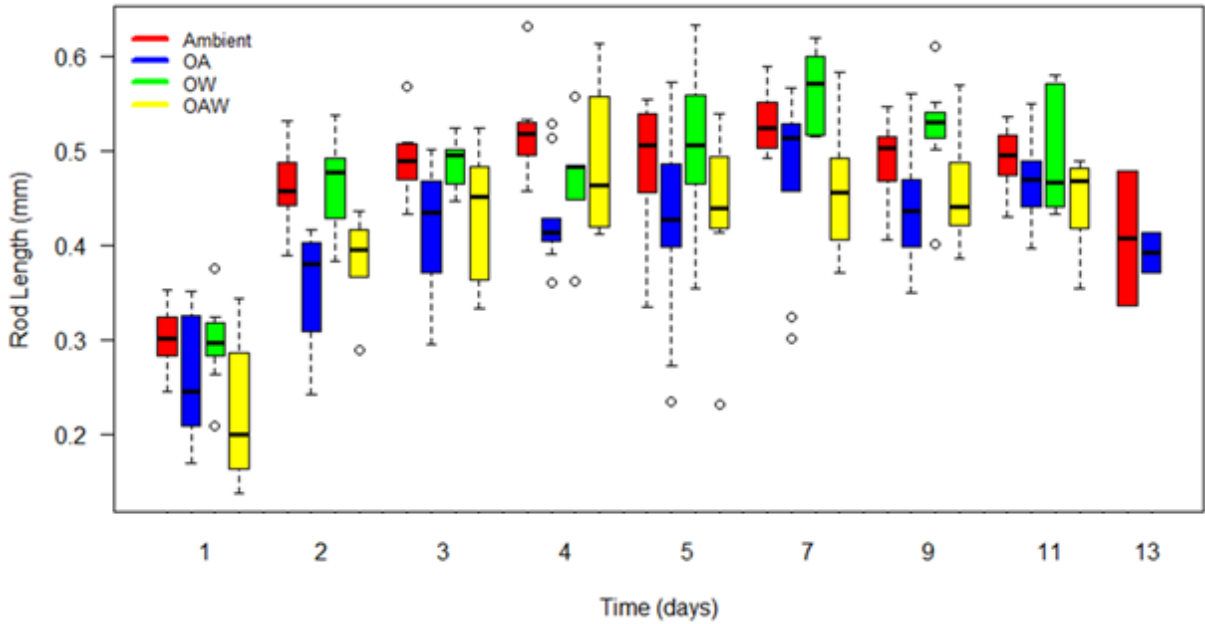
Figure 7: Average rod length (mm) for each treatment over time (days).

Total body width of *L. variegatus* larvae changed significantly over time (p= 7.09×10^{-12}) and with temperature (p= 9.86×10^{-3}), but not with pH (p= 0.53). The interaction between time and temperature was significant (p= 0.04); however, all other interactions between time, temperature and pH were not significant (p= 0.14, p=0.78, p=0.39, respectively for Time x Temperature, Time x pH, Temperature x pH and Time x Temperature x pH). Under warmer conditions (OW and OAW), average total body width of larvae after day 4 was higher than in larvae reared under current temperatures (ambient and OA) (Figure 8).
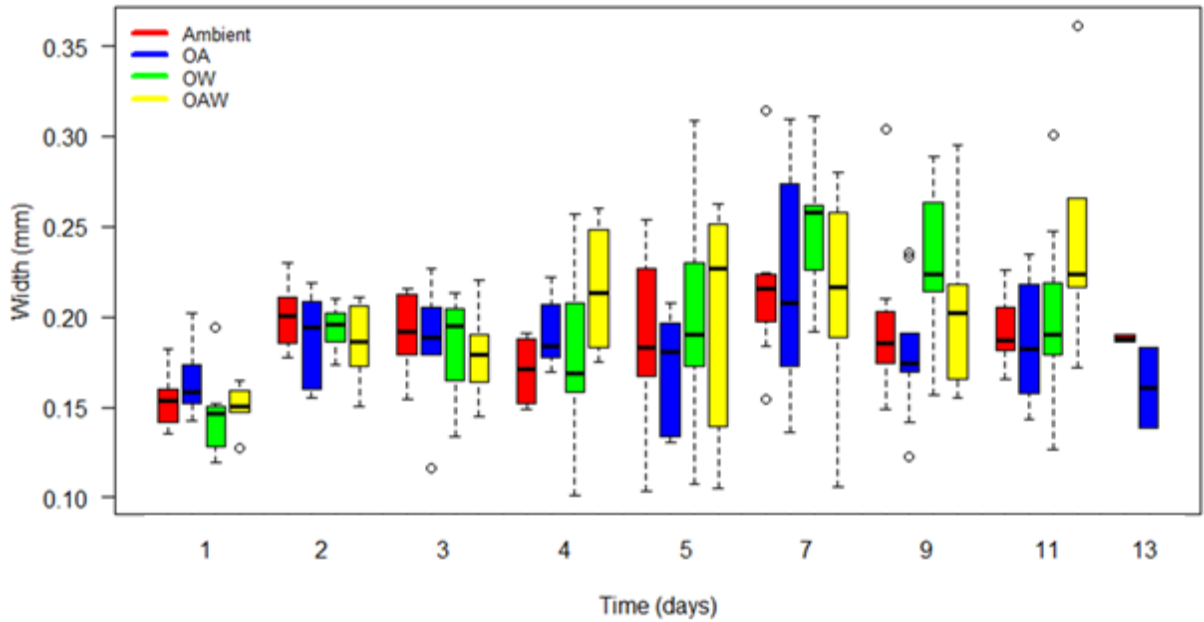
Figure 8: Total body width (mm) for each treatment over time (days).

Asymmetry between rods in *L. variegatus* larvae increased significantly over time (p= 0.01) and with decreased pH (p= 2.28×10^{-4}), but not temperature (p= 0.36). All interactions between time, temperature and pH were not significant (p= 0.60, p=0.34, p=0.50, p=0.88, respectively for Time x Temperature, Time x pH, Temperature x pH and Time x Temperature x pH). Larvae reared under low pH (OA and OAW treatments) were significantly more asymmetric than larvae reared under current pH (ambient and OW treatments, Figure 9).
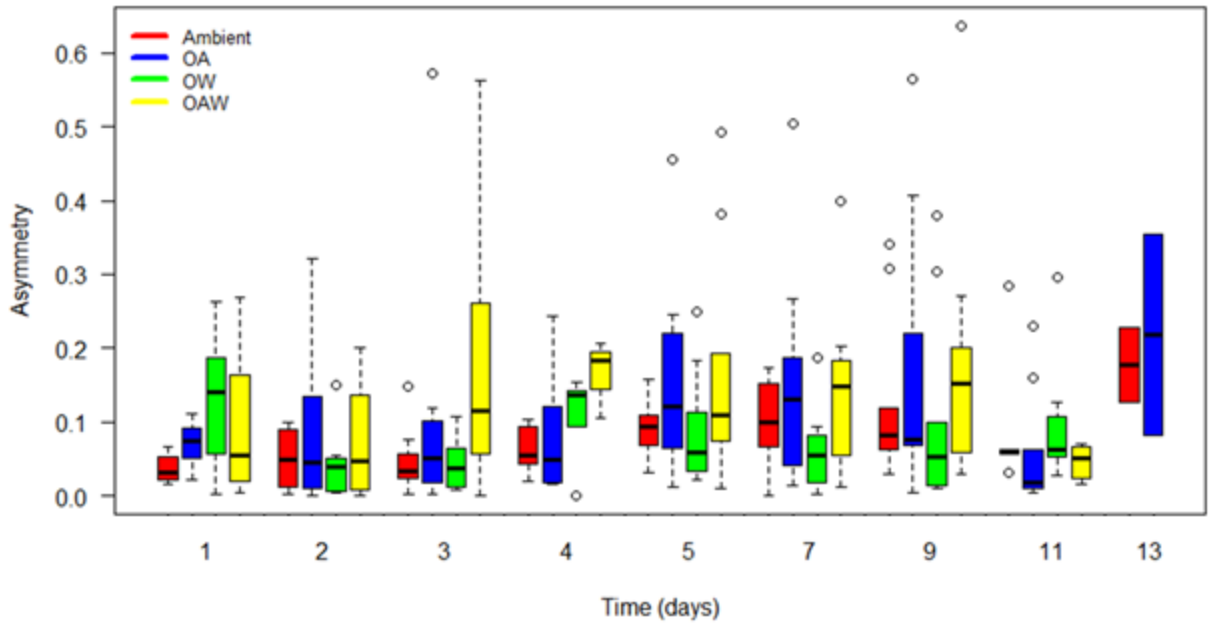
Figure 9: Larval asymmetry in each treatment over time (days).

At 28°C and pH 7.8, larval development of *L. variegatus* (Figure 9) was delayed, as reflected by the greater variation in development 1-day post-fertilization, and because this treatment had more larvae in earlier stages of development than in the ambient treatment (Figure 10). At 31°C (OW and OAW treatments), larvae exhibited minor delays in development on day 2 but clearly increased development starting on day 5 relative to ambient (Figure 10), suggesting that increased temperature accelerated development. Development did not differ substantially between OW and OAW treatments (Figure 10). Thus, increased temperature appears to outweigh the negative effects of pH on development.
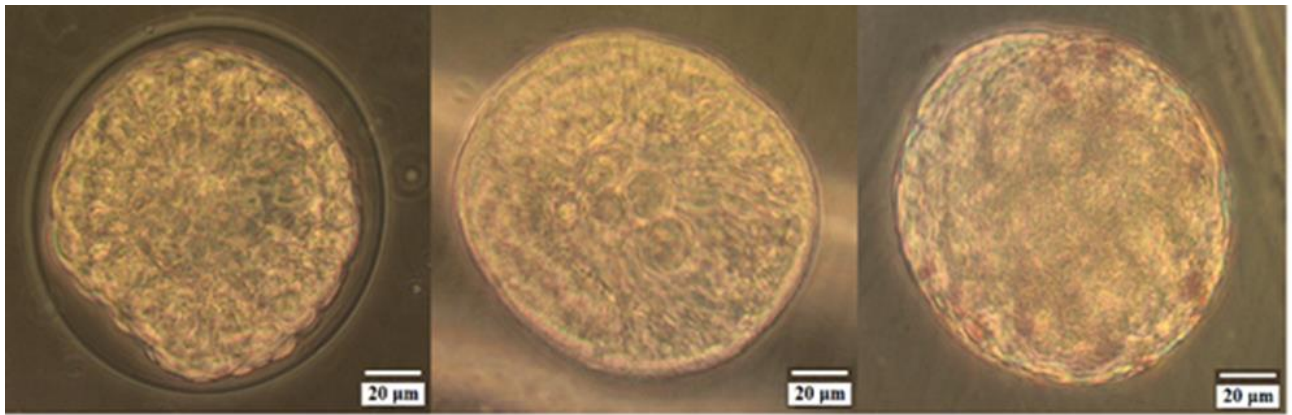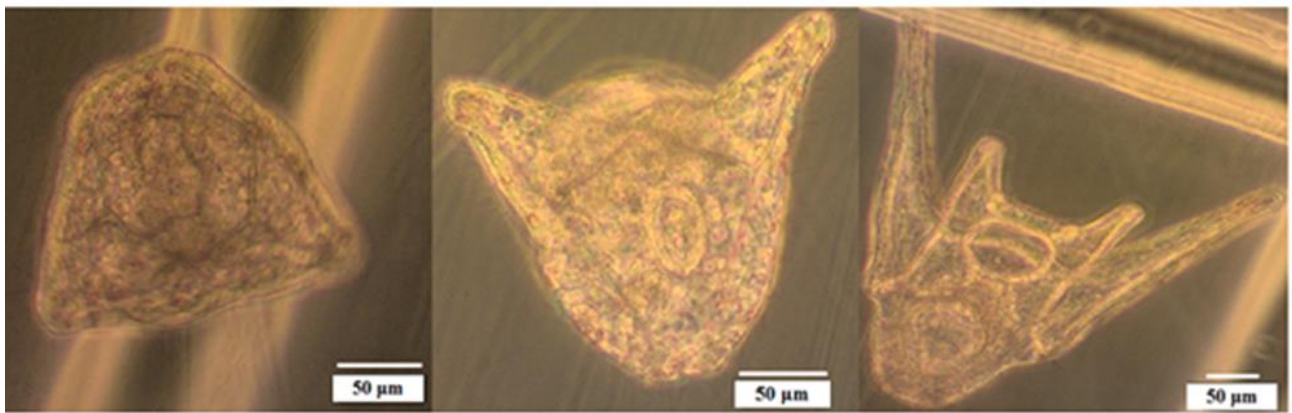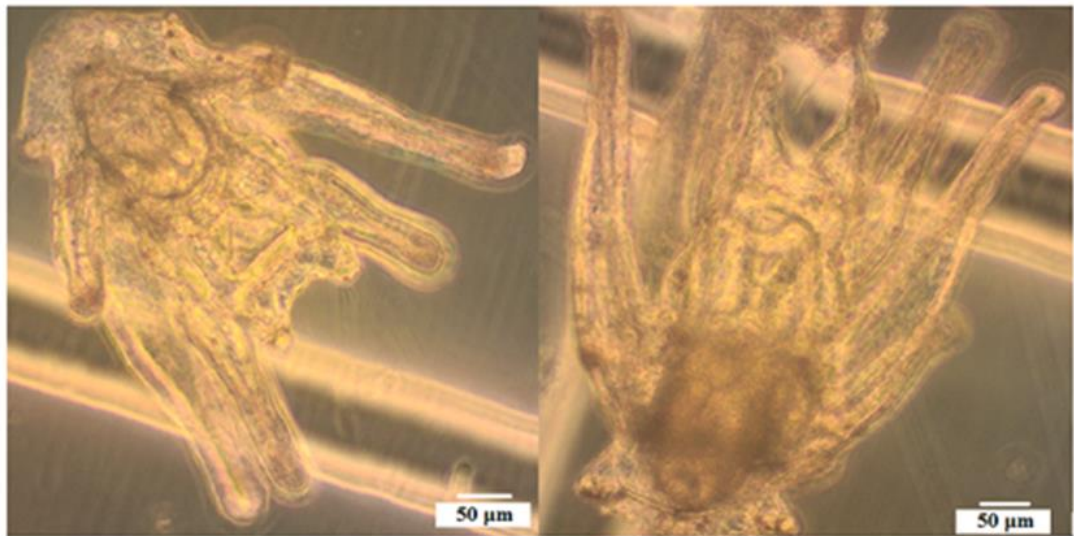
Figure 10: Developmental stages of *L. variegatus* echinopluteus larvae.
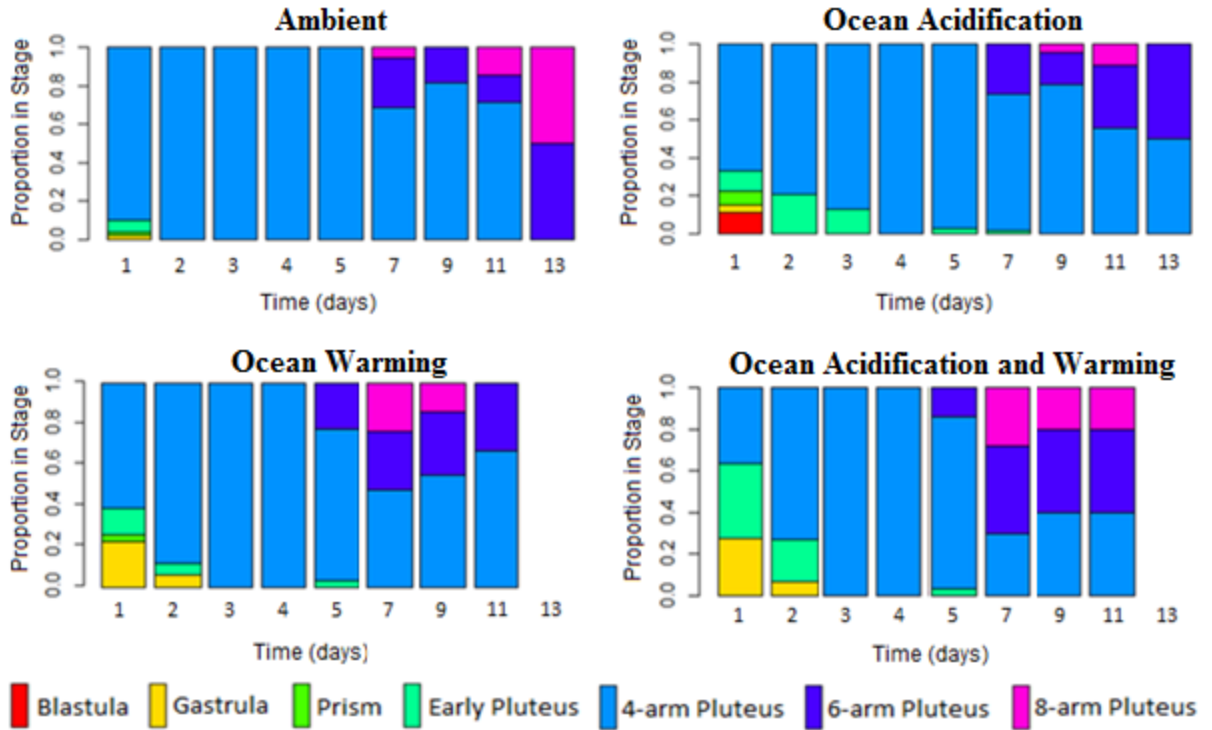
Figure 11: Larval Development of *L. variegatus* larvae displayed by proportion of larvae in each stage over time (days) for each treatment.

4.3 Experiment 3: Larval Diet

Larvae fed *Nannochloropsis* Instant Algae did not survive past day 10, whereas those fed EZ Larva survived until day 13. Diet significantly affected total body length ($p=1.59 \times 10^{-10}$), average rod length ($p=2.21 \times 10^{-11}$) and total body width ($p=2.22 \times 10^{-13}$). Specifically, larvae fed EZ Larva grew significantly larger in terms of total body length, rod length and body width than when fed *Nannochloropsis* Instant Algae (Figure 11). Larvae fed *Nannochloropsis* Instant Algae did not develop past the 4-arm pluteus stage (Figure 12).
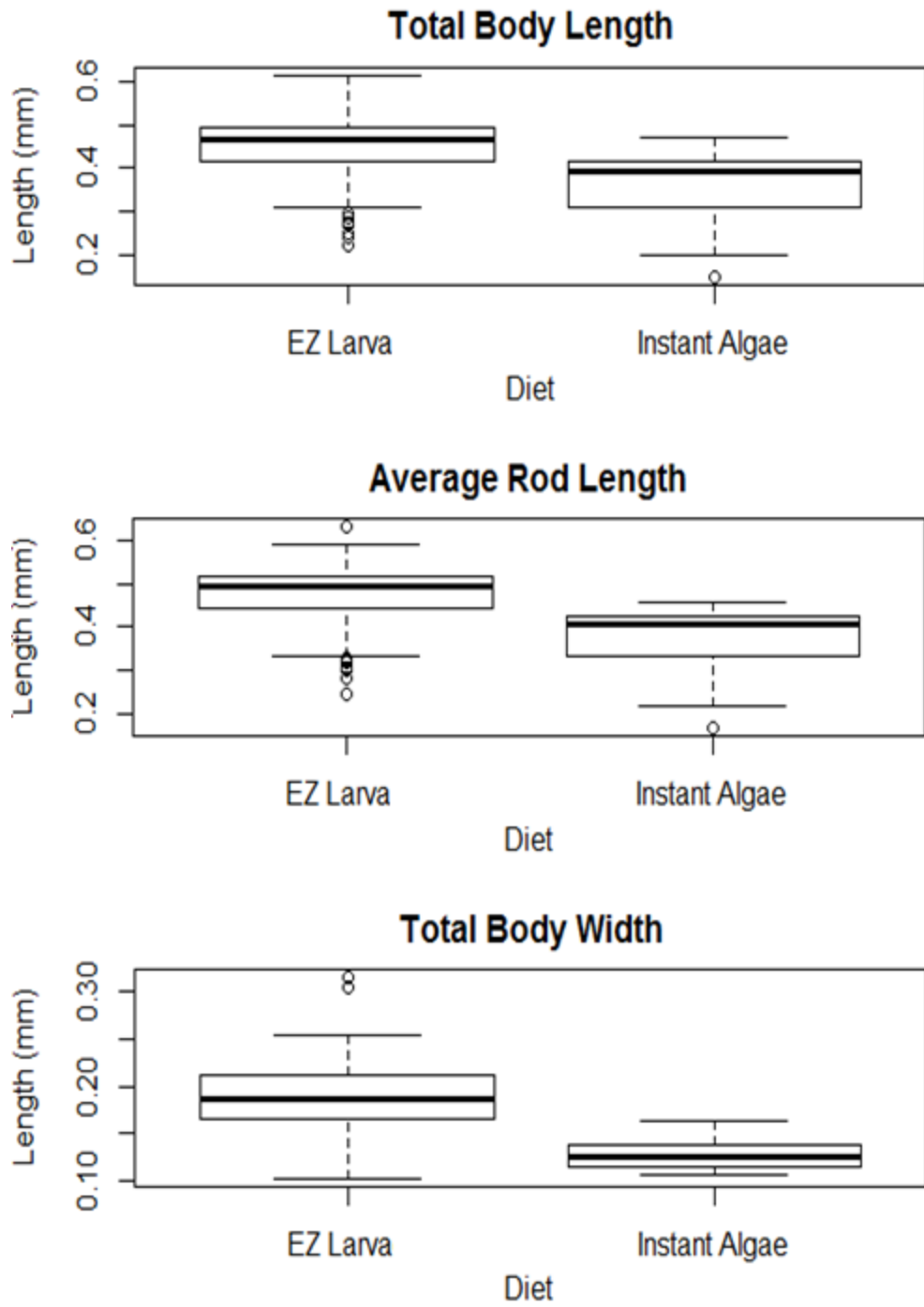
Figure 12: Total Body Length, Average Rod Length and Total Body Width of *L. variegatus* larvae fed two different diets.

EZ Larva   *Nannochloropsis* Instant Algae

50 μm   50 μm

Day 9: 6-arm Pluteus   Day 9: 4-arm Pluteus
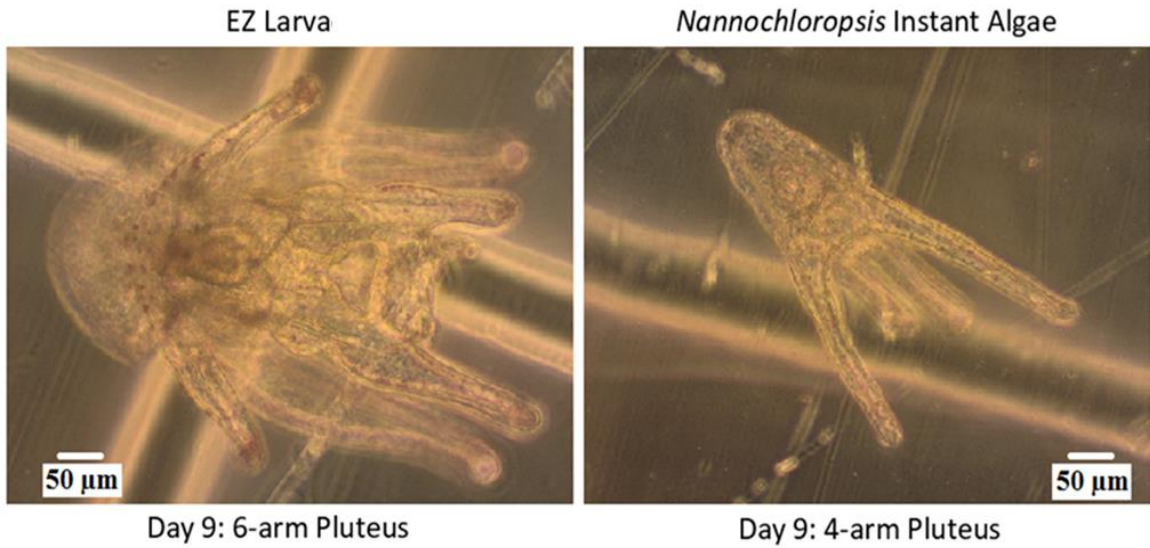
Figure 13: Most advanced development stage of *L. variegatus* larvae on day 9 when fed different diets under the same ambient conditions.

4.4 Experiment 4: Larval Respiration

Respiration differed significantly between temperatures (p= 0.02486); *L. variegatus* larvae consumed more oxygen at 31°C than at 28°C (Figure 13).
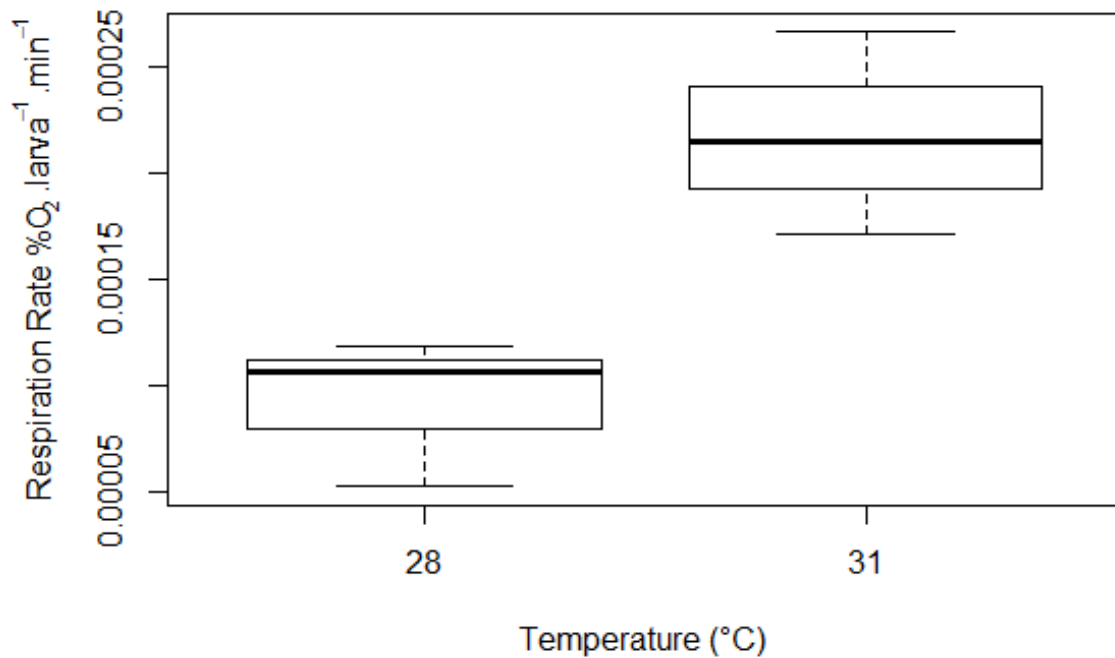
Figure 14: Respiration of *L. variegatus* larvae ($\%O_2.larva^{-1}.min^{-1}$) at ambient and warm temperature.

5. Discussion

Lowered pH (OA) had no effect on fertilization success of *L. variegatus* but delayed larval development, stunted growth, and caused larvae to become more asymmetric. Elevated temperature (OW) decreased fertilization success at a low sperm to egg ratio (1,847:1) and accelerated larval development, but had no significant effect on larval growth. Under the treatment combining both stressors (OAW), fertilization success was reduced, larval growth stunted, and larvae became more asymmetric. Although larvae developed more rapidly under OAW conditions, suggesting that the negative effects of reduced pH were outweighed by the accelerated effect of increased temperature, they remained smaller than under ambient conditions.

An end-of-the-century pH (7.8) did not affect fertilization success of *L. variegatus*, most likely because this species evolved in an environment where daily pH fluctuations, driven by photosynthesis and respiration, are similar to anticipated pH changes resulting from ocean acidification projected for surface ocean waters by 2100. The pH of seawater in coral reef ecosystems fluctuates throughout the day and is typically lower before dawn and higher during early evening (Hofmann et al. 2011). For example, *L. variegatus* occurs in Tampa Bay, where pH currently averages 8.1 but declines as low as 7.95 in mid-afternoon when photosynthesis peaks (Yates et al. 2007). Heavy rains can expose this coastal species to even lower pH levels, particularly near inlets. Previous studies have found that low levels of pH can inhibit the acrosomal reaction of sea urchin sperm (Gregg and Metz 1976) and lower sperm motility and respiration hindering internal ATPase activity (Christen et al. 1983). Reduced sperm motility can in turn decrease fertilization success, as reported in several sea urchin species (Kurihara et al. 2004, Havenhand et al. 2008, Moulin et al. 2011, Kapsenberg et al. 2017). However, such negative effects of pH on fertilization success in sea urchins have only been reported for pH levels ranging from 6.8 to 7.7, which represent scenarios projected for approximately the years 2150-2300+ (Caldeira and Wickett 2003, Turley 2011). In this study, we only tested the effects of pH for a near-future scenario (2100) because, beyond that point, species will either have acclimated/adapted, or died out.

Increased temperature decreased fertilization success under a low sperm to egg ratio, but not under a high ratio, likely because reduced sperm longevity at higher temperatures, and consequent negative impact on fertilization success, only becomes evident when the chances of encounter between gametes are reduced. Paternity may have been a factor in determining fertilization success, however, in this experiment the sperm of all males were combined to maximize sperm stock, thus eliminating the ability to differentiate between paternal effects. It seems most likely that the differences of fertilization success observed between trials occurred due to sperm and egg concentrations. Fertilization success is typically greater under high sperm to egg ratios, because it increases the chances of encounter between egg and sperm (Byrne et al. 2010). At low sperm to egg ratios, environmental factors, such as temperature, may boost or reduce the frequency of encounters between egg and sperm. Temperature increases boost fertilization success, because warmer temperatures increase sperm velocity (Mita et al. 1984, Alavi et al. 2005, Dadras et al. 2017). However, a tradeoff between sperm velocity and longevity influences fertilization success in several urchin species (Alavi et al. 2005, Dadras et al. 2017), including *Lytechinus variegatus* (Levitan 2000), whereas increased sperm velocity is associated with a reduced duration of sperm motility. The results here suggest that reduced fertilization success in *L. variegatus* under warmer conditions was more likely influenced by the reduced longevity of sperm motility, rather than increased sperm velocity. This decreased longevity did not affect fertilization success under high sperm to egg ratio conditions (as in Trial 2). However, a low sperm to egg ratio (as in Trial 1), which reduced sperm and egg encounter rates, combined with decreased longevity of sperm motility, lowered fertilization success.

Polyspermy may have occurred in this experiment, however, the results of fertilization success under high sperm concentrations do not indicate any evidence of polyspermy occurring directly post-fertilization as the fertilization rates were all high, although latent effects may be possible. Polyspermy, which occurs when an egg has been fertilized by more than one sperm, can cause the sea urchin egg to remain unfertilized, burst or become fertilized with latent effects. These latent effects caused by polyspermy can result in a decrease in development occurring a couple days post-fertilization. Studies have shown that increasing sperm concentration can lead to a decrease in developmental

success in zygotes most likely due to polyspermy (Levitan et al. 2007). This may explain why there is some initial delayed development observed in all treatments on Day 1 and 2 of larval development. Polyspermy may have occurred during fertilization and caused latent effects, resulting in a decrease in development as observed in the first couple of days of all treatments. However, these latent effects of polyspermy were not tested directly.

Under OAW conditions, warmer temperatures accelerated larval metabolism and consequently larval developmental rate in *L. variegatus*, outweighing the negative effects of reduced pH on larval development and skeletogenesis. As in other sea urchin species (Brennand et al. 2010, Stumpp et al. 2011, Byrne et al. 2013a), reduced pH slowed larval development in *L. variegatus*, likely because of down-regulation of genes central to energy metabolism and biomineralization (O'Donnell et al. 2010). However, the positive effect of temperature counteracted this negative effect of reduced pH on metabolism and larval development rate. At warmer temperatures, *L. variegatus* larvae consumed nearly twice as much oxygen than those at ambient temperature, reflecting an accelerated metabolism. Warm temperatures increase enzymatic activity, which in turn accelerates physiological processes, such as metabolism and cell division (Savage et al. 2004). As observed in other sea urchin species (Brennand et al. 2010, Byrne et al. 2013a), faster metabolism and rates of cell division caused by warmer temperatures led to faster larval development, i.e. a quicker progression to more complex larval stages. However, increases in metabolic rates decrease the scope for growth, (Stumpp et al. 2011) because larvae typically allocate energy primarily to survival, then to development (complexity), and only then to growth (size). When larval metabolism increases, larvae may consume energy reserves more quickly than they can replenish them through feeding. Thus, while the larvae *L. variegatus* developed faster under warmer conditions, this increase in complexity was not accompanied by a faster growth.

Elevated temperature did not affect total body length, average rod length, and asymmetry of *L. variegatus* larvae, but reduced pH decreased larval length and increased asymmetry, likely due to lower availability of calcium carbonate to form their calcifying skeleton. Sea urchin larvae are vulnerable to OA (O'Donnell et al. 2010, Stumpp et al.

2011), because they have a calcium carbonate skeleton (Heatfield 1970). Under decreased pH, calcium carbonate in sea water is less available for uptake, because free carbonate ions tend to form bicarbonate ions (Fabry et al. 2008, Doney et al. 2009). Ocean acidification also alters the expression of genes related to skeletogenesis in sea urchins. Specifically, genes central to energy metabolism and biomineralization are down-regulated in larvae of *L. variegatus* and other sea urchins reared in a lower pH, reducing growth rates (O'Donnell et al. 2010, Stumpp et al. 2011, Raven et al. 2005, Kroeker et al. 2010), i.e., larvae have smaller total body and average rod lengths. In addition, decreased pH also causes an increase in asymmetry in *L. variegatus* and other sea urchin larvae (Byrne et al. 2013a, Uthicke et al. 2013, Lamare et al. 2016). Under low pH, larvae allocate the few available calcium carbonate ions disproportionally between rods leading one rod to grow more than the other. The mechanism that regulates this differential allocation of carbon ions to the rods remains to be studied. Larval asymmetry may compromise a larva's ability to feed and control movement and placement in the water column (Strathmann 1975). The prevalence of rod asymmetry and decreased growth in sea urchin larvae due to elevated acidity suggests that ocean acidification will impair the ability of *L. variegatus* larvae to develop, grow and produce a normal, symmetric larval skeleton, and ultimately compromise their survival, dispersal and recruitment.

Reduced pH level did not affect total body width of *L. variegatus* larvae, however larvae reared under warmer conditions had a greater total body width, suggesting that total body width better reflects developmental stage, rather than growth. Over time, echinoplutei increase in width as they develop into more complex larval stages (Figure 8). At an increased temperature, larvae progressed into more developed stages more rapidly than at the ambient temperature, and their total body width increased concomitantly. These results contrast with findings on another sea urchin species, *Arachnoides placenta,* in which total body width decreased as temperature increased (Chen and Chen 1992). Also, whereas reduced pH did not alter total body width of *Lytechinus variegatus* larvae, it decreased this measure in *Lytechinus pictus* (O'Donnell et al. 2010) and increased it in *Strongylocentrotus droebachiensis* (Dorey et al. 2013). These contrasting outcomes suggest that differences in the effects of pH and temperature on sea urchin larval body width may vary among species.

EZ Larva was a better food source for the *L. variegatus* larvae than *Nannochloropsis* Instant algae, as it promoted faster larval development and growth. EZ Larva is a microencapsulated liquid larval diet with particles ranging from 10 to 50 µm and is typically used feed to shrimp larvae. It consists (dry weight) of 36.7% crude protein, 20% crude fat, 3.3% fiber, and 0.7% phosphorus. *L. variegatus* larvae have previously been raised on this diet, which suggests that it provides at least the minimal nutritional requirement for complete early development of the larvae of this species (George et al. 2004). On the other hand, *Nannochloropsis* Instant Algae particles range from 1.5-2 µm and have a greater protein (58.6%) and carbohydrate content (20%) but less lipids (14.5%) (Reed Mariculture, Campbell, CA). *Nannochloropsis* Instant Algae is a high-yield rotifer feed that has never previously been used as an artificial feed for sea urchins. In this experiment, *Nannochloropsis* Instant Algae reduced larval survival and growth. Additionally, none of the larvae fed this diet developed past the 4-arm pluteus stage, indicating that this diet is unable to provide some of the nutrients, likely lipids, required to support larval development past this point.

Species' tolerance for changes in environmental conditions depend in part on the magnitude of daily/annual fluctuations in the region in which they evolved; however, this is not always certain. Here, we studied the fertilization, larval development and growth under future OAW conditions of a sea urchin species from tropical/subtropical waters. We found that temperature affected larval development more than pH, although larval growth decreased under low pH, but was not affected by warmer temperature. Considering the effects of warming and acidification on sea urchin larvae have only been studied in fewer than ten species (Brennand et al. 2010, Byrne et al. 2009, Foo et al. 2012, Byrne et al. 2013a, Byrne et al. 2013c), it is hard to reach conclusions on the relative susceptibility of species to OAW based on differences in magnitude of annual temperature and pH variations between latitudes or proximity to coastlines where they evolved. In some animal groups (e.g. terrestrial ectotherms such as lizards, Tewksbury et al. 2008), it has been hypothesized and documented that, because temperate species have a wider temperature tolerance range, they should be more tolerant to ocean warming than tropical species. So far, the results of this and previous studies in sea urchin larvae seem to point to the opposite: higher tolerance to warming in tropical sea urchin species

relative to temperate species. The larvae of a tropical species, *Tripneustes gratilla*, grew faster as a result of a 3°C increase (Brennand et al. 2010). In addition, larval growth was recorded as optimal between 27-34°C in the tropical sea urchin *Echinometra lucunter*, with the maximum at +6°C above ambient temperature (Sewell and Young 1999). By contrast, a temperature increase of 4-6°C above maximum ambient decreased growth and development in larvae of two temperate species (Fujisawa 1989, Byrne et al. 2009). Tropical regions experience narrower sea surface temperature fluctuations, but values remain at the high end of the spectrum year-round. In temperate regions, temperatures vary much more widely throughout the year but reach high levels only during a much shorter period. This difference in duration of annual exposure to higher temperatures may explain why tropical sea urchins may be more tolerant to increases in temperature than temperate species.

For pH, differences in tolerance between species should not be correlated with latitude, but instead be site-specific. For example, species inhabiting coastal areas typically experience greater pH fluctuation than in the open ocean (Cai et al. 2011); therefore, sea urchins in coastal regions may be expected to tolerate greater changes in pH than non-coastal species. However, this and other studies (O'Donnell et al. 2010, Stumpp et al. 2011, Byrne et al. 2013c) documented that pH had a deleterious effect on the growth of all sea urchin species, regardless of proximity to the coastline. Thus, coastal sea urchins may be just as sensitive to reductions of pH as non-coastal species.

Warming and acidification are affecting the early life stages of many different marine organisms. For sea urchins, the combined effects of warming and acidification reduced fertilization success, accelerated larval development, reduced growth and increased asymmetry. Smaller and asymmetric larvae will not be able to properly to swim and feed, or control their position in the water column. Together with a reduced fertilization success, larvae will suffer greater mortality, disperse less and have a harder time to settle on a suitable environment. These deleterious effects are likely to have severe impacts on the persistence of the species, because they affect rates of connectivity and gene flow between populations and thus rates of recovery after disturbances.

Assessing the impacts of OAW on both adult and larval stages of key ecosystem species, such as sea urchins, are critical for accurately predicting the future of marine ecosystems.

Acknowledgements

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