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# The Effects of Ocean Acidification on Sea Urchin Larval Survivorship and Development in Lytechinus variegatus and Arbacia punctulata

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# THE EFFECTS OF OCEAN ACIDIFICATION ON SEA URCHIN LARVAL SURVIVORSHIP AND DEVELOPMENT IN *LYTECHINUS VARIEGATUS* AND *ARBACIA PUNCTULATA*

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## ABSTRACT

Accumulated carbon dioxide in the atmosphere is one of the driving factors in ocean acidification as oceanic absorption of carbon dioxide alters ocean chemistry. Lower concentrations of carbonate ions and higher concentrations of hydrogen ions in the water adversely affect marine organisms, including sea urchin larvae, that use calcium carbonate in their skeletal structures. While there is a wide body of literature demonstrating an impact of lowered pH on sea urchin larval development and survival, it is unclear if the method of pH manipulation and the species being studied influences the results. To address this, we compared two commonly employed pH manipulation methods, hydrochloric acid addition and carbon dioxide bubbling, for impacts on sea urchin development in *Lytechinus variegatus*. We also compared the effects of projected acidic ocean pH on larval development in *L. variegatus* and *Arbacia punctulata*. Regardless of species and pH manipulation method survivorship and aspects of skeletal features decreased as pH decreased. Counts of developmental abnormalities increased with decreasing pH. However, changes in size of specific skeletal features and prevalence of types of abnormalities observed varied with both pH manipulation method and species. Our results are consistent with previous studies showing a decrease in survivorship and skeletal size with lowered pH but indicate that the methods used to study effects of acidification on sea urchin larval development can affect the experimental outcomes and hinder making broad conclusions.

***Keywords***: Sea urchin larval development, decrease ocean pH, *L. variegatus*, *A. punctulata*

## INTRODUCTION

Increased environmental carbon dioxide from anthropogenic activities poses a major threat to global climate and diversity and is the driving factor in ocean acidification (Keeling 1997). Carbon dioxide ($CO_2$) is absorbed by the ocean and interacts with water to form carbonic acid ($H_2CO_3$), which dissociates into hydrogen (H+) and bicarbonate ions ($HCO_3^-$) (Keeling 1997). The H+ ions bind with carbonate ions ($CO_3^{2-}$) that are naturally found in seawater. These $CO_3^{-2}$ ions are used by calcifying organisms to build

calcium carbonate ($CaCO_3$) structures such as shells and skeletons. With an increase in atmospheric $CO_2$, more $CO_2$ is absorbed by the ocean, which leads to lower concentrations of free $CO_3^{-2}$ ions in the water (Tanhua et al. 2015). This in turn can cause potential adverse effects for numerous marine organisms including corals, mollusks, and sea urchins, that use $CaCO_3$ in their skeletal structures (Kroeker et al. 2013).

Most predictions, based on current atmospheric $CO_2$ levels and ocean pH, project the open ocean pH will decrease by 0.6 to 0.8 pH units in the next 100 years (Jiang et al. 2019). The ocean pH is currently about 8.1, which is slightly alkaline, and there is no atmospheric or geochemical evidence to suggest that over the past 300 million years the ocean pH was ever more than 0.6 pH units lower than its present level (Caldeira and Wickett 2003). Recent evidence suggests that during the Paleocene – Eocene Thermal Maximum (PETM, ~56 mya) rapid acidification of the oceans caused a mass extinction of benthic foraminifera (Zachos et al. 2005). Projected anthropogenic $CO_2$ input in the next 300 years is expected to be more rapid than during the PETM suggesting more profound adverse effects on marine organisms (Zachos et al. 2005). Guinotte and Fabry (2008) suggest that these effects will likely spread through various trophic levels and can cause irreversible damage to marine food webs.

Sea urchins are important marine organisms both ecologically and economically. As herbivores, adult sea urchins play an essential role in regulating algal growth in numerous marine habitats including coral reefs and kelp forests (Steneck et al. 2003; Dang et al. 2020). The depletion of herbivorous sea urchins from coral reefs allows rapidly growing algae to outcompete and kill corals, thereby disrupting the entire reef ecosystem (McCook et al. 2001). In a study of kelp forests, Donham et al. (2021) found significant decreases for sea urchins in both calcification rate and algal grazing rate under high $pCO_2$ (acidic conditions) as compared to sea urchins grown under ambient $pCO_2$ (normal ocean pH). Additionally, sea urchin larvae constitute an important component of pelagic zooplankton communities (Lampert 1997). Adult sea urchins are a large part of commercial invertebrate fishery, with much of the catch sent to Asia for human consumption. For example, the 2013 sea urchin harvest in the United States was valued at eleven million dollars however the once robust fisheries have been declining in recent years, due to a combination of overharvesting, habitat alteration, and climate change, of which acidification is a part (Medellin–Ortiz et al 2022).

Sea urchin larvae have been widely studied in the context of ocean acidification because their small size makes them particularly vulnerable to changes in pH (Yamada and Ikeda 1999), although interspecies plasticity in response has been observed (Dupont et al. 2010). For example, at similar pH, Gonzalez-Bernat (2013) found no effect of pH on the ratio of postoral arm (POA) length:body length (BL) in *Arachnoides placenta* while Dorey et al (2013) found a statistically significant decrease in POA:BL in *Strongelocentrotus droebachiensis*. Similarly, Chan and Tong (2020) found no statistically significant effect of pH on body length in *Heliocidaris crassispina,* while Hernandez et al (2020) found a statistically significant decrease in body length in *Diadema africanum* as pH declined. Understanding the consequences of this natural biological variation is complicated by methodological variation, including variation in

both method of pH manipulation and measured variables (Byrne et al. 2013). In laboratory studies, researchers typically alter seawater pH using either hydrochloric acid (HCl) or $CO_2$. Adding drops of HCl is simple and cost–effective while bubbling $CO_2$ creates more environmentally realistic conditions but requires complex apparatus to control $CO_2$ flow and bubble rate. Previous work by Kurihara and Shirayama (2004) compared these two pH modification methods for effects on fertilization rate, cleavage rate, developmental speed, and pluteus morphology in two tropical Pacific species, *Hemicentrotus pulcherrimus* and *Echinometra mathaei*. While both methods caused decreases in fertilization rate, cleavage rate, and developmental speed, the effects on fertilization rate were more pronounced in embryos exposed to carbon dioxide (Kurihara and Shiryama 2004). Interspecies differences in fertilization rate were also observed, regardless of pH manipulation methodology employed (Kurihara and Shiryama 2004).

The goal of the current work was to further study acidification effects on sea urchin larvae. We aimed to do this, first, by using one sea urchin species and altering pH manipulation method two different ways and second, by using one pH manipulation method and examining effects on two different species. By doing so, we built upon the work of Kurihara and Shiryama (2004) by considering impacts of pH manipulation method on two Atlantic Ocean & Caribbean Sea species. To meet the first aim, we compared HCL and $CO_2$ acidification methods for impacts on *Lytechinus variegatus* development. To achieve our second aim of contributing to the understanding of species-specific differences in ocean acidification, we compared the effects of lowering pH (via $CO_2$ bubbling) on larval development in two Western Atlantic/Caribbean species, *L. variegatus* and *Arbacia punctulata*. Both species are abundant on rocky and sandy bottoms or in seagrass beds throughout the Western Atlantic and Caribbean Sea *(*Hill and Lawrence 2003). *Lytechinus,* whose reproduction and development have been well documented in response to ocean acidification (Albright et al. 2012; Lenz et al. 2019), tends to inhabit shallow waters and is mainly herbivorous. The Mediterranean *Arbacia* congener, *A. lixula*, has also been well studied (Visconti et al. 2017; Garcia et al. 2018), while less is known about *A. punctulata's* developmental response to pH change. *Arbacia puntulata* is sympatric with *L. variegatus* in the Gulf of Mexico but it can also be found at much greater depths and has a more omnivorous lifestyle. By testing various pH manipulation methods and sea urchin species, while holding other variables constant, we hope to provide more context for the interpretation of the wide – ranging ocean acidification literature.

## MATERIALS AND METHODS

### *Specimen collection*

Adult *L. variegatus* and *A. punctulata* species were acquired from the Gulf Specimens Marine Lab in Panacea, Florida. Specimens were shipped overnight in an insulated container with saltwater. Upon arrival at the University of North Georgia, the adult sea urchins were equilibrated to a 120-gallon saltwater tank (pH 8.4, 19 °C, 27ppt) for an hour before being released into a saltwater tank.

### *Spawning and experimental design*

Two sets of studies were conducted: one comparing the effects of different pH manipulation methods on larval development and a second set comparing the response of larvae from two species to acidification. Gametes were spawned by intraceolomic injection with 3 ml of 0.53 M KCl. Dry sperm was collected from males by pipetting the sperm into a 1.5 ml microcentrifuge tube. Eggs from female sea urchins were spawned directly into a beaker of pH 8.4 artificial sea water (ASW) and then transferred to six dishes containing ~1 liter of ASW. Dry sperm was diluted 1:100 in ASW and added to the eggs until at least 80-100% fertilization rate was reached in each dish. The excess sperm was washed out with ASW, and fertilized eggs were monitored for 24 hours post fertilization (hpf) to ensure the larvae were developing normally.

At ~24 hpf, density of prism-stage cultures were quantified, and larvae from the fertilization dishes were equally distributed among gallon glass treatment jars at a final density of ~3 larvae per mL in 2 liters of pH-controlled ASW. There were nine treatment jars total because there were three replicates of the three pH treatments used: pH 8.4 (control, current pH in Gulf of Mexico (GOM) where sea urchins were collected), pH 8.0, and pH 7.6 (range of projected GOM pH levels by 2100) (Dee et al. 2019). Three replicates per treatment were set up with gentle agitation, using a paddle system, similar to Hubbard et al. (2003) and shown in Figure 1. pH was monitored using a handheld aquarium pH meter. Starting at 48hrs, larvae were fed 0.5ml of a liquid Chlorella culture every 24-48 hrs.

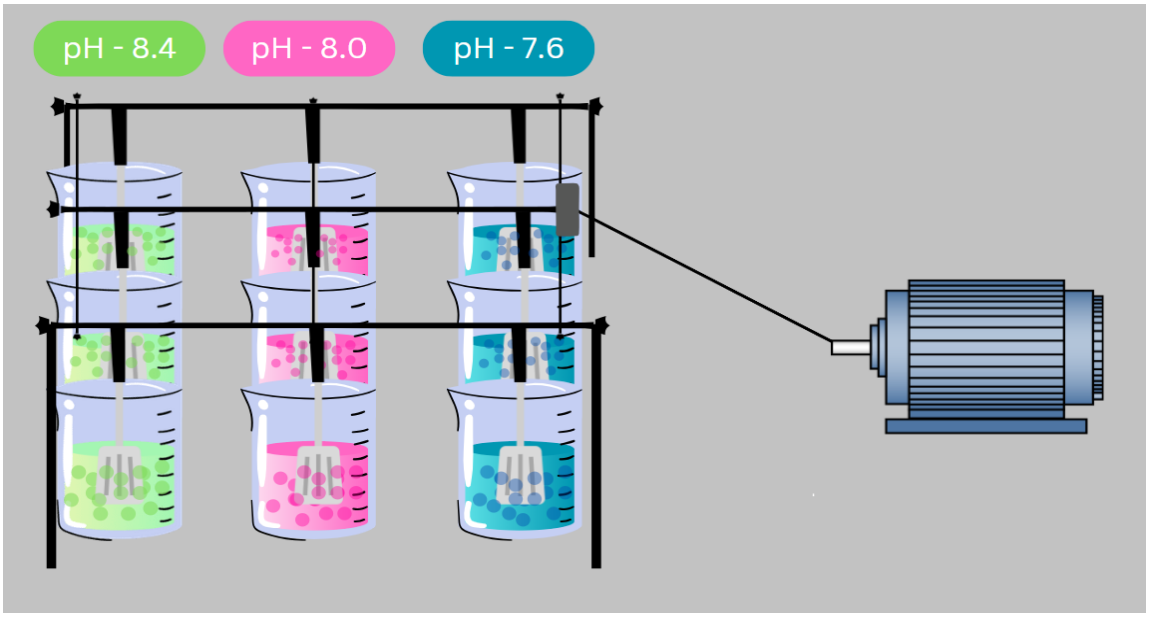


***Figure 1.*** Experimental apparatus. A motor was attached to a series of paddles that swept back and forth in the water of larval cultures to keep larvae suspended.

### *Comparison of pH Manipulation Methods*

*Lytechinus variegatus* larvae were used in the pH manipulation experiment. The pH was experimentally altered two different ways. 1.) Drops of 0.3M HCl were slowly added to three 8 L containers of ASW (salinity = 27 parts per thousand) until desired pHs of 8.4, 8.0, and 7.6 were reached in each of the three containers. These stock solutions were then split into the appropriate treatment jars with 2L of pH-adjusted water per jar. 2.) Pure $CO_2$ was bubbled directly into individual treatment jars to create $H_2CO_3$ in the water over multiple days preceding the experiment until pH stabilized. Carbon dioxide flow rate was about 3 bubbles/second, and pH was checked every 60 seconds until desired pHs were reached in each of the three containers.

### *Comparison of Species*

Effects of lower pH on larval survivorship and development were compared in two Western Atlantic/Caribbean sea urchin species, *L. variegatus* and *A. puntulata*. To compare species, pure carbon dioxide was bubbled directly into individual treatment jars for both species.

### *Larval Survivorship*

To determine survivorship, one mL samples were collected from each of three replicate jars for each of the three treatment groups and put in twelve-well plates. Samples were viewed under a stereoscopic microscope to visually identify living and dead larvae. For *L. variegatus* cultures whose pH were adjusted with HCl, 20 larvae were observed in each of the three replicate jars for a combined 60 larvae per treatment per day, and for *L. variegatus* adjusted with $CO_2$ and *A. punctulata* adjusted with $CO_2$, a total of 40 larvae were observed in each of the three replicate jars for a combined 120 larvae per treatment per day.

### *Larval Development*

Every 24 or 48 hours, 10 mL of culture from each replicate from each treatment group was collected in 15 mL test tubes and fixed in ~3% glutaraldehyde. Samples of *L. variegatus* larvae were collected every 48h *or 9* dpf for *A. punctulata* when larvae in the pH 8.4 culture were at the 4-arm pluteus stage. Larvae from the fixed samples were put in twelve-well plates and photographed on either an EVOS inverted fluorescent microscope (ThermoFisher Scientific, Waltham MA) or a VWR VistaVision inverted compound microscope, both at 25 x magnification. From these pictures, skeletal features were measured, including the right and left postoral arms, right and left anterolateral arms, body width and body length were measured using Image J (Figure 2) (Rasband 1997-2019). Additionally, these pictures were used to estimate the percentage of developmental abnormalities observed in cultures of each treatment.

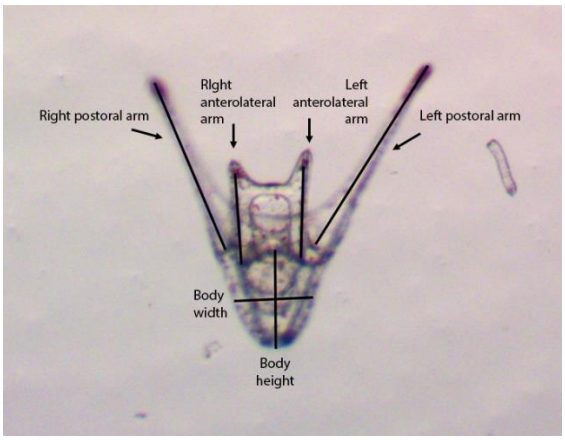


***Figure 2.*** Representative example of larval skeletal measurements.

### *Statistical Analysis*

All statistical analysis was performed in SPSS v29 (IBM, Armonk NY). To analyze the effect of pH on mortality, we calculated percent mortality separately for each of our three replicate jars within each treatment. Then we used a 2-way ANOVA with treatment (pH7.6; pH8.0; pH8.4) and time (days post fertilization) to see if there was an effect of these variables. We report only the results of treatment because it is expected that mortality will increase with time, and there was no significant interaction of time and treatment for any of the species. Similarly, to examine the impact of pH manipulation method in *L. variegatus* mortality, treatment was fully nested within pH manipulation method, so we ran a 2-way ANOVA and report the full results. Because skeletal measurements were taken at one point in time, we ran a 1-way ANOVA to analyze the effect of pH on skeletal measurements. Statistically significant ANOVA results were followed with a post-hoc Tukey's test. Lastly, to understand the relationship between pH and number of abnormal larvae, we used a 2 x 3 contingency table. Among counts of different abnormalities, we did not perform additional statistical analysis because single larvae could have multiple abnormalities, therefore abnormal larvae were not mutually exclusive for a type of abnormality.

## RESULTS

### *Larval Survivorship*

#### *Species comparison*

Survivorship curves follow similar trends for both species when water pH was adjusted with CO2, with initial decreases in survivorship starting between day 4 and day 6 post fertilization (Figure 3). In both *L. variegatus (L)* and *A. punctulata (A)*, the larvae exposed to the control pH (8.4) followed a similar course; survivorship began decreasing on day five. In both species, pH has a statistically significant effect on survivorship,

wherein there was a general decrease in survivorship with a decrease in pH (L: F=4.767, DF=2; p=0.010 and A: F=42.632; DF=2; p<0.001 respectively).

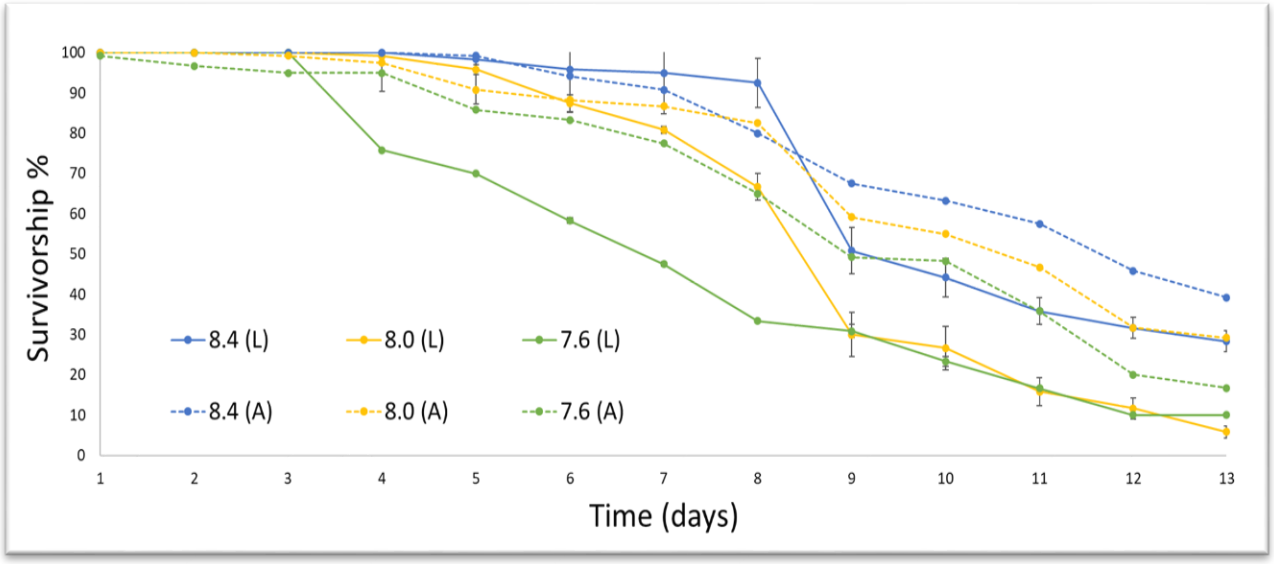


***Figure 3.*** Effect of $CO_2$ adjusted pH on survivorship of *L. variegatus (*L) and *A. punctulata* (A). *Lytechinus* (L) N=60/treatment 2 *Arbacia* (A) N= 120. Error bars =1 SD. No error bars indicate a SD of 0.

### *Method of pH manipulation comparison*

When pH was manipulated either through direct addition of HCl or through creating carbonic acid by bubbling $CO_2$ into the water, there was a statistically significant effect of pH on survivorship in *L. variegatus* larvae (Figure 4; F=4.767; DF=2; p=0.011). *Arbacia punctulata* were not used in this experiment. As in the interspecies comparison study, survivorship decreased with decreasing water pH. However, there was no significant difference in *L. variegatus* survivorship whether pH was manipulated with HCl or $CO_2$ (F= 0.001, DF=1; p=0.976). There was also no significant interaction between water pH and pH manipulation method when considering larval survivorship (F=1.212; DF=2; p=0.302).

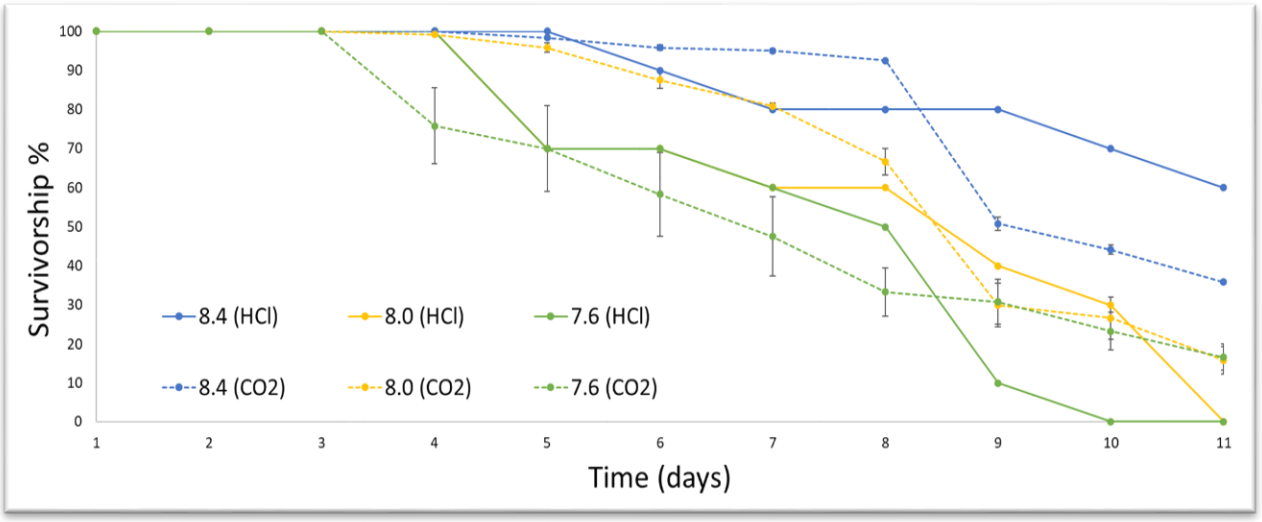


***Figure 4.*** Effect of different pH manipulation methods, HCl and CO2, on *L. variegatus* survivorship. N=120. Error bars = 1 SD. No error bars indicate a SD of 0.

## *Larval Development*

### *Skeletal measurements*

Across species and pH manipulation methods, decreased pH resulted in smaller skeletal features (Table 1.) which is consistent with other studies (Lentz 2019, Chang and Tong 2020) However, the specific nature of the change varied among pH manipulation methods and species so that the pattern is not straightforward. For example, both *L. variegatus* and *A. punctulata* had statistically significantly shorter postoral arms in lower pHs ($CO_2$ bubbling method), but *L. variegatus* showed no statistically significant difference in postoral arm length in lower pH (HCl drip method). So for postoral arm length, the pattern of skeletal change is consistent among species when pH was manipulated the same way (with $CO_2$).

However, when considering other body measurements, the pattern differs. For example, only *A. punctulata* exhibited statistically significantly shorter average anterolateral arms lengths. There was not statistically significant difference for *L. variegatus* regardless of pH manipulation method (Table 1). Statistically significant differences in body length among treatments did not correlate with either species of pH, and under no experimental conditions did we observe a statistically significant difference in body width (Table 1).

***Table I.*** Results of analysis of larval body measurements at the 4-arm pluteus stage at 8 and 9 days post fertilization (dpf). N.s. denotes a non-significant One-way ANOVA results where p<0.05. Post-hoc result significance is indicated by inequality signs. Carbon dioxide and HCl indicate method by which pH was manipulated. N=number of larvae from which body measurements were taken.

| | **Average Postoral Arm** | **Average Anterolateral Arm** | **Body Width** | **Body Length** |
|---|---|---|---|---|
| ***L. variegatus*** **8 dpf** **CO2** **(n=10)** | P=0.004<br>8.0 < 8.4<br>7.6 < 8.4 | n.s. | n.s. | n.s. |
| ***L. variegatus*** **8 dpf** **HCl** **(n=5)** | n.s. | n.s. | n.s. | P=0.05 |
| ***A. punctulata*** **9 dpf** **CO2** **(n=15)** | P<0.001<br>7.6<8.0<8.4 | P<0.001<br>7.6<8.4<br>7.6<8.0 | n.s. | P=0.002<br>7.6<8.4<br>7.6<8.0 |

## *Developmental abnormalities*

In addition to skeletal measurements, larvae were visually assessed for developmental abnormalities at either 8 dpf (*L. variegatus*) or 9 dpf (*A. punctulata*) during the 4-arm pluteus stage. Relative to the pH 8.4 control cultures (Figure. 5a) 5 general categories of abnormalities were observed (Figure. 5b-5f). These categories include: "yeeting" of postoral arms (arms jutting at irregular angles, Figure. 5b), curving of postoral arms (Figure 5c), short/missing postoral and/or anterolateral arms (Figure 5d), unequal or warped anterolateral arms (Figure 5e), pointed aboral side (Figure 5f). Multiple abnormalities could be present in a single larva. Anterolateral arms were visually assessed for a greater than 20% difference in arm length, when the difference between arm length rose about 20%, we classified the sea urchin as having unequal anterolateral arms (Figure 5e). Curving of postoral arms was assessed by using image J (Rasband 1997-2019) by drawing a straight line from one end of the postoral arm to the other end. Curving was indicated by the postoral arm deviating from the straight line (Figure 5c).

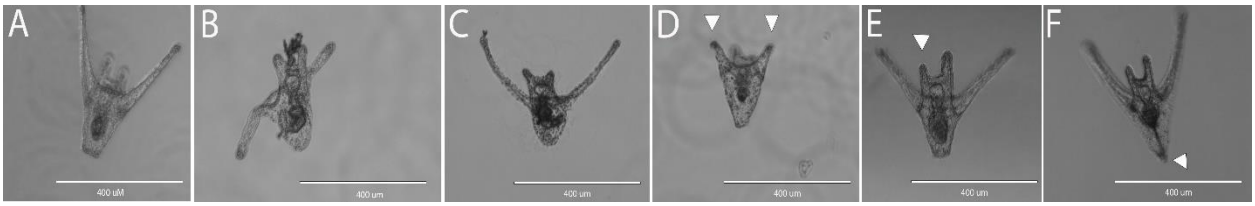


***Figure 5.*** Representative *L. variegatus* developmental abnormalities at 8 dpf. Pictures were taken at 25X magnification and 400 uM scale bar is shown for size reference. White arrows highlight abnormalities in some panels.

Skeletal abnormalities were also visually assessed for *A. punctulata* cultures relative to larvae from the pH 8.4 control (Figure 6). General types of abnormalities were similar to *L. variegatus* and included curving of postoral arms (Figure 6b), short/missing postoral and/or anterolateral arms (Figure 6c), unequal or warped anterolateral arms (Figure 6d), pointed aboral side (Figure 6e). There were two ways in which abnormalities differed among species. First, yeeting of any of the arms was never observed in *A. punctulata*. Second, abnormalities on the aboral side of *A. punctulata* other than just being pointed were observed (Figure 6f).

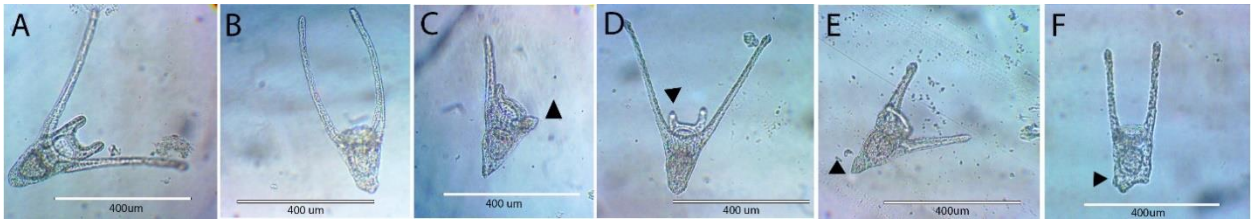


***Figure 6.*** Representative of abnormalities in *A. punctulata* at 9 dpf. Pictures were taken at 25X magnification and 400 uM scale bar is presented for size reference. Black arrows highlight abnormalities in some panels.

For both species, examination of the frequency with which these abnormalities occur show that as pH decreased, counts of abnormalities increased (Table 2). However, there was only a statistically significant increase in the number of abnormal larvae as pH decreased in *L. variegatus* when pH was adjusted with HCl ($X^2$=388; df=2;p<0.001) and *A. punctulata* when pH was adjusted with CO2 ($X^2$= 230.629; df=2; p<0.001). There was not a statistically significant effect of treatment on number of abnormal larvae with *L. variegatus* when pH was manipulated with CO2 ($X^2$= 2.895; df=2; p=0.235)

Some individual larvae exhibited more than one abnormality, therefore statistical tests comparing counts within specific categories of abnormalities which would assume independence could not be performed.

***Table II.*** Counts of observed developmental abnormalities. Species, dpf on which abnormalities were assessed, pH manipulation method and sample size are indicated in the left column. Total number of normal and abnormal larvae are first listed. Then, counts of abnormal larvae within each category are provided. Counts within each category of abnormality do not sum to the total number of abnormal larvae because multiple abnormalities could be observed in a single larva.

| | | pH 7.6 | pH 8.0 | pH 8.4 |
|---|---|---|---|---|
| *L. variegatus* $CO_2$ 8 dpf (N=87-151 larvae/treatment) | Total normal larvae | 64 | 66 | 45 |
| | Total abnormal larvae | 87 | 63 | 42 |
| | Yeeting arms | 0 | 2 | 2 |
| | Curved post oral arms | 2 | 5 | 2 |
| | Missing arms | 54 | 38 | 28 |
| | Unequal arms | 13 | 14 | 12 |
| | Aboral abnormalities | 18 | 4 | 1 |
| *L. variegatus* HCl 8 dpf (N=200 larvae/treatment) | Total normal larvae | 5 | 22 | 174 |
| | Total abnormal larvae | 195 | 178 | 26 |
| | Yeeting arms | 90 | 68 | 0 |
| | Curved post oral arms | 108 | 45 | 24 |
| | Missing arms | 103 | 68 | 3 |
| | Unequal arms | 186 | 178 | 16 |
| | Aboral abnormalities | 179 | 93 | 26 |
| *A. punctulata* $CO_2$ 9 dpf (N=200 larvae/treatment) | Total Normal larvae | 12 | 29 | 166 |
| | Total Abnormal larvae | 188 | 172 | 34 |
| | Yeeting arms | 0 | 0 | 0 |
| | Curved post oral arms | 84 | 53 | 14 |
| | Missing arms | 113 | 78 | 8 |
| | Unequal arms | 176 | 157 | 10 |
| | Aboral abnormalities | 142 | 106 | 13 |

## DISCUSSION

The effects of ocean acidification on marine organisms have been well documented, particularly in echinoderms (Clark, et al. 2009; Kurihara and Shiriyama 2004; Lenz et al. 2019). However, generalizations about acidification impacts are difficult to make since the methodology used to decrease water pH in controlled laboratory studies varies. Furthermore, among echinoderms, specifically sea urchins, there appears to be a range of species – specific responses to lower pH (Clark, et al. 2009; Palombo et al. 2023; Passarelli et al. 2017). To address these variables, we compared effects of two commonly used methods of pH manipulation, HCl addition and $CO_2$ bubbling, on sea urchin larval survival and development. We also examined acidification effects on larvae from two eastern North American sea urchin species, *L. variegatus* and *A. puntulata*. *L. variegatus* has been well studied in terms of larval developmental responses to ocean acidification

(Albright et al. 2012; Lenz et al. 2019). In contrast, *A. punctulata's* development is less studied in general and in response to ocean acidification in particular, despite a similar distribution to *L. variegatus* in the Gulf of Mexico. Much like Kurihara and Shiriyama (2004), we found that both pH manipulation method and species studied played important roles in the observed results and their interpretation.

### *Survivorship*

Our studies demonstrated that while lower than environmentally normal pHs had a significant impact on larval survivorship in both species, the method in which pH was altered (HCl drops versus $CO_2$ bubbling) was immaterial to overall larval survivorship. The lower survivorship (increased mortality) at lower seawater pH levels we observed was consistent with the literature on ocean acidification effects (Byrne et al. 2013; Dupont et al. 2010; Guinotte et al. 2008). Collectively, this body of work raises an alarm for the fate of marine populations. As more atmospheric carbon dioxide is absorbed by oceans, water pH will continue to decline, and marine organisms may be in jeopardy. Jiang et al. (2019) have reported a 0.1 unit drop in seawater pH since the industrial revolution and predict another 0.3 to 0.4 units drop by 2100.

### *Larval Development*

While larval survivorship showed a clear trend regarding seawater pH, the metrics of larval development that we assessed, skeletal measurements and counts of developmental abnormalities, were not as straightforward. Some aspects of skeletal measurements were consistently impacted across species. When pH was manipulated with $CO_2$, postoral arms were significantly shorter as compared to control (pH8.4) larvae, for both *L. variegatus* and *A. punctulata*. Similar findings in *L. variegatus* were reported by other researchers (Lenz 2019), but our study is the first to document the lowered pH effects on skeletal measurements in *A. punctulata* larvae to our knowledge. The consistency of our findings with prior literature suggests that at least shortened postoral arms is a robust consequence of ocean acidification for sea urchin larvae. Postoral arms play a critical role in larval feeding by driving water flow and directing food towards the mouth (Strathmann 1971). Shorter arms, resulting from decreased pH, could result in larvae obtaining less food and thus having lower survivorship.

Unlike our uniform findings with postoral arm length, the impact of decreasing pH on other larval skeletal measurements, such as anteriolateral arms and body width, were more variable. However, it is unclear to what degree these slight variations in skeletal morphology affect fitness. A recent study by Chan et al (2015) highlighted the importance of careful endpoint selection and the broad interpretation of modest morphological alterations.

Counts of developmental abnormalities (Fig 6) also exhibited variation based on species and pH manipulation method. There was not a statistically significant effect on total number of abnormal larvae in *L. variegatus* when pH was manipulated by $CO_2$bubbling. Conversely, there was a statistically significant effect of pH on number of abnormal alrvae in *A. punctulata* where pH was manipulated by $CO_2$ and in *L.*

*variegatus* where pH was manipulated by HCl (pH manipulation by HCl addition was not tested in *A. punctulata* larvae). The disparity between *A. punctulata* and *L. variegatus* suggests that there is a species-specific response to decreasing pH, which may be attributed to differences in larval phenotypic plasticity between the two species. Phenotypic plasticity is considered crucial in organism resilience to a changing environment. For example, Chen and Adams (2022) found that larval postoral arm length varied with food concentration in *A. punctulata* but not in *L. variegatus*. A study by Carr et al. (2006) observed different pH tolerances (reported as percent normal development) in *A. punctulata* populations from opposite ends of their geographic range. These populations experience dissimilar hydrographic conditions and likely demonstrate phenotypic plasticity variance akin to that seen between species.

When considering differences between species, variation in their responses may be correlated with native habitats. *L. variegatus* and *A. punctulata* occupy a similar geographic range on the coastal shelf in the Gulf of Mexico and southwestern Atlantic Ocean, however they inhabit distinct microhabitats. Whereas *L. variegatus* tends to remain in sandy areas and turtle grass beds, *A. punctulata* is localized to rubble beds (Hill and Lawrence 2003). Furthermore, *L. variegatus* exhibits a strong negative phototaxis, while *A. punctulata* is more tolerant of light and may be found in full sunlight at low tide (Sharp and Gray 1962). While these factors may not completely explain the disparate results observed between species, they support the need to continue widely exploring ocean acidification impacts since not all species respond in the same way. Understanding even small differences in response may help scientists more accurately predict local impacts and focus conservation efforts as ocean acidification increases.

Inconsistency in abnormality counts with our two pH manipulation methods may be attributed to the seawater chemistry of hydrochloric acid and carbonic acid. When $CO_2$ is added to seawater, H+, $HCO_3^-$, and $H_2CO_3$ concentrations increase while CO3- 2 concentrations decrease. HCl addition likewise increases [H+] and [$H_2CO_3$], however, $HCO_3^-$ and $CO_3^{-2}$ concentrations decreased in this case (Doney et al. 2009). The $CO_3^{-2}$ ion plays a critical role in seawater calcium chemistry by buffering free H+ ions and in the calcium availability for organisms with calcium carbonate structures, such as the sea urchin skeleton (Doney et al. 2009; Tanua et al. 2015). The observed differences in abnormalities in sea urchins exposed to either HCl and $CO_2$ – treated seawater may be attributed to subtle variation in seawater chemistry, notably in carbonate ion concentration, with these different pH altering mechanisms. While, to our knowledge there have been few studies specifically focused on comparing pH manipulation methods in sea urchin larvae (most recently Kurihara and Shiriyama, 2004), our results are consistent with their findings in that abnormalities were more severe as pH decreased. However, there was more variability in skeletal measurements among our species in response to pH manipulation method. For example, Kurihara and Shiriyama (2004) found that acidification via CO2 bubbling had more severe impacts on skeletal measurements for postoral arms, anterolateral arms and body width consistently at lower pHs, but they present skeletal measurements only for post Our results indicate

more variation in the region of the skeleton measured (Table 2) and suggest that the impact of pH manipulation method depends on species.

Overall, our results support previous literature showing that, in general, ocean acidification has negative impacts on sea urchin larvae. Further, the methodology used to study ocean acidification impacts have a significant effect on results. While this confounds the ability to make sweeping conclusions across species, it highlights the importance of continued research in a broad range of conditions and organisms.

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