

## Effects of Ocean Acidification on *Lottia scutum* Settlement

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### Abstract

The effects of ocean acidification on calcifying marine organisms are becoming more pronounced as atmospheric CO<sub>2</sub> levels have increased due to anthropogenic carbon emissions (Etheridge et al., 1996). Studies on these effects have also increased over time. Ocean acidification (OA) has been shown to affect the feeding behavior and metabolic rates of larvae in a number of species (Vargas et al., 2013; Pan et al., 2015). Metabolic changes can significantly influence developmental rates, but little is still known about consequences of OA for non-feeding marine invertebrate larvae. In this study, we focus on the effects of OA conditions on the larval stage of *Lottia scutum*, a Pacific rocky intertidal limpet species that ranges from Alaska to southern California. Larvae were exposed to OA conditions (pH 7.3) at competency stage and monitored for settlement behavior and metamorphosis. Our results indicate that *L. scutum* larvae were able to successfully settle in OA and ambient seawater treatments. We did not find a negative effect of the specific OA conditions used in this study on the settlement of *L. scutum*. These findings provide insight into how environmental stress might affect early life stages, as well as how marine invertebrate larvae from regularly low pH environments fare in OA conditions.

## Introduction

Ocean acidification (OA) is a phenomenon brought on by the increasing dissolution of anthropogenic CO<sub>2</sub> in seawater. There is consensus amongst the scientific community that average seawater pH will decrease by 0.3–0.4 units by the end of the century (Feely et al., 2009). This 0.3 drop in pH will not only impact the physiology of marine invertebrates, but also their saturation states (Waldbusser et al., 2015; Albright et al., 2008). There are particularly concerning consequences for calcifying marine organisms, whose carbonate structures can weaken, form abnormally, and break down with exposure to acidified conditions (Fabry et al., 2008). OA has been shown to negatively influence the settlement behavior of many marine invertebrate species (Espinel-Velasco et al., 2018; Kripa et al., 2014). OA conditions also negatively impact the metabolic rates and feeding behaviors of many larvae (Vargas et al., 2013; Pan et al., 2015), and by extension, developmental rates. We set out to investigate how OA affects the settlement behavior of *Lottia scutum* larvae in order to determine if non-feeding larvae also experience negative developmental effects. Although an increasing number of studies have investigated the consequences of OA on many calcifying marine organisms, including limpets (Gazeau et al., 2007; Wang et al., 2018), the effects of OA on the non-feeding larvae of limpets have yet to be thoroughly studied.

Limpets graze in the rocky intertidal in ways that can influence algae and microfauna species composition (Coleman et al., 2006; Crowe et al., 2011). *L. scutum* is a Pacific rocky intertidal limpet species that ranges from Alaska to southern California (Rathke, 1833). It undergoes a planktonic veliger stage during early larval development that possesses a calcified shell (Page, 2001), making it potentially vulnerable to drastic changes in pH. These planktonic larvae experience a critical life history stage in which they settle on substrata and metamorphosize

into juveniles. The non-feeding veliger's shell and cilia are altered and lost respectively during metamorphosis (Kay and Emlet, 2002). Larvae settle when exposed to both biotic and abiotic cues (Kay, 2001), and can settle directly onto adult habitat or recruit indirectly by settling outside of the adult habitat and then migrating into it later (Kay, 2001).

The aim of this study is to investigate how OA affects the metamorphosis and settlement behaviors of *L. scutum*. Such insights are critical to assessing how climate change-related processes such as OA will impact the ecology of rocky intertidal habitats as the early life stages of key grazers experience environmental stressors.

## **Methods**

### *Experimental Design*

We conducted this experiment at Friday Harbor Laboratories on San Juan Island, WA (FHL). *L. scutum* adults were collected from Lime Kiln Point, WA on June 5, 2023 and kept at 11°C in the laboratory. Spawning occurred on June 6, 2023, approximately 15 h after collection. The resulting larvae were transferred to 4-L glass jars (approximately 2000 larvae per jar) and kept at 11°C. Water was changed every 2 d.

Larvae were transferred to a wet laboratory and allowed to acclimate to ambient seawater temperature (12-13°C, 30 ppt) for 24 hours before trials began. The larvae were gently mixed in a 4-L glass jar using an acrylic paddle connected to a low-RPM electric motor (Strathmann, 1987). *L. scutum* larvae reach competency approximately 5 d after spawning (Kay and Emlet, 2002). Larvae were 15 d old when trials began. On the first day of trials, larvae were collected from the jar using a turkey baster and placed in glass custard bowls for inspection. Larvae were examined

under dissecting microscopes for healthy swimming and crawling behavior, as well as intact shells and cilia.

Four treatments were used in this study: 1) no settlement cue + no OA (control), 2) no settlement cue + OA, 3) settlement cue + no OA, and 4) settlement cue + OA. Each treatment had five replicates. The environmental cue used in this study came from biofouled microscope slides, which we considered to be a proxy for environmental settlement cue and substrate (Harder et al., 2002). Small rocks covered in biofilm were collected from limpet-

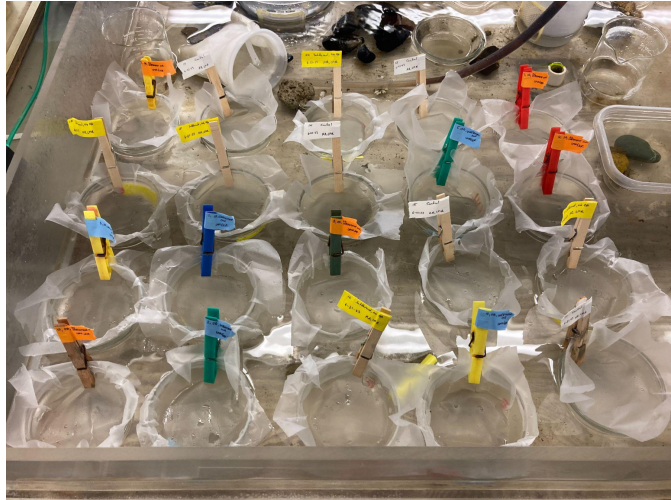
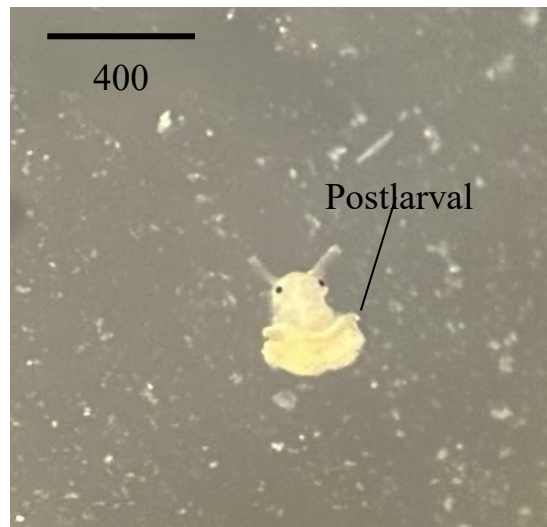


Figure 2. An image of the 20 custard dishes used in this study sitting in a seawater table.

covered tidepools at Fourth of July Beach, WA on June 18, 2023, brought back to the laboratory after removing adult limpets and other visible fauna, and kept in a bucket in unfiltered seawater. Microscope slides were then placed in the same bucket and left to collect biofilm until the beginning of trials on June 21.

### *Developmental Stage-Tracking*

On the first day of trials, 200 larvae were selected based on healthy appearance (Kay and Emlet, 2002). These larvae were divided into twenty groups of 10, each of which was then placed in a  $9 \times 4$  cm glass dish. The dishes were filled with 100 ml of either filtered seawater (7.6 pH; Treatments 1 & 3) or acidified seawater (pH 7.3; Treatments 2 & 4), and biofouled slides were placed in settlement cue-treatment dishes. Replacement water was collected each day from the FHL OA laboratory where it was tripled-filtered using sand, UV, and CO<sub>2</sub> scrubbers. Each bowl was covered with plastic film that covered the water's surface to reduce gas exchange. All replicates were partially immersed in a seawater table to maintain stable temperature, between 12-13°C. Over the course of 5 days, each replicate was removed from the seawater table and examined for larvae daily. When larvae were found, they were transferred to a microscope slide and inspected under a compound microscope (4X) for presence of cilia or post-larval shell (Kay and Emlet, 2002). Once larvae were inspected and both health and metamorph status were recorded, they were transferred to a new dish with fresh seawater (either filtered ambient or acidified) and their corresponding biofouled slide. Larvae were considered damaged if their shells were not intact. When larvae were not found, they were considered dead. We determined that mortality was a probable cause of missing larvae because we often found empty larval shells and shell



**Figure 4. A dissection microscope image of an 18-day old metamorph.**

fragments. We used the numbers of damaged vs. healthy larvae as a metric die-off observed in the treatments over time.

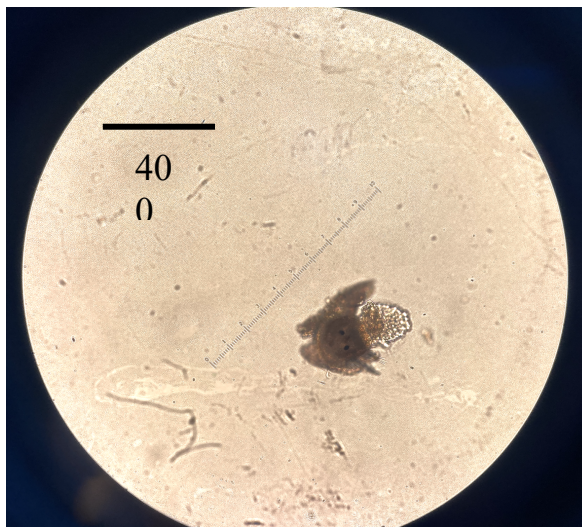
### *Carbonate chemistry*

Water samples (50 mL) were taken on the first and last day of trials to calculate total pH and alkalinity of the treatments. Total-scale pH and alkalinity were measured in the seawater used for this experiment. The remaining carbonate chemistry parameters were calculated via

CO2SYS software (Lewis et al., 1998). Spectrophotometric pH and alkalinity measurements were performed via the Guenther method (Guenther et al., 2022). A YSI ProODO Optical Dissolved Oxygen probe was used to measure the pH of both ambient and acidified seawater during daily water changes. Ambient and acidified seawater pH levels were recorded as 8.1 and 7.7 respectively, but spectrophotometer and titrator measurements showed that those water sources were much closer to 7.6 and 7.3.

### *Statistical analysis*

To assess the effects of OA on settlement, we performed a Kruskal-Wallis rank sum test due to the data not following a normal distribution with treatment and settlement cue as factors and settlement as the response variable in R. A post-hoc Dunn test was performed as well in order to identify which treatments were different from each other in terms of the settlement of metamorphs in R.



**Figure 3. A compound microscope image of a “damaged” veliger. Shell is fragmented and much of the body is exposed.**

## Results

### *Ocean acidification*

Settlement was observed only in treatments that included a settlement cue (Table 2, Figure 5). The least amount of surviving larvae at the end of the experiment was observed in the treatments without a settlement cue (Figure 6). Settlement was highest in the low pH (OA) and settlement cue treatment, with the OA-settlement cue treatment having significantly the most metamorphs initiate settlement (Table 2., Figure 5). The majority of the 200 larvae died or could not be found by the end of the experiment, with only the OA-settlement cue having the most surviving individuals followed by the OA, Control, and settlement cue treatment (Figure 6). The percentage of damaged larvae (Table 4.) fluctuated day to day due to the changes in the total number of larvae found in each dish per day which may have been a result of our treatments having a much lower pH than anticipated (7.3 and 7.6). This occurrence can be inferred based on the saturation rates (less than 1) that were calculated using the CO2SYS software shown in Table 5. Overall, *L. scutum* larvae settled faster and had the most percentage of metamorphs in the OA-settlement cue treatment compared to the settlement cue treatment (Figure 8).

**Table 1. Kruskal-Wallis rank sum test testing for the effect of OA on the number of metamorphs that settled. DF: degrees of freedom. Bold results indicate significance.**

<b>ChiSquare</b>	<b>df</b>	<b>p</b>
11.593	3	<b>0.00895</b>

**Table 2. Dunn Kruskal-Wallis Multiple Comparison test for the effect of OA on the number of metamorphs that settled. DF: degrees of freedom. Bold results indicate significance.**

<b>Comparison</b>	<b>Z</b>	<b>P. unadj.</b>	<b>P. adj.</b>
Control - OA	0.0000000	1.000000000	1.00000000
Control - OA, settlement cue	-2.6470618	0.008119451	<b>0.04871671</b>
OA - OA, settlement cue	-2.8633184	0.004192291	<b>0.02515375</b>
Control – settlement cue	-1.7757109	0.075780585	0.45468351
OA - settlement cue	-1.9150699	0.055483595	0.33290157
OA, settlement cue - settlement cue	0.8979861	0.369192941	1.00000000

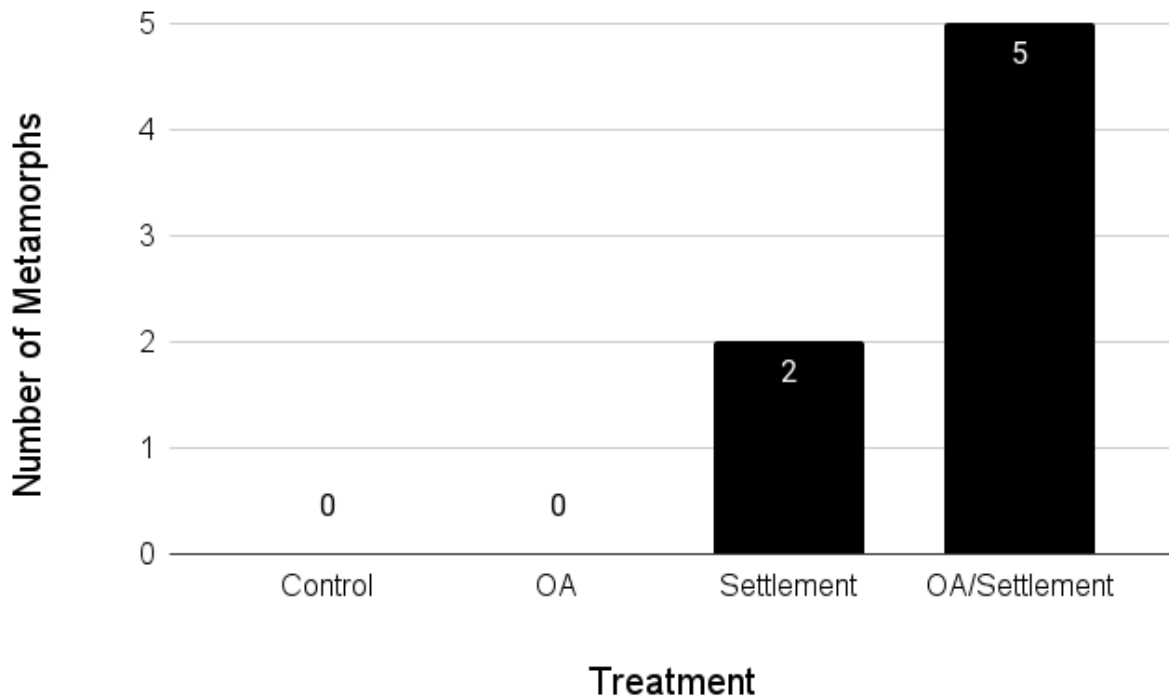


Figure 5. Total number of *L. scutum* metamorphs in each treatment that initiated settlement, *L. scutum* significantly settled more in the OA/settlement cue treatment (Table 1. and 2.).

**Table 3. Percentage of animals that had metamorphosed in each treatment per day.**

<b>Treatment</b>	<b>6-22-23</b>	<b>6-23-23</b>	<b>6-24-23</b>	<b>6-25-23</b>	<b>6-26-23</b>
<b>Control</b>	0%	0%	0%	0%	0%
<b>OA</b>	0%	0%	0%	0%	0%
<b>Settlement</b>	0%	0%	0%	20.00%	40.00%
<b>OA/Settlement</b>	0%	0%	21.43%	26.67%	37.50%

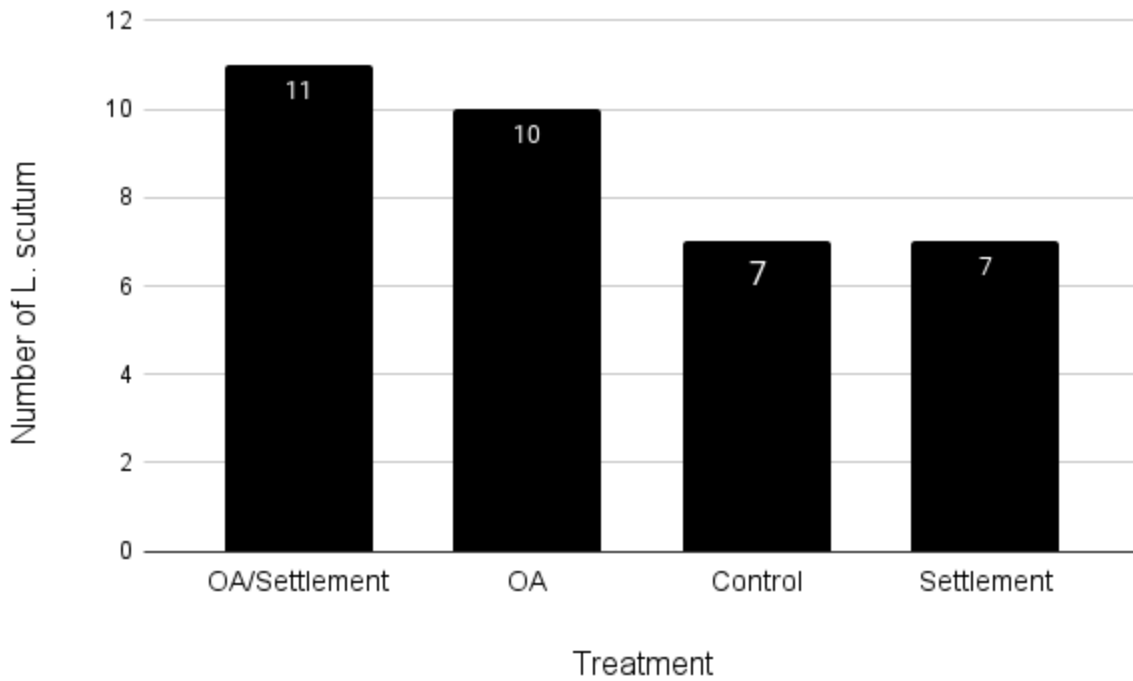


Figure 6. Total number of surviving *L. scutum* larvae per treatment at the end of the experiment.

**Table 4. Percentage of damaged larvae present per treatment by day.**

<b>Treatment</b>	<b>6-22-23</b>	<b>6-23-23</b>	<b>6-24-23</b>	<b>6-25-23</b>	<b>6-26-23</b>
<b>Control</b>	28.57%	56.52%	42.86%	44.44%	57.14%
<b>OA</b>	20.00%	43.24%	50.00%	78.57%	85.71%
<b>Settlement</b>	43.75%	37.14%	61.54%	33.33%	66.67%
<b>OA/Settlement</b>	14.71%	40.74%	35.29%	26.67%	28.57%

**Table 5. Carbonate Chemistry parameters calculated via CO2SYS software**

<b>Treatment</b>	<b>pH (total)</b>	<b>Total Alkalinity (μmol/kg SW)</b>	<b>pCO<sub>2</sub> (μatm)</b>	<b>Ω<sub>Ar</sub></b>	<b>Ω<sub>Ca</sub></b>
<b>Ambient</b>	7.642	2226.0	1083.0	1.42	0.90
<b>Ambient</b>	7.649	2213.3	1058.4	1.43	0.91
<b>OA</b>	7.333	2226.3	2283.7	0.72	0.46
<b>OA</b>	7.332	2223.6	2286.4	0.72	0.46

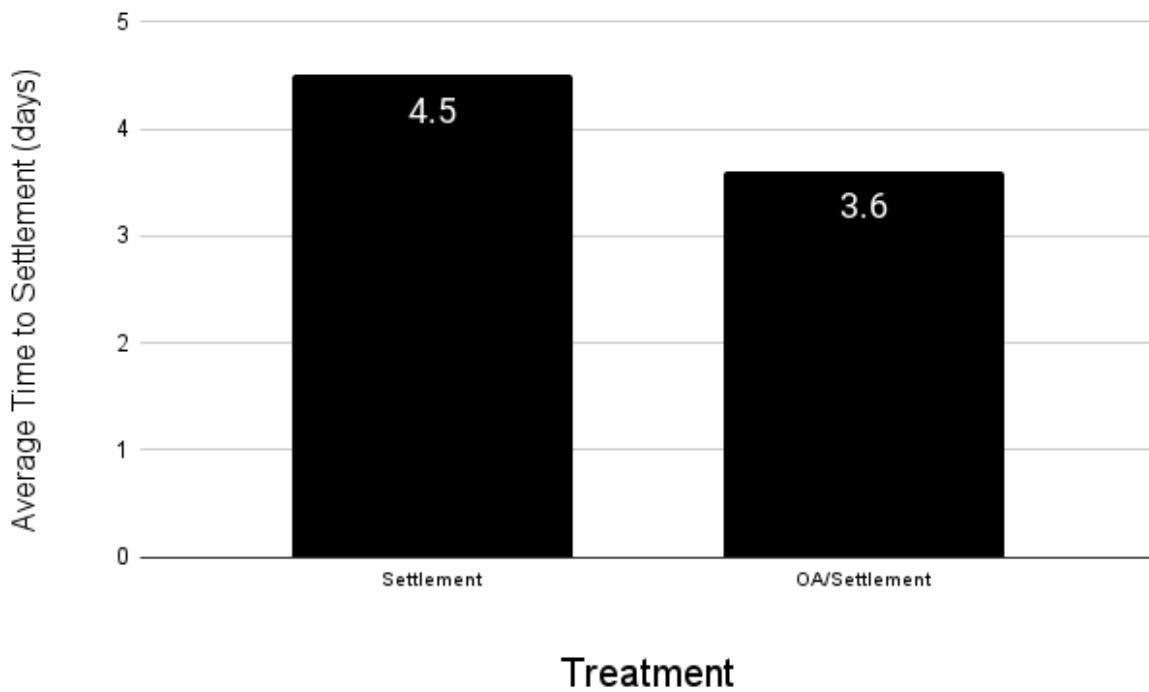


Figure 8. Average number of days for larvae to settle by treatment, for treatments in which settlement was observed.

## Discussion

The aim of this study was to investigate the impacts of OA on settlement behavior in *L. scutum*. Our results suggest that there was no negative impact of OA conditions on the settlement behavior of *L. scutum* in our experiment. Comparison of the total number of metamorphs observed revealed that larvae settled at a significantly higher rate in the OA treatments with settlement cues than in the non-OA treatments with settlement cues (Fig. 5., Table 1 and 2,  $p < 0.05$ ). Throughout the experiment, we noted the deterioration of larval health by way of cracked and/or missing larval shells. This may have been a result of our adjusted (7.3 for OA and 7.6 for ambient) and desired pH (7.4 for OA and 7.7 for ambient) being lower than it actually was for both our ambient and OA treatments. Carbonate chemistry calculations revealed saturation rates were lower than 1 (Table

5.) and explains why we observed increased deterioration of shells (Bednaršek et al., 2017). However, once metamorphs began to exhibit settlement behavior, their post larval shells were growing without any indications of damage that had been observed in other gastropod larvae (Hickman, 2001). The effect of OA on settlement behavior has been studied in other larvae, where exposure to OA negatively impacted settlement behavior and elicited a range of carry-over effects to the next life history stage (Hettinger et al., 2012; Velasco et al., 2018). However, exposure to slightly-acidified conditions has also increased settlement rates in other marine invertebrate species (Dooley et al., 2015, Wangsteen et al., 2013). Coinciding with our findings, the treatment in which we observed the most settlement behavior was in our OA-settlement cue treatment.

Marine environments in the Pacific Northwest are naturally more acidic on average than the global average as a result of constant mixing occurring in the water column (McElhany et al., 2021). Larvae that live in and are exposed to acidic environments throughout critical life history stages, such as those of *L. scutum*, may be more resilient to dramatic changes in pH. Due to the low ambient ocean pH of the San Juan Islands, larvae in this region may require more extreme conditions in order to exhibit a stress response. We exposed our larvae to a pH of 7.6 (ambient) and 7.3 (acidic), but our acidic pH treatment may not have been low enough to elicit a stress response in our larvae. Moreover, our results provide us with some insight into the level of resilience marine invertebrates possess, specifically those that undergo their entire life history in periodic-high stress.

The higher rate of settlement in the OA-settlement treatment than in the ambient-settlement treatment does not necessarily indicate that the pH level of the first treatment is beneficial to *L. scutum* larval settlement. Therefore, more larvae, younger larvae, more replicates, and a longer trial period would all allow for this observation to be investigated in greater detail. The larvae used

in this study were close to the age at which other studies have observed settlement (Kay and Emlet, 2002). Using younger larvae, which become competent after 5 d, could reveal preferences larvae have for settlement cues and/or pH conditions. Future studies could use rocks or other natural substrates from the intertidal as a settlement cue in order to make inferences on the settlement behavior of these larvae.

## **Conclusion**

From this study, it appears that a pH of 7.3 does not elicit a significantly different settlement response from *L. scutum* larvae than ambient seawater pH in the presence of a biofilm cue. In order to truly observe and understand how OA may impact the early life stages of marine invertebrates, further research should consider studying post-metamorphic life stages under OA conditions as settlement is not necessarily an indicator of future health. Additionally, this study provides the opportunity to fill critical knowledge gaps on the impacts of OA on larval species that inhabit periodically high stress environments.

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