

**The effect of pH on natural settlement and metamorphosis in the invasive limpet,
*Crepidula fornicata***

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Abstract. The average pH of ocean surface waters has dropped by about 30%, due to absorption of anthropogenic CO₂ emissions. In the past decade, much research has been conducted examining the effect of this “ocean acidification” on marine organisms. Larvae seem especially sensitive. Acidification has been shown to affect chemosensory mechanisms and behavior of clownfish larvae, but little is known about how acidification may affect the sensory biology of marine invertebrate larvae. Like many marine invertebrates, the gastropod *Crepidula fornicata* settles and metamorphoses in response to chemical cues associated with favorable habitat for juveniles. Other studies on marine invertebrates have found decreased settlement and metamorphosis at lower pH, but none have measured how pH affects settlement and metamorphosis in response to cues from adult conspecifics. We tested the effect of pH on settlement and metamorphosis of four broods of larvae of *C. fornicata* that were derived from different parents. pH had a significant overall effect on both settlement and metamorphosis in the presence of adults, but not in the direction expected. Larvae settled and metamorphosed at higher frequency at pH 7.5 and 7.7 than at pH 8.0. While this pattern was seen in three of the four broods tested, response to pH treatment varied between broods. Further research is needed to determine if differences between broods remain consistent throughout the larval period, and might therefore reflect adaptations to variable pH regimes in estuarine environments.

Introduction

Anthropogenic CO₂ emissions have been increasing since the beginning of the Industrial Revolution. Uptake of CO₂ by the oceans has subsequently increased, dropping the surface pH by as much 0.1 units in the past two centuries (Orr et al., 2005). Even

further decline is predicted within the coming centuries (Ross et al., 2011). Acidification has been found to have deleterious effects on many marine organisms, especially calcifying organisms.

Estuarine environments experience highly variable levels of $p\text{CO}_2$ as a result of high levels of respiration and upwelling (Cai and Wang, 1998). Values can dip as low as 50 ppm during algal blooms and reach as high as 9000 ppm (Borges and Frankignoulle, 2002). With the low alkalinity of estuaries, these variations in $p\text{CO}_2$ can have even more of an effect on pH than in the open ocean (Wong, 1979). The variability of pH in estuarine environments adds additional complexity to studying the effect of ocean acidification; Organisms living in these environments already experience relatively extreme pH conditions, so it seems useful to understand how well they tolerate the extremes and how they will respond to increased variability.

Recent research has focused extensively on the effect of acidification on early life stages (e.g. Byrne and Przeslawski, 2013; Byrne et al., 2010; Harris et al., 1999). Larval stages have been found to be particularly sensitive to acidification stress (Ross et al., 2011). Beyond affecting growth and development of larvae, studies have found that acidification can affect important behavioral mechanisms of larvae. Acidification has been found to impair the ability of marine fish larvae to detect and respond to olfactory cues of adult habitats and predators (Dixson et al., 2010; Munday et al., 2009); this has implications for their ability to find suitable locations to settle and to avoid predation, both of which are important functions during the larval stage.

The effect of acidification on chemosensory mechanisms of marine invertebrates is not well understood. Such organisms use chemical cues to mediate all levels of behavior,

from finding food and mates to avoiding predators (Reviewed by Hay, 2009). In many species, cues from adult habitat have been found to induce settlement and metamorphosis in larvae, facilitating preferential settlement among their own species (Reviewed by Hadfield and Paul, 2001). The veliger larvae of the estuarine limpet, *Crepidula fornicata* (Linné 1758), can be induced to metamorphose using seawater conditioned by exposure to conspecific adults for several hours (McGee and Targett, 1989). Adult cues likely facilitate settlement on the semi-permanent adult “stacks” that are formed by these sessile organisms (Collin, 1995). Mating occurs among individuals of a stack, so finding a stack to settle upon is crucial to reproductive success (Collin, 1995). It is possible that acidification may affect larvae’s ability to utilize adult chemical cues and therefore they may not settle on the adults as readily or may metamorphose at a lower rate under lower pH conditions.

C. fornicata is native to the eastern coast of the United States, but have had remarkable success as an invasive species throughout the Northern Hemisphere (Blanchard, 1997). As a consequence of oyster farming, *C. fornicata* was first introduced into the Puget Sound in Washington more than 80 years ago (Blanchard, 1997). They have since become abundant. The Puget Sound experiences extreme ranges in $p\text{CO}_2$, with a single location fluctuating from less than 100 to nearly 1,000 $\mu\text{mol CO}_2 \text{ mol}^{-1}$ (PMEL Carbon Program, 2015). During the current reproductive period of *C. fornicata*, the $p\text{CO}_2$ at the Twanoh buoy has ranged from 320 to 560 $\mu\text{mol CO}_2 \text{ mol}^{-1}$ (PMEL Carbon Program, 2015). Understanding the environmental limitations of *C. fornicata* could be crucial to predicting their further spread and continued success in their current habitats.

A challenge faced in testing how pH alters the ability of *C. fornicata* to respond to metamorphosis-inducing cues is that preparation of adult-conditioned water by the usual method, as described in McGee and Targett (1989), does not allow for the control of seawater chemistry necessary for ocean acidification experiments. Adult respiration would alter the quantity of CO₂ in the water and the chemicals they emit could serve to buffer the water. Additionally, the dissolution of their shells would buffer any low-pH treatment and make it difficult to maintain undersaturated conditions. Therefore, developing a method for using adults to measure natural settlement and metamorphosis in a flow-through system where seawater chemistry could be controlled and quantified would be vastly useful. In this study, we sought to use the flow-through system at the FHL Ocean Acidification Environmental Laboratory (OAEL) in order to investigate whether natural settlement and metamorphosis vary over a pH range typically experienced by *C. fornicata* in their invaded habitat in the southern Puget Sound.

Materials and Methods

Organism Collection and Adult Maintenance

Crepidula fornicata were collected from Totten Inlet, Thurston County, Washington in June and July of 2015. *C. fornicata* lives in stacks of individuals, with larger, immobile females usually making up the base of the stacks and a few smaller, more mobile males making up the top (Collin, 1995). During collection, adult stacks with visible males were chosen for larval collection. Small stacks of two or three individuals were collected for use as substrate and natural cues in the metamorphosis assays. All stacks were transported to Friday Harbor Laboratories (FHL) in Friday Harbor, Washington. Stacks to be used for metamorphosis assays were kept in an aerated five-

gallon bucket of natural unfiltered seawater. They were fed 0.75 mL of Reed Mariculture Shellfish Diet 1800 several times daily and their water was changed daily. Stacks for obtaining larvae were maintained individually in aerated gallon jars with two liters of natural unfiltered seawater. Adults were maintained in these jars for no more than two weeks to ensure that any broods released were oviposited before collection.

Larval Rearing

C. fornicata broods its larvae in egg masses before releasing them simultaneously. When a brood of larvae was released in one of the gallon jars, they were siphoned into a 150 μm Nitex sieve in a beaker. Stranded larvae at the bottom of the sieve were then washed into a small bowl of 1 μm -filtered seawater. If more than one egg mass was released in the same jar on the same date, the larvae were not used to ensure that all larvae in a brood were siblings.

Gallon glass jars were filled with two liters of 1 μm -filtered seawater of pH 8.0. Five hundred larvae were counted using a pipette under a microscope and then placed into each jar. The cultures were then reared on the benchtop at room temperature (21-23°C). The water within the culture jars was changed every other day, when the larvae were fed *Isochrysis* sp. (clone T-ISO) at concentrations of 12×10^4 cells mL^{-1} . When necessary, jars were cleaned with Bon Ami and hot water to prevent a buildup of biofilm. Under these conditions, larvae become competent for metamorphosis after approximately 12 days and at a size of about 800 μm (Pechenik, 1980). For this experiment, larvae were used between the age of 11 and 16 days at an approximate size of 900 to 1200 μm .

Metamorphosis Assays

Stacks of adults served as natural cues for metamorphosis while being held in flow-through containers that maintained stable conditions of pH (7.5, 7.7, and 8.0) and temperature (20° C). Before being used in metamorphosis assays, adult stacks were cleaned of anything growing on them with a toothbrush to make larval/juvenile observation easier. Dental wax was lodged into the crevices of the empty shell that formed the bottom of each stack to prevent larvae and juveniles from disappearing into the shell. Each stack was then secured with a nylon zip-tie to prevent the adults from consuming the larvae, as adults have been found to filter larvae out of the water (Pechenik et al., 2004). Stacks were then placed into approximately 400-mL polyethylene wide-mouth jars that were modified to have a 160 µm Nitex bottom to allow water to flow through the containers. Four replicate containers were placed into each treatment tank at the OAEL, which regulated temperature and pH as described by Timmins-Schiffman et al. (2013). A constant flow-through of pH and temperature-controlled (20°C) water into the containers was maintained using irrigation drippers at a rate of 50 ml min⁻¹. Twenty competent larvae were placed into each container to be tested for settlement and metamorphosis. After one hour, the containers were removed from the microcosms. The adult stacks were removed and washed into a glass bowl using a squirt bottle. The zip-tie was then removed. The adults were examined under a microscope to remove any remaining larvae or juveniles. All individuals that were on the adult when it was removed from the container were counted as settled. Settlement (%) was calculated as:

$$\text{Settlement} = \frac{\text{no. settled}}{\text{no. initial larvae}} \times 100$$

All individuals were then examined to determine if they had metamorphosed.

Metamorphosis was defined as the complete loss of velum by a larva (Figure 1).

Metamorphosis (%) was calculated as:

$$\text{Metamorphosis} = \frac{\text{no. metamorphosed larvae}}{\text{no. initial larvae}} \times 100$$

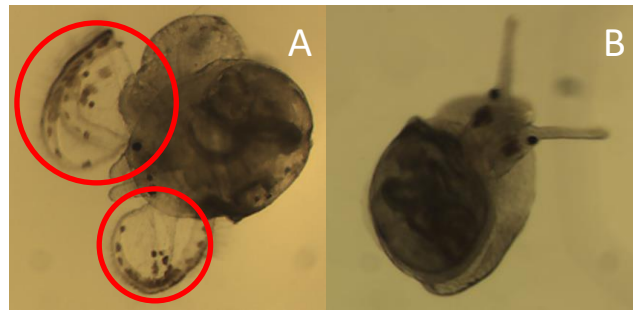


Figure 1. Examples of the larval and juvenile stage of *C. fornicata*. **A.** A larva (velum circled in red). **B.** A metamorphosed juvenile (clearly lacks velum).

Any missing larvae were not counted toward the initial number of larvae when calculating settlement and metamorphosis as it was unknown if they would have settled/metamorphosed had they not been eaten. This process was repeated with a total of four broods. During the course of the investigation, the treatment tanks were changed twice such that each pH treatment was in a different tank after each change. This ensured that any results were not site-dependent, as metamorphosis assays at each pH were performed in three different tanks over the course of the experiment.

Carbonate Chemistry

The pH of the culture jars was measured before and after water changes using a Durafet III pH probe (Honeywell, Morristown, New Jersey). During metamorphosis assays, the pH of the 3-L microcosms was measured at the end of each assay. The probe was verified periodically using the m-cresol purple spectrophotometric method of

Clayton and Byrne (1993). The salinity and temperature before and after water changes were measured using a conductivity meter. Water samples for total alkalinity were taken from the 3-L microcosms when the adults were present during a metamorphosis assay. Alkalinity was then measured using the open cell titration method described by Dickson et al. (2007). CO2Calc version 1.2.0 for Windows (US Geological Survey, www.idn.ceos.org) was used to calculate $p\text{CO}_2$ and the saturation states of calcite and aragonite.

Analysis

Prism was used for statistical analysis. ANOVAs were used to distinguish the effect of brood identity and treatment on settlement and metamorphosis. Tukey multiple comparisons were used to compare settlement and metamorphosis at the different treatments.

Results

Metamorphosis Assay

There was a significant effect of pH treatment on settlement and metamorphosis ($p=0.0322$ and $p=0.0346$, respectively). Settlement at pH 8.0 was significantly lower than at 7.5 but not at 7.7 ($p=0.0247$ and

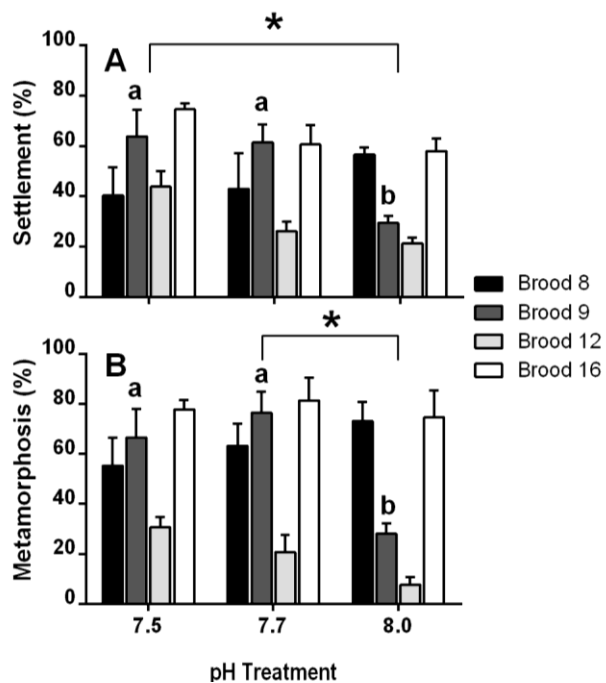


Figure 2. Rates of settlement and metamorphosis of *Crepidula fornicata* for four different broods at three pH treatments: 7.5, 7.7, and 8.0. **A.** Settlement. **B.** Metamorphosis. Comparisons made using ANOVA and Tukey multiple comparison intervals ($\alpha = 0.05$). Different letters between treatments in Brood 9 indicate statistical differences. Asterisk (*) indicates statistical difference between treatments. Error bars represent +1 SEM.

p=0.4375, respectively; Figure 2). Metamorphosis at pH 8.0 was significantly lower than at 7.7 but not at 7.5 (p=0.0376 and p=0.1132, respectively; Figure 2). Neither settlement nor metamorphosis were significantly different at pH 7.5 or 7.7 (p=0.3000 and p=0.8661, respectively). While settlement and metamorphosis were lowest at pH 8.0 in all broods except Brood 8, Brood 9 was the only brood within which this trend was significant (settlement: 7.5 vs. 8.0, p=0.0061, 7.7 vs. 8.0 p=0.0107; metamorphosis: 7.5 vs. 8.0, p=0.0048, 7.7 vs. 8.0, p=0.004; Figure 2). Settlement and metamorphosis varied between broods (p<0.0001) and there was no significant effect of treatment in any of the other broods (p>0.05). In most cases, not all of the original 20 initial larvae were recovered. Most frequently, 15 individuals (75%) were recovered (Table 1).

Table 1

Descriptive statistics for number of larvae recovered from metamorphosis assays (\pm standard deviation).

<u>Average Recovered</u>	<u>Minimum Recovered</u> (out of 20)	<u>Maximum recovered</u>	<u>Mode</u>
15.5 \pm 2.66	6 (1)	20 (3)	15 (11)

The frequency of minimum recorded, maximum recorded and mode are indicated in parentheses.

Table 2

Seawater properties during larval culturing and metamorphosis assays (\pm standard deviation).

	Larval culturing	Metamorphosis assay		
	pH 8.0	pH 7.5	pH 7.7	pH 8.0
Temperature ($^{\circ}\text{C}$)	21.2 ± 1.1	19.7 ± 0.2	19.8 ± 0.3	19.7 ± 0.2
Salinity	30.0 ± 0.3	30.1 ± 0.3	30.1 ± 0.2	30.1 ± 0.3
TA ($\mu\text{mol kg}_{\text{SW}}^{-1}$)	2114 ± 23 (2)	2110 ± 12 (3)	2106 ± 7 (3)	2106 ± 8 (3)
pH _{Total}	7.96 ± 0.06	7.51 ± 0.02	7.71 ± 0.03	7.96 ± 0.05
$p\text{CO}_{2\text{calc}}$ (μatm)	574 ± 7	1481 ± 115	910 ± 86	491 ± 60
Ω_{calcite}	2.93 ± 0.05	1.27 ± 0.08	1.95 ± 0.16	3.18 ± 0.35
$\Omega_{\text{aragonite}}$	1.89 ± 0.04	0.82 ± 0.05	1.25 ± 0.10	2.04 ± 0.22

$p\text{CO}_{2\text{calc}}$, Ω_{calcite} and $\Omega_{\text{aragonite}}$ calculated from the measured values of total alkalinity (TA), pH, temperature, and salinity.

The number of samples for TA is indicated in parentheses.

Salinity and temperature remained consistent between treatments. Intended pH values were achieved in each treatment during the metamorphosis assays with no overlap between different treatments. Calcite was supersaturated in all treatments. Aragonite was undersaturated in the pH 7.5 treatment but supersaturated in the pH 7.7 and 8.0 treatment (Table 2).

Discussion

Viability of Flow-Through Method for Natural Settlement Experiments

This study explored a unique method of measuring settlement and metamorphosis in response to adults. Cahill performed a comparable experiment measuring how adult density affected larval recruitment in the field, but, to date, our experiment seems to be the only one that has performed such an experiment in a laboratory setting (2015). This method is promising but has clear weaknesses. Even when adult stacks were zip-tied to prevent gaping in the shell, all twenty larvae were recovered on only three occasions and

on one occasion, only six were recovered. However, on average, the recovery rate was approximately 77 percent and settled larvae/juveniles were clearly visible and easy to retrieve under a dissecting scope. Greater recovery could be possible in species that do not consume their larvae, or consume them at a slower rate (Pechenik et al., 2004). Additionally, *C. fornicata* have been found to consumer larvae at a slower rate in the presence of other food, so simultaneously feeding the adults while running the metamorphosis assays could increase recovery (Pechenik et al., 2004). More work should be done to see how recovery rate is affected by the initial number of individuals and time.

Settlement and Metamorphosis

pH had a significant effect on both settlement and metamorphosis. However, this effect was in the opposite direction of what was expected. In a similar study where veliger abalone larvae were exposed to various pH after being raised to competence under ambient conditions, metamorphosis was significantly reduced from controls at pH 7.61 and below (Guo et al., 2015). Studies on the Pacific oyster (*Crassostrea gigas*) and the Olympia oyster (*Ostrea lurida*) also found a decrease in metamorphosis with decreased pH (Hettinger et al., 2013; Ko et al., 2014). Several studies with coral have found a decline in settlement at low pH, sometimes after as little as two hours of exposure (Albright, 2011; Doropoulos and Diaz-Pulido, 2013). To the contrary, in this study, where significant differences were present, settlement and metamorphosis at pH 8.0 were always lower than at the other treatments, though this was the highest pH and the conditions were closest to the ambient conditions under which the larvae were raised.

Even though the pH 8.0 treatment showed lower settlement and metamorphosis, these measures remained relatively robust across all treatments. These pH are within the

range that *C. fornicata* would experience during the reproductive period in the southern Puget Sound, consistent with their abundance in this region (PMEL Carbon Program, 2015). Their ability to tolerate a range of conditions may explain how they continue to perform throughout their native and invasive ranges while other mollusks, such as oysters, are showing a decrease in larval recruitment if spawning coincides with naturally-occurring low pH events (Barton et al., 2012). It would be informative to explore further the range of pH at which settlement and metamorphosis remain robust to understand how future decrease in pH could affect this population.

There was a high degree of variability in response between broods. A study with the oyster, *Saccostrea glomerata*, found variability in response to elevated $p\text{CO}_2$ within and between populations (Parker et al., 2012). Genetic variability between organisms within a population allows the population to adapt to changes in the environment (Applebaum et al., 2014). This can have implications for the genetic composition of future populations, as individuals who are “unaffected” or “thrive” in future conditions will continue to pass on their genetic material, but those that “suffer” will not (Applebaum et al., 2014). Since each brood used for this study was only used in one assay, it is unclear whether the variability seen is the result of genetic variation between broods or other unexplained sources of variation within the experiment. In order to discern the cause of this variability, multiple metamorphosis assays should be performed within single broods to see if their responses are repeatable, and therefore indicative of variation between broods.

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