

# Effect of pH on the Early Development of the Biofouling Ascidian *Ciona robusta*

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Ocean acidification (OA) impacts the survival, fertilization, and community structure of marine organisms across the world. However, some populations or species are considered more resilient than others, such as those that are invasive, globally distributed, or biofouling. Here, we tested this assumption by investigating the effect of pH on the larval development of one such tunicate, *Ciona robusta*, which is currently exposed to a wide range of pH levels. Consistent with our hypothesis, *C. robusta* larvae developed and metamorphosed at a rate comparable to control (pH 8.0) at modest near-future conditions (pH 7.7) over a 58-hour period. However, development was stunted at the extreme low pH of 6.8 such that no embryo progressed beyond late cleavage after 58 hours. Interestingly, piecewise regression of the proportion of embryos at the most advanced stage at a given time point against pH identified a breakpoint with the highest pH (~pH 7.6) at around hatching. The variation in breakpoint pH throughout ontogeny highlighted that the sensitivity to decreasing pH differs significantly between developmental stages. More broadly, our results show that even a cosmopolitan, biofouling, invasive species could be negatively impacted by decreasing pH.

**Key words:** Tunicates, Tadpole, Larvae, Global climate change, Ocean acidification.

## BACKGROUND

As atmospheric carbon dioxide (CO<sub>2</sub>) levels continue to increase, the average pH of the surface ocean is predicted to drop from the pre-industrial value of 8.1 to about 7.7 by 2100 (Caldeira and Wickett 2005; Zeebe 2012). In coastal and upwelling locations, the pH could even reach as low as 7.2 (Feely et al. 2008; Hofmann et al. 2011). The process of ocean acidification (OA) has wide-reaching ecological consequences, from reducing density in coral reef skeletons (Mollica et al. 2018) to drastically elevating the mortality rate of bioturbating brittle stars (Dupont et al. 2008). However, not all species respond to OA negatively (Maboloc and Chan 2021; Pecquet et al. 2017). Biofouling species are known to be resilient to environmental stress, as

they disperse over long distances (Carlton et al. 2017; Rech et al. 2016; Ulman et al. 2019) and are successful when exposed to varying conditions—so much so that considerable financial resources are expended to combat their resilience (Oreska and Aldridge 2011). If invasive biofouling species are less vulnerable to OA than native sessile species, their relative success over more vulnerable natives could imply further changes in community dynamics (Lord et al. 2019; Sanford et al. 2014; Young and Gobler 2021). By fertilizing *Ciona robusta* gametes at four different pHs to simulate varying present-day average surface ocean pH (pH 8.0) to an extreme low (pH 6.8) and tracking development until metamorphosis, this study aims to identify a developmental tipping point of a fouling species.

Ascidians from the genus *Ciona* are widespread

biofouling, invasive species. The hermaphroditic, short-lived *C. robusta* is native to the Pacific Northwest but now has a disjointed worldwide distribution: in the North and South Atlantic Ocean, the South Pacific Ocean, the Indian Ocean, and the Mediterranean Sea (Bouchemousse et al. 2016b). This wide biogeographic distribution and its association with other biofoulers implies that *C. robusta* already experiences varying environmental conditions, including pH levels. Such pre-exposure suggests that they are highly tolerant to different environmental stressors. Indeed, the early development of this species can take place across a wide range of temperatures (15–25°C) and salinities (25–34 psu) (Kim et al. 2019; Malfant et al. 2017). There are, however, site-specific differences in the timing of larval settlement, which correspond to different ambient temperatures and suggest variations among populations (Bouchemousse et al. 2016a; Bouchemousse et al. 2016b; Clutton et al. 2021; Kim et al. 2019). Interpopulation variations in pH sensitivity in *C. robusta* are also plausible. *C. robusta* are found along the California upwelling current system, which shows a large fluctuation in pH (Chan et al. 2017; Hauri et al. 2013). Therefore, it is possible that tunicates found in this ecosystem exhibit local adaptation and are more resilient to reduced pH (Hofmann et al. 2014; Przeslawski et al. 2015). Furthermore, the recent work by Gallo et al. (2019) showed that decreasing the pH to 7.8, both through exposure to CO<sub>2</sub> vents and laboratory CO<sub>2</sub> additions, reduced *C. robusta* sperm motility and altered their morphology and physiology. However, the physiology of the sperm fully recovered after seven days of exposure. Such observations suggest that pre-exposure of the adults may confer resilience, making *C. robusta* less affected by reductions in pH.

While *C. robusta* have demonstrated resilience to a wide range of temperatures and salinities, it is unknown if their durability extends across various ontological stages under low pH conditions. An ecotoxicological assay with mineral acid and base additions showed that the closely related *C. intestinalis* develop normally across a wide range of pH, from pH 7.4 to pH 8.8 (Bellas et al. 2003). Therefore, we exposed *C. robusta* to a lower range of pH values, including an extreme low of pH 6.8, to identify a tipping point. We further hypothesized that the effect of reduced pH would vary across early development, as previous studies have found varying responses to environmental stressors across developmental stages in other marine invertebrates (Mak and Chan 2018; Pineda et al. 2012).

## MATERIALS AND METHODS

### pH manipulation and monitoring

A pH computer (American Marine, Inc.) was used to adjust the pH of the artificial seawater (Crystal Sea Bioassay Mix, ASW) through CO<sub>2</sub> bubbling immediately before spawning. Two 50 mL alkalinity samples for each experimental pH were taken from these newly prepared ASW. The alkalinity of the samples was determined through Gran titration (905 Titrando, Metrohm, Switzerland). Calibration was performed using the standard seawater provided by the Dickson Lab (Batch 181). Millivoltage and pH of the samples and a Tris/HCl buffer solution provided by the Dickson Lab (salinity = 33, Batch T36) were measured with a Metrohm 913 pH meter and unitrode with Pt1000 (Herisau, Switzerland). Salinity was measured with a handheld refractometer. Procedural controls, *i.e.*, independent flasks with ASW and larvae, were prepared, and their pHs were measured 24- and 48-hours after the initiation of the experiment. These control flasks were used to reduce disturbance caused by inserting a pH electrode. The CRAN package ‘seacarb’ was used to calculate the potentiometric pH of the water throughout the experiment. Carbonate system parameters ( $p\text{CO}_2$ ,  $\Omega_{\text{ar}}$ , and  $\Omega_{\text{ca}}$ ) were calculated from these measurements in CO2SYS using the dissociation constants from Mehrbach et al. (1973) as refitted by Dickson and Millero (1987).

### Spawning

The adult ascidians, *Ciona robusta*, were procured from M-Rep Ltd (California). Adults were kept in ASW at 16°C ( $\pm$  1°C) and a salinity of 32 psu ( $\pm$  2 psu). Five adult animals were dissected for their gametes. A 5 ♀  $\times$  4 ♂ cross was performed, where the eggs of a given individual were distributed into four 35mm-diameter glass dishes. The sperm of all four individuals were added into each dish, which held filtered ASW (0.22  $\mu\text{m}$ ) with one of the four experimental pH values (pH 8.0, pH 7.6, pH 7.2, and pH 6.8). pH 8.0 was chosen to represent present-day conditions and is deemed the optimal pH value for tunicate development (Bellas et al. 2003; Irvine et al. 2019), and pH 7.6, 7.2, and 6.8 are reduced pH conditions; these low pH levels were already recorded in fouling communities (Woolmington and Davenport 1983). The dishes were placed on a cold plate set at 16°C, and the gametes were mixed through gentle pipetting. After one hour, all dishes were checked under a dissecting microscope to ensure that > 95% fertilization had occurred by checking for first cleavage.

## Larval development

Three 25 mm<sup>2</sup> tissue culture flasks were used as replicates for each experimental pH. The embryos were then distributed to flasks of their respective pH treatment at a concentration of 10–15 embryos mL<sup>-1</sup>; this concentration was used to avoid overcrowding the flasks, which would contribute to a decrease in pH. All flasks were completely filled to minimize the potential of trapped air bubbles altering the gas equilibrium. Given the small volume used and to avoid disturbing the larvae with a pH probe, we set up two additional flasks for each pH treatment with the same larval concentration (procedural controls) and used them to measure pH at 24- and 48-hours post-fertilization (hpf). All flasks were placed into a water bath held at 16°C (Ecoline Silver, Luda).

At each of the eight observation time points (3, 6, 12, 21, 30, 39, 49, and 58 hpf), two 2 mL subsamples were taken from each replicate flask of each of the experimental pHs (4 pH conditions x 3 replicate flasks x 2 replicate subsamples x 8 time points). Before taking each sample, the flask was inverted 2–3 times to ensure we took a random, homogenized sample. Such inversions also potentially dislodged any larvae adhered to the sides of the flask. This “planktonic” sampling approach was confounded by the fact that a reduction in larval density could be due to mortality, larvae attaching to the flask, or a variation in degree of dislodgement. These time points were chosen to match a published *C. robusta* developmental schedule, with 58 hours marking the advancement of larvae into the metamorphosis period (Hotta et al. 2020 2007). The samples were fixed with 4% formaldehyde immediately following subsampling for subsequent processing. For each sample, the overall number of embryos was counted, and the stage of each embryo was recorded following Cahill et al. (2016) and Hotta et al. (2020 2007). Unfertilized eggs were also counted and noted; they were distinguished from eggs that were fertilized but did not reach cleavage, with only the latter being included in our staging analysis.

## Statistical Analysis

The relative density in each subsample of a given flask was defined as the number of larvae counted divided by the average count between the three replicate flasks during the first sampling for that pH, which took place at 3 hpf. It was essential to calculate relative density, as each flask started with slightly different initial counts of larvae, ranging from 10–15 individuals per mL. Given that Hotta et al. (2020) suggested the adhesion period occurs at 24–27 hpf at 18°C and that

our sampling method could not differentiate between larvae that died, dissolved, or were missing due to attachment, we only used the data collected from the first 21 hpf for the density related statistical analysis. For all other analyses, however, we included larval data up to 58 hpf.

After confirming the data met the normality and homoscedasticity assumptions with the Shapiro-Wilks and Levene’s tests, an ANOVA was used to examine the change in relative density over time, with pH as the fixed factor and time as the covariate. We also attempted a more commonly used regression approach to compare larval density (e.g., Chan et al. 2015). Since we used different replicate flasks at each time point, in contrast to sampling the same flask repeatedly, no statistical comparison in the regression slope could be made.

We conducted a piecewise regression to determine the exact pH at which the developmental rate at each time point altered significantly (referred to as the breakpoint). We used the most advanced developmental stage for each time point as a proxy for developmental rate; this was computed as the proportion of larvae that had reached a particular stage for each pH treatment. Samples in which less than 10 individuals were reported were excluded from this analysis. This “breakpoint” analysis, which was completed with the SLM - Shape Language Modeling Toolbox of MATLAB, enabled us to identify the pH at which there was a change in trend in the data.

## RESULTS

### Carbonate chemistry

The total scale pH within the small volume experimental flask changed over the experimental period in the presence of *Ciona* embryos. However, the relative difference between the pH treatments was stable over time (Table 1). While the nominal pH 7.2 and 6.8 were undersaturated in calcite throughout the experiment, only the pH 8.0 flask had an aragonite saturation state greater than 1.

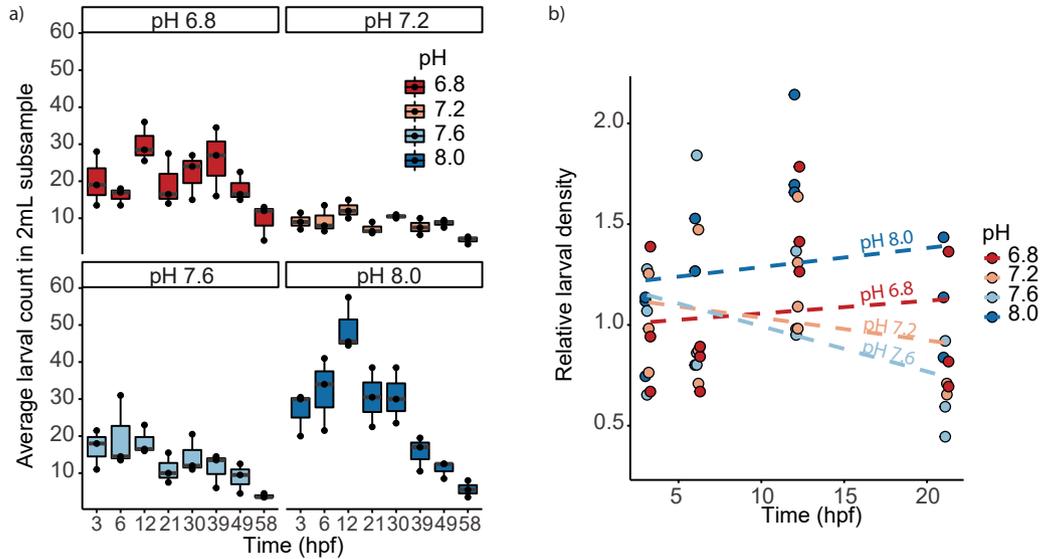
### Larval density over time

The raw count of larvae differed significantly between pHs at two time points ( $F_{1,91} = 37.011$ ,  $p < 0.001$  at 12 hpf;  $F_{3,91} = 22.641$ ,  $p < 0.001$  at 49 hpf, Fig. 1a). However, it is important to note that the flasks were not repeatedly sampled over time. Instead, different sets of flasks were used for every time point. Therefore, the density of larvae relative to the average between the three replicate jars at 3 hpf (starting time point) was

used to better access the effect of pH on survivorship accounting, although we estimated putting 10–15 individuals per mL (~30 individuals in 20 mL, some sets of replicates had excess). Once normalized, the relative larval density did not significantly change with time nor pH (Table 2, Fig. 1b). Within the first 21 hpf, larval density did not have a significant linear relation with time across all treatments (Table 2, Fig. 1b).

### Larval development and breakpoint analysis

At a given time point, the proportion of larvae reaching a more developed stage increased with pH (Fig. 2). For example, at 6 hpf, 86.7% ( $\pm 3.6\%$  S.E.) of larvae at pH 8.0 had reached the gastrula stage compared to 31.2% ( $\pm 3.2\%$ ) at pH 7.6 and none at the lower pH treatments. This trend continued at 30 hpf, where the



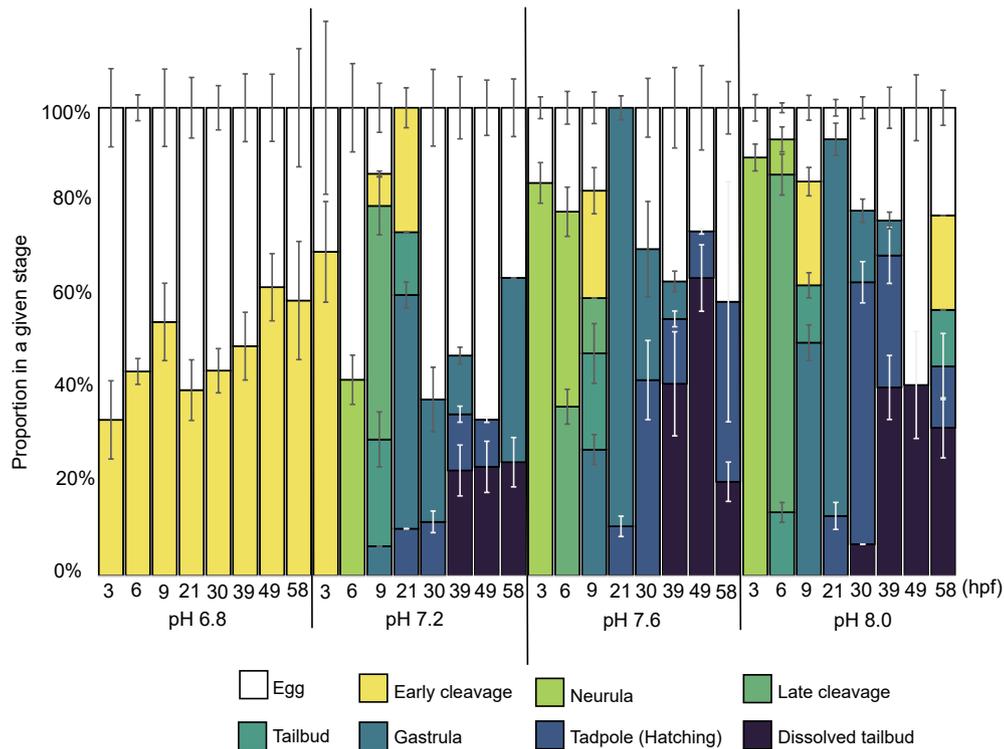
**Fig. 1.** Average number of individual *Ciona robusta* found in 2 mL subsample within each replicate flask (a) varied across pH and time (3–58 hpf). To enable comparison between treatments that had different initial concentration, larval count was normalized against the average of the triplicate flask of the given pH at 3 hpf. In other words, the average between the three replicates at 3 hpf for a given pH = 1. This survivorship estimate (b), indicated by the relative density across four pHs (pH 8.0, pH 7.6, pH 7.2, and pH 6.8) from fertilization to meta-metamorphosis (adhesion period at 21 hpf), did not vary with time or pH (ANCOVA, Table 2). Each dot represents a single replicate flask. The dotted regression lines were not significant but illustrate the general trends (Table 3).

**Table 1.** Carbonate chemistry of the artificial seawater used. Total scale pH was measured at the beginning of the experiment (freshly prepared ASW) and after incubating with tadpole larvae in the water bath for 24 and 48 hours. Duplicate samples of the prepared water were measured with Gran titration, and the average was used to compute the carbonate chemistry

	Nominal pH	Measured parameters			Calculated parameters	
		pHT	Alkalinity ( $\mu\text{mol kg}^{-1}$ )	$p\text{CO}_2$ ( $\mu\text{atm}$ )	$\Omega_{\text{ca}}$	$\Omega_{\text{ar}}$
Freshly prepared ASW	8.0	7.95	2310, 2217	511.7	3.15	2.02
	7.6	7.54	2492, 2534	1595.3	1.52	0.98
	7.2	7.23	2180, 2122	2912.9	0.62	0.40
	6.8	7.00	2183, 2195	5073.6	0.38	0.25
24 hpf	8.0	8.07	N.A.	371.2	3.97	2.55
	7.6	7.65	N.A.	1216.8	1.92	1.23
	7.2	7.26	N.A.	2712.7	0.67	0.43
	6.8	7.03	N.A.	4728.9	0.41	0.26
48 hpf	8.0	7.95	N.A.	517.6	3.09	1.98
	7.6	7.46	N.A.	1938	1.27	0.82
	7.2	7.22	N.A.	2955.3	0.63	0.41
	6.8	7.02	N.A.	4831.0	0.41	0.26

most larvae reached the late tailbud and hatched stages at pH 8.0 (60.6%, ± 1.6%), followed by pH 7.6 (42.7%, ± 9.9%) and pH 7.2 (8.1%, ± 3.5%). While larvae began to metamorphose during the later time points, more developed individuals were collected in the subsample from the pH 7.6 treatment than the other pH treatments;

at 49 hpf, 32% (± 6.3%) had reached the curled up stage in the pH 7.6 treatment, with only 17.3% (± 2.7%) in pH 8.0 and 5.3% in one flask of pH 7.2 reaching the same stage. Oddly, at 21 hpf, we recorded no eggs in pH 7.2, 7.4, or 8.0 but counted eggs in all of the following time points. More importantly, at pH 6.8, there was a



**Fig. 2.** Ocean acidification led to developmental delay in *Ciona robusta*. The mean and standard error for the proportion of embryo/larvae at a given stage relative to the total larval count at a particular time point were plotted. Note that 100% represents a different relative larval density for each bar (see Figure 1 for larval density).

**Table 2.** pH alone did not affect larval density over the first 21 hpf according to an analysis of covariance

Factor	d.f.	MS	F	p
Time	1	0.039	0.274	0.604
pH	3	0.226	1.596	0.204
Error	43	0.141		

**Table 3.** There was no significant linear regression between normalized larval density, a proxy for survivorship, and time within the first 21 hpf (see Fig 1). y represents the proportion of larvae that survived at the time of interest compared to the larval density recorded at 3 hpf, and x is the time of interest

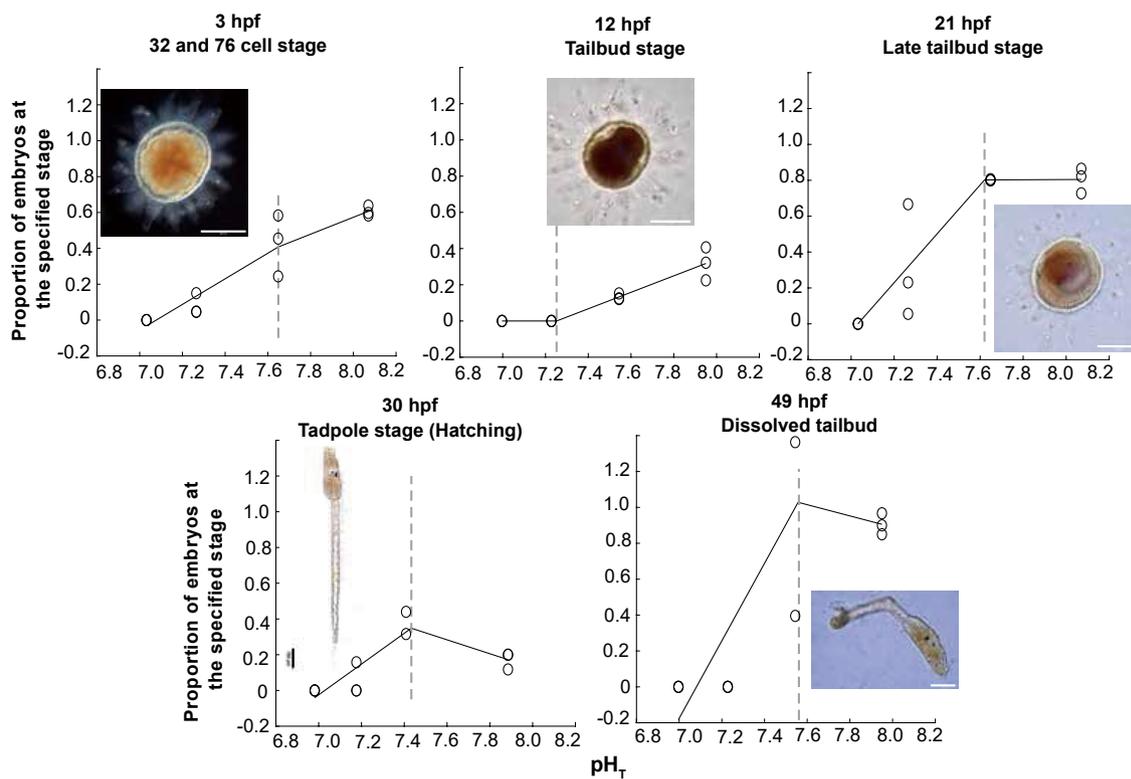
pH	Equation	r <sup>2</sup>	F	p
6.8	y = 0.006x + 0.996	0.015	0.154	0.703
7.2	y = -0.01x + 1.139	0.048	0.502	0.495
7.6	y = -0.022x + 1.216	0.181	2.215	0.168
8.0	y = 0.009x + 1.193	0.026	0.271	0.614

slower rate of development compared to the other three pHs, with no eggs developing past cleavage throughout the 58-hour experiment (Fig. 2).

All piecewise regressions were statistically significant and accounted for at least 56% of the variance observed in the data (Table 4). The breakpoint pHs differed across time: across 3, 12, 21, 30, and 49 hpf (Fig. 3). Earlier development, specifically the gastrulation and tailbud stages, was little affected by pH reduction and had breakpoints of about pH 7.3. In contrast, the breakpoint pH was the highest around ~ pH 7.6 when the larvae began hatching.

### DISCUSSION

Ocean acidification negatively affects many marine organisms, and early developmental stages are often considered particularly vulnerable (Kroeker et al. 2010; Przeslawski et al. 2015). In our observation, *Ciona robusta* fertilized, developed, hatched, and metamorphosed at the pH level predicted for the end of this century, pH 7.7 (Caldeira and Wickett 2005; Zeebe 2012). However, the developmental rate decreased with decreasing pH, with little to no metamorphosis observed after 58 hpf at pH 6.8. Sensitivity to changes in pH can



**Fig. 3.** The breakpoint pH, represented by the dotted line, varied throughout ontogeny. The breakpoint was identified by piecewise regression. Only five representative time points were presented (see Table 4 for the full regression statistics for all stages). The scale bars of the insets were 100  $\mu$ m.

**Table 4.** Piecewise linear regression detecting breakpoint pH for the proportion of larvae reaching the most advanced stage at each time point

Time (hpf)	Constant	Slope 1	Slope 2	r <sup>2</sup>	Breakpoint pH
3	-0.1827	1.028	0.906	0.676	7.56
6	0	0	0.866	0.99	7.31
12	0	0	0.317	0.922	7.25
21	0	0.820	0.8046	0.871	7.62
30	-0.0328	0.408	0.606	0.901	7.65
39	0	0.159	0.044	0.557	7.64
49	-.0405	0.348	0.173	0.754	7.43
58	0	0	0.494	0.895	7.43

differ between developmental stages in populations of the same species: the breakpoint for successful development was pH 7.4 for late tailbud stage but around pH 7.6 at hatching. While the early development of this biofouling species was unaffected by near-future ocean acidification, further reductions in pH could decrease their recruitment success.

Similar to other biofouling species, we did not observe a significant effect of pH on the mortality of *C. robusta* during the first 21 hpf at 16°C. In urbanized coastal systems, eutrophication and the resulting deoxygenation further drives pH values downward (Wallace et al. 2014). pH values beneath the macrofouling community can reach levels far lower than what is predicted for future acidification (Woolmington and Davenport 1983). It is therefore possible that *C. robusta* were pre-exposed and selected to survive lower pH conditions (Maboloc and Chan 2021; Sunday et al. 2014). Such relatively high resilience highlights this species as a likely winner in the future ocean. Indeed, a colonization study on the fouling community showed that ascidians' abundance can increase up to four-fold at pH 7.7, while other space competitors, such as the calcifying tubeworm, decrease in density (Peck et al. 2015). *In-situ* observations showed a reduction in overall diversity (Brown et al. 2016). *C. robusta*'s resilience to low pH might also be related to their mode of development. Pecquet et al. (2017) showed that the fouling bryozoan *Bugula neritina* survived and successfully settled amid delay in time even at pH as low as 6.5. Both *B. neritina* and *C. robusta* have non-feeding larvae with relatively short pelagic larval durations (< 48 hours). Our observation supports the speculation that non-feeding larvae may be an advantage in unpredictable and extreme environments (Byrne and Hernández 2020; Dupont et al. 2010).

The differences in the breakpoint pH across different time points suggest that some developmental stages are more sensitive to reductions in pH than others. In particular, the breakpoint exceeded pH 7.7 between 30 to 40 hpf, during which the most advanced larvae were hatching or swimming as tadpole larvae. More acidic conditions have been shown to negatively impact hatching success in other marine invertebrates (Espinel-Velasco et al. 2018), such as the stone crab (Gravinese 2018), the Tanner crab (Swiney et al. 2016), and the barnacle *Semibalanus balanoides* (Findlay et al. 2009). The oxygen consumption rate of the sister species *C. intestinalis* significantly increases at hatching and decreases after tail resorption (Ishikawa et al. 1972). Hypercapnia and/or acidosis associated with an elevated oxygen demand (Pörtner 2008) at hatching could explain why the reduction of pH disproportionately affects this stage. Nevertheless, the activity of purified

hatching enzymes extracted from *C. intestinalis* peaked at pH 8.5, and the activity level dropped off 50% of the peak at pH 7.5 (D'Aniello et al. 1997). Further immunolocalization and quantification of this enzyme (Scippa et al. 2006), combined with physiological measurements, e.g., oxygen consumption and protein turnover rate, could inform the mechanisms that underlie the sensitivity (Pan et al. 2015). The variation in sensitivity to pH across life history stages caution against making generalized predictions of organismal response to climate change based on observations made only on the more accessible adults (Collin et al. 2021).

While we fertilized the gametes at the four pH levels, the potential effect of reduced pH on sperm motility and egg fertilizability was not investigated. After acclimation to pH 7.8 for a week, the sperm motility of adult *C. robusta* returned to normal (Gallo et al. 2019). This observation suggests that extracting gametes without acclimating the adults could have magnified the effect of OA observed. If such acclimation applies to other development, the resilience of *C. robusta* to low pH would be higher than we have presented. Future transgenerational studies that mimic environmental conditions would provide a more accurate prediction of the performance of this fouling species to acidifying ocean, and subsequently, its interactions with other benthic species.

Aside from the lack of acclimation of the adults over gametogenesis, our current sampling approach (duplicate, 2 mL sub-sample per flask) was somewhat limiting, as illustrated by the lack of eggs at 21 hpf in the pH 7.6 treatment when we found unfertilized eggs before and after that said time point (Fig. 2). A complete count of every individual in a smaller container would be more ideal. Given that we only pipetted out free-floating individuals after inverting the flask, this sampling method also failed to accurately depict the pH-induced changes during the pre- and post-metamorphic period as the larvae adhere to the substrate. It is unclear how many individuals were dislodged by the inversion and if the rate of dislodgement was pH dependent. To address this question of changes in adhesion strength, future studies may model after Guenther et al. (2018), which used experimental flumes and shear flow to demonstrate that reductions in pH delayed macroalgal spore attachment and weakened the attachment strength of the spores. Another future research question is to test if reductions in pH decreases the rate of juvenile development in the post-metamorphic period. Placing settlement plates (e.g., glass sides) within experimental chambers of controlled pH could be a way to address said question (Pecquet et al. 2017).

## CONCLUSIONS

The cosmopolitan, biofouling tunicate *Ciona robusta* was able to complete development and settled at reduced pH level, similar to the predicted average surface ocean condition at the turn of this century (pH 7.7). However, development was delayed at pH levels below pH 7.6. Given the role of *C. robusta* as a model organism for ecotoxicology, our work suggests that the optimal range of pH for future experimentation should be kept above pH 7.7. This study adds to the growing evidence that organisms' vulnerability to decreasing pH differs between life history stages and that there are specific and critical stages in early development that are particularly sensitive to such changes in pH.

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**Authors' contributions:** Bailey S.C.L. Jones: Conceptualization, Investigation, Formal analysis, Writing original draft. Lauren A. Holt: Conceptualization, Investigation, Formal analysis, Writing original draft. K.Y. Karen Chan: Conceptualization, Formal analysis, Review and editing, Supervision.

**Competing interests:** BJ, LH, and KC declare that they have no conflict of interest.

**Availability of data and materials:** Data is available upon request.

**Consent for publication:** Not applicable.

**Ethics approval consent to participate:** Not applicable.

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