Zoological Studies **62:** 4 (2023)

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

Bailey S.C.L Jones[1,2,§], Lauren A. Holt[1,§], and Kit Yu Karen Chan[1,*]

[1]*Biology Department, Swarthmore College, 500 College Ave., Swarthmore, Pennsylvania, PA 19081, USA. *Correspondence: kchan1@swarthmore.edu (Chan).*
E-mail: baileycenlu@gmail.com (Jones); lholt2399@gmail.com (Holt)

[2]*Department of Ophthalmology & Visual Science, Yale University School of Medicine, 300 George St., New Haven, CT 06511, USA*

[§]BJ and LH contributed equally to this work.

(Received 30 May 2022 / Accepted 6 November 2022 / Published -- 2023)
Communicated by Benny K.K. Chan

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.

Citation: Jones BSCL, Holt LA, Chan KYK. 2023. Effect of pH on the early development of the biofouling ascidian *Ciona robusta*. Zool Stud **62:**04.

BACKGROUND

As atmospheric carbon dioxide (CO_2) 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 disjoint 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_2 vents and laboratory CO_2 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* little affected by reductions in pH.

While *C. robusta* have demonstrated resilience to a wide range of temperature and salinity, it is unknown if this 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_2 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, 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 (pCO_2, Ω_{ar}, and Ω_{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 (± 1°C) and a salinity of 32 psu (± 2 psu). Five adult animals were dissected for their gametes. A 5♀ × 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 μm) 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^2 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^{-1}; 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 meta-metamorphosis period (Hotta et al. 2020; Hotta et al. 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 after Cahill et al. (2016); Hotta et al. (2020); Hotta et al. (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 include 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.

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

		Measured parameters		Calculated parameters		
	Nominal pH	pH_T	Alkalinity ($\mu mol\ kg^{-1}$)	pCO_2 (µatm)	Ω_{ca}	Ω_{ar}
Freshly	8.0	7.95	2310, 2217	511.7	3.15	2.02
prepared ASW	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

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 relatively 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).

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		

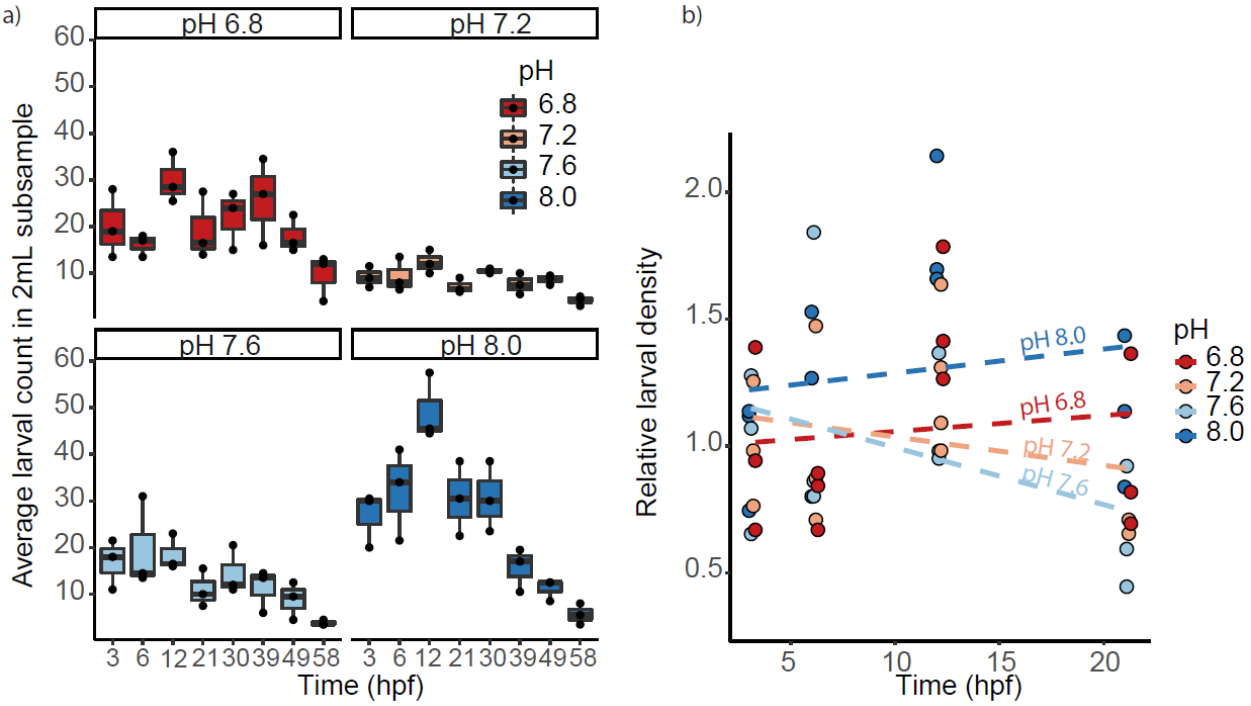

Fig. 1. Average number of individual *Ciona robusta* found in 2 mL subsample within each replicate flask (a) varied across pH and time (3- 58 hpf). To enable comparison between treatments that had different initial concentration, larval count was normalized against the average of the triplicate flask 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 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^2	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

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% (± 3.6% S.E.) of larvae at pH 8.0 had reached the gastrula stage compared to 31.2% (± 3.2%) at pH 7.6 and none at the lower pH treatments. This trend continued at 30 hpf, where the 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 count eggs in all of the following time points. More importantly, at pH 6.8, there was slower rate of development compared to the other three pHs, with no eggs developing past cleavage throughout the 58-hour experiment (Fig. 2).

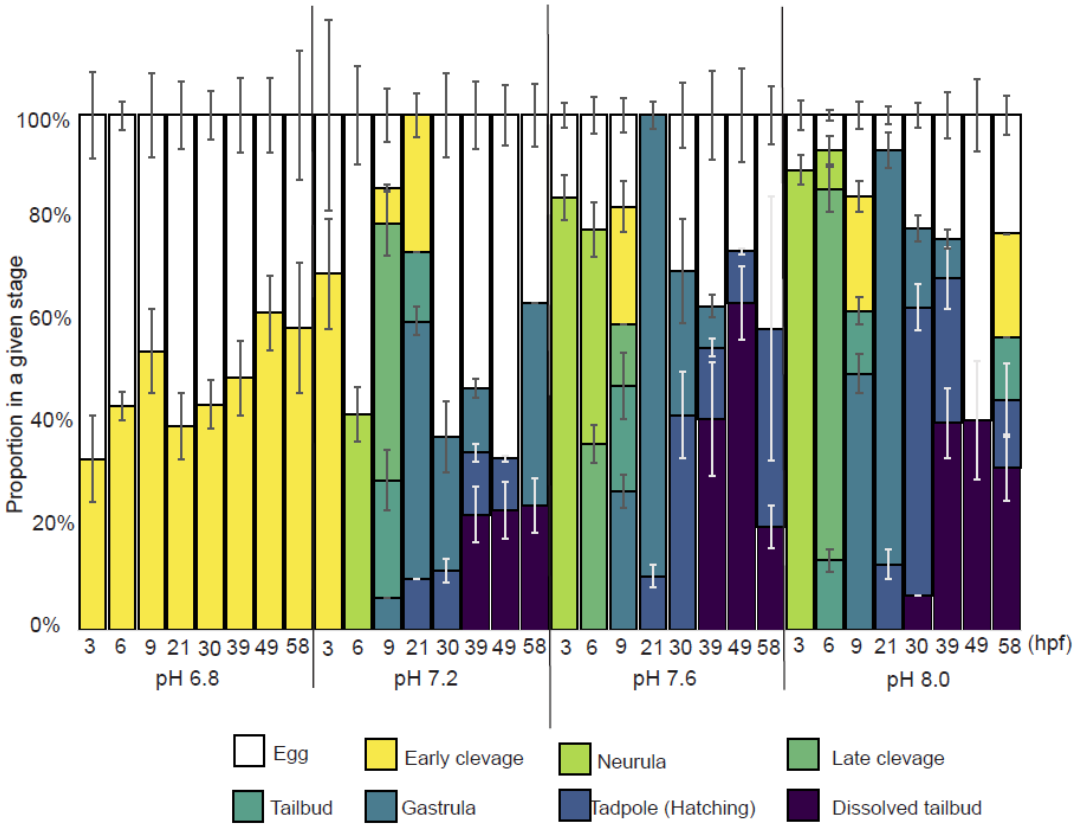

Fig. 2. Ocean acidification led to developmental delay in *Ciona robusta*. The mean and standard error for the proportion of embryo/larvae at a given stage relative to the total larval count at a particular time point were plotted. Note that 100% represents a different relative larval density for each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

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.

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^2	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

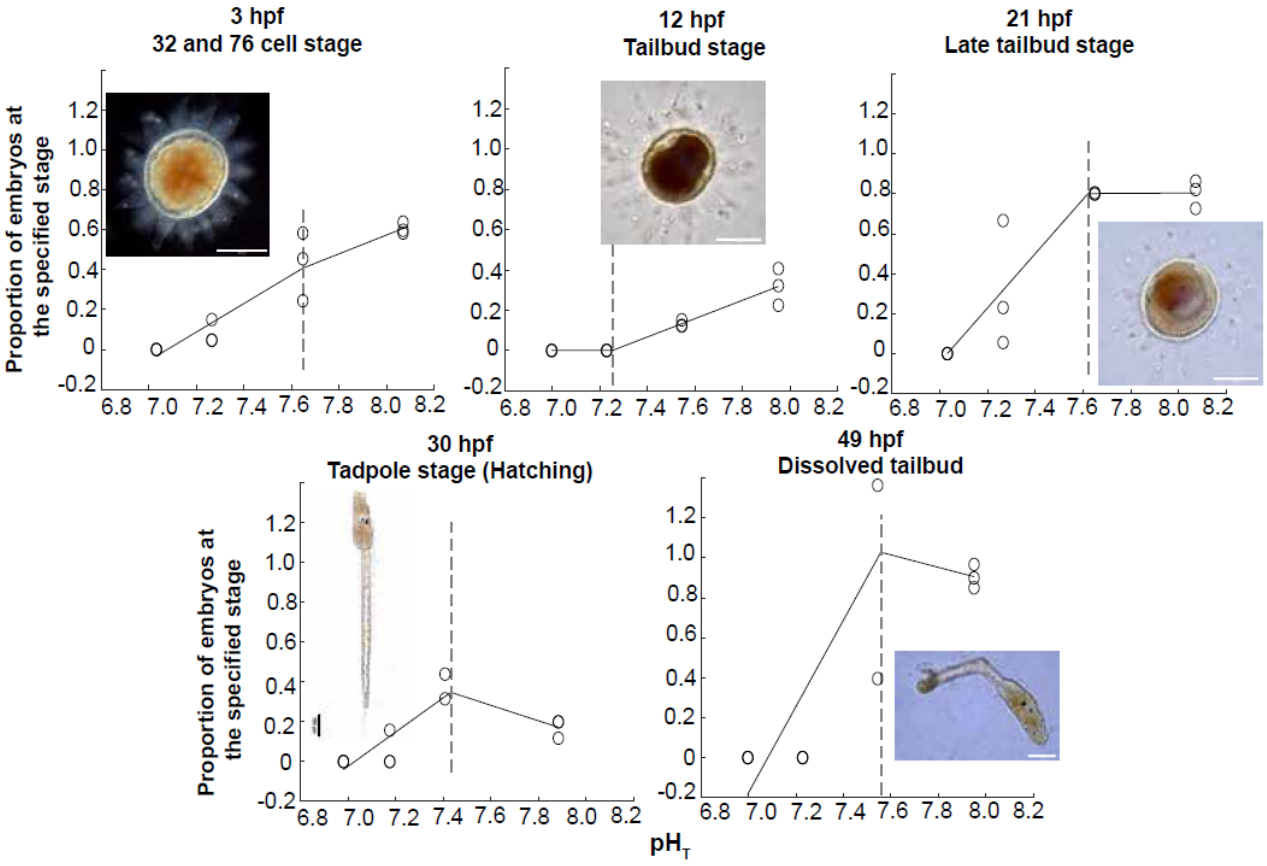

Fig. 3. The breakpoint pH, represented by the dotted line, varied throughout ontogeny. The breakpoint was identified by piecewise regression. Only five representative time points were presented (see Table 4 for the full regression statistics for all stages). The scale bars of the insets were 100 μm.

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 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 value downward (Wallace et al. 2014). pH value beneath 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 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 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 duration (< 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 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 pipette 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), who 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 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.

Acknowledgments: This work is partially supported by the Swarthmore Faculty Research Fund. We thank S. Le-Page, B. Davidson, C. J. Pickett, and G. Kannapel for their assistance.

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.

REFERENCES

Bellas J, Beiras R, Vázquez E. 2003. A standardisation of C*iona intestinalis* (Chordata, Ascidiacea) embryo-larval bioassay for ecotoxicological studies. Water Res **37:**4613–4622. doi:10.1016/S0043-1354(03)00396-8.

Bouchemousse S, Bishop JDD, Viard F. 2016a. Contrasting global genetic patterns in two biologically similar, widespread and invasive *Ciona* species (Tunicata, Ascidiacea). Sci Rep-UK **6:**24875–24875. doi:10.1038/srep24875.

Bouchemousse S, Liautard-Haag C, Bierne N, Viard F. 2016b. Distinguishing contemporary hybridization from past introgression with postgenomic ancestry-informative snp s in strongly differentiated *Ciona* species. Mol Ecol **25:**5527–5542. doi:10.1038/srep24875.

Brown NEM, Therriault TW, Harley CDG. 2016. Field-based experimental acidification alters fouling community structure and reduces diversity. J Anim Ecol **85:**1328–1339. doi:10.1111/1365-2656.12557.

Byrne M, Hernández JC. 2020. Sea urchins in a high CO_2 world: Impacts of climate warming and ocean acidification across life history stages. Developments in Aquaculture and Fisheries Science **43:**281–297. doi:10.1016/B978-0-12-819570-3.00016-0.

Cahill PL, Atalah J, Selwood AI, Kuhajek JM. 2016. Metamorphosis of the invasive ascidian *Ciona savignyi*: Environmental variables and chemical exposure. PeerJ **4:**e1739. doi:10.7717/peerj.1739.

Caldeira K, Wickett ME. 2005. Ocean model predictions of chemistry changes from carbon dioxide emissions to the atmosphere and ocean. J Geophys Res-Oceans **110:**C09S04.

Caputi L, N Andreakis, F Mastrototaro, P Cirino, M Vassillo, P Sordino. 2007. Cryptic speciation in a model invertebrate chordate. Proceedings of the National Academy of Sciences **104:**9364.

Carlton JT, Chapman JW, Geller JB, Miller JA, Carlton DA, McCuller MI et al. 2017. Tsunami-driven rafting: Transoceanic species dispersal and implications for marine biogeography. Science **357:**1402–1406. doi:10.1126/science.aao1498.

Chan F, Barth JA, Blanchette CA, Byrne RH, Chavez F, Cheriton O et al. 2017. Persistent spatial structuring of coastal ocean acidification in the California Current System. Sci Rep **7:**1–7. doi:10.1038/s41598-017-02777-y.

Chan KYK, García E, Dupont S. 2015. Acidification reduced growth rate but not swimming speed of larval sea urchins. Sci Rep **5:**1–7. doi:10.1038/srep09764.

Clutton EA, Alurralde G, Repolho T. 2021. Early developmental stages of native populations of *Ciona intestinalis* under increased temperature are affected by local habitat history. J Exp Biol **224**. doi:10.1242/jeb.233403.

Collin R, Rebolledo AP, Smith E, Chan KYK. 2021. Thermal tolerance of early development predicts the realized thermal niche in marine ectotherms. Funct Ecol **35:**1679–1692. doi:10.1111/1365-2435.13850.

D'Aniello A, Denucé MJ, de Vincentiis M, Di Fiore MM, Scippa S. 1997. Hatching enzyme from the sea-squirt *Ciona intestinalis*:Purification and properties. BBA-Protein Struct M **1339:**101–112. doi:10.1016/s0167-4838(96)00222-1.

Dickson AG, Millero FJ. 1987. A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. Deep Sea Research Part A. Oceanographic Research Papers **34:**1733–1743.

Dupont S, Dorey N, Thorndyke M. 2010. What meta-analysis can tell us about vulnerability of marine biodiversity to ocean acidification? Estuarine, Coastal and Shelf Science **89:**182–185. doi:10.1016/j.ecss.2010.06.013.

Dupont S, Havenhand J, Thorndyke W, Peck L, Thorndyke M. 2008. Near-future level of CO_2-driven ocean acidification radically affects larval survival and development in the brittlestar *Ophiothrix fragilis*. Mar Ecol Prog Ser **373:**285–294. doi:10.3354/meps07800.

Espinel-Velasco N, Hoffmann L, Agüera A, Byrne M, Dupont S, Uthicke S et al. 2018. Effects of ocean acidification on the settlement and metamorphosis of marine invertebrate and fish larvae: A review. Mar Ecol Prog Ser **606:**237–257. doi:10.3354/meps12754.

Feely RA, Sabine CL, Hernandez-Ayon JM, Ianson D, Hales B. 2008. Evidence for upwelling of corrosive “acidified” water onto the continental shelf. Science **320:**1490–1492. doi:10.1126/science.1155676.

Findlay HS, Kendall MA, Spicer JI, Widdicombe S. 2009. Future high CO_2 in the intertidal may compromise adult barnacle *Semibalanus balanoides* survival and embryonic development rate. Mar Ecol Prog Ser **389:**193–202. doi:10.3354/meps08141.

Gallo A, Boni R, Buia MC, Monfrecola V, Esposito MC, Tosti E. 2019. Ocean acidification impact on ascidian *Ciona robusta* spermatozoa: New evidence for stress resilience. Sci Total Environ **697:**134100. doi:10.1016/j.scitotenv.2019.134100.

Gravinese PM. 2018. Ocean acidification impacts the embryonic development and hatching success of the Florida stone crab, *Menippe mercenaria*. J Exp Mar Biol Ecol **500:**140–146. doi:10.1016/j.jembe.2017.09.001.

Guenther R, Miklasz K, Carrington E, Martone PT. 2018. Macroalgal spore dysfunction: Ocean acidification delays and weakens adhesion. J Phycol **54:**153–158. doi:10.1111/jpy.12614.

Hauri C, Gruber N, Vogt M, Doney SC, Feely RA, Lachkar Z et al. 2013. Spatiotemporal variability and long-term trends of ocean acidification in the california current system. Biogeosciences **10:**193–216. doi:10.5194/bg-10-193-2013.

Hofmann GE, Evans TG, Kelly MW, Padilla-Gamiño JL, Blanchette CA, Washburn L et al. 2014. Exploring local adaptation and the ocean acidification seascape-studies in the california current large marine ecosystem. Biogeosciences **11:**1053–1064. doi:10.5194/bg-11-1053-2014.

Hofmann GE, Smith JE, Johnson KS, Send U, Levin LA, Micheli F et al. 2011. High-frequency dynamics of ocean pH: A multi-ecosystem comparison. PLoS ONE **6:**e28983. doi:10.1371/journal.pone.0028983.

Hotta K, Dauga D, Manni L. 2020. The ontology of the anatomy and development of the solitary ascidian *Ciona*: The swimming larva and its metamorphosis. Sci Repo **10:**1–16. doi:10.1038/s41598-020-73544-9.

Hotta K, Mitsuhara K, Takahashi H, Inaba K, Oka K, Gojobori T et al. 2007. A web-based interactive developmental table for the ascidian *Ciona intestinalis*, including 3D real-image embryo reconstructions: I. From fertilized egg to hatching larva. Dev Dyn **236:**1790–1805. doi:10.1002/dvdy.21188.

Ishikawa M, Numakunai T, De Vincentiis M, Lancieri M. 1972. Enzymatic activities relating to the respiration and the onset of metamorphosis of the ascidian tadpole. Development, Growth & Differentiation **14:**229–236. doi:10.1111/j.1440-169X.1972.00229.x.

Jofré Madariaga D, Rivadeneira MM, Tala F, Thiel M. 2014. Environmental tolerance of the two invasive species *Ciona intestinalis* and *Codium fragile*: Their invasion potential along a temperate coast. Biol Invas **16:**2507–2527. doi:10.1007/s10530-014-0680-7.

Kim MK, Kim DH, Park J-u, Kim DH, Yoon TJ, Kim DG et al. 2019. Effects of temperature and salinity on the egg development and larval settlement of *Ciona robusta* (Ascidiacea, Phlebobranchia, Cionidae). Ocean Sci J **54:**97–106. doi:10.1007/s12601-018-0056-5.

Kroeker KJ, Kordas RL, Crim RN, Singh GG. 2010. Meta-analysis reveals negative yet variable effects of ocean acidification on marine organisms. Ecol Lett **13:**1419–1434. doi:10.1111/j.1461-0248.2010.01518.x.

Li H, Huang X, Zhan A. 2020. Stress memory of recurrent environmental challenges in marine invasive species: *Ciona robusta* as a case study. Front Physiol **11:**94. doi:10.3389/fphys.2020.00094.

Lord JP, Harper EM, Barry JP. 2019. Ocean acidification may alter predator–prey relationships and weaken nonlethal interactions between gastropods and crabs. Mar Ecol Prog Ser **16:**83–94. doi:10.3354/meps12921.

Maboloc EA, Chan KYK. 2021. Parental whole life cycle exposure modulates progeny responses to ocean acidification in slipper limpets. Glob Change Biol **27:**3272–3281. doi:10.1111/gcb.15647.

Mak KK-Y, Chan KYK. 2018. Interactive effects of temperature and salinity on early life stages of the sea urchin *Heliocidaris Crassispina*. Mar Biol **165:**1–11. doi:10.1007/s00227-018-3312-4.

Mehrbach C, Culberson CH, Hawley JE, Pytkowicx RM. 1973. Measurement of the apparent dissociation constants of carbonic acid in seawater at atmospheric pressure[1]. Limnol Oceanogr **18:**897–907. doi:10.4319/lo.1973.18.6.0897.

Mollica NR, Guo W, Cohen AL, Huang K-F, Foster GL, Donald HK et al. 2018. Ocean acidification affects coral growth by reducing skeletal density. Proc Natl Acad Sci USA **115:**1754–1759. doi:10.1073/pnas.1712806115.

Oreska MPJ, Aldridge DC. 2011. Estimating the financial costs of freshwater invasive species in great britain: A standardized approach to invasive species costing. Biol Invasions **13:**305–319. doi:10.1007/s10530-010-9807-7.

Pan TCF, Applebaum SL, Manahan DT. 2015. Experimental ocean acidification alters the allocation of metabolic energy. P Natl A Sci **112:**4696–4701. doi:10.1073/pnas.1416967112.

Peck LS, Clark MS, Power D, Reis J, Batista FM, Harper EM. 2015. Acidification effects on biofouling communities: Winners and losers. Glo Change Biol **21:**1907–1913. doi:10.1111/gcb.12841.

Pecquet A, Dorey N, Chan KYK. 2017. Ocean acidification increases larval swimming speed and has limited effects on spawning and settlement of a robust fouling bryozoan, *Bugula neritina*. Mar Pollut Bull **124:**903–910. doi:10.1016/j.marpolbul.2017.02.057.

Pineda MC, McQuaid CD, Turon X, López-Legentil S, Ordóñez V, Rius M. 2012. Tough adults, frail babies: An analysis of stress sensitivity across early life-history stages of widely introduced marine invertebrates. PLoS ONE **7**:e46672. doi:10.1371/journal.pone.0046672.

Pörtner H-O. 2008. Ecosystem effects of ocean acidification in times of ocean warming: A physiologist's view. Mar Ecol Prog Ser **373:**203–217. doi:10.3354/meps07768.

Przeslawski R, Byrne M, Mellin C. 2015. A review and meta-analysis of the effects of multiple abiotic stressors on marine embryos and larvae. Glo Change Biol **21:**2122–2140. doi:10.1111/gcb.12833.

Rech S, Borrell Y, García-Vazquez E. 2016. Marine litter as a vector for non-native species: What we need to know. Mar Pollut Bull **113:**40–43. doi:10.1016/j.marpolbul.2016.08.032.

Sanford E, Gaylord B, Hettinger A, Lenz EA, Meyer K, Hill TM. 2014. Ocean acidification increases the vulnerability of native oysters to predation by invasive snails. P R Soc B-Biol Sci **281:**20132681. doi:10.1098/rspb.2013.2681.

Scippa S, De Candia A, Groppelli S, De Vincentiis M. 2006. Hatching enzyme immunolocalization during embryonic development of the ascidians *Ciona intestinalis* and *Phallusia mammillata*. Invertebr Reprod Dev **49:**121–123. doi:10.1080/07924259.2006.9652201.

Sunday JM, Calosi P, Dupont S, Munday PL, Stillman JH, Reusch TBH. 2014. Evolution in an acidifying ocean. Trends Ecol Evol **29:**117–125. doi:10.1016/j.tree.2013.11.001.

Swiney KM, WC Long, RJ Foy. 2016. Effects of high pCO_2 on tanner crab reproduction and early life history—part I: Long-term exposure reduces hatching success and female calcification, and alters embryonic development. ICES Journal of Marine Science **73:**825–835. doi:10.1093/icesjms/fsv201.

Ulman A, Ferrario J, Forcada A, Seebens H, Arvanitidis C, Occhipinti-Ambrogi A et al. 2019. Alien species spreading via biofouling on recreational vessels in the Mediterranean Sea. J Appl Ecol **56:**2620–2629. doi:10.1111/1365-2664.13502.

Wallace RB, Baumann H, Grear JS, Aller RC, Gobler CJ. 2014. Coastal ocean acidification: The other eutrophication problem. Estuarine, Coastal and Shelf Science **148:**1–13. doi:10.1016/j.ecss.2014.05.027.

Woolmington AD, Davenport J. 1983. pH and PO_2 levels beneath marine macro-fouling organisms. J Exp Mar Biol Ecol **66:**113–124. doi:10.1016/0022-0981(83)90033-3.

Young CS, Gobler CJ. 2021. Coastal ocean acidification and nitrogen loading facilitate invasions of the non-indigenous red macroalga, *Dasysiphonia japonica*. Biol Invasions **23:**1367–1391. doi:10.1007/s10530-020-02445-9.

Zeebe RE. 2012. History of seawater carbonate chemistry, atmospheric CO_2, and ocean acidification. Annu Rev Earth Pl Sc **40:**141–165. doi:10.1146/annurev-earth-042711-105521.