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Effects of low and high pH on sea urchin settlement, implications for the use of alkali to counter the impacts of acidification

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ABSTRACT

Respiration, photosynthesis, and calcification of cultured organisms and biological substrata can substantially alter the pH and other carbonate parameters of water in aquaculture systems. One such example is the diel cycle of photosynthesis and respiration by diatoms and seaweeds growing on 'settlement plates' used to induce metamorphosis of invertebrate larvae and as food for post-larvae. We documented low pH and high pH conditions in nursery raceways and simulated settlement tanks that were as much as 0.26 pH units lower and 0.52 pH units higher than the pH of the source seawater supplied to the systems. To better understand whether the low pH and high pH conditions commonly found in aquaculture culture systems affected the success of the settlement stage of the sea urchin *Centrostephanus rodgersii*, we induced larvae to settle at pH 7.6, 7.8 (created by injecting CO_2), 8.1 (ambient), 8.2, and 8.3 (created by raising total alkalinity), and followed post-settlement growth, development, and survival for 16 d. At metamorphosis, low pH significantly increased the occurrence of abnormalities and reduced the number and length of the sea urchins' spines and pedicellaria, but did not affect settlement rate or size compared to ambient pH. In contrast, high pH generally had little effect on morphological traits, but settlement was significantly reduced by 14–26% compared to ambient and low pH treatments. After 16 d, juveniles in the low pH treatments were as much as 7% smaller, had 2–4 fewer and 9–13% shorter spines, and had less-developed digestive systems compared to juveniles in ambient or high pH treatments, and there was a non-significant trend towards lower survival in low pH treatments. Our results highlight that the low pH and high pH conditions in invertebrate settlement and nursery culture systems have the potential to hamper production through reduced settlement or growth rates. We need to understand the impacts of fluctuating pH in culture systems, especially day-night oscillations. Treating seawater with alkali chemicals to stabilise pH and counter acidification should be done with caution. Due to the potential for deleterious effects on settlement, dosage regimens will need to be optimised.

1. Introduction

Aquaculture of low trophic-level organisms is essential for the sustainable supply of seafood to a burgeoning global human population (Duarte et al., 2009; Pauly et al., 2003). However, the culture of many low trophic-level marine invertebrates is hampered by a substantial bottleneck; low survival in the transition from pelagic to benthic life stages. Mortality rates are often 60–99% during this period (e.g. Chao et al., 2010; Grosjean et al., 1998; Mos et al., 2011). One explanation for high mortality rates during settlement could be a heightened sensitivity to environmental stressors (Gosselin and Qian, 1997), but little is known about the ways in which many stressors impact growth and survival during the settlement period (Azad et al., 2010). Understanding the effects of environmental stressors on settlement success is important to improve the viability and sustainability of aquaculture, particularly in the face of a changing global climate.

Marine invertebrates in culture are typically settled to plastic 'settlement plates' with a cover of diatoms and seaweeds such as crustose coralline algae (CCA) to provide a larval settlement cue and an initial source of food for juveniles (Lawrence et al., 2019; Mos et al., 2011). Diatoms and CCA have a diel physiological cycle where photosynthesis dominates during the day, resulting in the drawdown of CO_2 and a subsequent increase in the pH of surrounding water. Algae respire continuously, but respiration dominates during the night as photosynthesis stops, causing an increase in CO_2 levels and decrease in the pH of surrounding water. The extent to which the carbonate chemistry

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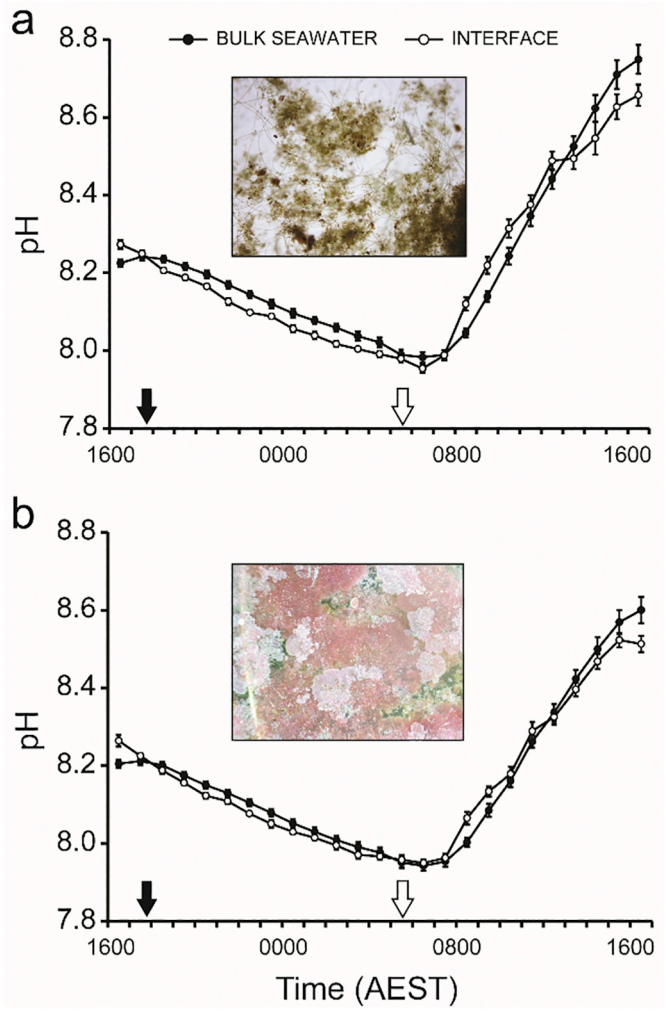

Fig. 1. The effect of (a) settlement plates with diatom-dominated biofilm and (b) settlement plates with crustose coralline algae on pH over 24 h. pH_{NIST} was measured in the bulk seawater surrounding the plates (closed markers) and at the interface of the settlement plates and the seawater (open markers) – see Materials and methods for further details. Black and white arrows indicate sunset and sunrise, respectively. AEST: Australian Eastern Standard Time. Insets are representative photographs of the settlement plates at (a) 40× zoom and (b) 8× zoom. Data are means ± SE, $n = 10$.

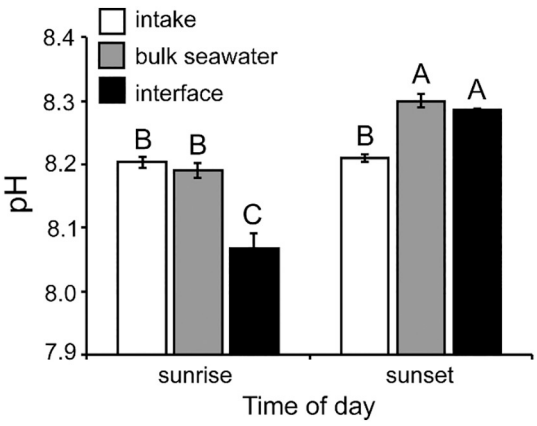

Fig. 2. The pH of culture water in 1350-L sea urchin nursery raceways at sunrise and sunset. pH_{NIST} was measured in the seawater supplied to the raceways (intake, white bars), the bulk seawater surrounding settlement plates in the raceways (bulk seawater, grey bars), and at the interface of the settlement plates and the seawater (interface, black bars) – see Materials and methods for further details. Bars with the same letters are not significantly different according to two-way ANOVA followed by post-hoc pairwise comparisons (Table S1). Data are means ± SE, $n = 3$.

of surrounding water is altered by algal metabolism is influenced by the amount of water flow. Variability in pH increases as water flow decreases (Hurd et al., 2011). When invertebrate larvae are introduced to settlement plates in hatchery culture, seawater flow is often turned off to retain larvae (Lawrence et al., 2019; Mos et al., 2011). As the settlement process can take up to 48 h, larvae may metamorphose into benthic juveniles in low pH or high pH conditions. Even though flow is resumed after settlement, newly settled juveniles are exposed to low and high pH levels that occur in the DBL (diffusive boundary layer, sensu Hurd et al., 2011) at the interface of settlement plates and surrounding seawater (Figs. 1, 2, Table S1).

In addition to algal respiration on settlement plates, invertebrate culture systems can be acidified due to high CO_2 levels in source water as shown for the influence of upwelling conditions in oyster hatcheries (Barton et al., 2012). Organisms in culture can also reduce pH through respiratory CO_2 or taking up carbonates for calcification and internal pH buffering (Mos et al., 2015, 2016). Recirculating systems that use biofiltration to detoxify ammonia may have particular problems with acidification as aerobic nitrification by bacteria results in a considerable draw down in total alkalinity (A_T) and production of CO_2 (Timmons et al., 2018). Declines in production of marine invertebrates due to acidification during the settlement period can be a substantial problem for industry (Barton et al., 2012; Clements and Chopin, 2017), and this is being exacerbated by climate change, eutrophication, and other anthropogenic impacts which alter the carbonate chemistry of source water (Clements and Chopin, 2017; Narita et al., 2012).

Among the invertebrates most vulnerable to acidification during settlement are commercially important bivalves, corals, and sea urchins which exhibit low settlement rates, poor post-settlement growth and development, and decreased survival when raised in low pH/high CO_2 conditions (Byrne et al., 2017; Kroeker et al., 2013; Mos et al., 2019). The negative effects of acidification on settlement are thought to be driven by hypercapnia, which reduces metabolic rates (Moya et al., 2012; Nakamura et al., 2011), as well as decreased growth and survival due to metabolic suppression and reduced saturation of the calcium carbonate minerals required for calcification (Byrne and Fitzer, 2019; Edmunds et al., 2013). Changes in larval behaviour are also reported as acidification can inhibit settlement by interrupting sensory systems or the production of chemical cues that trigger settlement (Ashur et al., 2017; Espinel-Velasco et al., 2018). Conversely, exposure to low pH during the settlement period can be beneficial for some species. For example, newly settled *Acanthaster* sp. and *Crassostrea gigas* grow faster at pH 7.6 and pH 7.4, respectively, than at pH 8.1 (Kamya et al., 2016; Ko et al., 2013). Given the inconsistent effects of low pH on settlement success within phyla (Espinel-Velasco et al., 2018; Kroeker et al., 2013), further studies examining the effects of low pH on the settlement success of commercially important species are warranted.

Alkali chemicals are often used to counter acidification of water in recirculated, low exchange, and static culture systems (Boyd and Tucker, 1998; Timmons et al., 2018), or to counter high CO_2 in source water (e.g. Barton et al., 2015). The alkali chemicals commonly used in aquaculture, sodium hydroxide (NaOH), sodium bicarbonate ($NaHCO_3$), calcium hydroxide ($Ca(OH)_2$), and calcium oxide (CaO), boost pH, lower dissolved CO_2 levels, and increase the saturation states of carbonate species by increasing the A_T of seawater (Boyd et al., 2016; Summerfelt et al., 2000). The effects of the use of alkali chemicals and associated changes in the carbonate chemistry of culture water on settlement success have been tested for four marine clams, one sea urchin, and one coral using carbonate-rich substrata, one marine clam and two prawns (*Penaeus*) using NaOH, one oyster using $Ca(OH)_2$, and one estuarine clam using sodium carbonate (Na_2CO_3) (Table 1). The

Table 1
Results of published single-species manipulative studies investigating the effects of alkali chemicals on settlement success or early post-settlement growth and survival of estuarine and marine invertebrates. C = ambient pH or control treatment. T = treatments where pH was increased. – no data available.

Phylum, species	pH levels tested	Method	Outcome	Reference
Arthropoda				
Penaeus chinensis	T: 7.8, 8.0, 8.5	Added NaOH/HCl to culture water	- Reduced survival at 8.5	Wang et al. (2002)
Penaeus vannamei	C: 8.1; T: 8.6, 9.1	Added NaOH/HCl to culture water	- Reduced growth at 9.1 - No effect on survival	Pan et al. (2007)
Cnidaria				
Pocillopora damicornis	–	Compared settlement substrates	- Increased recruitment when coral rubble added to concrete tiles compared to concrete and inert tiles	Lee et al. (2009)
Echinodermata				
Centrostephanus rodgersii	C: 8.1; T: 8.2, 8.3	Added mix of Na_2CO_3, $NaHCO_3$, and $Na_2B_4O_7$ to culture water	- Reduced settlement at 8.2 and 8.3 - No significant effect on post-settlement growth or survival after 16 d	This study
Tripneustes gratilla	–	Compared settlement substrates	- Lower settlement on concrete than granite or greywacke - In general, juveniles on concrete were larger, and had longer spines and higher survival rates than on greywacke or granite, respectively	Mos et al. (2019)
Mollusca				
Argopecten irradians	C: 7.1–7.7; T: 8.0	Added Na_2CO_3 to culture water	- No effect on metamorphosis	Gobler et al. (2014)
Mercenaria mercenaria	C: –; T: +0.3	Added crushed clam shell to sediment	- Increased recruitment by ~3 fold after 35 d	Green et al. (2013)
Mya arenaria	C: 7.0; T: 7.3	Added crushed clam shell to sediment	- Increased recruitment by ~3 fold after 14 d	Green et al. (2009)
	–	Added crushed oyster or clam shell to sediment	- No effect on recruitment or survival	Ruesink et al. (2014)
Panopea japonica	C: 8.0; T: 8.4, 8.8, 9.2	Added NaOH/HCl to culture water	- Metamorphosis reduced at pH 9.2 - Reduced survival at pH 8.8 and 9.2	Huo et al. (2019)
Ruditapes philippinarum	–	Added crushed oyster or clam shell to sediment	- No effect on recruitment or survival	Ruesink et al. (2014)
	C: 7.51 & 7.19; T: 7.60 & 7.39	Added oyster shell hash to sediment	- No effect on recruitment, growth, or survival	Greiner et al. (2018)
	–	Added crushed oyster shell hash to sediment	- No effect on growth or survival	Dethier et al. (2019)
Saccostrea glomerata	C: 8.19; T: 9.24	Added $Ca(OH)_2$ to the surface of settlement plates	- Plates with added $Ca(OH)_2$ induced higher settlement than inert control	Anderson, 1996
Tridacna squamosa	–	Added crushed crustose coralline algae to concrete tiles	- No effect on recruitment	Neo et al. (2009)

majority (10 of 14) of these studies reported neutral or negative effects on settlement rates or early post-settlement growth and survival (Table 1). Given alkali chemicals are already widely used to counter acidification in commercial culture and are likely to be increasingly employed in the face of ocean acidification (Clements and Chopin, 2017; Narita et al., 2012), greater understanding of the consequences of alkali dosing for larval and juvenile invertebrates is required.

This study examined the effects of culture water with low pH or high pH (high alkalinity) on settlement success and early post-settlement development, growth, and survival of the sea urchin, *Centrostephanus rodgersii* (A. Agassiz, 1864). This species is an ecosystem engineer in the habitats it occupies and supports two growing fisheries in Australia (Byrne and Andrew, 2020). A total of ~628 t was landed in 2015, valued at ~AU$800,000 (Byrne and Andrew, 2020). Fertilisation and embryonic larval stages of *C. rodgersii* appear to be robust to reduced pH (Foo et al., 2012; Pecorino et al., 2014) but later larval stages experience increased mortality rates and reduced growth rates at pH 7.8 or below (Doo et al., 2012; Pecorino et al., 2014). The effects of low pH and high pH on settlement success and early post-settlement growth and survival of *C. rodgersii* are unknown.

We were particularly interested in understanding the way in which pH directly affects settlement rates and early post-settlement fitness of invertebrates in culture systems in contrast to the way in which pH influences larvae and post-larvae through changes in settlement substrata (i.e. indirect effects). To do this, we partitioned the direct effects of pH on larvae and post-larvae from indirect effects on settlement plates by exposing *C. rodgersii* to low and high pH conditions in the absence of settlement plates, and used an ecologically relevant chemical inducer to trigger settlement and metamorphosis. Larval *C. rodgersii* swim towards the substratum and metamorphose when exposed to concentrations of histamine equivalent to levels found in seawater near seaweeds in adult habitats (Mos and Dworjanyn, 2016; Swanson et al., 2012). To create low and high pH conditions (pH 7.6, 7.8, 8.1 – ambient, 8.2, and 8.3), we manipulated seawater carbonate chemistry in two ways. We lowered pH by the addition of CO_2, representing scenarios where larvae and juveniles may be exposed to culture water

acidified by respiration by algae on settlement plates, biogenic acidification (sensu Mos et al., 2015), aerobic nitrification, and high CO_2 levels in source water. We increased pH by the addition of alkali chemicals, which simultaneously raised A_T and calcite saturation state (ΩCa) and lowered the partial pressure of dissolved CO_2 (pCO_2) (Table S2). High pH treatments represented scenarios where alkalinity augmentation is used to counter acidification of source or culture water and have similar pH to levels measured near settlement plates during the day (Figs. 1, 2). We induced *C. rodgersii* to settle in seawater at pH 7.6, 7.8, 8.1 (ambient), 8.2, and 8.3, and measured settlement rates and the morphology of newly settled juveniles. We then followed growth, development, and survival of juveniles in the five pH treatments to 16 d post-settlement.

2. Materials and methods

2.1. Effect of settlement plates on pH and A_T in static conditions

To investigate the carbonate chemistry of the water surrounding settlement plates during the static conditions that often occur when settlement is induced in hatchery culture, pH and A_T of the seawater surrounding plates were measured over 24 h. Two types of settlement plates were tested; diatom-dominated (Fig. 1a) and crustose coralline algae (CCA) dominated (Fig. 1b) communities on corrugated polycarbonate plates (80 × 70 mm) which had been cultivated for 6 months in an outdoor raceway. Six plates were placed vertically in a clear plastic container (95 mm H, 125 mm Ø) which held 0.9 L of seawater. The ratio of the surface area of plates to the volume of the containers was chosen to simulate the ratio found in commercial scale settlement raceways. There were ten replicate containers for each type of settlement plate (diatom or CCA) ($n = 10$). Ten containers without settlement plates were used as autogenic controls. The containers were placed in a covered outdoor raceway with flowing seawater (85 mm deep), which acted as a water bath to maintain a stable temperature (18.9 °C ± 0.2 SE, $N = 24$). Light intensity (photosynthetically active radiation) was 0.0 μmol m^{-2} s^{-1} between sunset and sunrise (Fig. 1) and reached 127 μmol m^{-2} s^{-1} at 1330 h, measured at the level of the containers using a LI-COR® LI-250A light meter.

Temperature and pH of the seawater in the containers with and without settlement plates was measured each hour for 24 h using a Hach® HQ40d multi-controller and Hach® PHC101 pH probe calibrated with high precision buffers (Oakton®) with the probe held at least 20 mm from the nearest settlement plate. All pH measurements were recorded on the NIST scale (pH_{NIST}). At each sampling time, a seawater sample (~ 0.5 mL) was also collected from the surface of 1–3 settlement plates in each container using a pipette. Water samples were used to measure pH variability in the diffusive boundary layer (DBL), the interface between the seawater and the surface of a biofilm. DBL samples were measured using a Thermo Scientific 8200BNWP pH microprobe and Tunze® 7070/2 controller calibrated with high precision buffers (Oakton®). Differences in the outputs of the two pH probes when measuring the same sample of seawater simultaneously were always 0.02 pH units or less. At the beginning and end of the experiment, 25-mL water samples were collected via syringe from each container, fixed using 10 μL of saturated $HgCl_2$, and stored in borosilicate glass vials at 4 °C. Total alkalinity (A_T) was determined from water samples by potentiometric titration using a Metrohm® 888 Titrando calibrated using a certified reference material (Batch 116) (Dickson et al., 2007).

2.2. pH in nursery raceways

The pH_{NIST} of culture water was measured in three flow-through (1.7 L min^{-1}) 1350-L raceways (2.5 L × 1.1 W × 0.5H m, water depth ~ 0.35 m) housing approx. 225 vertical corrugated polycarbonate settlement plates (~ 300 × 450 mm) colonised by CCA and diatoms. Measurements were taken at sunrise and sunset in the seawater supplied to each of the three raceways by submerging the probe in the outflow and in the bulk seawater in each raceway (~ 100 mm depth, centre of each raceway) using a TPS AQUA-pH controller and HANNA® HI1083 pH microprobe calibrated with high precision buffers (Oakton®). At these times pH levels are at their minimum and maximum. Measurements were also taken at sunrise and sunset in 0.5-mL samples pipetted from the surface (i.e. DBL) of six haphazardly-selected plates in each raceway, and the mean pH of the six plates for each of the three raceways used for statistical analysis ($n = 3$).

2.3. Larval production

Adult *Centrostephanus rodgersii* (3 F, 5 M) were collected near Coffs Harbour (30°12.5′S, 153°16.1′E) in late August and induced to spawn using 1–5 mL intracoelomic injection of 1.0 *M* KCl. Sperm was introduced to the eggs, and when > 95% of eggs had a fertilisation envelope, excess sperm was removed by washing the embryos in filtered seawater (filtered to 1.0 μm, and UV sterilised: hereafter FSW). Embryos (7 embryos mL^{-1}) were added to gently aerated 300-L cylindro-conical culture tanks containing FSW (21–23 °C) which was exchanged twice daily. Larvae were fed after each water change with *Proteomonas sulcata* (1–4 × 10^4 cells mL^{-1}) from 3 d post-fertilisation. Larval density was reduced to 0.2–1.0 larvae mL^{-1} by five weeks to accommodate increases in the arm length of larvae. Larvae were considered to be competent to settle and used in the experiment at 40 d post-fertilisation when > 60% of larvae possessed multiple pedicellaria (Mos and Dworjanyn, 2016).

2.4. Effects of pH treatment on settlement success and morphology

To assess the effect of seawater carbonate chemistry on the settlement of *C. rodgersii*, larvae were induced to settle under five pH_{NIST} conditions, 7.6, 7.8, 8.1 (ambient control), 8.2, and 8.3 (Table S2). Seawater of pH 7.6 and 7.8 were created by adding pure CO_2 to FSW using an automatic CO_2 injection system (Tunze®) and vortex mixer (Red Sea®). Seawater with pH 8.2 and 8.3 were created by increasing the A_T of FSW by addition of a concentrated carbonate buffer solution (Brightwell Aquatics™ Alkalin 8.3 KH buffer; active ingredients: Na_2CO_3, $NaHCO_3$, and $Na_2B_4O_7$) until the desired pH was reached. For each treatment, temperature and pH were measured using the previously described Hach pH probe, salinity was measured using a Hach® CDC101 conductivity probe, and A_T was measured from 25 mL water samples as previously described. Values for pCO_2, ΩCa, HCO_3^-, and CO_3^{2-} (Table S2) were calculated from temperature, pH_{NIST}, salinity, and A_T data using CO2SYS (Pierrot et al., 2006) using the dissociation constants of Mehrbach et al. (1973) as refitted by Dickson and Millero (1987).

Settlement assays were done in 70-mL specimen containers (Techno Plas, 57 mm H × 44 mm Ø) completely filled with the treatment seawater and sealed to prevent outgassing of CO_2. There were ten replicates for each treatment ($n = 10$). Competent larvae (20–40) were added to each replicate and induced to settle by the addition of histamine dihydrochloride (Sigma-Aldrich) to a final concentration of 100 μM (Swanson et al., 2012). Preliminary trials showed pH of the five seawater treatments in these containers decreased by 0.01–0.03 pH units after 24 h and 0.02–0.06 pH units after 48 h. Dissolved oxygen (DO) was always above 95% (> 6.5 mg L^{-1}). There was no measurable effect of the addition of histamine on seawater pH (< 0.01 pH units). Replicates were haphazardly assigned positions on a bench in a temperature-controlled laboratory (22.5 °C) under a 12:12 h light/dark photoperiod.

Settlement was recorded after 48 h. Larvae were scored as metamorphosed if they were attached to a surface, had everted their rudiment, and partially or fully absorbed their larval structures. All other larvae were classed as not metamorphosed. Settlement was calculated as the proportion of larvae that metamorphosed out of the total number

of larvae added to each replicate and reported as a percentage. There was no settlement by *C. rodgersii* in any pH treatments in the absence of histamine (data not presented). Settled juveniles were gently removed using a pipette and photographed. Digital photographs were used to score settled urchins on the occurrence of abnormalities, test diameter (TD), presence, number, and length of spines, and the number of pedicellaria. Individuals were scored as abnormal if they had an irregular shape due to malformation of the test or bulges of tissue through the integument, abnormal colouration, or were > 50% smaller than juveniles in the same treatment (Mos and Dworjanyn, 2016). Test diameter and spine length of juveniles were measured from the digital photographs by Feret's Diameters of circles encompassing their tests using ImageJ (NIH, USA). Spine length (SL) was calculated using the formula:

$$SL = ½(FD_S - FD_T)$$

where FD_S and FD_T were Feret's Diameters of circles encompassing the urchins' spines and tests, respectively. No distinction was made between juvenile and adult spines during measurements of spine length or spine counts. Mean occurrence of abnormalities, TD, presence, number, and length of spines, and the number of pedicellaria were calculated for each container, and used as the data for statistical analyses.

2.5. Effects of pH treatment on post-settlement growth, morphology, and survival

To follow post-settlement survival and development, settled juveniles were placed into new 70-mL sealed containers, and maintained at their respective pH treatment for a further 14 d in a temperature-controlled laboratory (22.6 ± 0.1 °C, $N = 12$). Seawater in the replicates was exchanged daily using FSW at the respective pH, prepared daily as described above. Post-settlement survival was assessed every second day. After 14 d, surviving juveniles were photographed, and TD and the number and length of spines were measured (as above). The number of juveniles that had a well-developed digestive system, visible through the test as a dark U-shaped mass (Fig. 4), was also recorded.

2.6. Statistical analysis

Data on the effects of time of day (sunrise, sunset) on pH of culture water in sea urchin nursery raceways were analysed by two-way permutational analysis of variance (PERMANOVA, Anderson, 2001), with factors time of day (random), location (fixed), and the interaction between these factors in a fully crossed design. The data on the effects of pH on settlement, morphology, and post-settlement growth and survival of *C. rodgersii* were analysed by one-way PERMANOVA with pH treatment as a fixed factor. Pairwise comparisons were used as post-hoc tests if PERMANOVA results indicated that there were significant differences among treatments. Analyses were done using Primer 6 (Primer-E, Plymouth) with PERMANOVA$^+$ extension (v.6.1.11) software.

3. Results

3.1. Effects of settlement plates on pH and A_T in static conditions

There was substantial variation in seawater pH_{NIST} in the presence of diatom-dominated and CCA-dominated settlement plates under static conditions (i.e. no water flow) (Fig. 1a,b), but not in autogenic controls where pH and A_T were stable (pH_{NIST} = 8.09 ± 0.003 SE; A_T = 2291.0 ± 1.0 μmol kg^{-1}, $n = 10$). Mean pH in the bulk seawater above the diatom-dominated plates fell to pH 7.98 at night and rose to pH 8.75 by day, representing a 0.25 pH unit decrease and 0.52 pH unit increase, respectively, compared to the initial (pH 8.23) (Fig. 1a). Similarly, mean pH in the bulk seawater above the CCA-dominated plates fell to pH 7.94 at night and rose to pH 8.60 by day, representing a 0.26 pH unit decrease and 0.40 pH unit increase, respectively, compared to the initial (pH 8.20) (Fig. 1b). At the interface of the settlement plates and surrounding seawater (i.e. in the DBL - diffusive boundary layer), pH followed the diel pattern of decrease and increase measured in the bulk seawater (Fig. 1a,b).

Total alkalinity (A_T) was 2282.9 ± 4.1 SE μmol kg^{-1} and 2038.9 ± 29.7 μmol kg^{-1} in the diatom-dominated plate and CCA-dominated plate treatments, respectively, after 24 h. A_T was reduced by 0.6% in the diatom-dominated plate treatment ($F_{1,18}$ = 11.70, p = .0018) and 9.7% in the CCA-dominated plate treatment ($F_{1,18}$ = 50.89, p = .0001) compared to the initial A_T (2297.1 ± 0.8 μmol kg^{-1} and 2258.9 ± 8.4 μmol kg^{-1}, respectively).

4. pH in nursery raceways

There were substantial differences in the pH_{NIST} of culture water between sunrise and sunset in nursery raceways housing settlement plates even though they were supplied with flow-through seawater (Fig. 2, Table S1, significant time of day × position interaction, followed by post-hoc pairwise comparisons). At sunrise, pH in the raceways (i.e. bulk seawater) was not different to the pH of the seawater supplied to the raceways (i.e. inflow), but the pH of the seawater in the DBL at the surface of the settlement plates was 0.14 pH units lower than both the bulk seawater in the tank and inflow seawater (Fig. 2, Table S1, DBL < bulk = inflow). At sunset, the pH of the bulk seawater and the DBL at the surface of the settlement plates was 0.07–0.09 pH units higher than in the seawater supplied to the raceways (Fig. 2, Table S1, DBL = bulk > inflow).

4.1. Effects of pH treatment on settlement success and morphology

After 48 h, settlement was significantly higher in pH 7.6, 7.8, and 8.1 treatments than in pH 8.2 and 8.3 treatments ($F_{4,45}$ = 5.69, p < .0016, post-hoc pairwise comparisons, 7.6 = 7.8 = 8.1 > 8.2 = 8.3, Fig. 3a). Settlement was not different among pH 7.6, 7.8, and 8.1 treatments (mean 78, 74, and 76%, respectively), and there was no difference in settlement between pH 8.2 and 8.3 treatments (64 and 58%, respectively).

There were significantly more abnormal juveniles in the pH 7.6 and 7.8 treatments than in pH 8.1, 8.2, and 8.3 treatments ($F_{4,45}$ = 10.56, p < .0001, post-hoc pairwise comparisons, 7.6 = 7.8 > 8.1 = 8.2 = 8.3, Fig. 3b). The percentage of abnormal juveniles was not significantly different between pH 7.6 and 7.8 treatments (mean 36 and 46%, respectively), and not different among pH 8.1, 8.2, and 8.3 treatments (22, 21, and 17%, respectively).

There were significantly fewer juveniles with spines in the pH 7.6 and 7.8 treatments than in the pH 8.1 and 8.3 treatments ($F_{4,45}$ = 3.77, p < .0098, post-hoc pairwise comparisons, 7.6 = 7.8 = 8.2 < 8.2 = 8.1 = 8.3, Fig. 3c). The percentage of juveniles with spines was not different between pH 7.6 and 7.8 treatments (mean 74 and 70%, respectively), and not different between pH 8.1 and 8.3 treatments (86 and 88%, respectively). The percentage of juveniles with spines in the pH 8.2 treatment (78%) was not statistically different than any other treatment (Fig. 3c).

Juveniles in the pH 7.6 and 7.8 treatments had the fewest spines (mean 10 and 11 spines ind.$^{-1}$, respectively), fewer than juveniles in the pH 8.1 treatment (14 spines ind.$^{-1}$), which had fewer spines than juveniles in pH 8.2 and 8.3 treatments (17 and 18 spines ind.$^{-1}$, respectively) ($F_{4,37}$ = 32.02, p < .0001, post-hoc pairwise comparisons, 7.6 = 7.8 < 8.1 < 8.2 = 8.3, Fig. 3d). There was no difference in the number of spines per juvenile between pH 7.6 and 7.8 treatments, or between pH 8.2 and 8.3 treatments (Fig. 3d).

The spines of juveniles in pH 7.6 and 7.8 treatments were significantly shorter than those of juveniles in pH 8.1 and 8.3 treatments ($F_{4,40}$ = 9.66, p < .0001, post-hoc pairwise comparisons, 7.6 = 7.8 < 7.8 = 8.2 < 8.2 = 8.1 = 8.3, Fig. 3e). Spine length was

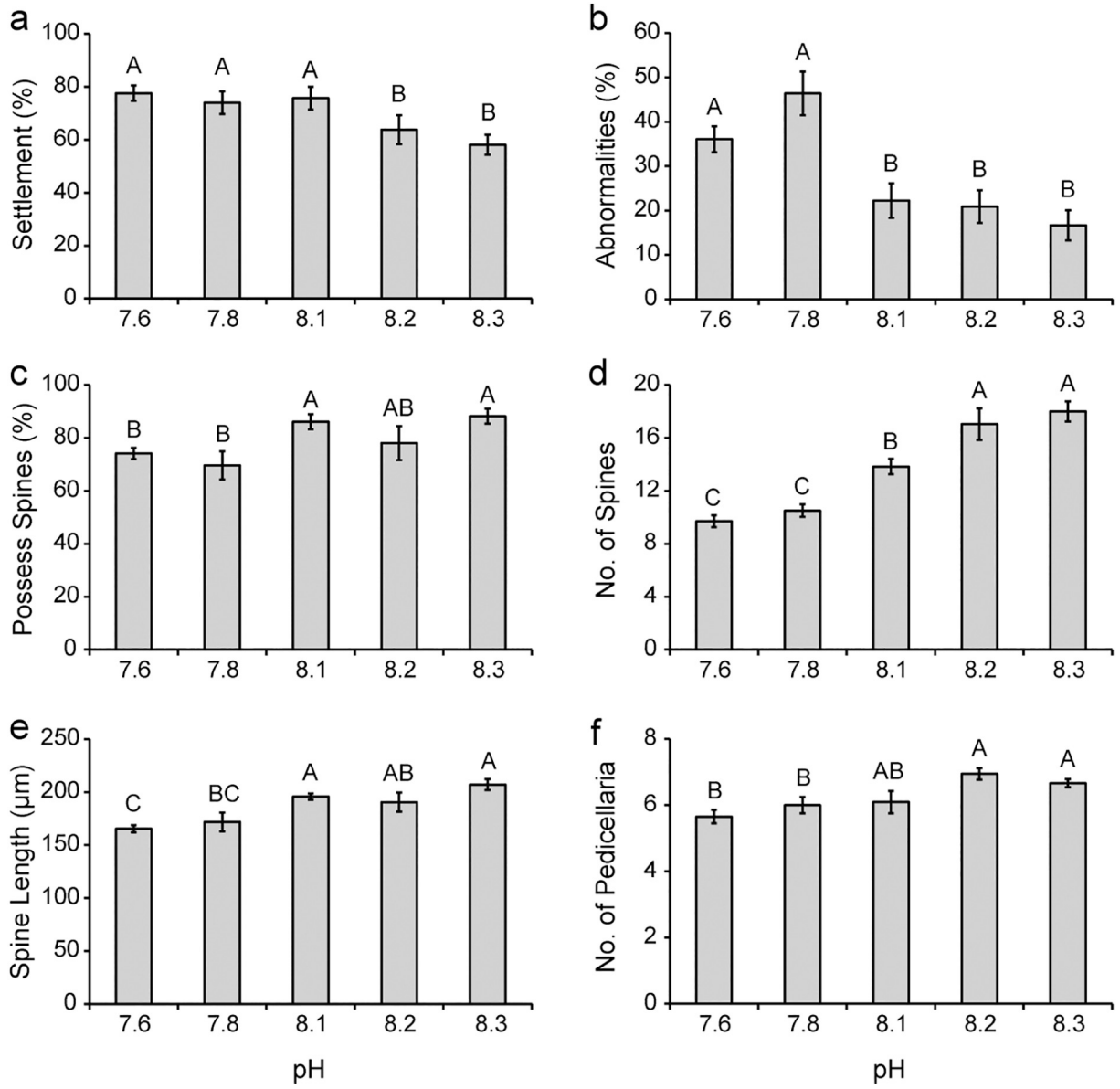

Fig. 3. The effect of pH and alkalinity treatments on settlement and post-settlement morphology of *Centrostephanus rodgersii* induced to settle by 100-µM histamine after 48 h. (a) Percentage of larvae that attached to a surface and metamorphosed, (b) Occurrence of abnormal juveniles, (c) Percentage of juveniles that possessed spines, (d) Number of spines possessed by juveniles, (e) Spine length, and (f) Number of pedicellaria possessed by juveniles. pH values on the X-axis correspond with the pH_{NIST} of untreated seawater (8.1) or seawater treated by addition of CO_2 (7.6, 7.8) or alkali chemicals (8.2, 8.3) – see Materials and methods for further details. Bars with the same capitalised letter are not significantly different (ANOVA, in text, followed by post-hoc pairwise comparisons). Data are means ± SE, $n = 10$, except for the pH 8.2 treatment in b–f where $n = 5$.

not different between pH 7.6 and 7.8 treatments (mean 165 and 172 µm, respectively), and not different between pH 8.1 and 8.3 treatments (191 and 207 µm, respectively). The spine length of juveniles in the pH 8.2 treatment (190 µm) was not statistically different than any other treatment except pH 7.6 (Fig. 3e).

Juveniles in pH 7.6 and 7.8 treatments had ~1 fewer pedicellaria than those in the pH 8.2 and 8.3 treatments ($F_{4,37} = 3.93$, $p < .0096$, post-hoc pairwise comparisons, 7.6 = 7.8 = 8.1 < 8.1 = 8.2 = 8.3, Fig. 3f). The number of pedicellaria per juvenile was not different between pH 7.6 and 7.8 treatments, and not different between pH 8.2 and 8.3 treatments (Fig. 3f). The number of pedicellaria per juvenile in the pH 8.1 treatment was not statistically different than any other treatment (Fig. 3f).

Mean test diameters (TD) of newly settled juvenile *C. rodgersii* after 48 h were 645 ± 6 µm SE (pH 7.6), 658 ± 12 µm (pH 7.8), 657 ± 4 µm (pH 8.1), 652 ± 16 µm (pH 8.2) and 671 ± 5 µm (pH 8.3). There was no effect of pH treatment on TD ($F_{4,40} = 1.43$, $p = .2398$).

4.2. Effects of pH treatment on post-settlement growth, morphology, and survival

Survival of *C. rodgersii* to 16 d ranged from 55 to 76% (Fig. 5a). There was a trend towards lower survival in the pH 7.6 and 7.8 treatments compared to all other treatments (Fig. 5a). However, ANOVA was unable to detect differences among the pH treatments ($F_{4,40} = 2.46$, $p = .061$).

There were substantial differences in the morphology of juveniles in the pH treatments at day 16 (Fig. 4, Fig. 5b–e). Juveniles in the pH 7.6 and 7.8 treatments had significantly smaller TDs than those in the pH 8.1 and 8.3 treatments ($F_{4,40} = 0.005$, $p < .0022$, post-hoc pairwise comparisons, 7.6 = 7.8 = 8.2 < 8.2 = 8.1 = 8.3, Fig. 5b). There

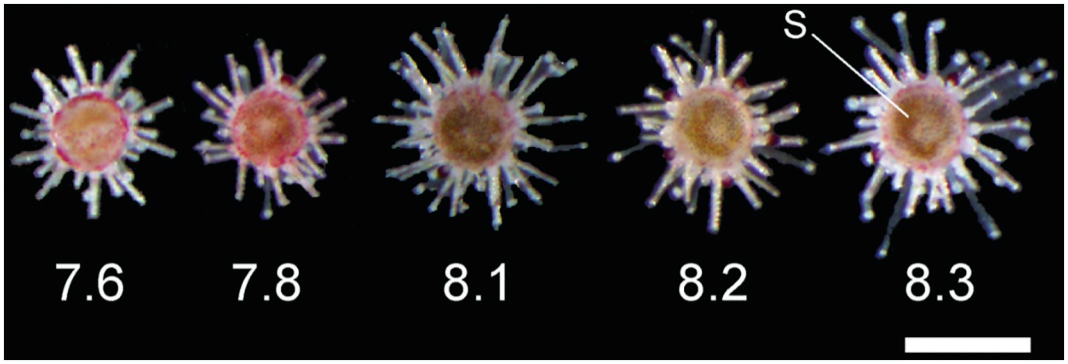

Fig. 4. Representative *Centrostephanus rodgersii* juveniles at day 16 in five pH treatments. Size (test diameter) and the number of spines possessed by juveniles were reduced in pH 7.6 and 7.8 treatments compared to all other treatments (ANOVA, in text, followed by post-hoc pairwise comparisons). Spine length was reduced in the pH 7.8 treatment compared to the pH 8.1, 8.2, and 8.3 treatments (ANOVA, in text, followed by post-hoc pairwise comparisons). S identifies the dark U-shaped mass indicative of a well-developed digestive system. Scale bar = 1000 μm.

was no difference in TD of juveniles between pH 7.6 and 7.8 treatments (mean 651 and 630 μm, respectively), and no difference between pH 8.1 and 8.3 treatments (680 and 685 μm, respectively). The TD of juveniles in the pH 8.2 treatment (652 μm) was not statistically different than any other treatment (Fig. 5b).

Juveniles in the pH 7.6 and 7.8 treatments had significantly fewer spines than those in the pH 8.1, 8.2, and 8.3 treatments ($F_{4,40} = 7.46$, $p < .0001$, post-hoc pairwise comparisons, $7.6 = 7.8 < 8.1 = 8.2 = 8.3$, Fig. 5c). The number of spines per juvenile was not significantly different between pH 7.6 and 7.8 treatments (mean 17 and 16 spines, respectively), and not different among the pH 8.1, 8.2, and 8.3 treatments (19, 20, and 20 spines, respectively).

Juveniles in the pH 7.8 treatment had the shortest spines (381 μm), significantly shorter than those of juveniles in the pH 8.1, 8.2, and 8.3 treatments ($F_{4,40} = 0.005$, $p < .0218$, post-hoc pairwise comparisons, $7.8 = 7.6 < 7.6 = 8.1 = 8.2 = 8.3$, Fig. 5d). There was no difference in the spine length of juveniles among pH 8.1, 8.2, and 8.3 treatments (mean 433, 428, and 437 μm, respectively). The spine length of juveniles in the pH 7.6 treatment (417 μm) was not statistically different than any other treatment (Fig. 5d).

There were significantly fewer juveniles with a well-developed digestive system in the pH 7.6 and 7.8 treatments than in the pH 8.1, 8.2, and 8.3 treatments ($F_{4,40} = 5.58$, $p < .0017$, post-hoc pairwise comparisons, $7.6 = 7.8 < 8.1 = 8.2 = 8.3$, Fig. 5e). The percentage of juveniles that had a well-developed digestive system was not different between the pH 7.6 and 7.8 treatments (mean 67 and 88%, respectively), and not different among pH 8.1, 8.2, and 8.3 treatments (97, 100, and 100%, respectively).

5. Discussion

Settlement plates containing diatoms or CCA reduced the pH of surrounding water by as much as 0.26 pH units and increased pH by as much as 0.52 pH units under the static conditions that are used when settling larvae. Even in flow-through nursery raceways, pH levels at the surface of settlement plates were 0.14 pH units lower and 0.09 pH units higher than the pH of the seawater supplied to the raceways. Importantly, exposure to low pH or high pH during the settlement period was a key determinant of the fitness of *C. rodgersii*. At settlement, low pH had no effect on settlement success but resulted in more abnormal post-larvae, and juveniles with fewer and shorter spines. High pH (and alkalinity) reduced settlement by 14–23%, but the resulting post-larvae had more spines. After 16 days, pH treatments did not have a clear effect on survival, but low pH resulted in smaller juveniles with fewer spines and less-developed digestive tracts. High pH had little effect on growth or development. Our results demonstrate the potential for low pH and high pH to influence the number and morphology of sea urchins transitioning through the settlement period, and suggest that controlling pH in culture systems might help to avoid low settlement rates and poor post-settlement growth and survival that are substantial problems for industry (Mos et al., 2011).

In static conditions, settlement plates altered the pH of all of the water in which they were sitting. In the flowing seawater of the nursery raceways, the highest variability in pH was in the diffusive boundary layer (DBL) of the settlement plates. Similar patterns of pH variability occur at the surfaces of seaweeds and coral (Comeau et al., 2019; Hurd et al., 2011). Variation in the pH of DBLs surrounding settlement plates (or seaweeds) is particularly relevant for marine invertebrates as attachment, metamorphosis, and the first weeks of post-settlement growth occur in this layer, highlighting the importance of measuring pH at the scale of larvae and post-larvae. Our knowledge of the dynamics of the carbonate system in DBLs in recirculating aquaculture systems is particularly lacking. The diel oscillation in pH in the DBL of settlement plates was driven by the interplay of photosynthesis and respiration (Hurd et al., 2011). The same processes are also responsible for fluctuations in dissolved oxygen across light and dark cycles in abalone culture systems (Searcy-Bernal, 1996). It would be interesting to see whether water quality in invertebrate culture systems can be controlled by manipulating light intensity. For instance, providing continuous low-intensity lighting to promote photosynthesis may avoid acidification of culture water and stabilise pH and oxygen levels when using settlement plates to provide food for newly settled juveniles.

We partitioned the direct effects of low pH on settlement of *C. rodgersii* by inducing larvae to settle using an ecologically relevant chemical settlement cue, histamine (Swanson et al., 2012) in the absence of settlement plates. Settlement of *C. rodgersii* was not affected by low pH. It appears that indirect effects of low pH on settlement substrata play the primary role in determining settlement rates of echinoderms (also see Uthicke et al., 2013), and this might explain why most studies on echinoderms have found little effect of low pH on settlement rates (e.g. Foo et al., 2016; Wangensteen et al., 2013; but see Hu et al., 2018). Settlement of sea urchins (Dworjanyn and Pirozzi, 2008) and many other marine larvae (reviewed by Hadfield, 2011) is triggered by an association between bacterial biofilms and seaweeds. While acidification alters bacterial assemblages (Webster et al., 2013) and can reduce or bolster the growth of seaweeds (Graba-Landry et al., 2018; Kuffner et al., 2008; Poore et al., 2016), how this relates to settlement cues remains unclear.

We found no effect of low pH on the size (TD) of *C. rodgersii* at settlement, similar to Mos et al. (2019) who found newly settled sea urchins, *Tripneustes gratilla*, settled at pH 7.9 and 7.7 were similar in size (TD) or larger than those that settled at pH 8.1. In contrast, Wangensteen et al. (2013) found TD of the sea urchin *Arbacia lixula* at

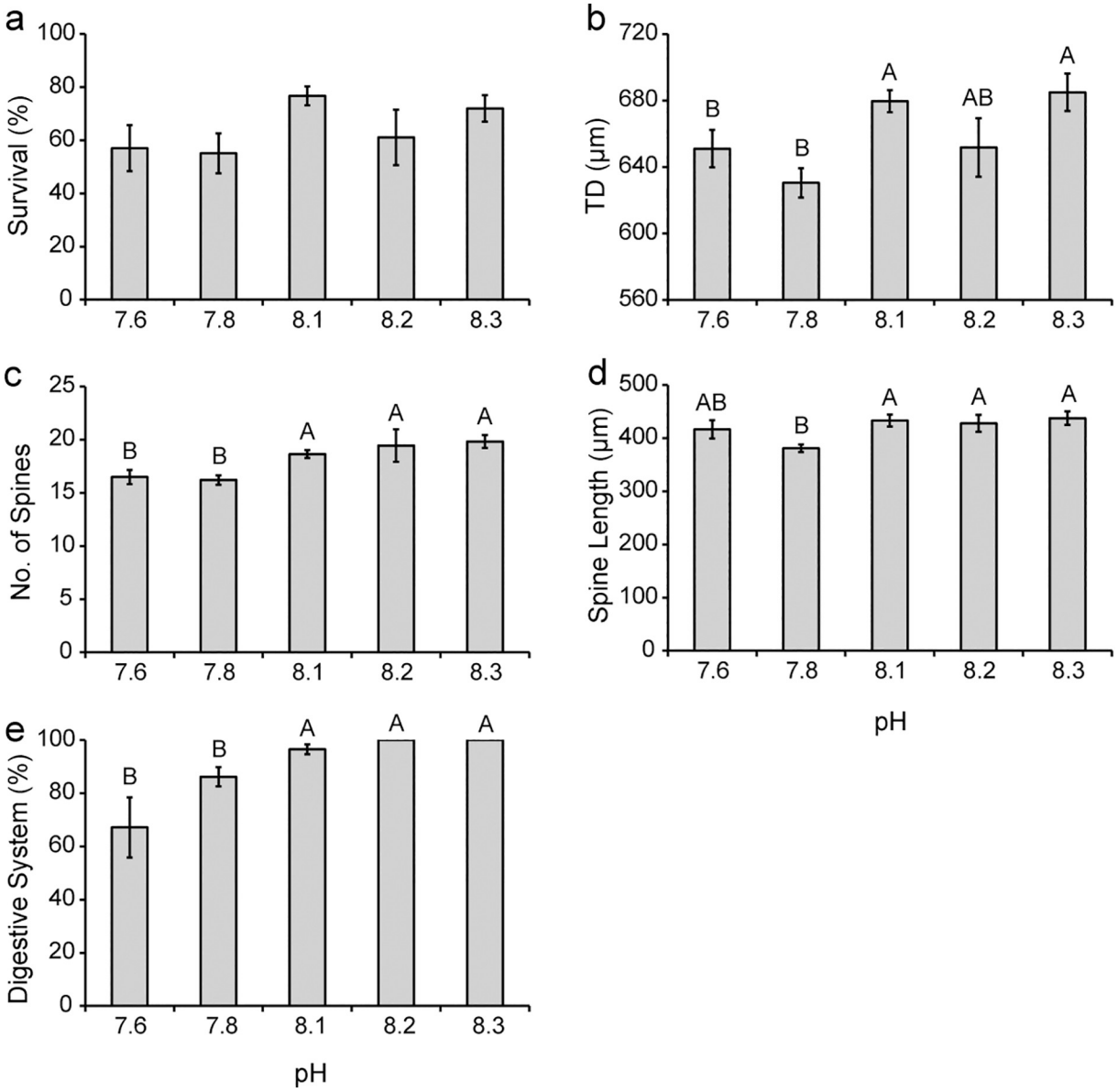

Fig. 5. The effect of pH and alkalinity treatments on early post-settlement survival and morphology of *Centrostephanus rodgersii* 16 d after metamorphosis in response to 100 µM histamine. (a) Survival, (b) Test diameter (TD), (c) Number of spines per juvenile, (d) Spine length, and (e) Percentage of juveniles that had a well-developed digestive system as evidenced by a dark U-shaped mass visible through the test (Fig. 4). pH values on the X-axis correspond with the pH_{NIST} of untreated seawater (8.1) or seawater treated by addition of CO_2 (7.6, 7.8) or alkali chemicals (8.2, 8.3) – see Materials and methods for further details. (a) There was a trend towards lower survival at day 16 in pH 7.6 and 7.8 treatments compared to other treatments, but this was not significant (ANOVA, $F_{4,40} = 2.46$, $p = .0611$). (b,c,d,e) Bars with the same capitalised letter are not significantly different (ANOVA, in text, followed by post-hoc pairwise comparisons). Data are means ± SE, $n = 10$, except for the pH 8.2 treatment where $n = 5$.

settlement was reduced by 11% when larvae metamorphosed at pH 7.69 compared to pH 8.09. Differences between this study and Wangensteen et al. (2013) may be because we used larvae that had been cultured under ambient conditions (pH ~8.1). Exposure to low pH during the larval stage can inhibit the growth of calcified structures and compromise feeding, which can result in smaller post-larvae because the size of post-larvae is often determined by the amount of energy and resources gathered during the larval stage (Byrne and Hernández, 2020).

We found there was a trend towards lower survival to 16 d post-settlement in low pH treatments ($p = .06$). Low pH also reduced early post-settlement survival of the sea urchins *Strongylocentrotus droebachiensis* and *T. gratilla* (Dupont et al., 2013; Mos et al., 2019), but there was no effect of low pH on survival of *A. lixula* and *Heliocidaris erythrogramma* (Wangensteen et al., 2013; Wolfe et al., 2013; Wolfe et al., 2013a). Differences in survival among echinoids in low pH conditions might be a consequence of species-specific responses to acidification during the settlement period (Espinel-Velasco et al., 2018), as well as the environment that they are adapted to (e.g. intertidal vs. subtidal, García et al., 2018b), the presence of additional stressors (e.g. extreme temperatures, hypoxia, Espinel-Velasco et al., 2018), and their mode of development (planktotrophy vs. lecithotrophy, Hardy and Byrne, 2014). Survival to 16 d post-settlement in this study was high (up to 76%), whereas many sea urchins in culture experience high mortality rates in the first weeks after metamorphosis (e.g. Grosjean et al., 1998; Mos et al., 2011). As post-larvae rely on energy and materials derived from maternal provisioning or planktotrophic feeding while they develop their digestive system (Byrne et al., 2008a, 2008b), inadequate maternal or larval provisioning could provide a better explanation for high mortalities in the first weeks after

settlement rather than seawater carbonate chemistry per se as tested here.

Low pH treatments had 1.6–2.0 times more abnormal post-larvae than any other treatment, and post-larvae in pH 7.8 and 7.6 had 3–4 fewer and 10–15% shorter spines and ~ 1 fewer pedicellaria than those in pH 8.1. After 16 d, *C. rodgersii* in pH 7.6 and 7.8 treatments were generally smaller, had fewer spines, and their digestive systems were less developed than juveniles in other treatments. Newly settled *T. gratilla* also experience sublethal effects of low pH on morphology, possessing fewer and shorter spines when grown for 14 d in pH 7.9 and 7.7 compared to pH 8.1 (Mos et al., 2019). In contrast, low pH conditions similar to those used in this study did not affect the morphology of newly settled *H. erythrogramma* or *S. droebachiensis* (Dupont et al., 2013; Wolfe et al., 2013; Wolfe et al., 2013a). In 'acidified' water, CO_2 is thought to be the primary species that limits growth and calcification of echinoids via hypercapnic suppression of metabolism (Byrne et al., 2013; Dubois, 2014; Holtmann et al., 2013). However, growth rates of calcified structures are correlated with carbonate saturation states, suggesting a role for reduced carbonate availability to limit growth and enhance dissolution of carbonate structures in low pH conditions (Byrne et al., 2013). The vulnerability of calcified structures to reduced carbonate saturation states increases with the Mg^{2+} content of the structure (Bischoff et al., 1987), and the Mg^{2+} content of echinoid skeletons tends to decrease with increasing latitude (McClintock et al., 2011; Smith et al., 2016; Weber, 1973). This may, in part, explain why the morphological effects of low pH were apparent for the tropical *T. gratilla* and the subtropical *C. rodgersii* population tested here in contrast to the temperate *H. erythrogramma* and *S. droebachiensis*.

The negative effects of low pH treatments on the morphology and development of *C. rodgersii* during settlement are likely to carry over to subsequent life stages. Reductions in the size and number of spines and pedicellariae could lower survival and growth rates because these structures play important roles in defence, adhesion, locomotion, feeding, anti-fouling, and cleaning (Ghyoot et al., 1987; Strathmann, 1981). Likewise, slowed development of the digestive system (especially teeth) after metamorphosis delays the onset of feeding (Moss and Tong, 1992), reducing energy reserves (Byrne et al., 2008a). Juveniles may acclimatise to low pH over time, but this might not compensate for reduced size or growth in the weeks after settlement. Reduced size and low energy reserves at early juvenile stages are linked to poorer survival and slower growth as juveniles and adults (Dworjanyn and Byrne, 2018; Emlet and Hoegh-Guldberg, 1997).

The carbonate chemistry conditions in our low pH treatments (pH 7.6 and 7.8) are similar to values predicted by 2100 for oceanic waters near south-eastern Australia (Lenton et al., 2015). This level of acidification during the settlement period results in smaller, less well-defended sea urchins, and may reduce natural recruitment with flow-on effects to the ecology of temperate marine systems. *C. rodgersii* is an important ecosystem engineer along the south-east coast of Australia and New Zealand (Byrne and Andrew, 2020), maintaining 'barrens' habitats which are a key driver of biodiversity (Curley et al., 2002). Predicted increases in atmospheric CO_2 are also likely to hamper growth of this species in culture, especially where culture water has reduced buffering capacity due to uptake of carbonates for calcification (Mos et al., 2015, 2016).

To date, only one study has tested the effects of using alkali chemicals for a sea urchin during the settlement period (Table 1). There was no difference in settlement rates of *T. gratilla* on an alkali substratum (concrete) compared to comparatively inert granite or greywacke, except at pH 7.7 where fewer larvae settled on concrete (Mos et al., 2019). However, juveniles generally had better post-settlement growth or survival on concrete than on the other substrata (Mos et al., 2019). We found the addition of alkali chemicals to boost pH levels above current ambient (~ pH 8.1) had some benefits in reducing the occurrence of abnormalities and increasing the number of spines and pedicellaria of newly settled *C. rodgersii*, although these effects were transient. At day 16, juveniles in pH 8.2 and 8.3 treatments were not larger or more developed than those in the pH 8.1 treatment, and survival rates were not different among ambient and high pH treatments. Indeed, our results suggest alkali chemicals can have negative effects, such as reducing the number of larvae that metamorphose as also found by Huo et al. (2019) for the geoduck clam *Panopea japonica*.

Settlement of *C. rodgersii* was 16% and 23% lower in the pH 8.2 and 8.3 treatments respectively compared to ambient pH (8.1). Settlement in high pH treatments may have been inhibited because these treatments interfered with the ability of larval receptors to detect histamine or disrupted the ability of receptors to signal to the nervous system and initiate metamorphosis. Larval receptors are sensitive to changes in ion concentration (Cameron et al., 1989; Pearce and Scheibling, 1994), and high pH treatments likely had higher concentrations of sodium ions than other treatments (from Na_2CO_3, $NaHCO_3$, and $Na_2B_4O_7$). However for the sea urchin *Lytechinus variegatus*, excess sodium does not affect settlement rate (Cameron et al., 1989). Alternatively, high pH conditions might have modified the protonation state of histamine, altering its function similar to the way in which low pH conditions alter the function of peptide signalling molecules (Roggatz et al., 2016). Additional studies to better understand the consequences of using alkalinising chemicals during the settlement period are warranted, especially given that these chemicals are expected to be employed with increasing frequency to combat acidification of source water associated with climate change, eutrophication, runoff from acid sulphate soils, and other anthropogenic impacts that are becoming increasingly prevalent (Barton et al., 2015; Reid et al., 2019).

6. Conclusion

This study examined the effects of pH on the settlement and post-settlement stages of sea urchins housed in relatively stable pH conditions. In 'real world' conditions, larvae settling in culture systems are likely to be exposed to variable pH conditions that oscillate between low and high extremes over short temporal periods (e.g. hours, Figs. 1, 2). It is possible that variability in pH promotes acclimation to adverse pH conditions through repeated exposure or mitigates the negative effects of low or high pH by periodically relieving physiological stresses (Hofmann et al., 2011). However, studies that have tested the consequences of fluctuating acidification during the settlement period have found periodic reductions in pH have equivalent or more adverse effects on growth and survival than a stable low pH (García et al., 2018a; Jiang et al., 2019). The consequences of exposure to alternating low and high pH conditions reflecting natural day-night flux during the settlement period have yet to be examined. This points to the need for future studies to investigate the consequences of fluctuating seawater carbonate chemistry in culture systems on growth and survival during the settlement period.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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