

Response of sea urchin pluteus larvae (Echinodermata: Echinoidea) to reduced seawater pH: a comparison among a tropical, temperate, and a polar species

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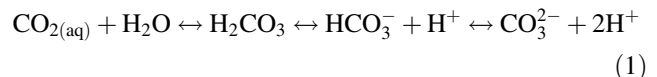
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Abstract Ocean acidification, as a result of increased atmospheric CO₂, is predicted to lower the pH of seawater to between pH 7.6 and 7.8 over the next 100 years. The greatest changes are expected in polar waters. Our research aimed to examine how echinoid larvae are affected by lower pH, and if effects are more pronounced in polar species. We examined the effects of lowered pH on larvae from tropical (*Tripneustes gratilla*), temperate (*Pseudechinus huttoni*, *Evechinus chloroticus*), and a polar species (*Sterechinus neumayeri*) in a series of laboratory experiments. Larvae were reared in a range of lower pH seawater (pH 6.0, 6.5, 7.0, 7.5, 7.7, 7.8 and ambient), adjusted by bubbling CO₂ gas. The effect of pH on somatic and skeletal growth, calcification index, development and survival were quantified, while SEM examination of the larval skeleton provided information on the effects of seawater pH on the fine-scale skeletal morphology. Lowering pH resulted in a decrease in survival in all species, but only below pH 7.0. The size of larvae were reduced at lowered pH, but the external morphology (shape) was unaffected. Calcification of the larval skeleton was significantly reduced (13.8–36.9% lower) under lowered pH, with the exception of the Antarctic species, which showed no significant difference. SEM examination revealed a degradation of the larval skeletons of *Pseudechinus* and *Evechinus* when grown in reduced pH. *Sterechinus* and *Tripneustes* showed no apparent difference in the skeletal fine structure under

lowered pH. The study confirms the need to look beyond mortality as a single endpoint when considering the effects of ocean acidification that may occur through the 21st century, and instead, look for a suite of more subtle changes, which may indirectly affect the functioning of larval stages.

Introduction

Ocean acidification, the phenomenon of decreasing ocean pH due to the uptake of anthropogenic carbon dioxide (CO₂), has been identified as an important consequence of rising CO₂ emissions worldwide (Feely et al. 2004; Orr et al. 2005; Fabry et al. 2008). The oceans are the largest active sinks of carbon on Earth, with an estimated 30% of anthropogenic carbon emissions produced since 1980 taken up by oceans (Sabine et al. 2004). This uptake of CO₂ decreases ocean pH through the conversion of dissolved CO₂ into carbonic acid (H₂CO₃) that further dissociates into bicarbonate (HCO₃⁻), carbonate (CO₃²⁻) and hydrogen ions (H⁺) in the following way:



As carbonic acid dissociates into bicarbonate and carbonate the pH of the water is lowered by the release of H⁺ ions. Consequently, surface ocean pH has dropped by 0.1 units since the industrial revolution (Caldeira and Wickett 2003; Cicerone and Orr 2004) and if current trends continue, is predicted to decrease a further 0.3–0.5 units by 2100 (Caldeira and Wickett 2005; Fabry et al. 2008).

Ocean acidification is expected to have a significant effect on marine ecosystems and, in particular, on

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calcifying invertebrate species, such as corals, bryozoans, molluscs and echinoderms (Orr et al. 2005). Calcifying marine organisms take up Mg^{2+} , Ca^{2+} and HCO_3^- or CO_3^{2-} ions and synthesize a magnesium calcite skeleton through a series of biochemical pathways (Killian and Wilt 2008). Presently, the production of calcareous structures is carried out in surface waters that are supersaturated for $CaCO_3$ (Raven et al. 2005). A decrease in pH however, lowers the saturation state of $CaCO_3$ because the carbonate ions are converted to bicarbonate in order to balance the excess H^+ ions that result when CO_2 dissolves in water. This decreases the concentration of carbonate ions in seawater, which reduces the calcite and aragonite saturations states and shallows the saturation horizon depth.

Reduced calcification is expected to result in weaker skeletons, reduced growth rates and, in corals, an increase in susceptibility to erosion (Raven et al. 2005). A reduction in calcification has been shown in corals (Marubini and Thake 1999; Langdon et al. 2000; Marubini et al. 2003), foraminifera (Spero et al. 1997), and coccolithophores (Riebesell et al. 2000; Zondervan et al. 2001) in response to reduced carbonate concentrations. For example, a doubling of atmospheric CO_2 from pre-industrial levels from 280 to 560 ppm CO_2 resulted in a 5–45% reduction in calcification rates in coccolithophores (Riebesell et al. 2000; Zondervan et al. 2001). Calcification rates of the edible mussel (*Mytilus edulis*) and the Pacific oyster (*Crassostrea gigas*) declined linearly with increasing ρCO_2 under levels within the range predicted by the IPCC for 2100 (Gazeau et al. 2007). Reduced calcite production was also found under elevated CO_2 concentrations in two species of coccolithophores (Riebesell et al. 2000), and was accompanied by an increase in the proportion of malformed coccoliths and incomplete coccospheres. A 6-month study by Shirayama and Thornton (2005) found that a moderate CO_2 increase of 200 ppm adversely affected the growth of gastropod (*Strombus luhuanus*) and echinoids (*Hemicentrotus pulcherrimus* and *Echinometra mathaei*).

These studies confirm that elevated CO_2 levels may lead to reduced calcification rates in many calcifying organisms. Decreasing calcification that result in changes in species survival or growth could lead to changes in community structure, reproduction and overall community function. Non-calcifying species may be favoured, which could lead to shifts in species composition and dominant organisms. Changes on this scale would be expected to have cascading effects throughout food webs and ecosystems. Research into ocean acidification is at an early stage and studies need to be carried out to identify species and ecosystems most at risk from ocean acidification and the levels of pH change that they can withstand. This would include the initial

processes of identifying the nature and degree of effects of lowering seawater pH on marine organisms.

In this study, sea urchin larvae were used as a model organism to investigate the effects of ocean acidification on calcifying organisms. Sea urchin embryos and larvae have been used extensively as a model in developmental studies; therefore, their embryonic and larval development is well understood. They have a high-magnesium calcite skeleton which is easily identified under a light or polarising microscope (Lowenstam and Weiner 1989). Skeletal deposition in sea urchin larvae may be particularly sensitive to lower seawater pH as skeletal elements form through an amorphous calcium carbonate precursor stage that has been found to be 30-times more soluble than calcite (Beniash et al. 1997; Politi et al. 2004). Other studies on the effects of ocean acidification on sea urchin larvae have found fertilization rate, cleavage rate, hatching rate, developmental speed and pluteus larval size all decrease with increasing CO_2 concentrations (Kurihara et al. 2004; Kurihara and Shirayama 2004; Havenhand et al. 2008; Kurihara 2008).

The larval stage is a critical period in the life history of a species with planktonic larvae because recruitment success is primarily determined by the survival of the embryos and larvae (McEdward and Miner 2007). Therefore, any decrease in the survival rates of this key life history stage will reduce the long-term viability of affected populations. Sea urchins are often the dominant grazer in many communities (Lawrence 2007) and can act as a keystone species, therefore changes in their survival or growth may lead to ecosystem scale shifts.

Previous researchers have examined the effect of pH on sea urchin embryos and larvae (see review by Kurihara 2008), however, more information is required on the nature of responses of marine larvae to future pH decreases, especially in equivalent larvae found across a range of marine environments. Therefore, an over-riding objective of the present study was to explore potential differences in the effects of lower seawater pH in larvae ranging from tropical to polar regions. This is of interest because calcification is already less favourable energetically at higher latitudes (i.e. a higher apparent solubility product for calcite, Clarke 1983) due to the cold sea temperatures and the upwelling of CO_2 -rich water, and these areas have been identified as regions where ocean acidification effects may appear earliest (Orr et al. 2005). For this reason, we determine the effects of low pH conditions on skeletal development in the Antarctic sea urchin *Sterechinus neumayeri* (Echinidae), the New Zealand temperate sea urchins *Pseudechinus huttoni* (Temnopleuridae) and *Evechinus chloroticus* (Echinometridae) and the tropical sea urchin *Tripneustes gratilla* (Toxophneustidae). Although these sea urchin species are not from the same family they

are phylogenetically similar (within the same Class) and have equivalent ecological roles (relatively abundant shallow sub-tidal grazers). Among these species we determined the effect that pH levels predicted for 2100 (pH 7.6–7.8) would have on the skeletal development and survival of sea urchin larvae. This also entailed determining pH thresholds for survival of sea urchin larvae between pH 8.2 and 6.0. We determined these effects by examining changes in larval survival, skeletal growth, calcification index and skeletal structure.

Materials and methods

Species and study sites

Larvae from four echinoid species were studied; the Antarctic *S. neumayeri* Meissner (Family: Echinidae), the New Zealand *E. chloroticus* Valenciennes (Family: Echinometridae) and *P. huttoni* Benham (Family: Temnopleuridae) and the tropical *T. gratilla* Linnaeus (Family: Toxopneustidae). Sea urchins were collected from three locations; McMurdo Sound, Antarctica (77.057°S, 164.416°E, average seawater pH 8.01 ± 0.01 SE), Doubtful Sound, New Zealand (42°25'S, 170°56'E, average seawater pH 8.1 ± 0.01 SE) and Aitutaki Lagoon, Cook Islands (18.85°S, 159.75°E, average seawater pH 8.2 ± 0.02).

Sea urchin spawning and larval culture

Spawning and experimentation was carried out during the period when each species was ripe (see Table 1 for dates of experiments). Adult sea urchins were induced to spawn by an inter-coelomic injection of 0.5 mol l^{-1} KCl using standard techniques (Lamare et al. 2006). Eggs were fertilised by adding several drops of dilute sperm, and only batches of eggs with a fertilization rate $\geq 95\%$ were accepted for use. All spawning and fertilization was carried out in ambient seawater pH. Water temperature was kept at -1.0°C during the Antarctic experiments, 10 or 15°C during the New Zealand experiments and 26°C during the tropical experiments. Larvae were not fed during the Antarctic experiments due to the slow metabolism of the larvae, or during the tropical experiments due to the short period of the experiments. For these two species, when the experiment was terminated the larvae had developed through to a functionally feeding stage. During the New Zealand experiments larvae were fed *Dunaleilla* spp. algae every third day at a concentration of $4,000\text{--}6,000 \text{ cells ml}^{-1}$. Feeding was achieved by injection of a small volume ($\leq 1 \text{ ml}$) of concentrated algae through a sealed port on the lid of the experimental containers.

pH treatment

We reduced the ambient pH of seawater at each of our study sites by pH 0.4, giving rise to an experimental pH in McMurdo Sound of 7.6, Doubtful Sound and Otago Harbour of 7.7 and Aitutaki Lagoon of 7.8. The pH (NBS scale) of the seawater was adjusted by bubbling CO_2 gas through it, and after 1–2 h transferred to sealed containers to prevent re-equilibration with the atmosphere. The pH was checked using a pH meter (Mettler Toledo MP220) calibrated to buffers of pH 4.0, 7.0 and 9.2 (Labserv Pro-analys, Biolab New Zealand). All pH measurements were adjusted for differences in seawater temperature, and the pH was checked again 1–2 h after adjustment to ensure the pH had not drifted (values <0.1 pH units were accepted for use). The water was changed every 6 days during the Antarctic experiments and every 3 days during the New Zealand experiments by filtering larvae onto a $50 \mu\text{m}$ sieve and filling the containers with new $1 \mu\text{m}$ -filtered seawater adjusted to the appropriate pH for each treatment. Between water changes, pH varied less than 0.1 pH units. Seawater was not changed during the tropical experiments due to the short experimental time period (4 days).

Seawater carbonate chemistry

Determining carbonate conditions in our experimental treatments required at least two measurements (i.e. pH, alkalinity, ρCO_2). While we could determine pH, it was not practical to measure other variables due to the remote locations of the study sites. Instead, we measured pH, temperature and salinity, from which alkalinity was estimated using methods described by Lee et al. (2006). This, in turn, allowed us to indirectly determine the carbonate chemistry of the seawater during experiments.

The pH (NBS pH scale) of the seawater was measured as described previously, while total alkalinity was calculated using standard algorithms for five global regions with an area-weighted uncertainty of $\pm 8 \mu\text{mol kg}^{-1}$ (Lee et al. 2006). The calculation of the total alkalinity was based on CTD measurements of salinity and temperature of seawater used in laboratory experiments. We assumed that the total alkalinity did not change between ambient and lowered pH treatments because the pH of the seawater was adjusted using CO_2 gas, and not by acid treatment. The partial pressure of CO_2 (ρCO_2) and the saturation value omegas for calcite (Ω_{C}) and aragonite (Ω_{A}) were calculated using a computer programme SWCO₂ (http://neon.otago.ac.nz/research/mfc/people/keith_hunter/software/software.htm) which calculates various parameters of the carbonate system in seawater under specified conditions of temperature and salinity using any two of the normal CO_2 system parameters or the concentrations of the individual species.

Table 1 Location, experimental period, temperature (°C), salinity (PSU), calculated total alkalinity (A_T), pH measured on the NBS scale, equilibrium CO_2 partial pressure (ρCO_2), $CaCO_3$ saturation value omega for calcite (Ω_C) and $CaCO_3$ saturation value omega for aragonite (Ω_A) for the seawater used in the ambient and low pH treatments of each experiment

Species	<i>Sterechinus</i>		<i>Pseudechinus</i>		<i>Evechinus</i>		<i>Tripneustes</i>	
Location	McMurdo Sound, Antarctica		Otago Harbour, New Zealand		Otago Harbour, New Zealand		Aitutaki Lagoon, Cook Islands	
Latitude/longitude	77.06°S/164.42°E		45.41°S/176.93°E		45.41°S/176.93		18.85°S/159.75°E	
Experimental period	24 October to 14 November 2007		13 August to 15 September 2007		14 to 26 January 2008		20 May to 1 June 2008	
Temperature (°C)	−1.9		12		15		26	
Salinity (PSU)	34.6 ^a		33.2 ^b		33.2 ^b		35.3 ^c	
A_T ($\mu\text{mol kg}^{-1}$)	2,336		2,229		2,224		2,315	
Treatment	Ambient	Low	Ambient	Low	Ambient	Low	Ambient	Low
pH _(NBS)	8.0	7.6	8.1	7.7	8.1	7.7	8.2	7.8
ρCO_2 (μATM)	521	1,380	429	1,282	438	1,320	395	1,119
Ω_C	1.64	0.66	3.28	1.29	3.58	1.42	5.45	2.56
Ω_A	1.03	0.41	2.09	0.82	2.29	0.91	3.60	1.69

Seawater was taken from three locations; McMurdo Sound (Antarctica) Otago Harbour (New Zealand) and Aitutaki Lagoon (Cook Islands)

^a CTD measurements at Cape Armitage, November 2007

^b CTD measurements of the Otago Harbour, November, 2008

^c CTD measurements of the Aitutaki Lagoon, April/May, 2008

We determined seawater properties using CO_2 equilibrium constants given by Mehrbach et al. 1973 (as modified in Dickson and Millero 1987) as recommended by Wanninkhof et al. (1999).

Oxygen concentrations in CO_2 treated seawater were measured using a YSI Model 550A, prior to, immediately after, and hourly for 3 h after the addition of CO_2 gas to the experimental seawater. Concentrations of dissolved oxygen in treated seawater remained greater than 90% of ambient seawater concentrations, with the exception of pH 6.0 treatments where oxygen concentrations were reduced to between 80.7 and 89.2% depending on water temperature.

Larval measurements

Survival and skeletal abnormalities

Once hatched larvae were placed at a density of 10–40 larvae ml^{-1} (depending on species) in 500 ml airtight containers filled with 1 μm -filtered seawater. The pH of seawater in each container was previously adjusted to pH values of 6.0, 6.5, 7.0, 7.5, 7.8, 8.0, and unadjusted controls of the ambient pH for each location (either 8.0, 8.1 or 8.2). The Antarctic experiment had three replicates for each treatment while the New Zealand and tropical experiments had four.

The Antarctic experiment ran for 7 days, the New Zealand experiment ran for 9–13 days and the tropical experiment ran for 4 days. Survival rates were measured every 1–3 days during the Antarctic and New Zealand experiments and every day during the tropical experiments by taking ten, replicate 1 ml seawater samples from each container and counting the number of larvae in each.

Repeated measure ANOVA tested for a significant ($\hat{p} < 0.05$) effect of pH on the proportion of larvae surviving during larval culturing. Proportional data were arcsine square transformed prior to testing. To identify significant ($\hat{p} < 0.05$) differences in survival rate among pH treatments at the end of the larval rearing experiment within each species, a one-way ANOVA was performed, with Tukey's HSD Post-hoc test used to identify significant ($\hat{p} < 0.05$) differences among pH treatment levels.

Skeletal growth

Once larvae were placed at a density of five larvae ml^{-1} in 500 ml airtight containers filled with 1 μm -filtered seawater. The water in half of the containers was left at the normal seawater pH of the location (either 8.0, 8.1, or 8.2) while the seawater pH in the other half was lowered to the pH expected at each location in the year 2100 (either 7.6, 7.7 or 7.8). The experiments had three replicates for each

treatment for *Sterechinus* and *Pseudechinus*, and four replicates for *Evechinus* and *Triploneustes*.

The Antarctic experiment ran for 17 days, the New Zealand experiment ran for 9–13 days and the tropical experiment ran for 4 days. At the end of the experiments, ten larvae were taken randomly from each container and photographed under normal and polarized light (Fig. 1). Growth indices were measured using Image J software Version 1.38 \times and included total length (TL), width (TW), primary recurrent rod (R1), antero-lateral skeletal rod (R2), ventral transverse skeletal rod (R3), and post-oral skeletal rod (R4) (Fig. 1).

Within each species, a two-way ANOVA was used to test for significant ($\hat{\theta} < 0.05$) differences in the size of larval components in response to pH treatment. A non-significant ($\hat{\theta} > 0.05$) interaction between body components and pH was interpreted as indicating no effect of pH on larval morphology. All data were $\ln(x)$ transformed prior to analyses.

Calcification index

Once hatched, larvae were placed at a density of 10–40 larvae ml^{-1} in 20 l buckets filled with 1 μm -filtered seawater. The water in half of the buckets was left at the ambient seawater pH of the location (either 8.0, 8.1 or 8.2) while the seawater pH in the other half was lowered to the pH expected for each location in 2100 (either 7.6, 7.7 or 7.8). The experiment had three replicates for each treatment, except for *Evechinus* that had four.

The Antarctic experiment ran for 17 days, the New Zealand experiment ran for 9–13 days and the tropical experiment ran for 4 days. At the end of the experiment the larvae were collected, rinsed briefly in freshwater, and dried in a 60°C oven for at least 1 day. The dried material was weighed onto approximately 30 \times 30 mm pre-weighed tinfoil pieces and ashed in a muffle furnace at 450°C for 4 h. Ten replicate 30 \times 30 mm tinfoil samples were also ashed to account for loss of weight by the foil. After ashing the larvae were re-weighed and the inorganic weight (% of dry weight) was determined by dividing the post-ash weight of the larvae by the pre-ash weight. The

post-ash weight was determined using the following formula:

$$\text{Post-ash weight (mg)} = \text{ashed larvae and foil weight (mg)} - (\text{initial foil weight (mg)} \times P) \quad (2)$$

where P was the percentage decrease in the standard foil weights after ashing. The differences in mean calcification index in larvae reared in normal and lowered seawater pH were compared within each species using a Student t test. Percentage data were arcsine square transformed prior to comparisons.

Scanning electron microscopy of larval skeletons

Upon termination of the survival experiment, larvae were collected to examine skeletal abnormalities. A 4.8% solution of bleach was added to the samples for 1 h to remove any tissue and the larvae were then rinsed twice in distilled water to remove bleach residue. After drying in a 60°C oven for at least 24 h samples were gold coated and examined using scanning electron microscopy (SEM) for evidence of skeletal abnormalities such as pitting or eroding of the skeleton.

Statistical analysis

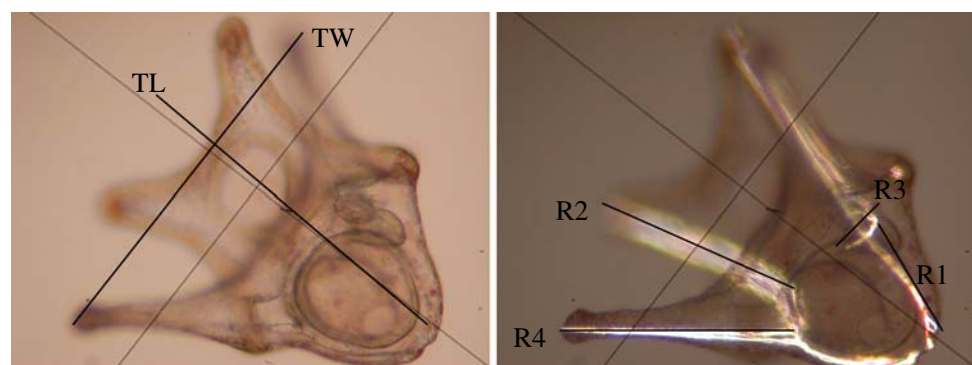
All statistical analyses were carried out using the computer software JMP7.0 statistical software (SAS Institute Inc.). Residuals were examined to check for significant departures from normality of data, while homogeneity of variances was tested using Cochran's C test (Underwood 1997).

Results

Seawater carbonate chemistry

The carbonate chemistry during experimental treatments were calculated from pH, temperature and salinity

Fig. 1 Morphometric measurements of sea urchin larvae, using *Sterechinus neumayeri* as an example. Measurements made are total length (TL), width (TW), primary recurrent rod (R1), antero-lateral skeletal rod (R2), ventral transverse skeletal rod (R3), and post-oral skeletal rod (R4). Larvae are viewed under normal and polarised light to highlight the larval skeleton



measurements, and estimated total alkalinity (Table 1). Under ambient seawater conditions, we calculated ρCO_2 to range from 395 μATM in Aitutaki to 521 μATM in McMurdo seawater. In the experimentally lowered pH seawater treatments, ρCO_2 ranged from 1,119 to 1,380 μATM . At all sites, both calcite and aragonite were saturated in ambient seawater. Calcite saturation levels increased from $\Omega_{\text{C}} = 1.64$ to 5.45 from the polar to tropical experiments, while aragonite saturation values ranged from $\Omega_{\text{A}} = 1.03$ to 3.60, also decreasing at higher latitudes. Under experimentally lowered pH, only the Antarctic treatments became under-saturated for calcite ($\Omega_{\text{C}} = 0.66$), remaining saturated in the temperate and tropical treatments ($\Omega_{\text{C}} = 1.29$ – 2.56). Aragonite was under-saturated in the polar and temperate experiments ($\Omega_{\text{A}} = 0.41$ – 0.91), but remained saturated in the tropical experiments ($\Omega_{\text{A}} = 1.69$).

Survival

For all four species, the in vitro survival of larvae varied among pH treatments (Fig. 2). For all species, the proportion of larvae surviving throughout the experiments, were greater than 52% in treatments with pH greater than pH 7.0. For all species, the pH 6.0 treatment resulted in $\approx 100\%$ mortality (0–1.7% survival), generally within 4–7 days, while pH 6.5 resulted in survival of 34.4% in *Pseudechinus* and 10.7% in *Tripneustes* over the course of the experiment (Fig. 2). Survival was greater than 74% in pH 6.5 in *Evechinus* and *Sterechinus*. Repeated measures ANOVA (Table 2) indicated that in the four species, the rate of survival was significantly dependent on seawater pH (Time \times pH interaction <0.001).

To identify differences in survival among pH treatment for each species, the rate of survival on the final day of sampling (Table 3) was compared using one-way ANOVA. In *Pseudechinus*, there was a significant difference [$F_{(5,12)} = 8.514$, $P = 0.0012$] in survival to day 9 among pH treatments, with a Tukeys Post-hoc test indicating that the difference was due to the lower survival in pH 6.0 and 6.5, which in turn differed significantly. Survival to day 4 in *Tripneustes* differed significantly [$F_{(6,21)} = 32.351$, $P < 0.0001$] among pH treatments, with pH 6.0, 6.5 and 7 having lower survival than the higher pH treatments. Among these low pH treatments, pH 7.0 had greater survival than pH 6.0 and 6.5. For *Sterechinus*, the significant difference [$F_{(4,10)} = 19.92$, $P < 0.0001$] in survival to day 22 among treatments was due to low survivorship in pH 6.0, with no difference in pH treatments between 6.5 and 8.0. A similar result was observed in *Evechinus*, where the significant difference [$F_{(5,18)} = 58.222$, $P < 0.0001$] among treatments was restricted the low survival to day 13 in the pH 6.0 treatment.

Growth

The size of larval body components was significantly affected by pH treatment, except in *Pseudechinus* (Fig. 3, Table 4). For the three significantly effected species, a lower pH treatment usually resulted in a significantly smaller body component. The exceptions were a larger primary recurrent rod (Rod 1) and transverse skeletal rod (Rod 3) in *Tripneustes* in lowered pH seawater. Overall reduction in the size of the larvae (total length) as a result of lowered seawater pH was lowest in *Sterechinus* (1.8%), intermediate in *Tripneustes* (3.2%) and *Evechinus* (4.2%), and greatest in *Pseudechinus* (18.3%).

Two-way ANOVA suggested that, while lower seawater pH reduced the size of the larvae, the morphology of the larvae was generally unaffected by pH. In this respect, a non-significant interaction term in all species (Body component \times pH, Table 4) indicated that the effect of pH was not dependent on the measurement (i.e. for the larvae, the size of the body components responded to a lower pH was in the same direction).

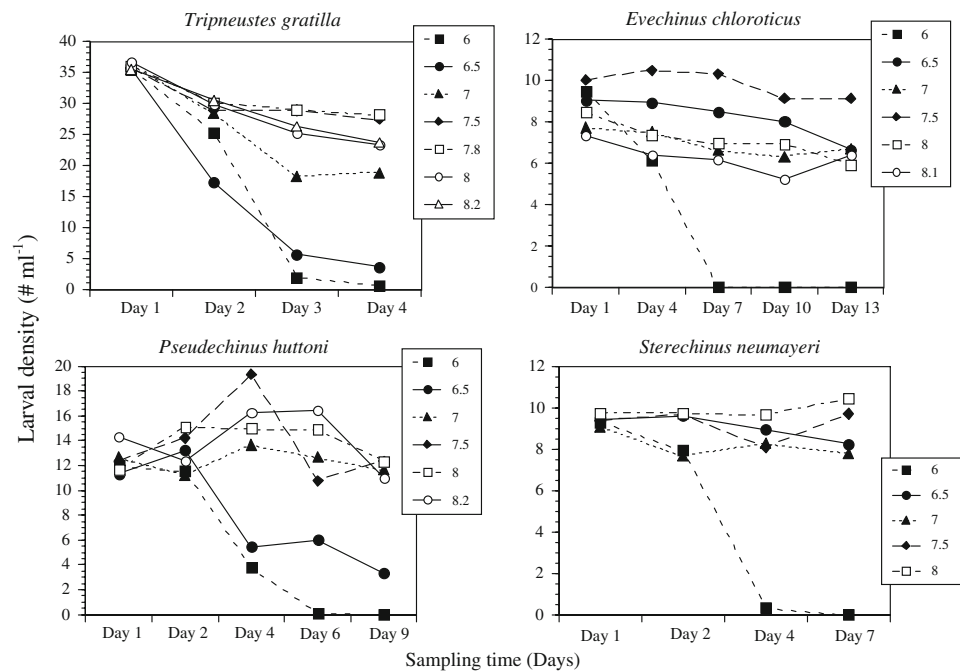
Calcification

The degree of calcification (percentage inorganic content of total dry weight) in larvae raised in normal seawater ranged from 67.4% in *Evechinus*, to 17.05% in *Sterechinus* (Table 5). When grown in lowered pH, three species showed a significant reduction in calcification index (Table 5), while *Sterechinus* showed a 3.9% (although not significant, $P = 0.4035$) lower calcification index. The percentage reduction in calcification was similar in *Pseudechinus* (36.9%) and *Evechinus* (30.6%), but less in *Tripneustes* (13.8%).

Larval skeleton fine structure

Seawater pH did not cause any change in the gross morphology of larval skeletons among species. It was apparent, however, that there were fine scale differences in skeleton structure as a result of pH treatment, although the effects were variable among the species (Fig. 4). In all four species, the skeletal rods from larvae grown in normal pH were characterised by a uniformly smooth surface (Fig. 4). When grown in low pH seawater however, there was an apparent loss of integrity in the surfaces of the skeletal rods) in the temperate species. For *Evechinus*, almost the entire surface of the larval skeletons was pitted, with only a small area with a smooth surface (Fig. 4a). *Pseudechinus* skeletons also had an eroded appearance (Fig. 4c), although the erosion surfaces were more angular compared with the rounded erosion observed in *Evechinus*. For *Pseudechinus* (Fig. 4c)

Fig. 2 *Tripneustes gratilla*, *Evechinus chloroticus*, *Pseudechinus huttoni* and *Sterechinus neumayeri*. Change over the experimental period in the average density of larvae of four species of sea urchin larvae experimentally reared in a range of seawater pH treatments. Sampling periods differ among species due to differences in the development rate of each. Error bars are excluded for clarity, but standard errors given for the final survival rates in Table 3). $N = 4$ for each data point, except for *Sterechinus* and *Pseudechinus* where $N = 3$



erosion appeared to be greatest on the surfaces of the cross-members of the fenestrated skeletal rods. In contrast to the former two species, there was no apparent difference in the surface structure of *Tripneustes* (Fig. 4b) or *Sterechinus* (Fig. 4d) skeletal rods from larvae grown in normal or reduced pH. In both species and treatments, skeletal rods were uniformly smooth.

We did not attempt to quantify differences in skeletal structure among species and treatments, as larval skeletons were almost always fragmented and impossible to assign to a known larva. Two semi-quantitative observations are noteworthy however. Firstly, in all four species, no larval skeletal elements from the ambient seawater treatments showed any loss of surface integrity and were uniformly smooth. Secondly, within each species there was a degree of variation in the amount of skeletal degradation in larvae reared in lowered pH, and it was possible to observe a range of responses (from the normal through to the severely malformed skeleton presented in Fig. 4).

Discussion

Seawater carbonate chemistry

This study investigated how ocean pH levels predicted to occur by the end of the 21st century could affect sea urchin larvae. Predicting the response of larvae to a lowered pH environment requires establishing appropriate experimental

conditions. We lowered pH (NBS scale) by 0.4 units below ambient levels, based on a generalized prediction of a drop of this magnitude in surface ocean waters by the year 2100 (Fabry et al. 2008). Predicted pH decreases between the years 2050 and 2100 vary in magnitude, and range from 0.3 to 0.5 (Caldeira and Wickett 2005). There will also be a degree of spatial and short-term temporal variation in pH changes, both across latitudes (Orr et al. 2005) and depth (Feely et al. 2004; Orr et al. 2005) and also at the local scale (Ohline et al. 2007). It was beyond the scope of the present study to account for these variations, with the aim to establish the general nature and magnitude of responses of sea urchin larvae to lowered pH in species from a range of latitudes.

We described the carbonate chemistry of our experiments based on measured pH, temperature and salinity, and modeled total alkalinity at each location. Based on this method, we obtained measures of carbonate parameters that were higher than in previous studies. For example, our estimates of ρCO_2 in ambient seawater were 521 μATM in McMurdo Sound, 429 and 438 μATM in Otago Harbour, and 395 μATM at the Cook Islands (Table 1). Previous measurements at McMurdo Sound are 410 μATM in November (Takahashi et al. 2002), up to 350 μATM for coastal Otago waters (Currie and Hunter 1998), and 370 μATM for South Pacific tropical waters of the same approximate latitude (Wanninkhof et al. 1999).

Our estimated ρCO_2 among treated seawater (Table 1) were also high (ranging from 1,119 to 1,380 μATM), and would result in calcite under-saturation only in the

Table 2 *Tripneustes gratilla*, *Evechinus chloroticus*, *Pseudechinus huttoni*, and *Sterechinus neumayeri*

Source of variation	$F_{(df)}$	P
<i>Tripneustes gratilla</i>		
Between-subjects		
pH	25.24 _(6,21)	<0.001
Within-subjects		
Time ^a	232.92 _(2,45,51.64)	<0.001
pH × time ^a	16.23 _(14,75,51.64)	<0.001
Mauchly criterion = 0.5106, $df = 9$, $P < 0.05$		
<i>Evechinus chloroticus</i>		
Between-subjects		
pH	56.89 _(5,18)	<0.001
Within-subjects		
Time	80.33 _(4,72)	<0.001
pH × time	20.26 _(20,72)	<0.001
Mauchly criterion = 0.5106, $df = 9$, $P = 0.273$		
<i>Pseudechinus huttoni</i>		
Between-subjects		
pH	14.04 _(5,12)	<0.001
Within-subjects		
Time	6.84 _(4,48)	<0.002
pH × time	3.36 _(20,48)	<0.003
Mauchly criterion = 0.2772, $df = 9$, $P = 0.146$		
<i>Sterechinus neumayeri</i>		
Between-subjects		
pH	9.07 _(4,10)	<0.0023
Within-subjects		
Time	15.87 _(3,30)	<0.001
pH × time	12.37 _(12,30)	<0.001
Mauchly criterion = 0.5247, $df = 5$, $P = 0.344$		

Univariate repeated measures ANOVA of the survival (proportional change over time in the density of larva during the experimental period) among seawater pH treatments. Repeated measurement of larval density varied in frequency and duration depending on species development rate. Tests for sphericity were made using the Mauchly's sphericity test

^a Degrees of freedom and P -value adjusted by Greenhouse-Geisser ϵ

McMurdo Sound experiments, and an under-saturation of aragonite in McMurdo Sound and temperate experiments, but not in the tropical treatments. Such a scenario is consistent with predictions of changes in the aragonite saturation state (Ω_A) for the year 2100, where Antarctic coastal waters may have $\Omega_A = 0.6 < 0.9$, and tropical waters remain saturated $\Omega_A = 2 < 3$ (Fabry et al. 2008). The under-saturation of seawater in the temperate regions was, however, not predicted by Fabry et al. (2008). Given our estimates of ρCO_2 in ambient seawater are higher than previously reported, it is possible that the temperate experiments were indeed saturated, as would be predicted for a pH 0.4 drop in 2100.

Effects of lowered pH on larvae

In most cases larval survival was not significantly affected until pH levels were below pH 7.0. These results are consistent with observations made for a range of marine organisms (review by Knutzen 1981), in which most species were not affected until pH levels dropped below 7.0. In our study the pH 6.0 treatment resulted in 100% mortality for all species within 4–7 days while survival in the pH 6.5 treatment was only significantly lower for *Pseudechinus* (34.4% survival) and *Tripneustes* (10.7% survival). As pH levels of 6.0 and 6.5 are well beyond what is predicted to occur over the next 100 years, direct extrapolation of our results would suggest that the pH levels expected for 2100 will not directly affect the survival of larvae of these four sea urchins species. It should be noted however, that our experiments only spanned a limited and early period of the larval stage, and Yamada and Ikeda (1999) have noted that the tolerable pH range for marine plankton decreases with increasing exposure time. The length of exposure times in our experiments only ranged from 4 to 22 days (to coincide with the completion of the four-armed pluteus stage), and it is possible that lethal effects may have occurred at higher pH levels if the experiments had run for longer.

Although ocean pH levels predicted for 2100 showed no direct effect on survival, more subtle changes were observed. With the exception of *Pseudechinus*, the growth of larval body components was significantly affected, with a lower pH treatment resulting in a significantly smaller body. For *Pseudechinus*, although not significant, the four growth indices examined were also smaller when larvae were raised at a lower pH. Kurihara et al. (2004) found that the size of *H. pulcherrimus* and *E. mathaei* pluteus larvae also decreased in response to increasing CO_2 concentrations. The reduced length of body components under low pH conditions suggests that the low pH levels or high ρCO_2 interfere with the growth of the larvae either through reduced calcification or via an acidosis effect where maintaining acid–base balance has an associated metabolic cost (i.e. Pörtner 2008).

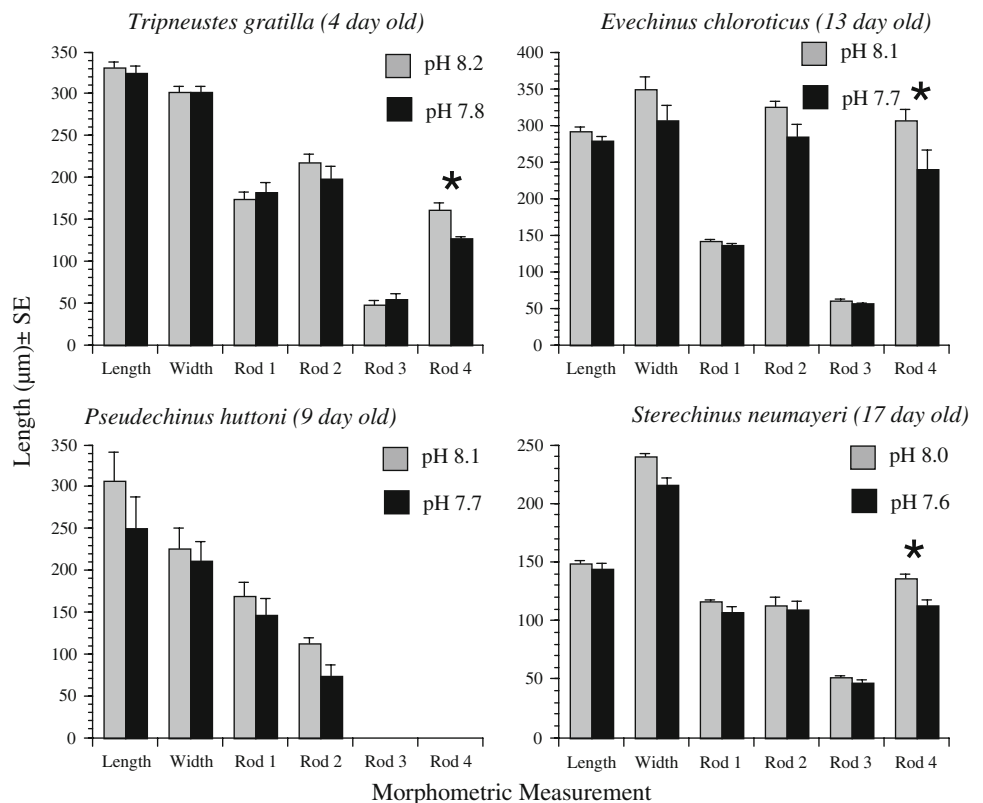
Three of our species studies showed a significant reduction in calcification index when larvae were grown under low pH conditions, with the exception of *Sterechinus*, which showed no significant difference between treatments. This decrease was greatest in the temperate New Zealand species, *Pseudechinus* (36.9%) and *Evechinus* (30.6%), and was more moderate in the tropical species *Tripneustes* (13.8%). The relationship of decreasing calcification index with increasing ρCO_2 or decreasing pH has been demonstrated in a number of other species including the blue mussel (Gazeau et al. 2007), the Pacific oyster (Gazeau et al. 2007), scleractinian corals (Reynaud et al. 2003; Marubini et al. 2003; LeClercq et al. 2000) coralline

Table 3 *Tripneustes gratilla*, *Evechinus chloroticus*, *Pseudechinus huttoni*, and *Sterechinus neumayeri*

pH treatment	Proportion survival at the end of the experiment (\pm SE)			
	<i>Tripneustes</i>	<i>Evechinus</i>	<i>Pseudechinus</i>	<i>Sterechinus</i>
6.0	0.017 (0.001)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
6.5	0.11 (0.052)	0.74 (0.02)	0.34 (0.07)	0.89 (0.076)
7.0	0.52 (0.1)	0.87 (0.015)	0.95 (0.131)	0.86 (0.021)
7.5	0.77 (0.017)	0.89 (0.021)	1.02 (0.059)	1.07 (0.082)
7.8	0.78 (0.044)	–	–	–
8.0	0.63 (0.034)	0.71 (0.018)	1.04 (0.131)	1.10 (0.063)
8.1 or 8.2	0.67 (0.068)	0.88 (0.039)	0.76 (0.015)	–
Experimental duration (days)	4	13	9	7

Proportion of larvae surviving from blastula to four-armed pluteus when reared in a range of seawater pH treatments. The duration of the experiment varied depending on species

Fig. 3 *Tripneustes gratilla*, *Evechinus chloroticus*, *Pseudechinus huttoni* and *Sterechinus neumayeri*. Length of morphometric variables in larvae of four species grown in either ambient pH seawater, or lowered pH seawater. While similar in developmental stage (4-armed pluteus), larvae varied in age. Asterisks indicates a significant differences ($P < 0.05$) between pH treatments within each measurement as identified by a student *t* test. $N = 40$ for each data point, except for *Sterechinus* and *Pseudechinus* where $N = 30$



algae (Gattuso et al. 1999; Gao et al. 1993; Borowitzka 1981) and coccolithophores (Sciandra et al. 2003; Riebesell et al. 2000). A reduction in the degree of calcification of this magnitude compared to the overall reduction in the size of larvae suggests a weaker skeleton. This is supported by the SEM photographs of *Evechinus* and *Pseudechinus* larval skeletons, which showed a loss of structural integrity (pitting or erosion) in skeletal elements grown under low pH conditions. Skeletal rods from *Sterechinus* appeared smooth under both treatments, consistent with the similar calcification indices observed under normal and reduced pH. The lack of any apparent loss of structural integrity in *Tripneustes* skeletons (despite a significantly reduced calcification index) is difficult to reconcile, and may simply

reflect a slowing of development under reduced pH where a normal skeleton is constructed but at a slower rate.

The degradation of the larval skeleton through reduced deposition or dissolution would be consistent with an acidosis stress on the larvae. In this respect, the buffering of tissues can be achieved through an increase in bicarbonate concentrations, which in calcified organisms, can be made through dissolution of calcite (such as the larval skeleton). Indeed, sea urchin larvae may be especially sensitive to acidosis. As osmo-conformers they have a low capacity for ionic regulation, which means they have a lesser ability to acid–base regulate through the process of ionic exchange used by many aquatic organisms (Pörtner 2008).

Table 4 *Tripneustes gratilla*, *Evechinus chloroticus*, *Pseudechinus huttoni*, and *Sterechinus neumayeri*

Source	df	Sum of squares	F-ratio	P
<i>Tripneustes gratilla</i>				
Body component	5	9.899	393.611	<0.001
pH	1	0.098	19.564	0.0001
Body component × pH	5	0.034	1.383	0.253
<i>Evechinus chloroticus</i>				
Body component	5	18.223	339.753	<0.001
pH	1	0.155	14.469	0.0005
Body component × pH	5	0.069	1.287	0.2906
<i>Pseudechinus huttoni</i>				
Body component	3	5.729	20.259	<0.001
pH	1	0.276	2.938	0.0994
Body component × pH	3	0.061	0.214	0.885
<i>Sterechinus neumayeri</i>				
Body component	5	19.065	135.593	<0.001
pH	1	0.122	4.608	0.0421
Body component × pH	5	0.131	0.981	0.449

Two-way ANOVA of the effects of seawater pH on the length of six body components in the larvae of four species. All data were $\ln(x)$ transformed prior to analysis

As concern has been expressed for the effects of lowered pH on high-latitude marine inhabitants (Orr et al. 2005), a wider aim of the present research was to assess the sensitivity of the polar species. We might expect larvae of the Antarctic sea urchin *Sterechinus* to be the most vulnerable to the effects of ocean acidification due to their slow metabolism (Shilling and Manahan 1994) and because they inhabit an environment which already has high levels of CO₂ as a result of polar seawater temperatures and upwelling of CO₂-rich water (Feely et al. 2004). However, of the four sea urchin species examined under the

experimental conditions described in this study, *Sterechinus* larvae were the least affected by low pH levels. Survival was only significantly reduced when pH levels dropped to 6.0. SEM photographs revealed no difference in the skeletal structure of rods between treatments and calcification rates not significantly lower in larvae raised under the low pH treatment. Evolving in an environment with historically higher levels of CO₂ (and a greater apparent solubility product for calcite) would have required *Sterechinus* to adapt to higher CO₂ conditions and, therefore, they may have greater capacity to acclimate to lowered seawater pH.

Indeed, the observation that in ambient seawater conditions, *Sterechinus* larvae have an overall lower calcification index (17.05%) compared with larvae of the remaining species (ranging from 45.7 to 67.4%) is consistent with previous suggestions that organisms living in water with a greater calcite solubility (deep seas or polar waters) potentially experience greater difficulty calcifying (Clarke 1983).

Conclusions

It appears that ocean pH levels predicted for 2100 will not have a direct effect on the survival of larvae of the sea urchin species examined in this study. Larvae may, however, exhibit decreased growth and calcification indices that could indirectly compromise their survival in the ocean. In this respect, in species with a planktonic larval stage, recruitment success is primarily determined by the survival of embryos and larvae, and therefore, the larval stage is a critical life history period. Mortality rates of larvae in the plankton are very high (Rumrill 1990; Lamare and Barker 1999) and any delay in the growth or

Table 5 *Tripneustes gratilla*, *Evechinus chloroticus*, *Pseudechinus huttoni* and *Sterechinus neumayeri*. Ash content (% of dry weight) in larvae grown in ambient pH seawater, and in seawater with a pH

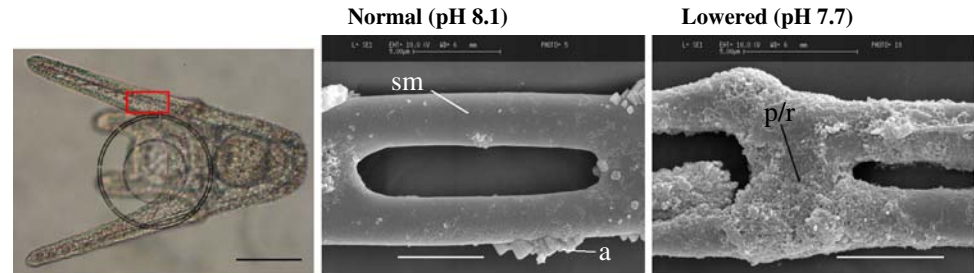
Species	Inorganic weight (% DW)		Percentage reduction (%)	T-test (T, P-value)
	Normal seawater pH (±SE)	Reduced seawater pH (±SE)		
<i>Tripneustes gratilla</i>	45.68 (±0.5)	39.35 (±0.5)	13.8	T = -8.88, P = 0.0004
<i>Evechinus chloroticus</i>	67.42 (±2.53)	46.82 (±4.79)	-30.6	T = -3.818, P = 0.007
<i>Pseudechinus huttoni</i>	54.91 (±1.81)	34.6 (±3.12)	-36.9	T = -5.518, P = 0.0053
<i>Sterechinus neumayeri</i>	17.05 (±0.9)	16.39 (±1.97)	-3.9	T = -0.268, P = 0.4035

The percentage reduction in calcification is given for each species, although for *Sterechinus*, an increase was observed

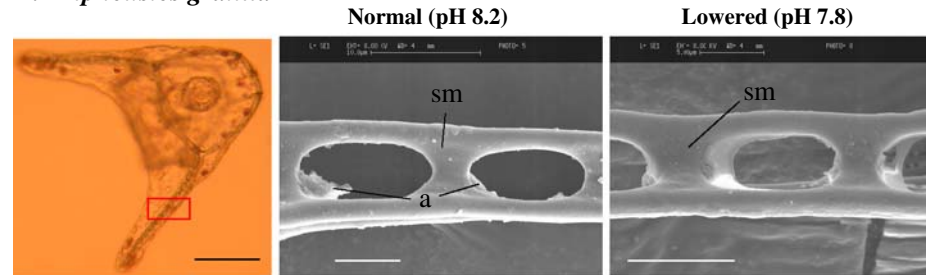
Experimental conditions: *Tripneustes gratilla* (26.7°C, Ambient pH average = 8.17, Lowered pH average = 7.81, duration = 4 days), *Evechinus chloroticus* (15°C, Ambient pH average = 8.16, Lowered pH average = 7.69, duration = 13 days), *Pseudechinus huttoni* (15°C, Ambient pH average = 8.14, Lowered pH average = 7.71, duration = 9 days), *Sterechinus neumayeri* (-1.0°C, Ambient pH average = 7.9, Lowered pH average = 7.51, duration = 17 days)

Fig. 4 *Evechinus chloroticus*, *Tripneustes gratilla*, *Pseudechinus huttoni*, and *Sterechinus neumayeri*. Scanning electron micrographs of larval skeletal rods from larvae of four species grown in normal pH seawater or lowered pH seawater. The region of the larval body the rods were extracted from are indicated on the representative larvae of each species. *sm* smooth surface, *p/r* pitted/eroded surface, *a* preparation artefact. Scale bars in whole larvae are 100 μm , and scale bars in SEM micrographs represent 5 μm

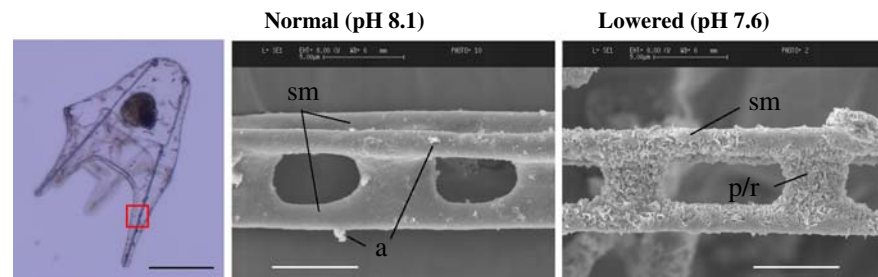
A. *Evechinus chloroticus*



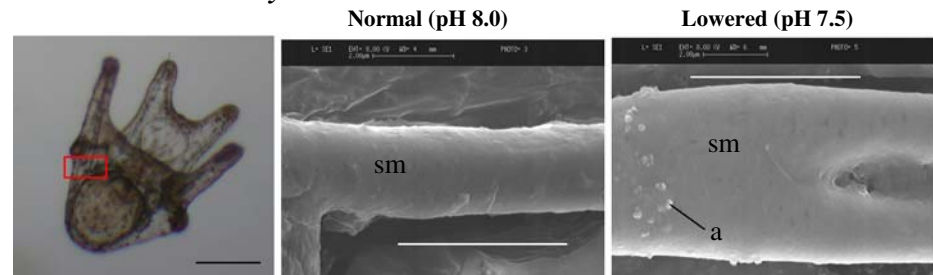
B. *Tripneustes gratilla*



C. *Pseudechinus huttoni*



D. *Sterechinus neumayeri*



development of larvae may mean they are exposed to higher mortality for extended periods. This will ultimately decrease the numbers of larvae completing development, and even if adults are not affected by increased CO_2 concentrations, the long-term viability of populations may be compromised if there is a significant reduction in larva surviving through to settlement.

Larvae of the Antarctic sea urchin *Sterechinus* did not appear to be more vulnerable to the effects of ocean acidification than species from lower latitudes, and if anything appeared to be more resistant. Further research within a similar suite of related species from a range of

latitudes will be required to confirm the generality of these observations. This study also shows, as others have previously, that any future research should look beyond mortality as an endpoint when considering the effects of ocean acidification, and should include examination of more subtle changes that may indirectly affect the functioning of an organism.

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