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No ocean acidification effects on shell growth and repair in the New Zealand brachiopod *Calloria inconspicua* (Sowerby, 1846)

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Surface seawaters are becoming more acidic due to the absorption of rising anthropogenic CO_2. Marine calcifiers are considered to be the most vulnerable organisms to ocean acidification due to the reduction in the availability of carbonate ions for shell or skeletal production. Rhychonelliform brachiopods are potentially one of the most calcium carbonate-dependent groups of marine organisms because of their large skeletal content. Little is known, however, about the effects of lowered pH on these taxa. A CO_2 perturbation experiment was performed on the New Zealand terebratulide brachiopod *Calloria inconspicua* to investigate the effects of pH conditions predicted for 2050 and 2100 on the growth rate and ability to repair shell. Three treatments were used: an ambient pH control (pH 8.16), a mid-century scenario (pH 7.79), and an end-century scenario (pH 7.62). The ability to repair shell was not affected by acidified conditions with >80% of all damaged individuals at the start of the experiment completing shell repair after 12 weeks. Growth rates in undamaged individuals >3 mm in length were also not affected by lowered pH conditions, whereas undamaged individuals <3 mm grew faster at pH 7.62 than the control. The capability of *C. inconspicua* to continue shell production and repair under acidified conditions suggests that this species has a robust control over the calcification process, where suitable conditions at the site of calcification can be generated across a range of pH conditions.

Keywords: calcification, carbonate saturation, climate change, CO_2, pH.

Introduction

Rising levels of anthropogenic CO_2 are affecting the carbonate chemistry of surface seawaters and causing our oceans to acidify (Caldeira and Wickett, 2003, 2005; Orr *et al.*, 2005; Gattuso and Hansson, 2011; IPCC, 2013). Excess atmospheric CO_2 since the industrial revolution has already caused a 0.1 pH unit decline and the rate of this change is predicted to increase considerably with a further decrease of 0.3–0.5 pH units by 2100 (Caldeira and Wickett, 2005; Orr *et al.*, 2005). Marine calcifying organisms such as corals, coccolithophores, molluscs, and brachiopods are considered to be the most susceptible to ocean acidification due to the predicted reduction in the availability of carbonate ions that is required for shell or skeletal production (Doney *et al.*, 2009; Byrne, 2011; Watson *et al.*, 2012; Byrne and Przeslawski, 2013; Kroeker *et al.*, 2013). Consequently, shell production and maintenance will likely become more difficult and more energetically expensive. Studies have shown variable responses of calcifying organisms to future predicted pH conditions with an increasing number of studies demonstrating tolerant species (Havenhand and Schlegel, 2009; Ries *et al.*, 2009; Parker *et al.*, 2012; Suckling *et al.*, 2014; Cross *et al.*, 2015).

Rhychonelliform brachiopods inhabit all the world's oceans from the intertidal to hadal depths (James *et al.*, 1992; Peck, 2001a). They are important organisms in shallow water communities as they provide a habitat for a broad range of epifauna, including other brachiopods, sponges, bryozoans, and algae, and may act as a significant carbon sink (Barnes and Peck, 1996; Rodland *et al.*, 2004). They have also been characterised as one of the most

calcium carbonate-dependent groups of marine organisms due to the very large proportion of their dry mass (>90%) accounted for by their calcareous skeleton and support structures (Peck, 1993, 2008). Most brachiopod research has focused on extinct taxa as they are one of the few phyla to be represented as fossils extensively throughout the last 500 million years (Richardson, 1986; James *et al.*, 1992; Pennington and Stricker, 2001). Despite a relatively recent increase in the literature on the distribution, ecology, and biology of extant brachiopods (Peck *et al.*, 1997, 2005; Peck, 2005; Harper *et al.*, 2009; Lee *et al.*, 2010; Peck and Harper, 2010), only two studies have investigated ocean acidification impacts (McClintock *et al.*, 2009; Cross *et al.*, 2015), both of which used the Antarctic brachiopod, *Liothyrella uva*, as their study species. McClintock *et al.* (2009) found significant shell dissolution after only 14 d in pH 7.4 conditions. Only empty valves, however, were used; so, the ability of *L. uva* to compensate any acidification impacts on their shells was not investigated. Cross *et al.* (2015) extended this research further by investigating future pH conditions on shell growth and repair in living *L. uva* and found no impact on calcification. The target taxon in the current research is the New Zealand brachiopod *Calloria inconspicua* (Sowerby, 1846), which is in the same order (Terebratulida) as *L. uva,* therefore providing a temperate comparison to the Antarctic species studied by Cross *et al.* (2015).

Calloria inconspicua is a small (maximum length reported is 28 mm; Stewart, 1981), epifaunal, sessile, suspension-feeding terebratellid brachiopod endemic to New Zealand (Doherty, 1979). It has a widespread distribution throughout New Zealand and is highly abundant in the intertidal and shallow subtidal regions, with reported densities of over 1000 ind. m^{-2} (Doherty, 1979). This species also has a broad temperature tolerance of 8–18°C (Lee, 1991). *Calloria inconspicua* is usually found individually or in conspecific clumps attached to hard substrata such as rock, other brachiopods, bivalves, bryozoans, gastropods, and corals (Lee, 1991). An early study reported slow growth in this species which is unevenly distributed throughout life; fastest during the first 4 years (up to 10 mm in length) and decreases after reproduction commences (Rickwood, 1977).

Growth is an indicator of an animal's well being in a particular environment as it represents the collective responses of physiological, cellular, and biochemical processes within the organism (Riisgård and Randløv, 1981). Another essential process to the existence of the vast majority of marine, shelled organisms is shell repair. Brachiopods become damaged in their natural environment due to impacts from a variety of causes including impacts from saltating clasts and predator attack (Harper *et al.*, 2009). Such damage requires quick shell repair to prevent the loss of body fluids, protect against predators, and prevent encounters with harmful substances (Harper *et al.*, 2012). Shell repair frequencies in *C. inconspicua* are variable between different localities with a maximum recorded frequency of 0.355 (number of damaged individuals/total number of individuals; E. M. Harper and L. S. Peck, unpublished data). Given the importance of maintaining shell production and repair, in addition to the limited research on ocean acidification effects on brachiopods, the aims of this study were to establish how shell growth and the ability to repair shell in *C. inconspicua* were affected by forecasted future pH conditions and to compare results for this temperate species with those for the Antarctic *L. uva* (Cross *et al.*, 2015). Growth rates and the frequency of shell repair following damage were measured in control conditions and predicted mid- and end-century pH levels.

Material and methods

Sample collection

Specimens of *C. inconspicua* were hand collected at low tide from under rocks in Portobello Bay, Otago Harbour, New Zealand (45° 82.000′S, 170° 70.00′E) in January 2013. Samples remained in their conspecific clumps and, to minimise disturbance, were only collected if they were attached to removable substratum to ensure that no pedicles were cut. Environmental conditions in Otago Harbour are surface seawater temperatures of 6.4–16.0°C (Roper and Jillett, 1981; Greig *et al.*, 1988), pH range of 8.10–8.21 (K. Currie, pers. comm.), and salinity is 32.5–34.8 (Roper and Jillett, 1981). Brachiopods were kept in seawater during the short transportation from the sampling site to Portobello Marine Laboratory, Otago Peninsula. Specimens were then immediately placed in the experimental system.

Experimental design

This study was conducted in a flow-through CO_2 perturbation system where seawater pumped from the harbour passed through sand filters (50 μm) and a finer cartridge filter (5–10 μm) before entering the system. Three treatments were used; a control at the average local current pH_{NIST} (8.1), the predicted oceanic pH by 2050 (pH 7.8), and the predicted pH by 2100 (pH 7.6) according to the IPCC "business-as-usual" scenario of the forecasted reduction of 0.3–0.5 pH units (IPCC, 2013) with three replicate 10 l tanks for each treatment. The pH of the acidified treatments was lowered in header tanks by intermittently bubbling CO_2 gas through a ceramic diffuser to maintain the pH at predetermined pH levels via a solenoid valve connected to a TUNZE 7070/2pH-controlled computer and electrode system. The experimental pH control system had an identical set up except that it lacked CO_2 injection. A circulating pump in each mixing header tank ensured a constant pH. This set up was shown to provide stable pH conditions over >200 d (Cunningham *et al.*, 2015). Seawater was gravity fed from each header tank at a rate of 1.05 ± 0.05 l min^{-1} into the experimental tanks.

Seawater temperature was not manipulated and was ambient for Otago Harbour. It was measured up to three times a day (°C, Digital Testo 106) with only small differences (<0.5°C) between treatments (Table 1) and no variation between replicate tanks in each treatment. Flow rate was also checked three times per day as was the computer-controlled pH. pH_{NIST} was measured in each treatment tank accurately twice weekly with a EUTECH instruments pH 5–10 pH/mV/°C meter and calibrated with pH buffers of pH 4.0, 7.0,

Table 1. Mean (± s.d.) seawater parameters in all three treatments during the 12-week experiment which follow the format recommended by Barry *et al.* (2010).

Seawater parameter	pH control	pH 7.8	pH 7.6
pH_{NIST}	8.16 ± 0.03	7.79 ± 0.06	7.62 ± 0.05
DIC (μmol kg^{-1})	2082.8 ± 22.0	2211.4 ± 8.3	2252.4 ± 25.3
Alkalinity (μmol kg^{-1})	2278.5 ± 18.8	2269.7 ± 9.2	2271.9 ± 6.5
pCO_2 (μatm)	464.8 ± 82.8	1130.2 ± 11.8	1535.6 ± 234.8
Ω calcite	3.5 ± 0.5	1.6 ± 0.0	1.3 ± 0.2
Ω aragonite	2.2 ± 0.3	1.0 ± 0.0	0.8 ± 0.1
Temperature (°C)	16.5 ± 1.7	16.9 ± 1.7	16.6 ± 1.7
Salinity	33.9 ± 0.2	33.9 ± 0.2	33.9 ± 0.2

Values for pCO_2, Ω calcite, and Ω aragonite were calculated using CO_2calc (Robbins *et al.*, 2010) with refitted constants (Mehrbach *et al.*, 1973; Dickson and Millero, 1987) as recommended by Wanninkhof *et al.* (1999).

and 9.2 (Pro-analys, Biolab, New Zealand). Salinity was measured once a week using a YSI data logger. A water sample from each treatment was fixed with saturated mercuric chloride ($HgCl_2$) at the beginning, middle, and end of the experiment. Dissolved inorganic carbon and total alkalinity were later determined by a Single Operator Multi-parameter Metabolic Analyser (SOMMA) and closed-cell potentiometric titration, respectively (Dickson *et al.*, 2007). Other carbonate system parameters, including the partial pressure of CO_2 (pCO_2) and the saturation values for calcite (Ω_C) and aragonite (Ω_A), were calculated using CO_2calc (Robbins *et al.*, 2010). Seawater properties were determined using CO_2 equilibrium constants from Mehrbach *et al.* (1973) refitted by Dickson and Millero (1987) as recommended by Wanninkhof *et al.* (1999).

Brachiopods were fed three times a week with microalgal concentrate of $\sim 397 \times 10^4$ cells mL^{-1} of *Tetraselmis* spp., which is within the natural range of phytoplankton cell abundance in Otago Harbour. Faeces and other debris were removed twice weekly by siphon.

Growth rates

One hundred and twenty-three specimens of *C. inconspicua* between 0.71 and 14.47 mm length (maximum shell dimension) were used in this experiment. At the start of the experiment, shell lengths of each individual >3 mm in length were measured to the nearest 0.01 mm using Vernier calipers. For individuals <3 mm, shell lengths were measured on a graticule eyepiece in a field microscope. The conspecific clumps of specimens were then divided evenly across all tanks ensuring a similar size range of specimens in each treatment. After 6 weeks and at the end of the 12-week experiment, the length of each individual was measured again and the shell edge photographed. Growth rates were calculated from the increase in length (presented as $\mu m\ d^{-1}$).

Shell repair frequencies

The largest (>14 mm in length) 10 or 11 individuals in each treatment were damaged by creating a 1–2 mm deep notch at the valve edge using a metal file. This style of injury replicated very similar damage seen in natural populations of rhynchonelliform brachiopods and interpreted as indicative of either predator attacks or abiotic stresses (Harper *et al.*, 2009). Notches of similar size and extent were made and care was taken not to break shells or cause other damage. After 6 and 12 weeks, the damaged section of each shell edge was photographed.

Statistical analyses

All data were analysed using Minitab (Statistical Software™ Version 15). Growth rate data for each treatment were all significantly different from normal (Anderson–Darling test; $p < 0.009$). These data were still not normally distributed after square root, logarithmic, and double logarithmic transformations because of the presence of zeros in the dataset. Non-parametric Kruskal–Wallis tests were thus used to determine whether treatment affected growth. When significant differences were found in the different datasets, further Kruskal–Wallis multiple comparison tests were used to identify which treatments were different from each other. A χ^2 test was used to establish any treatment effect on the percentage of undamaged individuals that did not grow throughout the experiment. χ^2 tests were also used to determine if treatment affected the percentage of damaged individuals that had completed shell repair at the different time periods.

Results

All seawater parameters in the control were within the ranges reported for shallow surface seawater (Table 1; Barry *et al.*, 2010), although seawater temperature was unusually high for Otago Harbour in January–April 2013 (MDL, pers. obs.) and slightly above the reported range of 6.4–16.0°C (Roper and Jillett, 1981; Greig *et al.*, 1988). Saturation states with respect to calcite and aragonite in both acidified treatments were just below the reported shallow surface seawater values ($\Omega < 1.9$ and <1.2, respectively); however, calcite was supersaturated ($\Omega > 1$) in both treatments and aragonite was supersaturated in pH 7.8 but undersaturated ($\Omega < 1$) in the pH 7.6 treatment. No mortality occurred throughout the 12-week experiment in any treatment. In addition, throughout the experiment, no individual demonstrated any signs of stress such as slow snapping responses, remaining closed for extended periods or wide gaping when open, and all specimens responded rapidly to physical stimulation when disturbed (Peck, 2001b; Cross *et al.*, 2015).

Shell repair frequencies

After 6 weeks, all damaged individuals had started to repair their notch and >36% of specimens had completed shell repair across all treatments (Table 2). Treatment had no effect on shell repair frequencies ($\chi^2 = 1.714$, $p = 0.424$). After 12 weeks, >80% of individuals had fully repaired their notch in every treatment (Table 2; Figure 1) with only three individuals (one specimen in pH 7.8 and two specimens in the pH 7.6 treatments) not completing shell repair. Treatment did not affect overall shell repair frequencies ($\chi^2 = 1.173$, $p = 0.556$). Although the majority of the damaged individuals managed to fully repair their shell, none of the large, notched individuals continued to produce new shell once the repair was complete.

Growth rates

The majority of individuals grew with growth rates ranging to values over 15 $\mu m\ d^{-1}$ in the pH control and the pH 7.6 treatment and up to 10.70 $\mu m\ d^{-1}$ in the pH 7.8 treatment (Figure 2). The only apparent ontogenetic trend was a slight decrease in growth rates for individuals >10 mm in length. Growth rates of undamaged individuals >3 mm in both acidified conditions were not significantly different from that of the pH control (Kruskal–Wallis, $H = 4.04$, $p = 0.133$).

Table 2. Shell repair frequencies after 6 weeks and after 12 weeks in the stated conditions.

Treatment	Number of individuals damaged at the start of the experiment	Length range of damaged individuals (mm)	Percentage of individuals that had completed repair	
			After 6 weeks	After 12 weeks
pH control	11	14.18–17.79 (mean = 15.59)	36% ($n = 4$)	100% ($n = 11$)
pH 7.8	10	14.14–17.62 (mean = 15.80)	40% ($n = 4$)	90% ($n = 9$)
pH 7.6	10	14.05–17.52 (mean = 15.31)	50% ($n = 5$)	80% ($n = 8$)

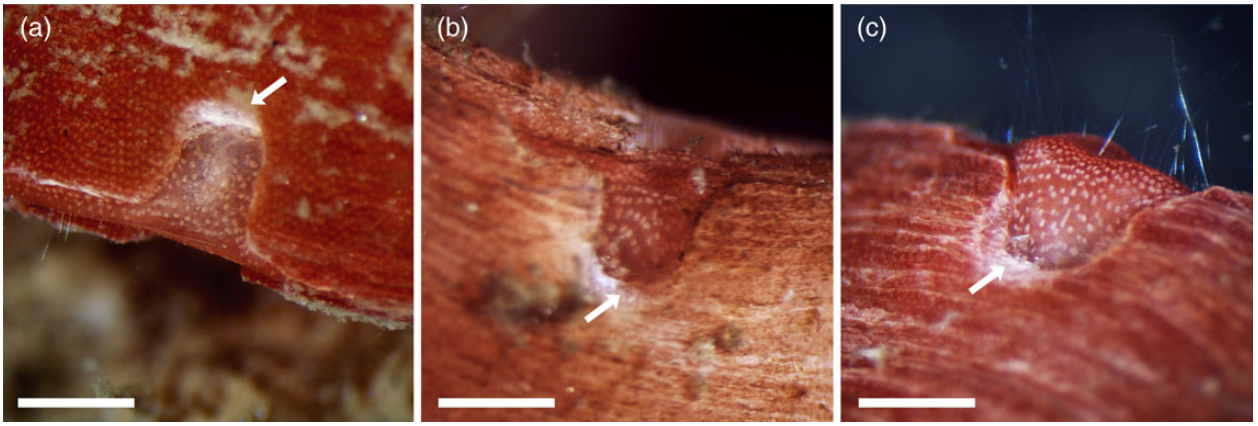

Figure 1. Examples of completed shell repair in damaged individuals after 12 weeks in the (a) pH control, (b) pH 7.8, and (c) pH 7.6 treatment. The arrow indicates the notch created at the start of the experiment. Scale bar = 100 μm.

Whereas growth rates were different among treatments in undamaged individuals <3 mm ($H = 6.23$, $p = 0.044$) and also when all undamaged individuals across the total size range were pooled ($H = 7.90$, $p = 0.019$). A further Kruskal–Wallis multiple comparison test on undamaged individuals <3 mm indicated that growth rates were higher in the most acidified treatment (pH 7.6; $Z = 2.488$, $p = 0.013$) compared with the moderately acidified treatment (pH 7.8) but not compared with the pH control ($Z = 0.826$, $p = 0.409$). Growth rates of undamaged individuals <3 mm in the moderately acidified treatment were not significantly different from the pH control ($Z = 0.746$, $p = 0.456$). A further Kruskal–Wallis Multiple Comparison test on all undamaged individuals indicated that in the most acidified treatment, growth rates were higher than in the pH control ($Z = 2.762$, $p = 0.006$) but not compared with the moderately acidified treatment ($Z = 1.918$, $p = 0.055$). Growth rates of all undamaged individuals in the moderately acidified treatment were also not significantly different from the pH control ($Z = 0.980$, $p = 0.327$). There was also no treatment effect on the proportion of undamaged individuals that did not grow throughout the experiment ($\chi^2 = 1.500$, $p = 0.472$).

Discussion

There were no signs of stress and no mortalities throughout the 12-week experiment, indicating that *C. inconspicua* are able to tolerate predicted mid- and end-century pH conditions. Similarly, mortality rates in other equivalent ocean acidification studies with brachiopods (*L. uva*; Cross *et al.*, 2015) and molluscs (*Arctica islandica*; Hiebenthal *et al.*, 2012) were low (3.9 and 3.3%, respectively). *Calloria inconspicua* will also be able to repair shell damage in the natural environment in the next 100 years as suggested by the ability of damaged individuals to continue shell repair under acidified conditions with >80% of injured specimens in all treatments completing shell repair after 12 weeks. Shell repair in the gastropod *Subninella undulata* was also unaffected by ocean acidification; however, the gastropod *Austrocochlea porcata* had a decreased shell repair rate, suggesting a species-specific response of marine shelled organisms in the ability to repair shell (Coleman *et al.*, 2014). Although the majority of the damaged individuals managed to repair their shell fully, none of them continued to produce new shell once the repair was complete. All damaged individuals of *C. inconspicua* in this study were, however, the largest in their treatment, all being above 14 mm. This size coincides with the reported 14–16 mm size of sexual maturity in *C. inconspicua* (Doherty, 1976; Lee and Wilson, 1979) indicating that growth rates were already low in these individuals due to a transfer of energy and resources from somatic growth to reproduction. Therefore, once the critical process of repair was complete, these individuals were much less likely to grow than the smaller juveniles. It remains unknown how smaller individuals will respond to the additional stress of shell repair in future oceans. Furthermore, shell production in *C. inconspicua* should be unaffected by changing pH levels in the natural environment up to the year 2100, as growth rates in undamaged individuals were either not affected (>3 mm in length) or positively affected (<3 mm in length) by acidified conditions. Shell growth studies in lowered pH conditions on molluscs have shown varied responses (Michaelidis *et al.*, 2005; Berge *et al.*, 2006; Nienhuis *et al.*, 2010; Thomsen *et al.*, 2010; Hiebenthal *et al.*, 2012), which could be due to the short- to medium-term duration (44 d) of some studies or the individual tolerance of different species (Ries *et al.*, 2009). The ability of *C. inconspicua* to continue shell production in low pH conditions suggests that this species has a strong control over their calcification process, similar to molluscs, by being able to generate suitable conditions at the site of calcification against a stronger concentration gradient (Ries, 2011; Gazeau *et al.*, 2013; Wittmann and Pörtner, 2013). Apart from the capability of marine calcifiers to elevate pH in calcifying compartments to facilitate precipitation of calcium carbonate, the mechanisms are poorly known, particularly in less-studied brachiopods.

This resilience to predicted future pH levels is also apparent in the Antarctic brachiopod, *L. uva* (Cross *et al.*, 2015). Similar large proportions (>90%) of identically damaged *L. uva* fully repaired their notch in the only other ocean acidification study to involve brachiopods (Cross *et al.*, 2015). The Antarctic study was conducted over 7 months, whereas the current work was a 12-week experiment, suggesting repair mechanisms may be faster in temperate brachiopods than Antarctic species as has been reported for growth (Peck *et al.*, 1997; Baird *et al.*, 2013). The high success rates of shell repair in lowered pH conditions in both a temperate and polar brachiopod suggest these species can maintain the rate of shell repair and regeneration in challenging chemical environments just as had previously been seen in the ophiuroid *Amphiura filiformis* (Wood *et al.*, 2008). Over 63% of all injured specimens in *L. uva* made new shell after repairing their notch (Cross *et al.*, 2015) further demonstrating the tolerance of this species. A wide size

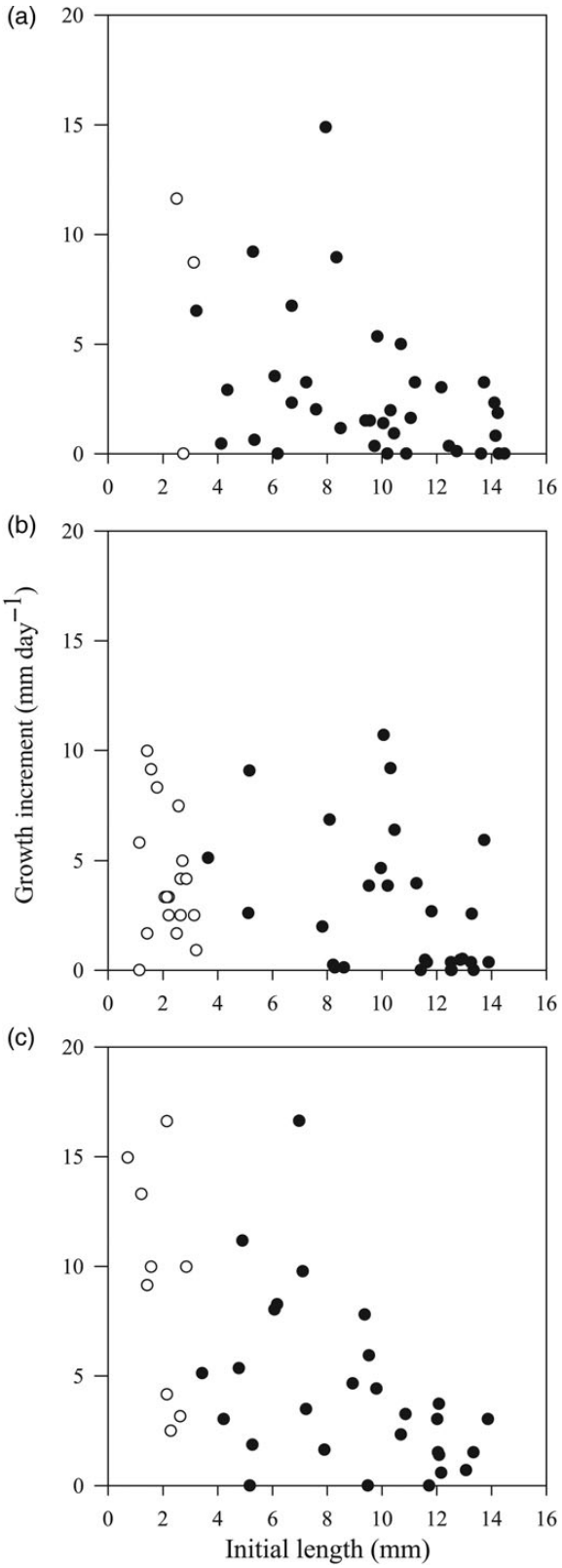

Figure 2. Growth rates of individuals <3 mm (open circle) and >3 mm (filled circle) that were left undamaged at the start of the experiment after 12 weeks in (a) the pH control (pH 8.16 ± 0.03), (b) pH 7.8 (pH 7.79 ± 0.06), and (c) pH 7.6 (pH 7.62 ± 0.05) treatments. Different symbols have been used for individuals above and below 3 mm for the two different methods used.

range (5.0–37.0 mm in length) of individuals had been damaged at the start of the Antarctic experiment, though compared with the limited size range used in the current experiment, specimens of all ages were included and therefore more were likely to continue shell deposition after repair. As here, shell growth rates of undamaged individuals of *L. uva* were not affected by acidified conditions. A 2°C increase in temperature, however, positively affected shell growth in *L. uva*.

Multiple stressors are becoming more widely used in ocean acidification studies where parameters such as temperature, food availability, and hypoxia have been shown to have a greater effect on marine organisms than lowered pH (Hiebenthal *et al.*, 2012; Thomsen *et al.*, 2013; Wolfe *et al.*, 2013; Hardy and Byrne, 2014; Hyun *et al.*, 2014; Noisette *et al.*, 2014; Cross *et al.*, 2015; Queiros *et al.*, 2015), although some studies demonstrate an interactive effect (Ericson *et al.*, 2012; Reymond *et al.*, 2013; Gobler *et al.*, 2014; Ko *et al.*, 2014). Research on the mollusc *Mytilus edulis* revealed that acidified conditions did not affect growth rate but rising temperature increased growth up to 20°C, which then sharply declined at 25°C, indicating that 25°C is above this species temperature tolerance limit (Pörtner, 2008; Hiebenthal *et al.*, 2012). Another study on the same species found that an abundant food supply outweighed the effects of ocean acidification on growth and calcification (Thomsen *et al.*, 2013). Oxygen availability, along with temperature but not pCO_2, were also found to be the dominating factors determining metabolic rate reductions in the squid *Dosidicus gigas* (Rosa and Seibel, 2008).

Overall, studies showing tolerance of marine species to ocean acidification are increasing, especially with the wider use of longer term experiments (Hazan *et al.*, 2014; Suckling *et al.*, 2014; Cross *et al.*, 2015; Queiros *et al.*, 2015). After 3 months, it was apparent that *C. inconspicua* is resilient to lowered pH in terms of shell growth and repair, highlighting the need for longer term ocean acidification studies to allow for acclimation and adaptation to better understand different species capabilities to respond to changing pH conditions. However, perhaps other biological processes could have been impacted by increasing acidity as seen in the ophiuroid *A. filiformis* where muscle wastage was reported in lowered pH treatments (Wood *et al.*, 2008). Further investigation is needed to determine such effects in *C. inconspicua*. Therefore, more environmentally relevant research, including several variables over long-term durations, is crucial to fully understand and predict how organisms will respond to near future changing environmental conditions.

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