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Seasonal variation in the effects of ocean warming and acidification on a native bryozoan, *Celleporaria nodulosa*

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Abstract Ocean warming and acidification are co-occurring stressors likely to affect marine biota through climate-driven change to the ocean. We investigated the effects of increased temperature and lowered pH, solely and in combination, on the growth of the endemic Australian bryozoan, *Celleporaria nodulosa*. Two temperatures and three pH levels were fully crossed in experimental treatments performed in winter 2008 (August) and summer 2009 (February/March). Fragments of *C. nodulosa* colonies (clones) were collected from Coffs Harbour, NSW, Australia, (30°18′S, 153°09′E) and elongation of colonies was assessed periodically over a 12-day incubation period. Lowered pH in winter significantly decreased growth. Elevated temperatures during the summer significantly impeded the growth of bryozoan colonies, possibly masking the effect of ocean acidification and discovering a maximal thermal tolerance at around 27 °C for *C. nodulosa*. The effects of decreased pH and increased temperature may be seasonally dependent and particularly acute during the summer months. Thermal stress may in fact be the initial stressor before ocean acidification, negatively affecting organisms in such a way that they are unable to survive before feeling the effects of ocean acidification.

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Introduction

The sequestration of carbon dioxide (CO_2) emissions by the ocean has been causing a change in ocean carbon chemistry (Kleypas et al. 1999; Caldeira and Wickett 2003; Feely et al. 2004). Current figures on ocean change estimate that pH has decreased by 0.1 since pre-industrial times, with an expectation that levels will decrease by a further 0.14–0.4 over the twenty-first century (Sabine et al. 2004; Fabry et al. 2008). Ocean temperatures have increased by 0.76 °C since the industrial revolution with a projection of a further 1–4 °C rise by 2100 (IPCC 2007). Carbonate saturation generally diminishes as pH levels fall, which may impede the ability of marine organisms to form essential structures such as shells and skeletons (Orr et al. 2005; Gazeau et al. 2007; Fabry et al. 2008; Przeslawski et al. 2008; Wood et al. 2008). Increased temperatures represent an additional biological stressor and 'hot spots' are predicted to develop within some areas, where temperatures will increase beyond average projections (Poloczanska et al. 2007). Marine organisms show varied responses to changes in ocean acidification and temperature, although some of this variation may be due to methodological differences (Byrne 2011). However, Langer et al. (2009) found genetic differences in populations of *Emiliania huxleyi* better explained variations in results rather than the method used to alter water carbon chemistry (addition of acid or bubbling of CO_2). Varying responses of organisms to ocean acidification suggests that a wide diversity of organisms need to be studied in order to understand ecosystem responses to climate change. The

effects of climate change cannot be reduced to changes in a single variable, and multi-stressor approaches (such as coupling pH and temperature treatments) are increasingly common (Feely et al. 2004; Byrne et al. 2009; Gooding et al. 2009; Martin and Gattuso 2009; Pistevos et al. 2011). To predict the biological consequences of these changes, it is important that we understand both their independent and interactive effects on a range of organisms.

Ocean acidification poses threats to many marine organisms, particularly calcifiers due to a reduction in ocean carbonate levels required for the calcification of protective structures such as shells (Guinotte and Fabry 2008). Several studies show reduced growth rates and smaller skeletons for organisms in low pH environments, including echinoderms and molluscs (Byrne et al. 2009; Gooding et al. 2009), brittle stars (Wood et al. 2008), corals (Hoegh-Guldberg 2007; De'ath et al. 2009) and bryozoans (Rodolfo-Metalpa et al. 2010; Pistevos et al. 2011). Fertilisation success and early life history processes may also be negatively affected by increased temperatures and decreased pH levels (Kurihara 2008; Byrne et al. 2009; Parker et al. 2009; Suwa et al. 2010; Albright and Langdon 2011; Anlauf et al. 2011).

The effects of increased temperature have been studied in echinoderms (Byrne et al. 2009; Gooding et al. 2009), bryozoans (O'Dea and Okamura 1999; Lisbjerg and Petersen 2001; Atkinson et al. 2006; Lombardi et al. 2006; Amui-Vedel et al. 2007; Lombardi et al. 2008), sea stars (Hutchins et al. 1996) and entire marine communities (Portner et al. 2005). Recent studies indicate that low levels of warming (+3 °C) buffer the negative impacts of low pH (pH 7.6) on calcification in larval and juvenile sea urchins (Brennand et al. 2010; Byrne et al. 2011). Studies such as this have helped us gain insight into the synergistic effects of temperature and pH (Albright et al. 2010; Anlauf et al. 2011; Lacoue-Labarthe et al. 2012).

Understanding genetic variability as a means for evolutionary adaptation to climate change is key to predicting outcomes (Hughes et al. 2008; Pandolfi et al. 2011; Sunday et al. 2011). Genetically diverse populations are more likely to contain individuals with advantageous genotypes, providing the potential for selection in the presence of environmental change (Price et al. 2003). In the absence of heritable variation, selection for tolerant genotypes cannot proceed and populations cannot adapt. Quantitative genetic studies determine the relationship between genotypes and the environmental variables organisms are subjected to. A requirement for a study such as this is that individuals used are genetically distinct from one another (Haworth and Plomin 2010; Winston 2010). We infer genetic variability from the mode of bryozoan reproduction: bryozoans produce sexually via broadcast spawning, followed by brooding and the subsequent release of newly formed larvae.

Seasonal change in environmental conditions, such as temperature and pH, can stress some organisms, and their sensitivity to other stressors may therefore vary seasonally (Hughes 1989; Love and Rees 2002). Many biological processes (e.g. reproduction) are also seasonally dependent (O'Dea and Okamura 1999; Lombardi et al. 2006), so may coincide with periods of stress. At such times, a trade-off between somatic maintenance and reproduction can occur, potentially jeopardising an organism's survival (Hughes 1989; Duckworth et al. 2004). Future climate scenarios predict that environmental change between seasons will increase (IPCC 2007), elevating stress to organisms during seasonal extremes. Climate change research should therefore consider an organism's response to stressors in multiple seasons.

Bryozoans play a major role in marine communities but are rarely the focus of climate change research (Atkinson et al. 2006; Amui-Vedel et al. 2007; Smith 2009; Rodolfo-Metalpa et al. 2010; Pistevos et al. 2011). They are found in almost every marine habitat globally and cover vast areas of ocean shelf, particularly in Australia and New Zealand (Smith 2009). Due to the manner in which they calcify (laying down calcite, magnesium calcite and aragonite), they are potentially sensitive to ocean acidification and suitable trackers of ocean chemistry change (Smith 2009). Furthermore, their colonial form makes them useful to examine the role of genetic variability in responding to climate change. Colonies can be fragmented to produce identical clones, allowing the one genotype to be exposed to many environmental treatments (Hughes 1989; Hughes et al. 2003; Atkinson et al. 2006; Winston 2010). This approach is essential to understand the adaptive capacity of oceanic organisms to future climate change (Hughes 1989; Smit and Wandel 2006; Hughes et al. 2008; Vezina and Hoegh-Guldberg 2008). Previous studies on bryozoans have found temperature to be one of the contributing factors that can limit growth (Hunter and Hughes 1995; Hughes et al. 2003; Rodolfo-Metalpa et al. 2010; Pistevos et al. 2011). When bryozoans were subjected to sustained periods of increased temperatures, growth ceased, and the simultaneous stress of decreased pH proved fatal (Rodolfo-Metalpa et al. 2010).

In order to determine the effects of increased temperature and ocean acidification, we conducted a multi-stressor climate change study on *Celleporaria nodulosa*, a common bryozoan endemic to Eastern Australia. We tested how projected near-future levels of ocean temperature and pH change (IPCC 2007) affect the growth and survival of the bryozoan in summer and winter, across multiple genotypes. This approach provides important insights into seasonally dependent effects of climate change, and the potential for genetic adaptation.

Materials and methods

Experimental design

Colonies were collected on two separate occasions (August and February), from boulders at Coffs Harbour on the central east coast of Australia (30°18′S, 153°09′E) at low tide in 1–3 m depths.

The first experimental period took place in the austral winter (August 2009) using 7 genotypes (colonies) of *C. nodulosa*. Each colony was cut into 18 clones, the highest number of pieces a colony could be divided into without resulting in clones too small in size, and placed into individual rearing containers (30 ml). The second experimental period was conducted in the austral summer (February/March 2010) using 13 genotypes cloned in an identical manner to winter experiments. One of the potential differences between experimental periods was the average ambient sea surface temperature (21.3° C in winter and 25.4° C in summer). In addition, *C.nodulosa* was gravid (reproductive) during summer. Clonal fragments were approximately 0.5 cm wide and 1–2 cm long. This size was kept consistent as possible throughout the fragmentation process, so as to not produce significantly varying clone sizes, which may have differing levels of regeneration success. One side of each piece had an intact growing edge to assist in the regeneration and development of zooids. After fragmentation, individuals were given a 24-h adaptation period in ambient water conditions before the system was turned on. Digital photographs were taken every 3 days using a dissecting microscope. The surface area of each colony was measured using the image analysis software ImageJ (NIH, USA). The mode of reproduction that bryozoans utilise results in the formation of offspring from sexually reproducing parents. This ensures that individuals collected from the field are genetically distinct, which is a requirement for quantitative genetic studies (Haworth and Plomin 2010; Winston 2010).

Monitoring of physical, chemical and biological parameters

Experiments were conducted in a flow through water system (flow rate, −0.13 ml/s) with filtered sea water (FSW; 1 μm) (ambient: pH, 8.06; temperature, (21.3 °C winter and 25.4 °C summer); salinity, 35–37 psu; dissolved oxygen, >90 %). Species living in estuarine environments experience fluctuation in various environmental variables over a year. This variation in salinity would reflect rainfall with seasonality, and as seasonal change is regular, it has been experienced for many years and potentially allowed adaptation to occur (Wong 1979). Experimental treatments consisted of two temperatures, with a control (21.3 °C in winter and 25.4 °C in summer) and a 3 °C increase from control levels, as well as three pH (NIST scale) levels with a control (averaging 8.06) and a 0.2 and 0.4 decrease from control levels. Both temperature and pH values were based on model projections for near-future (2100) surface ocean waters for south-east Australia (IPCC 2007). Three clonal fragments of each genotype (bryozoan colony) were haphazardly assigned to each of the 6 treatment combinations.

Experimental water conditions (temperature and pH) were adjusted in header tanks supplied with flow through FSW. Seawater pH_{NIST} (nominally, 7.6, 7.8) was adjusted by injection of pure CO_2 into header tanks and was controlled by a pH probe connected to a pH controller (TUNZE 7070/20), TUNZE solenoid valve, regulator and CO_2 cylinder. CO_2 injected into the header water was dissolved using a vortex mixer (Red Sea). In a third header tank, pH was not manipulated. Air was bubbled through all header tanks to aid mixing and maintain dissolved oxygen levels >90 %. Seawater from each header tank flowed to subtanks (11L) where temperature was controlled using in line 200-W aquarium heaters (Jager). After manipulation of pH and temperature (except for controls), water was supplied to each rearing container through a dripper tap system. Rearing containers were 100-ml plastic sample jars with an overflow window that maintained a constant volume of 30 ml. Water conditions (temperature and pH) were measured in 5 haphazardly selected containers per treatment with a WTW Multimeter (MultiLine P4) (See Table 1). Experimental ρCO_2 was determined from total alkalinity, pH_{NIST} and salinity data using CO_2 SYS, using the dissociation constants of Mehrbach and others (1973) as refitted by Dickson and Millero (1987) (see Table 1).

Nutrition

Throughout the experiment, clones were fed three times a day with the microalgae *Proteomonas sulcata* at a concentration of 10^5 cells/ml, concentrations used by Piola and Johnston (2006). During feeding, the flow through system was turned off for 1.5 h to retain algae in the experimental containers. Over this time, pH was monitored to ensure levels were not increasing. Colonies and containers were cleaned to remove debris and detritus every 3 days (Riisgard and Manriquez 1997; Lisbjerg and Petersen 2001).

Statistical analysis

A 3-factor analysis of variance (ANOVA) was used to test for effects of pH, temperature and genotype on the growth and survival of *C. nodulosa* (using initial and final surface area measurements). Data were analysed using the statistical software GMAV5 (Underwood and Chapman 1998). Genotype was considered as a random factor, and the

Table 1 Seawater parameters over both experimental periods, SE is shown in italics and parenthesis

Treatment (temp)	Experiment 1						Experiment 2					
	Ambient			Raised (+3 °C)			Ambient			Raised (+3 °C)		
Treatment (pH)	Control	7.8	7.6	Control	7.8	7.6	Control	7.8	7.6	Control	7.8	7.6
$pH_{(NIST)}$	8.06 (*0.01*)	7.81 (*0.01*)	7.61 (*0.01*)	8.06 (*0.01*)	7.79 (*0.01*)	7.59 (*0.01*)	7.94 (*0.01*)	7.78 (*0.01*)	7.64 (*0.01*)	7.95 (*0.01*)	7.76 (*0.01*)	7.62 (*0.01*)
Temperature (°C)	21.27 (*0.08*)	21.23 (*0.08*)	21.23 (*0.08*)	23.42 (*0.08*)	23.67 (*0.06*)	23.48 (*0.04*)	25.37 (*0.10*)	25.01 (*0.37*)	25.88 (*0.10*)	28.23 (*0.13*)	28.87 (*0.13*)	28.50 (*0.14*)
ρCO_2(μatm)	361.98	712.43	1189.15	360.25	733.04	1226.63	512.20	798.10	1575.60	793.50	836.40	1184.40
ΩCa	4.73	2.94	1.96	5.05	3.13	2.07	4.45	3.21	2.50	4.95	3.52	2.67
ΩAr	3.09	1.92	1.28	3.31	2.05	1.36	2.94	2.12	1.66	3.30	2.35	1.78
TA	2249.34 (*7.43*)						2308.39 (*9.67*)					

TA, total alkalinity; *ΩCa,* calcite saturation state; *ΩAr,* aragonite saturation state. $N = 10$ (TA, ΩCa, ΩAr, ρCO_2), $N = 52$ (Temperature and pH)

Table 2 3-factor analysis of variance of growth for *Celleporaria nodulosa* over a 12-day period (with outliers removed) across ambient and 3 °C above ambient seawater treatments (temp), with ambient (8.06), 7.8 and 7.6 pH treatments (pH)

Source	Winter				Summer			
	df	MS	*F*	*p*	*df*	MS	*F*	*p*
Temperature (Te)	1	0.1164	2.74	0.1585	1	0.4356	58.86	**<0.001**
pH	2	0.0883	3.55	**0.0333**	2	0.0034	0.46	0.6322
Genotype (Ge)	5	0.08	3.21	**0.0107**	12	0.0104	1.41	0.1648
Temp × pH	2	0.0178	0.54	0.597	2	0.0166	2.24	0.1086
Temp × Ge	5	0.0424	1.7	0.1432	12	0.0083	1.12	–
pH × Ge	10	0.0227	0.91	–	24	0.0062	0.84	–
Te × Ge × pH	10	0.0327	1.31	0.2373	24	0.0078	1.05	–
Residual	72	0.0252			156	0.0074		

Winter contained 7 genotypes (Ge) and pooled data. Summer contained 13 genotypes and unpooled data. Pooling occurred due to the large non-significant ($p \gg 0.05$) effect of pH*Genotype. Genotype is a fixed factor with pH and temperature both being random factors. Dashes for *p* values indicate that variables were pooled. Bold type indicates significant *p* values. $N = 7$ (winter), $N = 13$ (summer)

variables of pH and temperature were both fixed. Cochran's test and plots of the residuals were used to ensure that the assumptions of homogeneity of variance and normality were met. No transformations were required. One colony in each experimental period showed negative growth in the control treatment, and all data for these clones were omitted from the analysis. Strongly non-significant random sources of variance (i.e. sources including the random factor of genotype) were pooled when $p > 0.25$ (Underwood 1997).

Results

The response of *C. nodulosa* growth to experimental treatments differed between seasons. In winter, growth was reduced in lower pH environments, but there was no effect of temperature nor an interaction between pH and temperature (Fig. 1a, Table 2). Post hoc tests revealed a difference between control and 7.8 pH treatments, which showed highest and lowest growth, respectively ($p < 0.05$). Genotypes varied in growth (Fig. 1b), but did not differ in response to experimental treatments (Table 2).

In summer, there was a strong effect of temperature on the growth of *C. nodulosa* (Table 2, Fig. 2a). A 3 °C warming (from 25.4 to 28.5 °C) resulted in significantly reduced growth, regardless of genotype or pH treatment (Fig. 2b). There were no significant effects of pH in this experiment (Table 2).

Discussion

We aimed to investigate the tolerance of the native bryozoan *C. nodulosa* to changes in temperature and pH in two seasons and across many genotypes. Our results show tolerance to both temperature and pH was strikingly different between seasons, suggesting that effects of climate

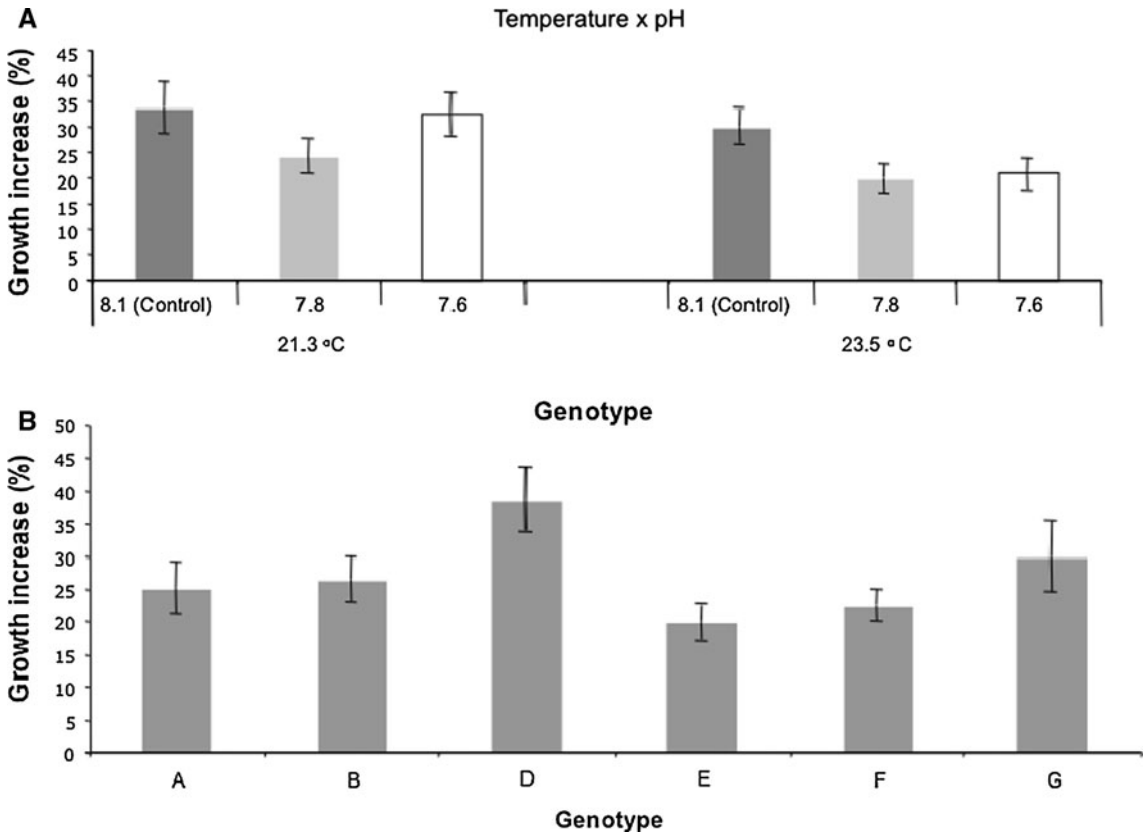

Fig. 1 *Celleporaria nodulosa*. Growth increase (%) over 12 days, under different temperature and CO_2 conditions for the winter (August) experimental period. **a** Mean growth for all genotypes across all pH and temperature treatments [21.3 and 23.5 °C (±SE)]. *Dark grey*: pH control, *light grey*: pH 7.8 and *white*: pH 7.6. **b** Mean growth over all treatments for each genotype (±SE). $N = 7$ (August/winter), $N = 13$ (February–March/summer)

change are seasonally dependent. Lower pH (7.8) resulted in reduced growth during winter, but during summer increased temperature strongly inhibited growth. The absence of a genotype by environment interaction in either season indicates little potential for adaptive evolutionary change within populations under strong directional selection from climate change stressors.

Results of reduced growth in the intermediate pH treatment compared to that of the control are counterintuitive to what we would expect to find given results from previous studies (Rodolfo-Metalpa et al. 2010; Pistevos et al. 2011). These studies show a reduction in growth at the lowest level of pH in comparison with control treatments. Our interpretation of our result is that *C. nodulosa* was not under thermal stress at this temperature, and therefore could withstand the lowered pH in this temperature treatment.

There are two main reasons why effects of climate change may vary seasonally. First, species may already be approaching the limits of their environmental tolerance during seasonal extremes, so have little capacity to cope with further environmental stress. This may have been the case in our experiment, since effects of increased temperature were most severe when ambient temperature was already high. Second, the life cycles and physiological attributes of many species are seasonally dependent, and some may be more sensitive to environmental stress at particular times of the year. In our experiment, *C. nodulosa* became reproductive during summer (ovicells were observed on every colony), so may have been more vulnerable to environmental change while undergoing this process.

Previous research has shown that elevated temperatures cause a reduction in zooid size, and bryozoans have the capacity to produce longer zooids in cooler seasons (O'Dea and Okamura 1999; Lisbjerg and Petersen 2001; Lombardi et al. 2006; Amui-Vedel et al. 2007). In a similar multi-stressor study, temperature caused a decrease in growth rate of the bryozoan *Celleporella hyaline* at 22 °C (Pistevos et al. 2011). Our results indicate that growth of *C. nodulosa* could be halted within the temperature range of 24–27 °C. Other studies have shown a reduction in bryozoan growth in acidification conditions (Rodolfo-Metalpa et al. 2010; Lombardi et al. 2011; Pistevos et al. 2011). Our results support this at lower winter temperatures, but not during summer when effects of temperature

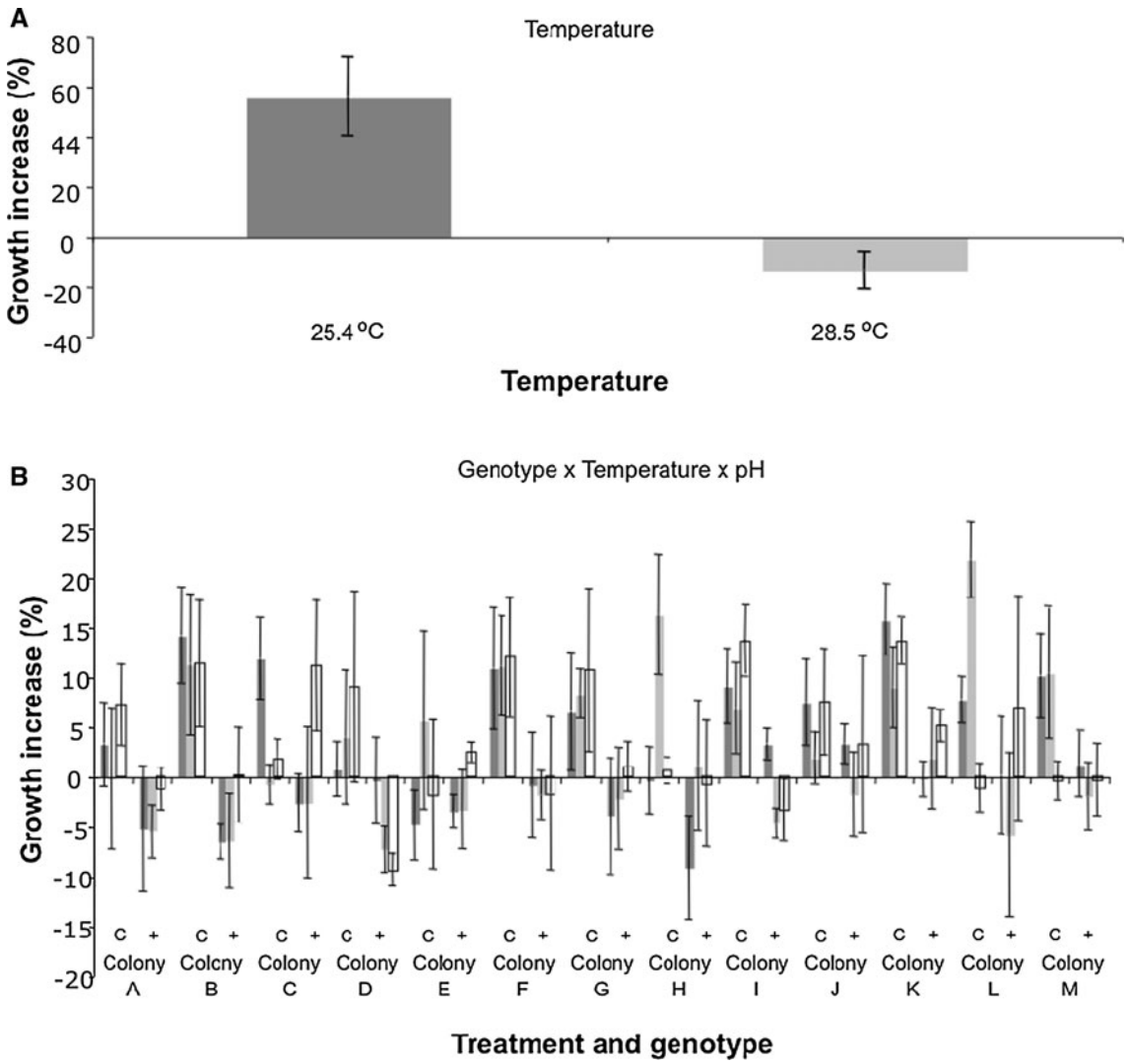

Fig. 2 *Celleporaria nodulosa*. Growth increase (%) over 12 days, under different temperature and CO_2 conditions for the summer (March) experimental period. **a** Mean growth for all genotypes and pH treatments across temperature treatments (25.4 and 28.5 °C (±SE)). **b** Mean growth for each genotype across all treatments (±SE), '*C*' (Control temperature treatment) and '+' (increased temperature treatment, +3 °C). $N = 7$ (August/winter), $N = 13$ (February–March/summer). *Dark grey*: pH control, *light grey*: pH 7.8 and white: pH 7.6

were paramount. It is likely that effects of temperature overshadowed potential effects of pH at this time, an outcome reported by Rodolfo-Metalpa and colleagues (2010). However, growth rates were generally low during summer and a measure of reproductive effort might have been a more sensitive indicator to pH stress (as observed by Pistevos et al. 2011).

Celleporaria nodulosa was reproductive over summer, which may have resulted in a trade-off between growth and reproduction. Such trade-offs have been noted in other bryozoans and typically result in negative growth (Hughes 1989). Many similar organisms experience trade-offs between metabolic, reproductive and somatic pathways, and the potential for these to interact with environmental stressors is an important avenue of research (Findlay et al. 2009). A previous study found that investment in reproductive pathways increased under higher temperatures and reduced pH conditions (Pistevos et al. 2011), suggesting that climate change stressors will influence the trade-off between reproduction and growth.

The capacity of a population to adapt to environmental change comes from the possession of advantageous genotypes. The more genetic variation within a population, the higher the chance that some individuals will survive adverse conditions (Price et al. 2003). Maternal provisioning can also improve the fitness of offspring, but experiments that span multiple generations are needed to observe such effects. Our study suggests that small

populations of *C. nodulosa* may have difficulty adapting to future climate change, since all genotypes in our experiment responded similarly. A similar result was observed by a previous study that examined the interaction between genetic variance and environmental conditions in *Mytilus chilensis* (Toro et al. 2004). It is possible that sourcing individuals from a wider range of locations or habitats would have identified greater genotypic variation in these traits. Toro et al. (2004) suggest their results may be explained by the collection of individuals from an environment of similar selection pressures.

Another explanation for the absence of a genotype by environment interaction is the sudden exposure to stress that we imposed on our test organisms. Studies that have shown a genotype by pH or temperature interaction have generally exposed organisms to gradual changes in environmental variables (Brakefield and Kesbeke 1997; Todd et al. 2004; Deutsch et al. 2008; Flint and Mackay 2009). Genotypes of the bryozoan *Celleporella hyalina* showed a variable response to acidification, but clones were prepared 15 days prior to experimentation and kept in ambient temperature conditions (Pistevos et al. 2011). If environmental change is too rapid, individuals may be unable to express their tolerance to the new conditions (Hoegh-Guldberg 2007; Veron et al. 2009). An expanded approach for this study would be to allow a longer acclimation period to ambient condition and also to treatments, providing time for colonies to display any adaptive abilities.

The aim of this study was to measure whether genetic variation in response to climate change stressors exists. This does not require correlation between genetic variability and morphology, only that individuals are genetically distinct from one another. We infer genetic variability from the mode of bryozoan reproduction: bryozoans produce sexually via broadcast spawning, followed by brooding and the subsequent release of newly formed larvae. Hence, we could say that every individual is the offspring of a sexually producing parent (Haworth and Plomin 2010; Winston 2010).

Species persistence in a changing environment will depend on phenotypic plasticity (acclimation) and evolutionary adaptation (Byrne 2011). Tolerance to climate change will be an increasingly important trait for many marine species (Todgham and Hofmann 2009). The need for a multi-stressor approach to climate research is becoming increasingly apparent, as many environmental changes will occur simultaneously (Feely et al. 2004). Multi-stressor studies improve understanding of complex additive and synergistic effects and aid in the development of appropriate adaptation and mitigation strategies. This study adds to knowledge of likely impacts on a major habitat-forming phylum, and further studies are now needed to explore the mechanisms of impacts. Given that *C. nodulosa* colonies were collected from a single area, our result should be interpreted in the context of genetic variability within a population from a single location.

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