Vol. 473: 235–246, 2013
doi: 10.3354/meps10058

MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser

Published January 21

Effects of ocean warming and acidification on embryos and non-calcifying larvae of the invasive sea star *Patiriella regularis*

Maria Byrne[1,*], Maria Gonzalez-Bernat[2], Steve Doo[3], Shawna Foo[3], Natalie Soars[3], Miles Lamare[2]

[1]Schools of Medical and Biological Sciences, University of Sydney, New South Wales 2006, Australia
[2]Department of Marine Science, University of Otago, Dunedin, New Zealand
[3]School of Medical Sciences, University of Sydney, New South Wales 2006, Australia

ABSTRACT: Little is known about the effects of potential synergies between concurrent ocean warming and acidification on marine benthos. We investigated the effects of warming and acidification on development to the non-calcifying larval stage in the sea star *Patiriella regularis*, in embryos reared from fertilization in present and future (2100+) conditions. Fertilization using gametes from multiple parents, to represent populations of spawners, was resilient to both stressors, as were cleavage stage embryos. Warming increased developmental rate across all pH levels. For blastulae, there was a complex interaction between stressors, with +4°C/pH 7.6 lethal to many embryos. A 4°C warming increased mortality by the gastrulation stage by 13 to 25% across all pH levels. In conjunction with warming, pH 7.6 increased mortality by 25 to 27% across all temperatures. For embryos that reached the 3 d bipinnaria stage, warming reduced the percentage of normal larvae and larval size, with no effect of acidification. These results highlight the importance of considering both warming and acidification, and effects on early embryos, in assessing life history responses to ocean change. Bipinnaria reared to Day 28 to determine the effects of acidification on non-calcifying feeding larvae provided a comparison with results for calcifying echinoplutei. pH 7.6 resulted in smaller larvae and increased mortality by 30%. After 24 d, near-future ocean acidification levels (pH 7.8) also resulted in smaller larvae. The effects of acidification in reducing growth in larvae that do not calcify indicates that the stunting response of echinoderm feeding larvae to pH/pCO_2 is strongly influenced by hypercapnic changes in metabolism and teratogenic effects. The results have implications for *P. regularis* in its invasive range in Australia, where this species is likely to be deleteriously affected by ocean warming.

KEY WORDS: Climate change · Ocean warming · Ocean acidification · Sea star · Non-calcifying larvae · Invasive species

INTRODUCTION

A great diversity of marine invertebrates have planktonic larvae that are being exposed to stressors from climate change in addition to those they already face. The oceans are on an irreversible trajectory of warming and acidification (Caldeira & Wickett 2005, IPCC 2007). Ocean warming is a pervasive global stressor, and deleterious effects on marine populations and ecosystems have been evident for decades (Brierley & Kingsford 2009, Hoegh-Guldberg & Bruno 2010). Mean sea surface temperatures (SST) are increasing, with a projected 2 to 4.5°C warming by 2100 (IPCC 2007). Due to increased atmospheric

*Email: mbyrne@anatomy.usyd.edu.au

CO_2, increased ocean pCO_2 will result in a decrease in surface ocean pH by 0.14 to 0.41 and 0.30 to 0.7 units, by 2100 and 2300, respectively (Caldeira & Wickett 2005, IPCC 2007, Doney et al. 2009).

Marine invertebrate development is highly sensitive to environmental perturbation (Pechenik 1987) and it is important to determine how embryos and larvae may fare in an ocean simultaneously warming and decreasing in pH, through multistressor studies (Byrne 2011). Development rates are directly tied to temperature, with larger larvae, larger skeletons, enhanced metabolism and shorter planktonic phases seen in warmer conditions, up to developmental thermotolerance limits (Chen & Chen 1992, Staver & Strathmann 2002, Widdicombe & Spicer 2008, Pörtner 2010, Sheppard Brennand et al. 2010). On the other hand, increased levels of pCO_2 in embryonic and larval tissues may perturb development due to hypercapnic changes in metabolism (Widdicombe & Spicer 2008, Pörtner 2010). Ocean acidification is accompanied by reduced availability of carbonate ions, with negative effects on skeleton formation in marine calcifiers (Doney et al. 2009, Dupont et al. 2010a, Hendriks et al. 2010, Hofmann et al. 2010, Byrne 2011).

Calcifying larvae reared in near future ocean acidification conditions are smaller and produce less skeleton, likely due to hypercapnic metabolic suppression, decreased mineral saturation, teratogenic effects or the diversion of energy to acid/base regulation and away from growth (O'Donnell et al. 2010, Parker et al. 2010, Sheppard Brennand et al. 2010, Martin et al. 2011, Stumpp et al. 2011, Uthicke et al. 2013). It has been suggested that non-calcifiers may have better survival in a future ocean and may have a competitive advantage with flow-on alterations to benthic assemblages (Attrill et al. 2007). Non-calcifying marine larvae are poorly studied in comparison to their calcifying counterparts (Byrne 2011, 2012). Here, we investigated the effect of ocean change stressors on non-calcifying sea star larvae.

Due to the 'developmental domino effect' (see Byrne 2011), exposure to stress early in development can result in latent deleterious downstream effects, because performance of later ontogeny depends on the success of early stages (Pechenik 2006). Understanding the interactive effects of warming and acidification on development remains a challenge and, thus far, has been investigated in the embryos and larvae of 7 species: 3 molluscs (*Crassostrea gigas*, *Saccostrea glomerata*, *Haliotis coccoradiata*), 2 echinoderms (*Tripneustes gratilla*, *Meridiastra calcar*) and 2 crustaceans (*Hyas araneus*, *Pandalus borealis*) (Parker et al. 2010, Sheppard Brennand et al. 2010, Walther et al. 2010, 2011, Byrne et al. 2011a, Nguyen et al. 2012, Arnberg et al. 2013). Warming diminishes the negative effects of acidification on 2 echinoids, an effect suggested to reflect its stimulatory influence on growth and calcification physiology (Sheppard Brennand et al. 2010, Byrne et al. 2011a). This interactive effect was not evident in molluscs, where both stressors have a negative impact on development (Parker et al. 2010, Byrne et al. 2011a). Biotic responses to ocean change are complex with potential antagonistic effects of stressors, e.g. hypercapnic suppression versus thermal stimulation.

Our first experiment investigated the interactive effects of near future warming and acidification in sea star *Patiriella regularis* embryos reared in experimental conditions from the onset of development (fertilization) to the 3 d bipinnaria larval stage. This study was designed to document the response of very early development (cleavage, blastulation, gastrulation, first larva) to these ocean change stressors. This is the first investigation of ocean change stressors on sea star development to the bipinnaria, the non-calcifying feeding larva characteristic of diverse asteroids. *P. regularis* is one of the most conspicuous intertidal and shallow subtidal invertebrates in New Zealand and is abundant in Tasmania, Australia, where it was introduced in association with oyster imports ~100 yr ago (Dartnall 1969). The interactive effects of ocean warming and acidification on early development to first larva in *P. regularis* were investigated in experiments using 3 temperature and 3 pH levels in all combinations. This study assessed if impacts would be evident in embryonic stages seldom investigated in ocean change studies, a potential mortality bottleneck in a changing ocean. We hypothesized that warming would increase developmental rate, a phenomenon widely reported in marine invertebrate embryos (Pechenik 1987), while acidification would have the opposite effect due to hypercapnic suppression (Widdicombe & Spicer 2008). As a 4°C increase above average SST approximates the developmental thermal threshold in many echinoderms (Byrne 2011), we expected that this treatment would be more deleterious to *P. regularis* than decreased pH. As temperature is likely to modulate the response to acidification, we expected a significant interactive effect of stressors.

In our second experiment, *Patiriella regularis* bipinnaria generated from fertilization in experimental conditions were reared for several weeks with the aim to assess the effects of ocean acidification on non-calcifying feeding echinoderm larvae. This

study provided data to compare with echinoplutei, where the stunting effect of ocean acidification on larval growth is attributed to impaired or delayed calcification due to decreased mineral availability and/or hypercapnia (Byrne 2011). Previous studies on sea star development found that near future acidification facilitated growth in the non-feeding, non-calcifying larvae of *Crossaster papposus* (Dupont et al. 2010b), an effect opposite to the stunting effect seen in echinoderm plutei. This facilitated growth was not seen in the non-feeding larvae of *Meridiastra calcar* (Nguyen et al. 2012). We hypothesized that due to absence of a larval skeleton, development in *P. regularis* would have a higher tolerance to near future levels of ocean acidification (pH 7.6 to 7.8) compared with echinoderm plutei.

MATERIALS AND METHODS

Specimen collection and spawning

Mature adult *Patiriella regularis* were collected during their reproductive season (December–January) (Byrne & Barker 1991) near Hobart, Australia (42° S, 147° E) at low tide (1.0 m depth) in December 2010 (Expt 1) and from Otago Harbor, New Zealand (45° S, 176° E), by SCUBA at ~5.0 m depth in December 2009 (Expt 2). The specimens from Australia were shipped the same day to the University of Sydney and maintained in aerated aquaria at ambient (Hobart) temperature (18 to 19°C) for 3 to 4 wk prior to experiments to provide an acclimation period. Specimens from New Zealand were placed in aquaria with flow through ambient seawater (15°C) from their habitat adjacent to the Portobello Marine Laboratory, University of Otago. Thus, they were held in recent field acclimatization conditions.

To induce spawning, females were injected with 10^{-5} M 1-methyl-adenine (1MA) in filtered seawater (FSW, 1 µm) at ambient temperature (19°C, Hobart; 15°C, Otago) and placed in individual dishes of FSW with a similar concentration of 1MA. Eggs were also obtained by placing dissected ovaries in 1MA. Eggs were examined for consistency in shape and germinal vesicle breakdown and transferred to fresh FSW. Testes were dissected from males and placed in a dish, covered and stored cool until use. Semen samples were examined for sperm motility. Each experiment used gametes from multiple males and females (see below) to establish populations of embryos. This approach was taken so that the outcomes might reflect the response of progeny generated from a population of spawners as observed in nature (Byrne & Barker 1991) and to avoid the strong maternal and paternal effects characteristic of single dam-sire crosses (Palumbi 1999, Foo et al. 2012).

Fertilization and early development to the bipinnaria larval stage

Experimental treatments were identified with respect to near future (2100) ocean change and regional projections (IPCC 2007, Hobday & Lough 2011). For the first experiment to Day 3, embryos were reared in 3 temperatures (19°C, 21°C [+2°C], 23°C [+4°C]) and 3 pH_{NIST} levels (pH_{NIST} 8.15, pH 7.8: −0.35 units, pH 7.6: −0.55 units) in all combinations (see Table S1 in the supplement at www.int-res.com/articles/suppl/m473p235_supp.pdf for water parameters and below for adjustment of water conditions).

Three fertilizations were undertaken using multiple males and females (Fertilization 1: 6 females, 4 males; Fertilization 2: 6 females, 4 males; Fertilization 3: 4 females, 4 males). For each fertilization, pooled eggs from all females were initially placed in 250 ml beakers and counted in ten 1 ml subsamples of the egg suspension to determine how many eggs were available. The eggs were then allocated to 250 ml sealed fertilization containers (density 10 ml^{-1}, total 2500 eggs per container), filled with experimental FSW at the 3 pH and 3 temperature levels in all combinations (Table S1) for 15 min, and were then fertilized with diluted sperm. Sperm density was determined in haemocytometer counts. A 50:1 sperm to egg ratio was used as previously determined to give optimal fertilization for *Patiriella regularis* (Byrne et al. 2010). After 10 min, the eggs were rinsed in experimental FSW to remove excess sperm. To ensure acceptable fertilization rates (≥80 %) in controls, fertilization was scored (envelope + cleavage) in counts of 50 embryos from controls at 2 h for each fertilization. Although the (lack of) effect of warming and acidification on fertilization in *P. regularis* is reported (Byrne et al. 2010), the effect of treatments on fertilization was addressed here for completeness, in a single count of 50 embryos from 1 container for each treatment across the 3 fertilizations (n = 3).

Embryos derived from the first 2 fertilizations in experimental conditions (as above) were transferred randomly into replicated rearing containers to document the influence of experimental treatments on developmental rate (cleavage, blastulation, gastrulation). These independent replicate embryo (10 ml^{-1}) populations (as generated from gametes of multiple

parents) were reared in separate containers (100 ml jars) to 3 time points (4 h, 9 replicates; 15 and 24 h, 6 replicates each). Thus for 4 h, we had 81 containers (2 temperature × 3 pH × 9 replicates) and 15 and 24 h each had 54 containers (2 temperature × 3 pH × 6 replicates). Fertilization 1 provided 6 replicates for 4 h and 3 replicates for each of 15 and 24 h. The rest of the replicates were obtained from Fertilization 2. The rearing containers were placed in water baths set at experimental temperatures, one for each temperature, with rotation between baths for different experimental runs. The jars were filled with experimental FSW with no headspace, avoiding air-water gas exchange. At each time point, the jars were removed, gently stirred, and the embryos sampled for scoring. The jars were discarded after scoring. Percentages of cleaving embryos (4 h), blastulae (15 h) and gastrulae (24 h) were scored in the first 50 randomly encountered embryos. For the 15 and 24 h time points, mortality (arrested and degenerating embryos) (Fig. 1) was also scored.

Separate populations of embryos derived from Fertilization 3 in experimental conditions were transferred randomly into 7 replicate rearing containers (total 63 containers) and reared to early 3 d bipinnaria in the same treatments. The embryos were handled as above, with daily renewal of experimental water. The water was removed by gentle reverse aspiration and replaced with fresh treatment water to ensure that pH and dissolved oxygen (DO) remained constant (Table S2). These larvae were not fed, as they are well provisioned by maternal nutrients to develop beyond Day 3 (Prowse et al. 2008). On Day 3, the larvae were harvested and placed briefly in 7.8% $MgCl_2$ to prevent contraction, prior to fixation in 1% glutaraldehyde-FSW. Photographs were taken of the first 50 larvae sampled using a digital camera mounted on a microscope and scored as normal or abnormal (e.g. arrested, incomplete gut) (Fig. 1). The length of larvae orientated flat to the plane of focus was measured using Image J (version 1.42q). For each replicate, the mean length of the first 30 larvae encountered was used as the data point for analysis. Mortality was not determined in the larval experiment, because many embryos in the 23°C treatments had disintegrated by Day 3 and so were not available for scoring.

Initial checks of pH levels in ~20 containers at 4 and 15 h confirmed that conditions were stable (data not shown). Thereafter, pH_{NIST} was measured at 24 h and prior to daily renewal of water in the 3 d experiment (Table S2).

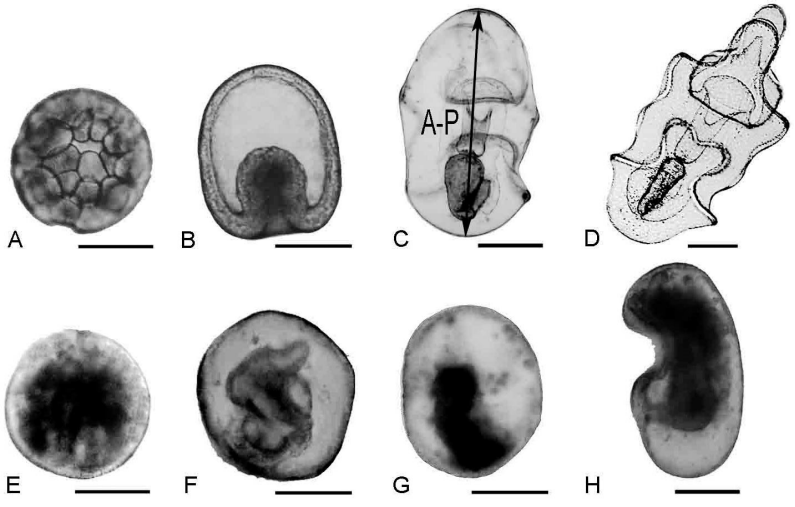

Fig. 1. (A–D) *Patiriella regularis.* Normal development: (A) cleavage, (B) gastrula, (C) 3 d bipinnaria with measurement line along the anterior–posterior (A–P) axis, (D) 24 d late bipinnaria. (E–H) Abnormal development: (E,F) arrested blastulae/gastrulae, (G,H) abnormal larvae with incomplete gut development. Scale bars = 100 µm

Larval development to Day 28

The larval rearing study used embryos generated by fertilization in experimental conditions (pH 8.1; 15°C) using the gametes of 5 males and 6 females (to avoid maternal and paternal effects). Eggs and sperm were counted as described above: the sperm:egg ratio was 4500:1. The eggs were transferred to experimental FSW in 400 ml containers at ambient temperature (15°C) maintained in a room under controlled temperature and 4 pH levels for 20 min prior to fertilization (Table S3). This was replicated 4 times. On Day 3.5, post-fertilization larvae (10 ml^{-1}) were transferred to rearing containers (400 ml) filled with experimental seawater and sealed to ensure no airspace. Larvae were reared for 28 d with change of experimental FSW every 2 d. pH was measured prior to exchange (Table S4). From Day 5, larvae were fed a mixture of *Dunaliella tertiolectra*, *Isocrysis galbana* or *Tetraselmis* sp. (~4000 cells ml^{-1}) every water change. Change in larval density was used as a measure of survival in counts of 10 random well-mixed 1 ml subsamples per replicate on Days 4, 8, 11, 16, 24 and 28. These samples were discarded. To document growth, the

first 12 larvae randomly collected from treatments on Days 6, 10, 16, 24 were photographed and their length measured.

Adjustment of seawater pH and temperature and measurement of seawater parameters

The first experiment of development to the Day 3 larval stage used 3 sources of freshly collected, open coast seawater (FSW, 1 µm) (mean pH_{NIST} 8.16, SE = 0.01; salinity 36.6, SE = 0.6; total alkalinity [TA] 2336.6, SE = 12.01). Large volumes (20 to 30 l) of seawater were equilibrated to the desired temperature/pH level to ensure that embryos or larvae from experimental replicates (sealed jars) were placed in the same conditions. Experimental pH_{NIST} was achieved by mixing CO_2 gas until the required pH was reached and remained stable, as determined by a pH meter (Wissenschaftlich-Technische Werkstätten 3400i) with a temperature compensating electrode calibrated with buffers (pH_{NIST} 4.0, 7.0, and 10.0 at 20°C, ProScitec). This method allows for rapid equilibrium of CO_2 to the required levels and stability (Table S3). Air was bubbled in parallel to maintain DO > 90% as measured by a meter (YSI 550A Dissolved Oxygen Meter). Experimental temperatures were maintained using temperature-controlled water baths or rooms (±0.5°C) monitored by temperature loggers (i-button, Thermodata).

For the larval rearing study, filtered ambient in-flow aquarium system water was used (pH_{NIST} 8.08, SE = 0.005, n = 11; salinity 34.97, SE = 0.007, n = 11; total alkalinity = 2288.7, SE = 10.1, n = 8). Four pH_{NIST} levels were used (pH 8.1: control, pH 7.8: –0.3 units, pH 7.6: –0.5 units, pH 7.0: –1.1 units) (Table S4), achieved as described above. The extreme pH 7.0 treatment (not a near future ocean acidification projection) was used to assess larval tolerance. The pH_{NIST} of each treatment was measured 40 to 44 times over the experiment (Table S4) using a pH meter (Eutech Instruments, P510) with a temperature-compensating electrode calibrated with buffers (pH_{NIST} 4.0 and 7.0 at 20°C; Proanalysis); DO was >90% (YSI 550A).

Seawater total alkalinity was determined for source FSW by closed cell potentiometric titration with reference standards (Dickson et al. 2007). Saturation values for calcite (Ω_C) and aragonite (Ω_A) and pCO_2 were calculated using CO2SYS (Pierrot et al. 2006) with data on temperature, salinity, pH_{NIST} and TA (Tables S1 & S3) using CO_2 equilibrium constants given by Mehrbach et al. 1973 (modified in Dickson & Millero 1987) as recommended by Wanninkhof et al. (1999).

Statistical analyses

Percentage data for the developmental stages (fertilization: 4 h, 64–120 cell+; 15 h, mid/late blastulae; 24 h, gastrulae), mortality, normal larvae and 3 d larval length were analysed using 2-way ANOVA with temperature and pH as fixed factors. Percentage data were arcsine transformed prior to analysis. Due to slower development of embryos at 19°C, these treatments had a high prevalence of zero 64–120 cell+ embryos at 4 h, and so the percentage cleavage data were transformed (arcsine – x + 1%) for analysis to achieve homoscedasticity. Percent mortality at 15 and 24 h were $\log_{10}$+1 transformed to achieve homoscedasticity. Six replicates in the 4 h 21°C/pH 7.6 treatment did not receive sperm and were removed from the analysis, which thus required an ANOVA with an unbalanced design. One outlier in the 15 h experiment, identified using visual analysis of the residuals, was removed from the analysis. For all ANOVAs, Levene's test confirmed homoscedasticity. Normality was confirmed for the 15 h percentage stage data, percentage mortality data (15 h, 24 h), percentage normal larvae data and the larval length data (Shapiro-Wilks test). The assumption of normality was not met for the 4 and 24 h percentage development stage data. As ANOVA is robust to deviations from normality with large data sets (Underwood 1997) these data were analysed. Tukey's HSD post-hoc test was used to determine treatment groups that differed.

Larval density collected at 7 time points and length data collected at 4 time points were analysed by repeated measures ANOVA (RMANOVA). Assumptions of sphericity were met with Mauchly tests. Midway through this experiment on Day 11, the survivorship data were analysed using 1-way ANOVA with pH as a fixed factor, and at Day 24, larval length data were also analysed using ANOVA. Bartlett test confirmed homoscedasticity. Tukey's HSD post-hoc test was used to determine treatment groups that differed. All statistical analyses were performed using JMP9 or SPSS.

RESULTS

Impacts of warming and acidification on early development to Day 3

Embryo development to gastrulation

Neither temperature nor pH affected percentage of fertilization (Temperature: $F_{2,18}$ = 0.68, p = 0.5199; pH: $F_{2,18}$ = 1.0, p = 0.3902). At 4 h, there was a signif-

icant effect of temperature ($F_{2,66}$ = 13.2, p < 0.0001) (Table S5) on the percentage of 64–120 cell+ embryos (Table S6) with more advanced embryos in the higher temperature treatment (Tukey's HSD: 19°C < 21°C = 23°C). The 21 and 23°C treatments (+2 to 4°C) had ~30 and 22%, respectively, more advanced cleavage stages than the 19°C treatments. There was no significant effect of pH/pCO_2 ($F_{2,66}$ = 2.6, p = 0.0841) and no interaction between stressors (Table S5).

By 15 h, most embryos were blastulae (Table S6). Warming and acidification were both significant (Temperature: $F_{2,44}$ = 3.5, p = 0.04; pH: $F_{2,44}$ = 6.5, p < 0.003) with no interactive effect of stressors (Table S5). The effect of temperature on the percentage of blastulae was equivocal (Tukey's HSD: 19°C = 21°C, 19°C = 23°C, 21°C > 23°C) (Table S5). The percentage of blastulae was lowest at pH 7.6 (Tukey's HSD: 8.15 = 7.8 > 7.6), with a reduction of the percentage of blastulae by ~40% at this pH compared with controls (Tables S5 & S6).

At 24 h, the percentage of early gastrulae was reduced by elevated temperature and reduced pH (Temperature: $F_{2,45}$ = 5.0, p = 0.0112; pH: $F_{2,45}$ = 34.7, p < 0.0001) with no interactive effect of stressors (Table S5). The percentage of gastrulae decreased (15 to 40%) across the 23°C group compared with controls (Tukey's HSD: 19°C = 21°C, 21°C = 23°C, 19°C > 23°C) (Table S6). Low pH (7.6) decreased the percentage of gastrulae (20% lower) compared with control pH 8.2 treatments (Tukey's HSD: pH 8.2 = 7.8 > 7.6).

For those embryos that were able to develop to 24 h in experimental treatments, elevated temperature accelerated development, as shown by the presence of late gastrulae in the 21 and 23°C treatments and absence of this stage in the 19°C treatments (Table S6).

Mortality

Mortality was low at 4 h across all treatments and so data for this stage were not analysed. By 15 and 24 h, mortality was conspicuous (Table S6, Fig. 2). At 15 h, temperature and pH both had a significant effect (Temperature: $F_{2,45}$ = 84, p < 0.0001; pH: $F_{2,45}$ = 14.24, p < 0.0001) (Table S7, Fig. 2A). There was a complex synergistic negative interaction between stressors ($F_{4,45}$ = 4.3, p < 0.005), particularly between increased temperature and pH 7.6 (Table S7). All temperature treatment groups differed (Tukey's HSD: 19°C < 21°C < 23°C), with a doubling of mortality at 21 and 23°C compared with controls. Mortality in pH 7.6 treatments at 21 and 23°C was 20 to 40% higher than in controls (Tukey's HSD: pH 8.15 = 7.8 < 7.6). In general, 15 h embryos at control temperature were tolerant to decreased pH (≤10% mortality), with a significant 2- to 4-fold increase in mortality in the increased temperature/lower pH treatments (Fig. 2A).

At 24 h (Fig. 2B), warming and acidification resulted in significant mortality (Temperature: $F_{2,45}$ = 6.33, p = 0.0038; pH: $F_{2,45}$ = 35.3, p < 0.0001) (Tables S6 & S7, Fig. 2B). Tukey's HSD test indicated

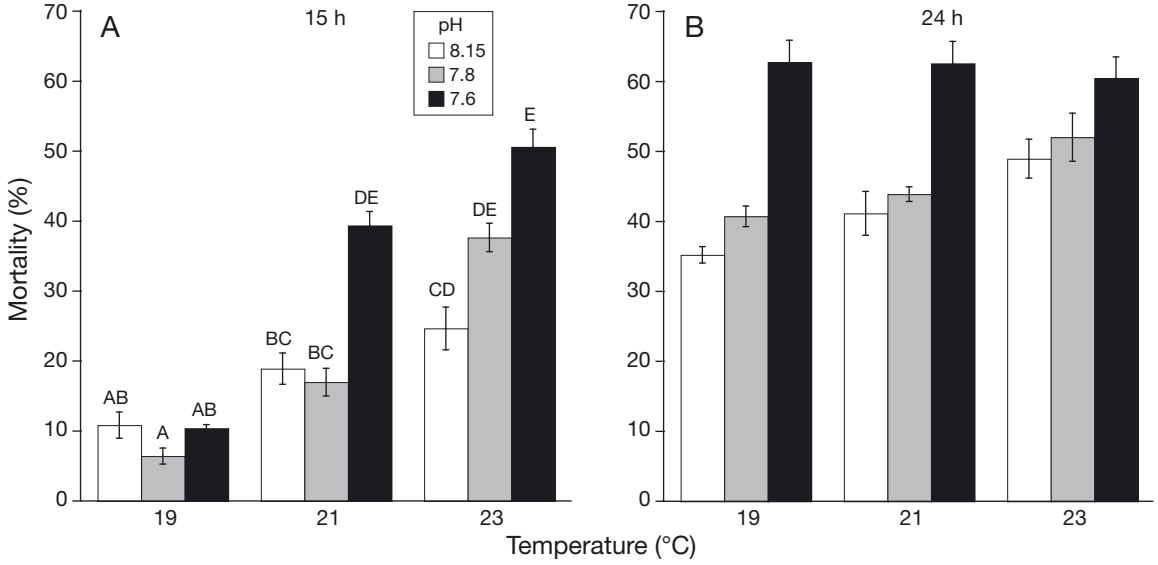

Fig. 2. Percentage (mean ± SE) of mortality in temperature-pH treatments: (A) 15 h, (B) 24 h. n = 9. There was a significant interaction between stressors at 15 h. Treatments with the same letter do not differ

increased mortality at 23°C (19°C = 21°C, 21°C = 23°C, 19°C < 23°C). At 23°C there was a 13 to 25% increase in mortality across all pH treatments compared with 19°C controls. pH 7.6 was deleterious, with higher mortality (25 to 27%) across all treatments compared with pH 8.15/19°C controls (Tukey's HSD: pH 8.15 = 7.8 < 7.6) (Fig. 2B). There was no significant interaction between stressors.

Early larval development on Day 3

By Day 3, there was a significant effect of temperature but not pH/pCO_2 on the percentage of normal larvae and larval growth (Normal larvae: $F_{2,32}$ = 6.3, p = 0.007; Length: $F_{2,54}$ = 6.9, p = 0.002) (Fig. 3). Tukey's HSD indicated that the percentage of normal larvae decreased with increased temperature (19°C = 21°C; 21°C = 23°C; 19°C > 23°C) (Fig. 3A, Table S8). The percentage of normal larvae at 23°C was 4 to 12% lower than in the controls. Larvae in the 23°C treatments were also the smallest (Tukey's HSD: Length, 19°C = 21°C, 21°C = 23°C, 19°C > 23°C), ~12% shorter than control larvae at 19°C.

Larval development to Day 28

Larval density in each pH treatment declined over time due to mortality (Fig. 4) and this differed among treatments (RMANOVA: pH × time, $F_{6,72}$ = 6.72, p < 0.0001) (Table S9). The divergence of control and pH 7.8 treatments from the pH 7.6 treatment was evident, with a ~50% decline at pH 7.6 by Day 11. The precipitous drop in density at pH 7.0 (~90% mortality) indicated that this pH exceeded tolerance (Fig. 4). Near the mid-time point of the experiment (Day 11), there was a significant difference among treatments (ANOVA: Day 11, $F_{3,15}$ = 60.47, p < 0.0001) (Table S10) due to a reduced number of larvae in low pH (Tukey's HSD: pH 8.1 = 7.8 < 7.6 < 7.0). At the end of the experiment (Day 28), there was a significant difference in larval densities among pH treatments (ANOVA: Day 28, $F_{3,15}$ = 115.7, p < 0.0001; Tukey's HSD: pH 8.1 = 7.8 > 7.6 > 7.0). On Day 28, mortality in the pH 8.1 and 7.8 treatments was 26 and 29%, respectively, while in the pH 7.6 and 7.0 treatments it was 62 and 97%, respectively.

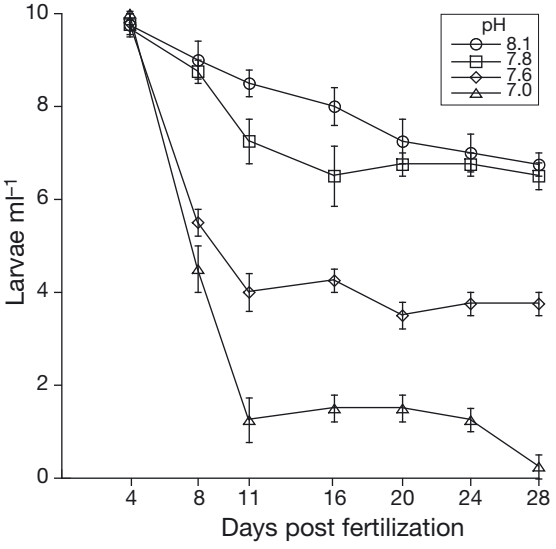

Fig. 4. Bipinnaria rearing experiment. Density (mean ± SE) of larvae in 4 pH treatments to Day 28. n = 4

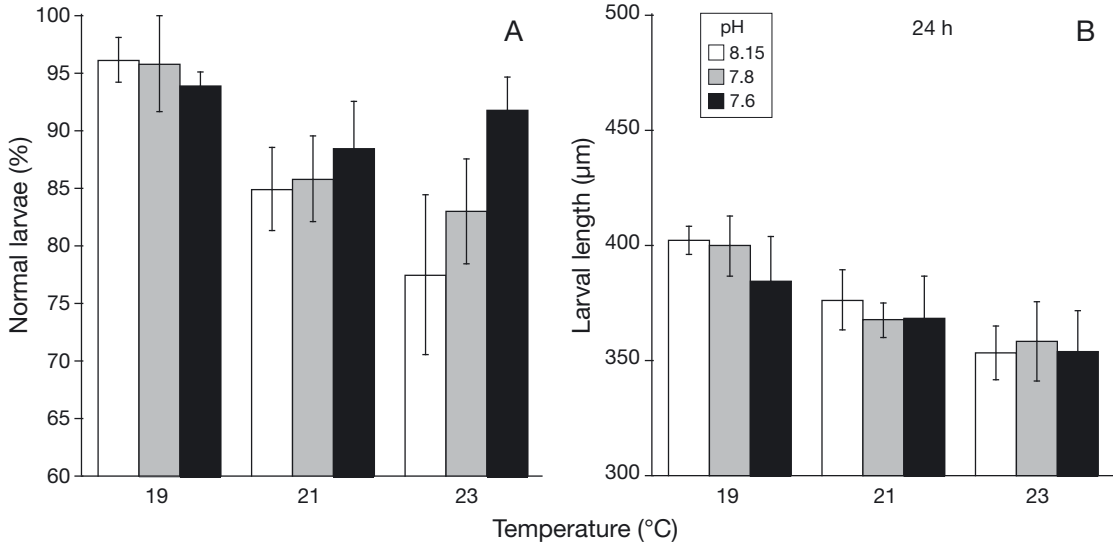

Fig. 3. Percentage (mean ± SE) of (A) normal 3 d bipinnaria and (B) larval length in 9 temperature-pH treatments. n = 7

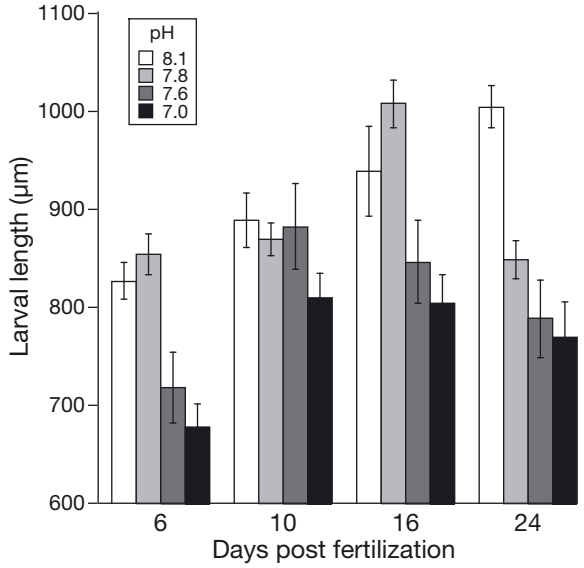

Fig. 5. Bipinnaria rearing experiment. Length (mean ± SE) of larvae on Days 6, 10, 16 and 24. n = 12

Larval length differed among treatments (Fig. 5) with a significant interaction between pH and time (RMANOVA: pH, $F_{3,44} = 17.96$, $p < 0.0001$; Time, $F_{3,42} = 17.23$, $p < 0.0001$; pH × Time, $F_{9,102} = 3.45$, $p = 0.001$) (Table S9). Larvae in control and pH 7.8 treatments were both similar in length to Day 16 and were larger than those reared at pH 7.6 and 7.0 (Fig. 5). By Day 24, larvae reared at pH 7.8 were also smaller than control larvae (ANOVA: Day 24, $F_{3,45} = 17.77$, $p < 0.0001$; Tukey's HSD: pH 8.1 > 7.8 = 7.6 = 7.0) (Fig. 5).

DISCUSSION

The first part of this study on the effects of near-future ocean change on a non-calcifying feeding echinoderm larva showed that development in *Patiriella regularis* is impacted by warming and acidification. Sensitivity of *P. regularis* development differed among embryonic and larval stages, with contrasting responses to warming and acidification. This emphasizes the importance of considering both major ocean change stressors and stress tolerance of early embryos. The significant stimulatory effect of warming on the timing of development and accelerated progression through embryonic stages was expected, as this is a well-documented phenomenon (Pechenik 1987, Byrne 2011). Deleterious effects of increased temperature and decreased pH occurred early in development of *P. regularis*. Even small reductions in single life history steps from adult spawning to larval settlement (i.e. 'transition probabilities') are multiplicative with respect to developmental success, and can reduce the ability of populations to maintain their size (Eckman 1996).

Fertilization, as indicated by the presence of embryos with a fertilization envelope, and early cleavage, as indicated by the presence of cell division, were resilient to warming and acidification. This is similar to results reported for several shallow water echinoderms in multiparent experiments, where resilience of fertilization is suggested to reflect the low pH of fertilization biology, multiple sire sperm competition and protective maternal mechanisms (e.g. stress proteins) (reviewed by Byrne 2011, 2012). At the blastula stage, even slight warming (+2°C) was deleterious, with significantly increased mortality. The most extreme temperature and low pH levels were not tolerated by many embryos. The 23°C and pH 7.6 treatments (2100+) were lethal to many hatched gastrulae. Early development in asteroids is sensitive to stressors (Lee et al. 2004, Byrne et al. 2009, 2011b, Nguyen et al. 2012), and may be a mortality bottleneck for progression to the early larval stage. In the sea star *Asterias amurensis*, a 5°C increase above ambient SST reduced gastrulation success by 60% (Lee et al. 2004). *Patiriella regularis* development was fairly robust to a 0.35 pH unit decrease in pH (pH 7.8), but not at the most extreme level tested, a 0.55 pH unit decrease (pH 7.6), conditions not expected for some time (ca. 2300) (Caldeira & Wickett 2005).

Larvae present on Day 3 survived the early period of mortality in warm-low pH conditions, indicating that the experimental populations included larvae that might be resilient to near-future ocean warming and acidification. For early pre-feeding larvae, temperature but not acidification exhibited a negative effect on development. The resilience of these larvae to decreased pH may be due to the presence of maternal (egg) nutrients that sustain *Patiriella regularis* larvae to ca. Day 5 (Prowse et al. 2008). Although many *P. regularis* bipinnaria present in the 23°C treatment appeared normal, they were also the smallest, similar to results reported for the bipinnaria of *Asterias amurensis* and the lecithotrophic larvae of *Meridiastra calcar* reared in similar levels of warming (Lee et al. 2004, Nguyen et al. 2012). While the pace of early development was facilitated by a 4°C warming above ambient SST, this level of temperature increase resulted in a significant 2- to 4-fold increase in mortality. Our prediction that +2°C would increase larval growth rates, as seen in echinoplutei reared in similar conditions (e.g. Sheppard Brennand et al. 2010), was not supported. It appears that 4°C above ambient SST approximates the upper temperature

tolerance threshold for *P. regularis* development, similar to other echinoderms (Byrne 2011, 2012).

The suggestion that *Patiriella regularis* larvae would be less impacted by near future ocean acidification (pH 7.8) than warming (+2 to 4°C) was supported for early development, but negative effects of pH 7.8 were evident by Day 24. Ocean warming is the major ocean change stressor of concern for early embryos, and future prospects for *P. regularis* will likely depend on the rate and level of SST increase in the region (Hobday & Lough 2011).

Growth of feeding *Patiriella regularis* bipinnaria in ambient conditions to Day 28 was similar to that determined previously (Byrne & Barker 1991). Larvae reared in near-future acidification (−0.3 pH units), at pH 7.8, were similar to controls up to Day 16. The pH 7.6 treatments, a level of acidification projected beyond 2100, resulted in a 50% reduction in survival and reduced growth, while the extreme pH 7.0 was toxic to larvae (~100% mortality). In previous studies of acidification and sea star development, the lecithotrophic larvae of *Crossaster papposus* and *Meridiastra calcar* were robust to ocean acidification (pH 7.6 to 7.7), with double growth rates recorded for the former species (Dupont et al. 2010b), but not the latter (Nguyen et al. 2012). Reasons for the difference between studies are not clear, but may be influenced by taxonomy and contrasting larval type (feeding vs. non-feeding) and experimental design, particularly the developmental stage at which embryo incubations are initiated (see Nguyen et al. 2012).

In its native range in New Zealand, *Patiriella regularis* has a broad distribution (34 to 37° S) encompassing a number of water masses (O'Loughlin et al. 2002, Waters & Roy 2004), and so embryos and larvae are likely to experience temperatures varying by 10°C (13 to 23°C, January SST). This range includes temperatures warmer than the control conditions used in our experiments in southern New Zealand. Thus, *P. regularis* development across its native range may be more resilient than our results would suggest. However, invasive populations of *P. regularis* in Tasmania experience a similar temperature range (11 to 24°C, January SST) (see Byrne et al. 2011b) and their sensitivity to a low level of warming (+2°C) indicates that this species may not fare well in this ocean warming hotspot (Hobday & Lough 2011), with potential for extinction. Data on the thermal tolerance of early development of *P. regularis* in New Zealand are required to determine if this level of temperature sensitivity is characteristic of this species in its native range.

Our study of the impact of ocean acidification on a non-calcifying feeding echinoderm larva provides a basis to assess stressor effects independent of the requirement to produce a larval skeleton. Our prediction that the larvae of *Patiriella regularis* would be less impacted by acidification than calcifying (plutei) larvae was not supported. A decrease to pH 7.6 reduced growth in *P. regularis*, similar to echinoplutei (Byrne 2011, 2012), where the stunting effect of low pH/high pCO_2 is suggested to be due to impaired calcification due to lower mineral saturation, hypercapnic delay, teratogenic effects and energetic constraints in acid-base regulation, or a combination of these (Sheppard Brennand et al. 2010, Chan et al. 2011, Martin et al. 2011, Stumpp et al. 2011). Reduced growth and increased abnormality in a larva that does not calcify indicates that the stunting response of feeding echinoderm larvae to pH/pCO_2 is strongly influenced by hypercapnic suppression as well as teratogenic effects (mortality, abnormality). Recent meta-analyses suggest that non-calcifying larvae are more resilient to ocean acidification than calcifying larvae (Hendriks et al. 2010, Kroeker et al. 2010). This trend is not supported by our results for *P. regularis.* However, as this is the first study of a non-calcifying echinoderm feeding larva, investigation of more species with this larval type from a range of latitudes and habitats in multi-stressor studies is required to address the suggestion that echinoderm larvae that do not require a larval skeleton will be more resilient, potentially competitive winners in a future ocean.

For free-spawning marine invertebrates, the role of pre- and post-settlement processes in controlling population dynamics and distributions has long been discussed (Eckman 1996, Gosselin & Qian 1997, Pechenik 2006), and these arguments could also be applied to understanding the effects of climate change processes on marine populations. While our results and a plethora of recent studies on the impact of ocean change stressors on marine life histories suggest that pre-settlement processes (e.g. fertilization, embryogenesis, larval development) are important (reviewed by Byrne 2011), any life history stage can represent a population bottleneck. It is also possible that certain life history stages can compensate for mortality at other stages. In this respect, while mortality of embryos and larvae may be greater in ocean change conditions, greater post-settlement growth rates and density-dependent juvenile survival may enhance recruitment into adult populations, as seen in the response of populations of the sea urchin *Strongylocentrotus droebachiensis* to

increased temperature (Hart & Scheibling 1988). In *S. purpuratus*, recruitment is controlled by post-settlement processes, and juvenile growth and survival are more important than initial settlement densities (Rowley 1990). Although less well understood, faster growth in warmer water may also lead to earlier reproduction and greater production of progeny over an individual lifetime, given the appropriate nutritive regime (Kalam Azad et al. 2010).

As the ocean will change over coming decades, more gradually than in our experiments, acclimation in adults resulting in the production of more resistant offspring is likely. Environmental history of echinoderm parents, particularly that of the female, has a strong influence on developmental tolerance to stress (O'Connor & Mulley 1977, Johnson & Babcock 1994, Byrne et al. 2011b). Long-term acclimation of fish to moderately elevated pCO_2 can result in trans-life-cycle resilience of progeny to reduced pH (Miller et al. 2012). In similar experiments with urchins variable results were obtained (Dupont et al. 2013, Uthicke et al. 2013) potentially influenced by gonad condition at the onset of experiments with adults. Progeny derived from oysters acclimated in elevated pCO_2 were more resilient to ocean acidification, suggesting potential for genetic adaptation, although maternal (phenotypic) effects were not discounted (Parker et al. 2012). In addition, quantitative genetics studies point to the presence of preadaptive traits to facilitate resilience and adaptation (genetic) to climate change (Sunday et al. 2011, Foo et al. 2012).

Acknowledgements. We thank K. Wolfe and H. D. Nguyen for assistance. Research was funded by grants from the Australian Research Council (M.B.), University of Otago Research (M.L.), the National Climate Adaptation Facility (S.F.) and New Zealand AID (M.G.-B.) and University of Sydney (S.F.) scholarships. The reviewers are thanked for their insightful comments. This is contribution number 76 of the Sydney Institute of Marine Science.

LITERATURE CITED

Arnberg M, Calosi P, Spicer JI, Tandberg AHS, Nilsen M, Westerlund S, Bechmann RK (2013) Elevated temperature elicits greater effects than decreased pH on the development, feeding and metabolism of northern shrimp (*Pandalus borealis*) larvae. Mar Biol (in press)

Attrill MJ, Wright J, Edwards M (2007) Climate-related increases in jellyfish frequency suggest a more gelatinous future for the North Sea. Limnol Oceanogr 52: 480–485

Brierley AS, Kingsford MJ (2009) Impacts of climate change on marine organisms and ecosystems. Curr Biol 19: R602–R614

Byrne M (2011) Impact of ocean warming and ocean acidification on marine invertebrate life history stages: vulnerabilities and potential for persistence in a changing ocean. Oceanogr Mar Biol Annu Rev 49:1–42

Byrne M (2012) Global change ecotoxicology: identification of early life history bottlenecks in marine invertebrates, variable species responses and variable experimental approaches. Mar Environ Res 76:3–15

Byrne M, Barker MF (1991) Embryogenesis and larval development of the asteroid *Patiriella regularis* viewed by light and scanning electron microscopy. Biol Bull 180: 332–345

Byrne M, Ho M, Selvakumaraswamy P, Nguyen HD, Dworjanyn SA, Davis AR (2009) Temperature, but not pH, compromises sea urchin fertilization and early development under near-future climate change scenarios. Proc Biol Sci 276:1883–1888

Byrne M, Soars NA, Ho MA, Wong E and others (2010) Fertilization in a suite of coastal marine invertebrates from SE Australia is robust to near-future ocean warming and acidification. Mar Biol 157:2061–2069

Byrne M, Ho M, Wong E, Soars NA and others (2011a) Unshelled abalone and corrupted urchins: development of marine calcifiers in a changing ocean. Proc Biol Sci 278:2376–2383

Byrne M, Selvakumaraswamy P, Ho MA, Nguyen HD (2011b) Sea urchin development in a global change hot spot, potential for southerly migration of thermotolerant propagules. Deep-Sea Res II 58:712–719

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

Chan KYK, Grünbaum D, O'Donnell MJ (2011) Effects of ocean-acidification-induced morphological changes on larval swimming and feeding. J Exp Biol 214:3857–3867

Chen CP, Chen BY (1992) Effects of high temperature on larval development and metamorphosis of *Arachnoids placenta* (Echinodermata, Echinoidea). Mar Biol 112: 445–449

Dartnall AJ (1969) New Zealand sea stars in Tasmania. Pap Proc R Soc Tas 103:53–55

Dickson AG, Millero FJ (1987) A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. Deep-Sea Res A 34:1733–1743

Dickson AG, Sabine CL, Christian JR (2007) Guide to best practices for ocean CO_2 measurements. PICES Special Publication 3. North Pacific Marine Science Organization, Sidney, British Columbia

Doney SC, Fabry VJ, Feely RA, Kleypas JA (2009) Ocean acidification: the other CO_2 problem. Ann Rev Mar Sci 1: 169–192

Dupont S, Ortega-Martínez O, Thorndyke MC (2010a) Impact of near future ocean acidification on echinoderms. Ecotoxicology 19:449–462

Dupont S, Lundve B, Thorndyke M (2010b) Near future ocean acidification increases growth rate of the lecithotrophic larvae and juveniles of the sea star *Crossaster papposus*. J Exp Zool B Mol Dev Evol 314B:382–389

Dupont S, Dorey N, Stumpp M, Melzner F, Thorndyke M (2013) Long-term and trans-life-cycle effects of exposure to ocean acidification in the green sea urchin *Strongylocentrotus droebachiensis*. Mar Biol (in press)

Eckman JE (1996) Closing the larval loop: linking larval

ecology to the population dynamics of marine benthic invertebrates. J Exp Mar Biol Ecol 200:207–237

Foo SA, Dworjanyn SA, Poore AGB, Byrne M (2012) Adaptive capacity of the habitat modifying sea urchin *Centrostephanus rodgersii* to ocean warming and ocean acidification: performance of early embryos. PLoS ONE 7:e42497

Gosselin LA, Qian PY (1997) Juvenile mortality in benthic marine invertebrates. Mar Ecol Prog Ser 146:265–282

Hart MW, Scheibling RE (1988) Heat waves, baby booms and the destruction of kelp beds by sea urchins. Mar Biol 99:167–176

Hendriks IE, Duarte CM, Álvarez A (2010) Vulnerability of marine biodiversity to ocean acidification: a meta-analysis. Estuar Coast Shelf Sci 86:157–164

Hobday AJ, Lough JM (2011) Projected climate change in Australian marine and freshwater environments. Mar Freshw Res 62:1000–1014

Hoegh-Guldberg O, Bruno JF (2010) The impact of climate change on the world's marine ecosystems. Science 328: 1523–1528

Hofmann GE, Barry JP, Edmunds PJ, Gates RD and others (2010) The effect of ocean acidification on calcifying organisms in marine ecosystems: an organism-to-ecosystem perspective. Annu Rev Ecol Evol Syst 41:127–147

IPCC (Intergovernmental Panel on Climate Change) (2007) Climate change 2007: The fourth assessment report of the Intergovernmental Panel on Climate Change (IPCC). Cambridge University Press, Cambridge

Johnson LG, Babcock RC (1994) Temperature and the larval ecology of the crown-of-thorns starfish, *Acanthaster planci.* Biol Bull 187:304–308

Kalam Azad A, McKinley S, Pearce CM (2010) Factors influencing the growth and survival of larval and juvenile echinoids. Rev Aquacult 2:121–137

Kroeker KJ, Kordas RL, Crim RN, Singh GG (2010) Meta-analysis reveals negative yet variable effects of ocean acidification on marine organisms. Ecol Lett 13: 1419–1434

Lee CH, Ryu TK, Choi JW (2004) Effects of water temperature on embryonic development in the northern Pacific asteroid, *Asterias amurensis*, from the southern coast of Korea. Invertebr Reprod Dev 45:109–116

Martin S, Richier S, Pedrotti ML, Dupont S and others (2011) Early development and molecular plasticity in the Mediterranean sea urchin *Paracentrotus lividus* exposed to CO_2 driven ocean acidification. J Exp Biol 214: 1357–1368

Mehrbach C, Culberson CH, Hawley JE, Pytkowicz RM (1973) Measurement of apparent dissociation constants of carbonic acid in seawater at atmospheric pressure. Limnol Oceanogr 18:897–907

Miller GM, Watson SA, Donelson JM, McCormich MI, Munday PL (2012) Parental environment mediates impacts of increased carbon dioxide on a coral reef fish. Nature Clim Change 2:858–861

Nguyen HD, Doo SS, Soars NA, Byrne M (2012) Non-calcifying larvae in a changing ocean: warming, not acidification/hypercapnia, is the dominant stressor on development of the sea star *Meridiastra calcar.* Glob Change Biol 18:2466–2476

O'Connor C, Mulley JC (1977) Temperature effects on periodicity and embryology, with observations on population genetics, of aquacultural echinoid *Heliocidaris tuberculata.* Aquaculture 12:99–114

O'Donnell MJ, Todgham AE, Sewell MA, Hammond LM and others (2010) Ocean acidification alters skeletogenesis and gene expression in larval sea urchins. Mar Ecol Prog Ser 398:157–171

O'Loughlin PM, Waters JM, Roy MS (2002) Description of a new species of *Patiriella* from New Zealand, and review of *Patiriella regularis* (Echinodermata, Asteroidea) based on morphological and molecular data. J R Soc NZ 32: 697–711

Palumbi SR (1999) All males are not created equal: fertility differences depend on gamete recognition polymorphisms in sea urchins. Proc Natl Acad Sci USA 96: 12632–12637

Parker LM, Ross PM, O'Connor WA (2010) Comparing the effect of elevated pCO_2 and temperature on the fertilization and early development of two species of oysters. Mar Biol 157:2435–2452

Parker LM, Ross PM, O'Connor WA, Borysko L, Raftos DA, Pörtner HO (2012) Adult exposure influences offspring response to ocean acidification in oysters. Glob Change Biol 18:82–92

Pechenik JA (1987) Environmental influences on larval survival and development. In: Giese AC, Pearse JS (eds) Reproduction of marine invertebrates. Academic Press, New York, NY, p 551–608

Pechenik JA (2006) Larval experience and latent effects — metamorphosis is not a new beginning. Integr Comp Biol 46:323–333

Pierrot D, Lewis E, Wallace DWR (2006) MS Excel program developed for CO_2 system calculations. ORNL/CDIAC-105a. Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, US Department of Energy, Oak Ridge, TN

Pörtner HO (2010) Oxygen- and capacity-limitation of thermal tolerance: a matrix for integrating climate-related stressor effects in marine ecosystems. J Exp Biol 213: 881–893

Prowse TAA, Sewell MA, Byrne M (2008) Fuels for development: evolution of maternal provisioning in asterinid sea stars. Mar Biol 153:337–349

Rowley RJ (1990) Newly settled sea urchins in a kelp bed and urchin barren ground: a comparison of growth and mortality. Mar Ecol Prog Ser 62:229–240

Sheppard Brennand H, Soars N, Dworjanyn SA, Davis AR, Byrne M (2010) Impact of ocean warming and ocean acidification on larval development and calcification in the sea urchin *Tripneustes gratilla.* PLoS ONE 5:e11372

Staver JM, Strathmann RR (2002) Evolution of fast development of planktonic embryos to early swimming. Biol Bull 203:58–69

Stumpp M, Wren J, Melzner F, Thorndyke MC, Dupont ST (2011) CO_2 induced seawater acidification impacts sea urchin larval development. I. Elevated metabolic rates decrease scope for growth and induced development delay. Comp Biochem Physiol A 160:331–340

Sunday JM, Crim RN, Harley CDG, Hart MW (2011) Quantifying rates of evolutionary adaptation in response to ocean acidification. PLoS ONE 6:e22881

Underwood AJ (1997) Experiments in ecology. Cambridge University Press, Cambridge

Uthicke S, Soars N, Foo S, Byrne M (2013) Effects of elevated pCO_2 and the effect of parent acclimation on development in the tropical Pacific sea urchin *Echinome-*

tra mathaei. Mar Biol (in press)
Walther K, Anger K, Pörtner HO (2010) Effects of ocean acidification and warming on the larval development of the spider crab *Hyas araneus* from different latitudes (54° vs. 79° N). Mar Ecol Prog Ser 417:159–170
Walther K, Sartoris FJ, Pörtner HO (2011) Impacts of temperature and acidification on larval calcium incorporation of the spider crab *Hyas araneus* from different latitudes (54° vs. 79° N). Mar Biol 158:2043–2053
Wanninkhof R, Lewis E, Feely RA, Millero FJ (1999) The optimal carbonate dissociation constants for determining surface water pCO_2 from alkalinity and total inorganic carbon. Mar Chem 65:291–301
Waters JM, Roy MS (2004) Phylogeography of a high-dispersal New Zealand sea-star: does upwelling block gene-flow? Mol Ecol 13:2797–2806
Widdicombe S, Spicer JI (2008) Predicting the impact of ocean acidification on benthic biodiversity: What can animal physiology tell us? J Exp Mar Biol Ecol 366: 187–197

Editorial responsibility: James McClintock, Birmingham, Alabama, USA

Submitted: April 3, 2012; Accepted: September 17, 2012
Proofs received from author(s): December 22, 2012