1. INTRODUCTION

Global warming and increased atmospheric CO₂ are causing the oceans to become warmer and acidic (Feely et al. 2004; IPCC 2007), the latter through the absorption of CO₂, which dissolves in sea water to form carbonic acid. This acid dissociates into protons and bicarbonate ions, thereby decreasing pH. Atmospheric CO₂ concentration will increase from present levels (approx. 300–380 ppm) to 450–1000 ppm by 2100, with a corresponding predicted decrease in ocean pH by 0.14–0.35 units, assuming the IPCC 'business-as-usual' scenario (Caldeira & Wickett 2003; Feely et al. 2004; IPCC 2007; Fabry et al. 2008).

Our understanding of the potential impacts of increased temperature and acidification on marine biota is impeded by the scarcity of empirical data and a focus on single stressor studies, many of which use stressor levels beyond the scarcity of empirical data and a focus on single stressor studies, many of which use stressor levels beyond the scarcity of empirical data and a focus on single stressor studies, many of which use stressor levels beyond the scarcity of empirical data and a focus on single stressor studies, many of which use stressor levels beyond the scarcity of empirical data and a focus on single stressor studies, many of which use stressor levels beyond the scarcity of empirical data and a focus on single stressor studies, many of which use stressor levels beyond the scarcity of empirical data and a focus on single stressor studies, many of which use stressor levels beyond the scarcity of empirical data and a focus on single stressor studies. In keeping with near-future climate change scenarios, we determined the interactive effects of warming and acidification on fertilization and development of this echinoid. Experimental treatments (20–26°C, pH 7.6–8.2) were tested in all combinations for the ‘business-as-usual’ scenario, with 20°C/pH 8.2 being ambient. Percentage of fertilization was high (>89%) across all treatments. There was no difference in percentage of normal development in any pH treatment. In elevated temperature conditions, +4°C reduced cleavage by 40 per cent and +6°C by a further 20 per cent. Normal gastrulation fell below 4 per cent at +6°C. At 26°C, development was impaired. As the first study of interactive effects of temperature and pH on sea urchin development, we confirm the thermotolerance and pH resilience of fertilization and embryogenesis within predicted climate change scenarios, with negative effects at upper limits of ocean warming. Our findings place single stressor studies in context and emphasize the need for experiments that address ocean warming and acidification concurrently. Although ocean acidification research has focused on impaired calcification, embryos may not reach the skeletogenic stage in a warm ocean.

Keywords: climate change; ocean warming; ocean acidification; sea urchin fertilization and development; Intergovernmental Panel on Climate Change; scenarios
extreme predicted for 2070–2100 (IPCC 2007; but see Whooten et al. 2008). Although recent focus on ocean acidification has been on biocalcification (Reynaud et al. 2003; Feely et al. 2004; Fine & Tchernov 2007; Hoegh-Guldberg et al. 2007; Wood et al. 2008), it is essential to determine whether benthic calcifiers will be able to reach the skeletogenetic developmental stage.

Sea water with high pCO2 and reduced pH has negative impacts on growth, reproduction and development of marine invertebrates due to direct pH effects and hypercapnia (Kurihara & Shirayama 2004; Michaelidis et al. 2005; Pörtner et al. 2005; Wood et al. 2008). Increased temperature has a direct impact on physiological function, increasing growth and developmental rates (Fujisawa & Shigei 1990; Palmer 1994; Reynaud et al. 2003; O’Connor et al. 2007). In a long history of research, numerous studies provide data on the influence of temperature or pH on echinoderm fertilization and development (e.g. Farmanfarmaian & Giese 1963; Chen & Chen 1992; Bay et al. 1993; Roller & Stickle 1993; Riveros et al. 1996; Kurihara & Shirayama 2004; Carr et al. 2006; Dupont et al. 2008; Havenhand et al. 2008), but there are no published data on the potential interactive effects of increased temperature and acidification. These data, within a range applicable to climate change scenarios, are crucial to understand the potential impact of climate change on basic biological processes essential for the integrity and persistence of all marine populations. We assessed the potential interactive impacts of ocean warming and acidification on the early life-history stage of a free spawning benthic invertebrate to enable predictions as to how marine biota may fare with respect to climate change.

2. MATERIAL AND METHODS

(a) Study organism and exposure to stressors

Heliocidaris erythrogramma were collected near Sydney, Australia and induced to spawn by injection of 1–2 ml of 0.5 M KCl. Experiments used gametes from at least two males and two females. Gametes were collected directly from the aboral surface using glass pipettes. The eggs were placed in filtered sea water (FSW; 0.2 μm) and sperm were collected dry. All experiments were conducted with freshly collected FSW, salinity 36 ppt, pH 8.25 (s.e. = 0.02, n = 5) and dissolved oxygen (DO) ≥85 per cent. Sea-water variables (pH, DO, temperature) were measured using a WTW multiline F/SET-3 multimeter. Total alkalinity (TA) was determined for each sea-water source (Sydney Water Monitoring Services). Experimental pCO2 values were determined from TA, pH and salinity data using the CO2 system calculation program (Lewis & Wallace 1998). Treatments (20, 24 and 26°C; pH 7.6, 7.8, 7.9 and 8.2; range pCO2 = 230–690 ppm) were tested in all combinations for the 2070–2100 ‘business-as-usual’ scenario and beyond (IPCC 2007). Temperature imprinting of reproduction and development plays a major role in echinoderm developmental tolerance (O’Connor & Mulley 1977; Fujisawa & Shigei 1990) and the 20°C control reflected the recent thermal history of the adults. Experimental temperatures, 24 and 26°C, were +4–6°C above ambient. A rangefinder study determined that temperatures ≥28°C are lethal to H. erythrogramma embryos (H. D. Nguyen 2007, unpublished data). The experimental pH 7.6, 7.8 and 7.9 (approx. 0.3–0.6 pH units below ambient) were achieved by bubbling CO2 gas into the water until the desired pH was reached. DO levels were maintained by the simultaneous bubbling of air in all water used.

(b) Fertilization

The total number of eggs for each experiment was measured from a 50 ml suspension determined through counts of 100 μl aliquots. For each experiment, the eggs were split into 12 beakers (500 ml) at three temperatures and four pH levels at densities of 3–4 eggs ml−1. The eggs were preincubated in experimental water for 20 min prior to fertilization. Water baths were used to maintain constant temperature. The sperm concentration was used, 104 sperm ml−1, as determined by haemocytometer counts. These conditions resulted in more than or equal to 90 per cent fertilization and high rates of normal development (≥75%) in procedural controls.

Just prior to fertilization, the sperm were placed in the experimental water for a few seconds at the concentration required to ensure appropriate fertilization conditions when added to the beakers containing eggs. After 15 min, the eggs were rinsed three times in experimental FSW to remove excess sperm and resuspended in fresh experimental FSW. Fertilization success was determined after 2 h as the proportion of eggs that had a fertilization envelope or exhibited cleavage, out of three samples of 50 eggs taken from the egg suspension. Five independent fertilizations were undertaken with full replication for each treatment.

(c) Development

Fertilized eggs were separated into replicate beakers (100 ml) containing experimental water at densities of approximately 3–4 eggs ml−1. For each independent fertilization, 72 beakers (three temperatures × four pH, two sampling times × three replicate beakers per sampling time) containing approximately equal numbers of embryos were reared in the same conditions used for fertilization. The beakers were covered with parafilm to minimize evaporation. At two time points (2–3 h cleavage and 19–20 h gastrulae), three beakers were removed from each treatment and the percentage of normal development was determined in the first 50 embryos collected from the beakers. The beakers were discarded after scoring. Experimental water was renewed 4–5 h post-fertilization and the beakers were incubated for 15–16 h overnight. H. erythrogramma is routinely reared to 20–24°C (Kobayashi 1980; Byrne et al. 2001), with the latter being the maximum summer SST. Sea-water pH in the treatments measured at 4–5 h did not change and pH data from the end of the experiment (19–20 h) is provided in table 1. Embryos from 20°C/pH 8.2 fertilizations were reared to the rudiment stage (4 days) to assess the quality of all embryo sources.

<table>
<thead>
<tr>
<th>temperature (°C)</th>
<th>pH 7.9</th>
<th>pH 7.8</th>
<th>pH 7.6</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>8.06 (0.23)</td>
<td>7.85 (0.1)</td>
<td>7.67 (0.02)</td>
</tr>
<tr>
<td>24</td>
<td>7.96 (0.01)</td>
<td>7.87 (0.02)</td>
<td>7.77 (0.02)</td>
</tr>
<tr>
<td>26</td>
<td>8.05 (0.04)</td>
<td>7.89 (0.04)</td>
<td>7.88 (0.07)</td>
</tr>
</tbody>
</table>

Table 1. Mean pH in treatments at the end (20 h) of the five experiments. Value in parentheses is the standard error.
Statistical analyses
All dependent variables (fertilization, cleavage and gastrulae) were analysed by two-way ANOVA with temperature and pH as fixed factors. The three beakers within runs were used to improve the precision of our estimates and means values were used as replicates in each experiment (n = 5). All percentage data were arcsine transformed prior to ANOVA and assumptions of the analysis were checked before proceeding.

Normality was assessed visually and Cochran’s test was used to ensure that the variances were homogeneous. Where significant differences were evident, the Newman–Keuls (NK) multiple comparison test was used for post hoc analyses.

Data analyses were run on SPSS.

3. EFFECTS OF OCEAN WARMING AND ACIDIFICATION ON FERTILIZATION IN H. ERYTHROGRAMMA
Percentage of fertilization was high (>89%) across all treatments with a slight decrease at upper warming (figure 1a), and this difference was significant (temperature \( F_{2,48} = 3.52, p = 0.05 \)) with no effect of pH (pH \( F_{3,48} = 1.40, p = 0.25 \)). There was no interaction between temperature and pH (pH \( F_{6,48} = 0.86, p = 0.53 \)). Multiple comparison analyses revealed that fertilization did not differ between the 20 and 24°C nor between the 24 and 26°C treatments, but did differ between the 20 and 26°C treatments. Thus, at the upper warming (+6°C) and pCO\(_2\) acidification (pH –0.6 units) scenarios, fertilization of H. erythrogramma was affected slightly by temperature but not pH.

4. EFFECTS OF OCEAN WARMING AND ACIDIFICATION ON THE DEVELOPMENT OF H. ERYTHROGRAMMA
Following fertilization in experimental conditions, temperature had a significant effect on the development of H. erythrogramma, but pH did not (cleavage: temperature \( F_{4,48} = 58.05, p < 0.0001 \), pH \( F_{3,48} = 1.63, p = 0.19 \); gastrulation: temperature \( F_{2,48} = 155.89, p < 0.0001 \), pH \( F_{3,48} = 1.23, p = 0.31 \); figure 1b,c). The percentage of normal cleaving embryos 2–3 h post-fertilization was significantly lower as temperature increased (NK 20 > 24 > 26°C), as was the percentage of normal gastrulae 24–26 h post-fertilization (NK 20 > 24 > 26°C; figure 1b,c). Hence, at the highest temperature (26°C), the percentage of embryos showing normal cleavage fell below 20 per cent and gastrulation fell to <4 per cent, while, at 20°C, the percentage developing normally was more than 65 per cent across all pH levels. Thus, temperature had a significant effect on developmental failure at the upper warming (+4–6°C) level, regardless of pH. In addition, there was no significant interaction between these factors (cleavage: \( F_{6,48} = 0.13, p = 0.99 \); gastrulation: \( F_{6,48} = 1.17, p = 0.34 \)). Control embryos reared for 4 days at 20°C/pH 8.2 produced a mean of 75 per cent (s.e. = 5, n = 5) rudiment stage larvae.

5. DISCUSSION
Temperature, as the single most important environmental factor controlling marine populations, has long been the

Figure 1. Effect of temperature and pH on the percentage of (a) fertilization, (b) normal cleaving embryos and (c) normal gastrulae of H. erythrogramma. The 20°C and pH 8.2 treatment represented ambient conditions. Black bars, 8.2; dark grey bars, 7.9; light grey bars, 7.8; white bars, 7.6.
focus of physiology, phenology, biogeography and life-history biology studies (Vernberg 1962; O’Conner & Mulley 1977; Pechenik 1987; Olive 1995), with recent interest in the influence of temperature change on range extensions and contractions (Hays et al. 2005; O’Conner et al. 2007; Visser 2007; Przeslawski et al. 2009).

As we report here for H. erythrogramma, single stressor studies of thermostolerance in a diverse suite of tropical and temperate sea urchins show that fertilization and early development are robust to temperatures well above ambient and the increases expected from climate change (Farmanfarmaian & Giese 1963; Chen & Chen 1992; Roller & Stickle 1993). Thermostolerance in sea urchin fertilization and early development is conveyed by protective heat shock proteins (Sconzo 1995; Yamada & Mihashi 1998) and potentially include (Farmanfarmaian & Giese 1963; Chen & Chen 1992; ambient and the increases expected from climate change and temperate sea urchins show that fertilization and early development within values predicted for environmental change, sea urchin fertilization and early development are robust to temperatures well above ambient and the increases expected from climate change (Farmanfarmaian & Giese 1963; Chen & Chen 1992; Roller & Stickle 1993). Thermostolerance in sea urchin fertilization and early development is conveyed by protective heat shock proteins (Sconzo 1995; Yamada & Mihashi 1998) and potentially include (Farmanfarmaian & Giese 1963; Chen & Chen 1992; ambient and the increases expected from climate change.

Table 2. pH tolerance of fertilization and early development, and pH levels producing impaired fertilization and development, in studies of sea urchins, where pH was lowered by the treatment of sea water with CO2 gas, with control fertilization ≥80–90% and development ≥75%. n.d., no data. (Asterisk indicates the individual cleavage stages scored.)

<table>
<thead>
<tr>
<th>species</th>
<th>pH range fertilization</th>
<th>pH fertilization &lt;60–70%</th>
<th>pH range normal early development</th>
<th>pH normal early development &lt;60–70%</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strongylocentrotus purpuratus</td>
<td>7.3–8.2</td>
<td>7.0</td>
<td>7.5–8.3</td>
<td>7.3</td>
<td>Bay et al. (1993)</td>
</tr>
<tr>
<td>Hemicentrotus pulcherrimus</td>
<td>7.4–8.0</td>
<td>7.0</td>
<td>7.6–8.0*</td>
<td>7.4</td>
<td>Kurihara &amp; Shirayama (2004)</td>
</tr>
<tr>
<td>Arbacia punctulata</td>
<td>6.9–8.6</td>
<td>6.8</td>
<td>7.4–8.6</td>
<td>6.8</td>
<td>Carr et al. (2006)</td>
</tr>
<tr>
<td>Heliocidaris erythrogramma</td>
<td>7.6–8.3</td>
<td>n.d.</td>
<td>7.8–8.3</td>
<td>n.d.</td>
<td>this study</td>
</tr>
</tbody>
</table>

Our experiments show that fertilization using gametes from H. erythrogramma adults acclimatized at 20°C (+6°C above ambient SST). In development, this tolerance was reduced to 24°C (+4°C above ambient SST), with developmental failure at 26°C, regardless of pH. Developmental arrest in the 26°C pH 8.2 treatment in hatched embryos followed transcription of the hatching enzyme, a zygotic genome product, indicating that arrested development occurred after switching on of the zygotic genome and depletion of protective maternal factors. This developmental threshold (+4°C) falls within the maximum environmental warming predicted for eastern Australia by 2070–2100 and also coincides with the maximum SST (24°C) that spawned gametes and dispersive embryos experience in a warm summer. It is not known whether gametes from H. erythrogramma adults acclimatized to 24°C would have successful development in a +4°C treatment (28°C).

Although this seems unlikely, the strong influence of phenotypic thermal acclimatization on sea urchin reproduction and development warrants further study with respect to future SST scenarios to determine the potential adaptive capacity of fertilization and development to ocean warming. For the closely related sympatric species Heliocidaris tuberculata, parental acclimatization in nature dramatically shifts temperature tolerance of developing embryos (O’Conner & Mulley 1977).

Comparative data (controls: >80–90% fertilization, >75% normal development) on the effect of increased pCO2 and decreased pH as a single stressor on sea urchin fertilization and development are available for five species (table 2). These studies show that sea urchin fertilization and early development are only affected by pH <7.4 (above 1000 ppm CO2), levels that are well below model predictions for ocean acidification. As for H. erythrogramma, several species show a broad pH/pCO2 tolerance of fertilization and development within values predicted for climate change. In Echinometra mathaei, Hemicentrotus pulcherrimus and Strongylocentrotus purpuratus fertilization dropped from more than or equal to 90 per cent in controls to 70, 60 and 60% at pH 7.4, 7.0 and 7.0, respectively (Bay et al. 1993; Kurihara & Shirayama 2004). With regard to development, the percentage of normal S. purpuratus embryos dropped from approximately 90 per cent in controls to 70 per cent at pH 7.3 (Bay et al. 1993). Studies on the influence of pH using acid or environmental samples to lower sea-water pH show a similar tolerance of echinoderm fertilization and development to acidification (Smith & Clowes 1924; Riveros et al. 1996; Kurihara & Shirayama 2004). For H. erythrogramma, a recent study observed a drop in fertilization success from 62 per cent (s.e. = 8%) at pH 8.1 to 51 per cent (s.e. = 8.4%) at pH 7.7, a drop attributed to decreased sperm motility (Havenhand et al. 2008). We do not know whether sperm motility was affected in our experiments. Owing to different fertilization conditions (control 70% fertilization rate; Havenhand et al. 2008), it is not possible to directly compare our study, but we note that fertilization success in our experiments exceeded 90 per cent. The weight of evidence (table 2) suggests that, within predicted values for environmental change, sea urchin fertilization and early development are robust to decreased pH. This may be due to the low pH naturally associated with echinoderm reproduction; the internal pH of activated sperm is initially at pH 7.6 and acid is released by echinoderm eggs at fertilization (Peaucellier & Doree 1981; Holland & Gould-Somero 1982), phenomena characteristic of marine invertebrate reproduction (Holland et al. 1984). Moreover, the early life history of H. erythrogramma and other intertidal invertebrates is likely to be adapted to the broad range of pH (e.g. pH 7.4–8.9) characteristic of this habitat due to photosynthetic activity (Whooten et al. 2008).
Although our results indicate that the early life-history stages of \textit{H. erythrogramma} may be robust to predicted ocean acidification by 2100, decreased pH may have a negative effect on larval calcification. Echinoplutei of \textit{H. pulcherrimus} reared at pH 7.8 and ambient temperature have a shorter arm skeleton, an effect that would negatively impact skeletogenesis at ambient SST through enhancing the rate of calcification in low pH conditions (Wood \textit{et al.} 2008). Clearly, more empirical data are needed to place the prospects of marine calcification in the context of climate change.

Our results indicate that within the context of projected environmental change for eastern Australia (CSIRO Climate System Model Mk3.5), increased SST will be a more serious embryonic teratogen than acidification despite both stressors being applied simultaneously. Projections for eastern Australia indicate a SST warming up to 2–4°C in summer off the New South Wales. If this coincides with current maximum SST, temperatures above 26°C may occur. Our data indicate that such an increase would be deleterious for development. Although recent investigations of the impacts of climate change on marine systems have focused on ocean acidification and biocalcification (Reynaud \textit{et al.} 2003; Feely \textit{et al.} 2004; Fine \& Tchernov 2007; Hoegh-Guldberg \textit{et al.} 2007; Wood \textit{et al.} 2008), if temperatures increase beyond the inherent thermal adaptive capacity of successful reproduction, marine embryos may not reach the calcifying stage. More importantly, increased temperature may impair early developmental mechanisms (e.g. cleavage, gastrulation) that are conserved across the Metazoa, with broad deleterious effects for all marine ecosystems.

Two major stressors set to change in the global ocean are temperature and pH. While it is important to understand the influence of these stressors in isolation, it is imperative to understand their interactive effects. Examination of these two stressors on the early life-history stages of \textit{H. erythrogramma}, and the results of other studies of benthic taxa where interactive effects of the two major climate change stressors were investigated (Reynaud \textit{et al.} 2003; Anthony \textit{et al.} 2008), emphasize the urgent need for experiments cognizant of current local conditions and stressors and within the range of projected values (Poloczanska \textit{et al.} 2007; Fabry \textit{et al.} 2008; Przeslawski \textit{et al.} 2008). Our study also highlights the potentiality that adaptive phenotypic plasticity may help buffer the negative effects of warming, as suggested for corals (Wooldridge \textit{et al.} 2005; Edmunds \& Gates 2008). In reality, however, marine gametes and embryos are exposed to multiple stressors in addition to those associated with global warming, and stressors are unlikely to act independently (Przeslawski \textit{et al.} 2005, 2008). This presents a considerable challenge as we attempt to predict how benthic marine invertebrates will fare as the oceans warm and acidify.

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REFERENCES


Holland, L. Z. & Gould-Somero, M. & Paul, M. 1984 Temperature control of larval dispersal and the impli-


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


