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Fertilization in a suite of coastal marine invertebrates from SE Australia is robust to near-future ocean warming and acidification

Maria Byrne · Natalie A. Soars · Melanie A. Ho · Eunice Wong · David McElroy · Paulina Selvakumaraswamy · Symon A. Dworjanyn · Andrew R. Davis

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Abstract Climate change driven ocean acidification and hypercapnia may have a negative impact on fertilization in marine organisms because of the narcotic effect these stressors exert on sperm. In contrast, warmer, less viscous water may have a positive influence on sperm swimming speed and so ocean warming may enhance fertilization. To address questions on future vulnerabilities we examined the interactive effects of near-future ocean warming and ocean acidification/hypercapnia on fertilization in intertidal and shallow subtidal echinoids (*Heliocidaris erythrogramma*, *H. tuberculata*, *Tripneustes gratilla*, *Centrostephanus rodgersii*), an asteroid (*Patiriella regularis*) and an abalone (*Haliotis coccoradiata*). Batches of eggs from multiple females were fertilized by sperm from multiple males in all combinations of three temperature and three pH/P_{CO_2} treatments. Experiments were placed in the setting of projected near-future conditions for southeast Australia, an ocean change hot spot. There was no significant effect of warming and acidification on the percentage of fertilization. These results indicate that fertilization in these species is robust to temperature and pH/P_{CO_2} fluctuation. This may reflect adaptation to the marked fluctuation in temperature and pH that characterises their shallow water coastal habitats. Efforts to identify potential impacts of ocean change to the life histories of coastal marine invertebrates are best to focus on more vulnerable embryonic and larval stages because of their long time in the water column where seawater chemistry and temperature have a major impact on development.

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M. Byrne (✉)
Schools of Medical and Biological Sciences, F13,
University of Sydney, Sydney, NSW 2006, Australia
e-mail: mbyrne@anatomy.usyd.edu.au

N. A. Soars · M. A. Ho · E. Wong · D. McElroy ·
P. Selvakumaraswamy
School of Medical Sciences, F13, University of Sydney,
Sydney, NSW 2006, Australia

S. A. Dworjanyn
National Marine Science Centre, Southern Cross University,
PO Box 4321, Coffs Harbour, NSW 2450, Australia

A. R. Davis
Institute for Conservation Biology, University of Wollongong,
Wollongong, NSW 2522, Australia

Introduction

Reproduction in most benthic marine invertebrates involves free spawning of gametes and fertilization in the water column followed by a dispersive larval stage. Due to their sensitivity to environmental perturbation, marine gametes have long been used as a model system for environmental monitoring of toxicants (Dinnel et al. 1987; Ringwood 1992; Bay et al. 1993; Carr et al. 2006; Byrne et al. 2008; Byrne 2010). With climate change, gametes are now being released into an ocean that is warming, acidifying and increasing in P_{CO_2} (Caldeira and Wickett 2003; IPCC 2007; Fabry et al. 2008; Pörtner 2008), exposing gametes to a new suite of anthropogenic stressors. If these climate change stressors impair fertilization, the foundation stage for species persistence, this would have dire consequences for marine life. Increased environmental hypercapnia (P_{CO_2}) suppresses metabolism and has a well-understood narcotic effect on sperm motility and so increased ocean P_{CO_2} may impair fertilization (Chia and Bickell 1983; Ward et al. 1985; Brokaw 1990; Havenhand et al. 2008; Morita et al. 2010). On the other hand, ocean

warming may enhance fertilization (up to a threshold) due to increased sperm swimming speeds in warmer, less viscous seawater and the direct influence of temperature on the acrosome reaction, phenomena reported for polychaetes, molluscs and echinoderms (Greenwood and Bennett 1981; Mita et al. 1984; Clotteau and Dubé 1993; Kupriyanova and Havenhand 2005). The interactive effects of ocean change stressors on fertilization in the sea are difficult to predict.

Our understanding of the impacts of ocean change on marine biota is impeded by the dominant focus on ocean acidification as a sole stressor (Hendriks et al. 2010). Studies of CO_2-driven acidification on fertilization in six echinoderm and mollusc species show no significant effects of near-future (ca. 2070–2100) ocean change (ca. to pH 7.6, P_{CO_2} 1000 ppm) (Reviews: Kurihara 2008; Byrne 2010; but see Havenhand et al. 2008). Negative effects are generally encountered in more extreme conditions (e.g. 2300 and beyond: pH 7.4, P_{CO_2} 2000+ ppm). Studies where seawater pH was adjusted by mineral acid show mixed results (Kurihara and Shirayama 2004; Parker et al. 2009; Byrne 2010). The interactive effects of ocean warming and CO_2-driven acidification in a near-future climate-relevant setting on fertilization has only been investigated for one echinoid and two oyster species (Byrne et al. 2009; Parker et al. pers comm). Experiments with an oyster species indicate vulnerabilities of fertilization to both pH and temperature (Parker et al. 2009) but this was not the case for a sea urchin species (Byrne et al. 2009, 2010a). There is an urgent need for empirical data on potential interactive impacts of ocean change stressors on marine life histories from diverse species as we endeavour to predict flow on effects to marine ecosystems.

The fertilization biology of the Echinodermata and Mollusca is well characterised as a model system that can be used to assess the impacts of climate change stressors on marine biota (Byrne 2010). Here we investigated the potential interactive effects of ocean warming and ocean acidification/hypercapnia on fertilization in phylogenetically diverse species, four echinoids (*Heliocidaris erythrogramma*, *H. tuberculata*, *Centrostephanus rodgersii, Tripneustes gratilla*), an asteroid (*Patiriella regularis*) and an abalone (*Haliotis coccoradiata*). We previously showed resilience of fertilization in *H. erythrogramma* to climate change stressors (Byrne et al. 2009, 2010a) and revisit this here in tests using optimal sperm to egg ratio conditions, an important consideration for fertilization tests (Dinnel et al. 1987; Cherr et al., 1990; Levitan et al. 1991; Clotteau and Dubé 1993; Baker and Tyler 2001). The six species investigated are abundant and ecologically important members of intertidal and shallow water marine communities of southeast Australia, including major herbivores and habitat modifying species (Edgar 2000; Andrew and Byrne 2007; Keesing 2007; Ling et al. 2009). This region is a global change hot spot where sea temperatures are warming considerably faster than the global average (Poloczanska et al. 2007). Ocean warming is the most serious contemporary climate change stressor in the region and has caused major change to local ecosystems (e.g. Ling et al. 2009).

The effects of ocean warming and increased acidification/P_{CO_2} on fertilization success in six species were investigated in multifactorial experiments with simultaneous exposure to both stressors within a climate and regionally relevant setting for near-future ocean change. To maintain ecological relevance in each experiment we scored fertilization in embryos generated from the gametes of multiple males and females to represent a population of spawners in the field. This multiple dam-sire approach was used to reduce variability among experimental replicates because studies of fertilization kinetics, sperm competition, quantitative genetics and ecotoxicology, across a range of taxa, show how variable sperm are in quality and the variable receptiveness of eggs (Cherr et al. 1990; Evans and Marshall 2005; Levitan and Ferrell 2006; Evans et al. 2007; Levitan et al. 2007; Marshall and Evans 2007; Song et al. 2009). We sought to address the question: Can fertilization in a phylogenetically diverse suite of species occur in the warm and acidic/high P_{CO_2} ocean conditions projected for 2070–2100 (IPCC 2007; Poloczanska et al. 2007).

Methods

Specimen collection and spawning

Heliocidaris erythrogramma (Valenciennes), *H. tuberculata* (Lamarck), *Centrostephanus rodgersii* (A. Agassizi) and *Tripneustes gratilla* (Linneaus) and the abalone *Haliotis coccoradiata* (Reeve), were collected along the coast of New South Wales during their natural spawning period, as determined from previous histological, gonad index and spawning studies of these species (Laegdsgaard et al. 1991; Byrne et al. 1998; Wong et al. 2010; Byrne, pers obs). Four of these species (*H. erythrogramma*, *H. tuberculata*, *C. rodgersii*, and *H. coccoradiata*) were collected from Little Bay (33°58′S, 151°14′E) on the open coast near Sydney. This location is also from where the seawater for the experiments was sourced. *Tripneustes gratilla* was collected near Coffs Harbour (30°12′S, 153°16′E). The sea star *Patiriella regularis* (Verrill) was collected near Hobart, Tasmania (42°50′S, 147°15′E). Mature specimens of *H. erythrogramma*, *C. rodgersii*, *H. coccoradiata* and *P. regularis* were collected at low tide (0–1 m depth). *Tripneustes gratilla* and *H. tuberculata* were collected using SCUBA from 5 to 15 m depth. Mature specimens of each species were maintained in

aquaria at ambient sea surface temperatures (SST). Ambient SST around the time of collection was monitored from a local reference station (http://www.mhl.nsw.gov.au). Adult acclimatization influences the thermotolerance of development (Bingham et al. 1997; O'Conner and Mulley 1977; Byrne et al. 2010b) and our control temperatures reflect the SSTs experienced for the month prior to collection. For experiments with *H. erythrogramma*, *H. tuberculata*, *T. gratilla* and *H. coccoradiata* the control temperature was 20°C. For *P. regularis* the control temperature was 22°C and for *C. rodgersii* was 18°C.

The urchins were induced to spawn by injection of 2–3 ml 0.5 M KCl. Sea star eggs were obtained by placing ovaries in dishes of filtered seawater (FSW, 1.0 µm) containing the ovulatory hormone 1-methyladenine (10^{-5} M in FSW). For abalone the hydrogen peroxide spawning method was used, modified from Morse et al. (1977). Sperm were collected dry from the surface of urchins or from dissected sea star testes. For abalone, concentrated sperm was collected as it was released through the respiratory pores. Sperm of each species was placed in a small dish, covered, kept cool and used within 30 min of collection. For each experiment, gametes were pooled from multiple males and females (at least three individuals of each sex). We used this population approach with multiple parents to reduce experimental variability in percentage of fertilization (see Evans and Marshall 2005) and because inherent differences in unique male–female pairs (Levitan and Ferrell 2006) was not a focus. For each species multifactorial experiments in all combinations of factors were repeated 3–5 times, each with a different source of gametes (see below).

Treatments

All experiments were conducted with freshly collected FSW, mean pH 8.25 (SE = 0.02, $n = 14$), salinity 36.6 psu (SE = 0.3, $n = 14$). Total alkalinity (TA, 2307, SE = 35, $n = 14$) was determined for each seawater source by potentiometric titration (University of Sydney Analytical Services or CSIRO Laboratories, Hobart). This mean value along with pHnbs was used to determine experimental P_{CO_2} using CO2SYS (Pierrot et al. 2006) (Table 1). The stressor levels used reflect projected near-future (2070–2100) ocean change for southeast Australian waters (A1F1 scenario, IPCC 2007; Poloczanska et al. 2007). Experimental pH 7.6–7.9 (ca. 0.3–0.6 pH units below ambient) and dissolved oxygen (DO, >90%) levels were achieved by simultaneous bubbling of CO_2 gas and air into FSW until the desired levels was reached. Experimental temperatures were maintained by using a temperature controlled room or water baths. Water baths were switched between runs to reduce the likelihood that inherent differences between water baths would confound the effects of temperature.

Table 1 Experimental water conditions

Temp	pH	P_{CO_2}	ΩCa	ΩAr
18	8.25	324 (22)	4.95 (0.2)	3.20 (0.13)
18	7.9	801 (12)	2.53 (0.04)	1.64 (0.03)
18	7.8	1033 (15)	2.06 (0.04)	1.33 (0.02)
18	7.6	1695 (25)	1.34 (0.02)	0.87 (0.02)
20	8.25	327 (22)	5.20 (0.21)	3.39 (0.13)
20	7.9	814 (12)	2.68 (0.05)	1.74 (0.03)
20	7.8	1051 (15)	2.18 (0.04)	1.42 (0.02)
20	7.6	1729 (25)	1.43 (0.02)	0.93 (0.02)
22	8.25	330 (23)	5.47 (0.21)	3.58 (0.14)
22	7.9	827 (12)	2.84 (0.05)	1.86 (0.03)
22	7.8	1069 (16)	2.31 (0.04)	1.51 (0.03)
22	7.6	1762 (26)	1.52 (0.03)	0.99 (0.02)
24	8.25	332 (23)	5.74 (0.22)	3.78 (0.14)
24	7.9	839 (12)	3.00 (0.05)	1.98 (0.03)
24	7.8	1087 (16)	2.45 (0.04)	1.61 (0.03)
24	7.6	1795 (26)	1.61 (0.03)	1.06 (0.02)
26	8.25	335 (23)	6.02 (0.23)	3.99 (0.15)
26	7.9	851 (12)	3.17 (0.05)	2.10 (0.04)
26	7.8	1104 (16)	2.60 (0.04)	1.72 (0.03)
26	7.6	1828 (27)	1.71 (0.03)	1.13 (0.02)

Temp temperature (°C), P_{CO_2} partial pressure of carbon dioxide (µatm), ΩCa calcite saturation, ΩAr aragonite saturation (±SEM)

Experiments with *H. tuberculata* ($n = 3$) and *T. gratilla* ($n = 3$) used all combinations of three temperatures (ambient SST, +4°C, +6°C) and four pH levels (ambient pH, −0.3 pH units; −0.4 pH units, −0.6 pH units) in multifactorial experiments. For *H. coccoradiata* ($n = 5$) a narrower temperature range was used (ambient SST, +2°C, +4°C) because embryos of this species do not develop at +6°C (Wong, pers obs). Experiments with *H. erythrogramma* ($n = 4$), *C. rodgersii* ($n = 3$) and *P. regularis* ($n = 3$) used a 3 temperature (ambient SST, +4°C, +6°C) and 3 pH (ambient pH, −0.4 pH units, −0.6 pH units) design. Seawater variables (temperature, DO, pH) were determined at the beginning and end (2 h) of the experiments using a WTW Multiline F/Set-3 multimeter. There was no change in the control pH 8.2 and pH 7.8 treatments and a slight change (0.00–0.03 pH units) in the pH 7.6 treatments. Temperature did not change and DO remained >90%.

Fertilization conditions

Optimal sperm to egg ratios, determined in pre-tests for each species (Byrne et al. unpublished), were used to ensure high fertilization, low polyspermy and acceptable

rates of normal development to the larval stage (≥75%) in procedural controls. Fertilization conditions in nature are poorly understood (see Mead and Epel 1995) and guidance on appropriate sperm concentrations was also gleaned from empirical and modeling studies of fertilization. The sperm concentrations used were at levels identified as non-saturation for sea urchin fertilization and favorable for survivorship of embryos post fertilization (ca. $<10^{1-3}$ sperm ml^{-1}) (e.g. Levitan et al. 2007; Allen and Pechenik 2010). They were also similar or more stringent, to those identified as optimal for fertilization in other studies of molluscs and echinoderms (Cherr et al. 1990; Levitan et al. 1991; Clotteau and Dubé 1993; Desrosiers et al. 1996; Baker and Tyler 2001; Song et al. 2009).

For *Heliocidaris tuberculata*, *Tripneustes gratilla* and *Centrostephanus rodgersii* sperm to egg ratios of 25:1 (10^2 sperm ml^{-1}) were used, while for *H. erythrogramma* the ratio was 500:1 (10^3 sperm ml^{-1}). For *Haliotis coccoradiata* the ratio was 20:1 (10 sperm ml^{-1}) and for *Patiriella regularis*, 50:1 (10^2 sperm ml^{-1}). Although fertilization tests and experiments are typically done in small volumes (ca. 2–10 ml) in test tubes, vials or multiwell plates (Baker and Tyler 2001; Styan et al. 2005; Byrne et al. 2008; Havenhand et al. 2008), we used larger volumes (500 ml beakers) to better reflect field conditions. The total number of eggs for each experiment to be placed in 500 ml of FSW was measured from a 50 ml suspension determined through counts of 100 µl aliquots. The number of sperm present in 1 µl of the mixed semen sample combined from the males was determined in haemocytometer counts and this calculation was used to determine how much of the semen sample to use.

In the 4 pH × 3 temperature experiments unfertilized eggs were placed into twelve 500 ml beakers of experimental FSW, one beaker for each temperature–pH combination. For experiments using the 3 pH × 3 temperature design, the eggs were placed in nine 500 ml beakers of FSW, one for each temperature–pH combination. The eggs were placed in experimental FSW for 15 min prior to fertilization. The amount of semen sample to achieve the sperm to egg ratio required was added to 10 ml of experimental water for a brief activation (1–2 s) just prior to addition of this 10 ml volume to the beakers containing eggs. The beakers were sealed with Parafilm. After 15 min, the fertilized eggs were rinsed in experimental FSW to remove excess sperm and re-suspended in fresh experimental FSW. The beakers were resealed with Parafilm.

For echinoderms fertilization was scored by the presence of a fertilization envelope (which was clear to see in all species) plus cleavage. For abalone where the envelope was difficult too see, fertilization was scored by the presence of pink cytoplasm at the vegetal pole which is due cytoplasmic movement and accumulation of yolk granules in this region of the embryo (Wong et al. 2010) plus cleavage. Scoring was thus based on fertilization success not solely on cell division because embryonic mitosis can exhibit different sensitivity to stress (see Allen and Pechenik 2010). From each beaker, three samples of 50 embryos were scored. The mean of these counts was used as the data point for statistical analysis.

Statistical analysis

The data were analysed by two-way factorial analysis of variance (ANOVA) to determine the effect of the fixed factors: temperature and pH/P_{CO_2} on the percentage of fertilization. The pH and P_{CO_2} of seawater covaries and so pH was used as the factor for statistical analysis. Percentage fertilization data were arc-sine transformed and Cochran's C test confirmed homogeneity of variance. The data obtained for *Heliocidaris erythrogramma*, *H. tuberculata*, *Tripneustes gratilla* and *Centrostephanus rodgersii* were normal while those for *Haliotis coccoradiata* and *Patiriella regularis* were close to normal. As ANOVA is robust to deviations from normality (Underwood 1997) we proceeded with the analysis. Statistical analyses were carried out using SPSS PASW Statistics 17.0.2 (2009). We present means with 95% confidence intervals.

Results

In 3–5 independent fertilizations with six species the mean percentage of fertilization was above 80–90% across all treatments (Fig. 1). The confidence intervals (Fig. 1) show low variance among independent fertilizations. We did not detect a significant effect of either temperature or pH on the percentage of fertilization in any species (temperature: $P = 0.056$–0.932; pH: $P = 0.140$–0.896, Table 2). There was also no interaction between temperature and pH ($P = 0.319$–0.998, Table 2). In the pH 7.6 treatments, percent fertilization in one species, the subtidal sea urchin *Heliocidaris tuberculata* dropped slightly, indicating sensitivity to acidification. This result was close to being significant ($P = 0.056$).

Discussion

Fertilization in the echinoderm and mollusc species investigated here exhibited a broad tolerance to near-future ocean warming and acidification. Neither climate change stressor significantly impaired fertilization in these species, as found in numerous single stressor studies of temperature or pH (set by manipulating P_{CO_2}) with the gametes of sea urchins and bivalves exposed to ocean change conditions projected for 2070–2100 (Reviews; Kurihara 2008; Byrne

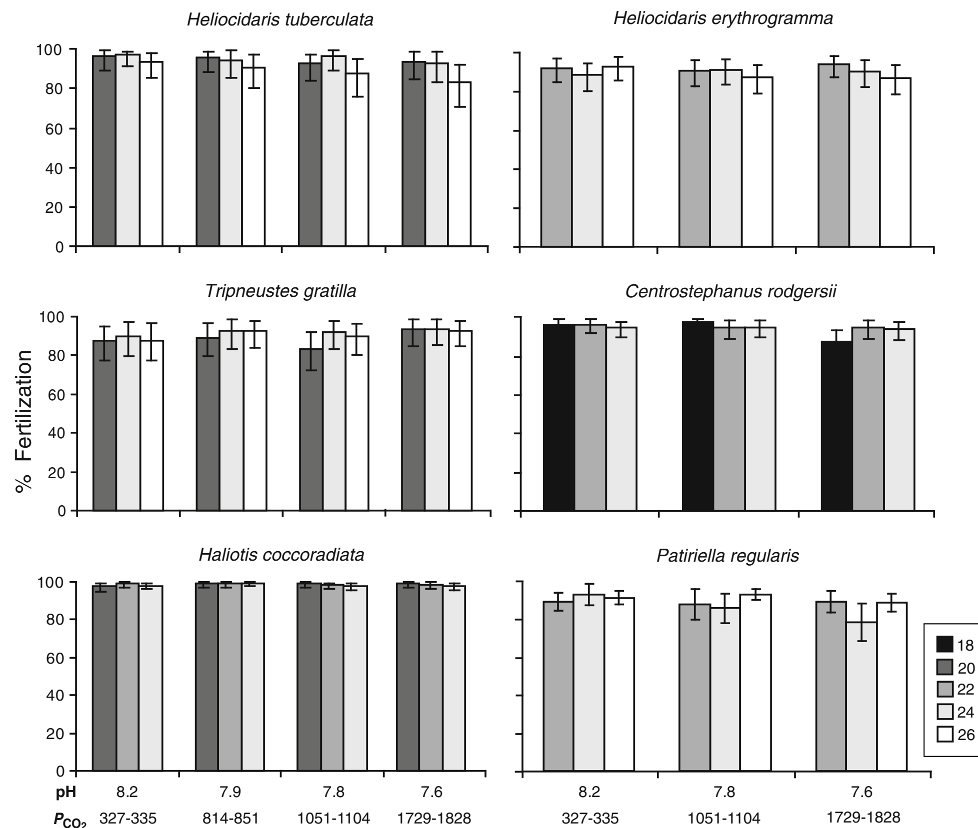

Fig. 1 Mean percentage of fertilization in echinoids, *Heliocidaris tuberculata*, *H. erythrogramma*, *Tripneustes gratilla* and *Centrostephanus rodgersii*, the asteroid *Patiriella regularis* and the abalone *Haliotis coccoradiata* in response to ambient and projected ocean change scenarios for year 2100 (A1F1, IPCC 2007; Poloczanska et al. 2007). Experiments in the left panel used 4 pH and 3 temperature levels while those in the *right panel* used 3 pH and 3 temperature levels. Experimental temperatures varied due to seasonal differences in the timing of gamete maturation in the different species. P_{CO_2} levels are indicated for each pH used. *Error bars* are 95% confidence intervals

Table 2 ANOVA *F* ratios for analyses of data on percentage fertilization for six species

Species	Temp (*df*)	pH (*df*)	Temp*pH (*df*)	*n*
H. erythrogramma	0.6 (2,2)	0.1 (2,4)	0.5 (4,27)	4
H. tuberculata	3.3 (2,3)	1.8 (3,6)	0.3 (6,24)	3
T. gratilla	0.6 (2,3)	1.2 (3,6)	0.2 (6,24)	3
C. rodgersii	0.1 (2,2)	2.2 (2,4)	1.3 (4,18)	3
P. regularis	0.3 (2,2)	0.7 (2,4)	0.32 (4,27)	4
H. coccoradiata	0.1 (2,3)	0.4 (3,6)	0.3 (6,48)	5

All *P* values were >0.1 (range 0.14–0.99) except for *H. tuberculata* (temperature $P = 0.056$)

Temp temperature (°C), *df* degrees of freedom

2010, but see Havenhand et al. 2008). We also did not observe interactive effects of the exposure of gametes to temperature and pH/P_{CO_2}.

In single stressor studies with increased temperature, fertilization among sea urchins drawn from a broad phylogeny (ca. 13 species) is robust to +≥8°C above ambient (Byrne 2010), with similar results reported for an asteroid (Lee et al. 2004). Fertilization in many mollusc species is also tolerant to +≥8°C warming (Clotteau and Dubé 1993; Desrosiers et al. 1996; Byrne 2010). Studies of echinoderms, molluscs and polychaetes show that the optimal temperature range for fertilization varies greatly among species and is broadest for tropical species and those with a wide latitudinal distribution (Rupp 1973; Clotteau and Dubé 1993; Sewell and Young 1999; Kupriyanova and Havenhand 2005; Byrne 2010; Byrne et al. 2010b; Parker et al. pers comm). Induction of spawning by thermal shock (+4–10°C above ambient) as routinely used to establish invertebrate larval cultures (e.g. Selvakumaraswamy and Byrne 2000; Parker et al. 2009) also indicates that invertebrate gametes tolerate thermal fluctuation. Thermal tolerance may be conveyed by protective factors in the eggs (e.g. heat shock proteins) and a thermal independent period post gamete binding (Yamada and Mihashi 1998; Hamdoun and Epel 2007).

In single stressor studies of the impacts of near-future CO_2-driven acidification, fertilization in sea urchin species is also robust to decreased pH (−0.6 pH units) (Kurihara

and Shirayama 2004; Carr et al. 2006; Byrne et al. 2009, 2010a), although conflicting results for the same species are also reported (cf. Havenhand et al. 2008; Byrne et al. 2009, 2010a). In *Heliocidaris erythrogramma* there was no effect of acidification (pH 7.6/P_{CO_2} 1800 ppm) even at very low sperm concentrations (10 sperm ml^{-1}) (Byrne et al. 2010a), whereas for *Strongylocentrotus franciscanus* fertilization was sensitive to acidification as sperm concentrations were lowered (Reuter et al. accepted manuscript). Impaired fertilization in *Strongylocentrotus purpuratus*, *Hemicentrotus pulcherrimus*, *Arbacia punctulata* and *Echinometra mathaei* is occurs at pH 7.0–7.4, acidification levels projected for 2300 and beyond (IPCC 2007). If we had used more severe conditions as in these other studies of far-future ocean conditions we may have seen differences in the resilience of fertilization in the species investigated here. Studies on the influence of pH using acid or samples of seawater from field sites known to have low pH show a similar tolerance of fertilization in echinoids (Smith and Clowes 1924; Riveros et al. 1996).

Near-future acidification levels did not significantly impair fertilization in *Haliotis coccoradiata*, as found for other molluscs (*Crossostrea gigas*, *Mytilus galloprovincialis*) where CO_2 was used to adjust pH (down to pH 7.4) (Kurihara 2008; Havenhand and Schlegel 2009), but not for *Saccostrea glomerata* where acid was used to adjust pH (Parker et al. 2009). Conflicting results for the same species are also reported (Kurihara 2008; Havenhand and Schlegel 2009; Parker et al. pers comm).

The gonads of marine invertebrates have a high internal P_{CO_2}/low pH that maintains gametes in a quiescent state prior to spawning reducing respiration and maintaining energy stores (Chia and Bickell 1983; Bookbinder and Shick 1986; Johnson et al. 1983). Increased P_{CO_2} thus decreases sperm swimming, as shown in motility studies (Havenhand et al. 2008; Morita et al. 2010). In nature the narcotic effect of hypercapnia on sperm motility is over ridden by the respiratory dilution effect of seawater (increased oxygen tension) (Chia and Bickell 1983). Egg jelly peptides also promote sperm motility at low pH, a phenomenon reported for molluscs, echinoderms and ascidians (Ward et al. 1985; Bolton and Havenhand 1996; Riffell et al. 2002; Darszon et al. 2008). The fertilization biology of marine invertebrates is characterised by acidic conditions (e.g. low gonad pH, low pH egg jelly, egg acid secretion) (Paucellier and Doree 1981; Holland et al. 1984). These features may have contributed to the tolerance of fertilization to low pH/high P_{CO_2} in the species investigated here.

The robust nature of fertilization in *Heliocidaris erythrogramma* to near-future ocean acidification (Byrne et al. 2009, 2010a, this study) contrasts with the findings of impaired fertilization in this species (Havenhand et al. 2008). Differences in experimental conditions may account for this contrast. The study by Havenhand et al. (2008) used single male–female crosses while we generated a population of embryos from multiple parents. Polyandry enhances fertilization in *H. erythrogramma* and reduces experimental variability in the percentage of fertilization (Evans and Marshall 2005). Strong maternal effects and intrinsic male effects can dominate the biology of single male–female crosses in *H. erythrogramma* and other species (Evans and Marshall 2005; Levitan and Ferrell 2006; Evans et al. 2007; Marshall and Evans 2007). Because polyandry occurs in the field and exerts strong selection on sperm performance (Levitan and Ferrell 2006), it seems the most appropriate model to test effects of climate change stressors on fertilization. Moreover, polyandry has the benefit of reducing experimental variability, lowering the number of replicates required. Different fertilization vessels and volumes (e.g. 500 ml in glass beakers used here vs. 2 ml in plastic well plates in Havenhand et al. 2008) may also contribute to different outcomes in fertilization studies. A recent comparison showed a higher fertilization in glass vials compared with plastic vessels (Lera et al. 2006). Fertilization success is also highly sensitive to gamete concentrations, gamete age, egg size, sperm to egg ratios and sperm-egg contact time (Levitan et al. 1991; Lera et al. 2006; Byrne et al. 2010a). As a result of the plethora of factors influencing fertilization rates and inter-laboratory variations encountered with fertilization tests for environmental monitoring (Bay et al. 1993; Lera et al. 2006), regulators moved to establish standard test protocols (e.g. ASTM 2004).

Thus far studies on impacts of climate change on fertilization in marine invertebrates are largely based on intertidal, estuarine and shallow water species that experience marked daily fluctuations in environmental pH (pH 6.9–10.1) and hypercapnia due to the diel interplay between respiration and photosynthesis (Ringwood and Keppler 2002; Björk et al. 2004; Wootten et al. 2008). These changes, together with marked thermal changes, far exceed projections for ocean surface waters by 2100 (IPCC 2007). Resilience of fertilization of the species investigated here may reflect their adaptation to the fluctuating temperature and pH conditions that they experience in nature. This would be especially true for the intertidal/shallow water species (*H. erythrogramma*, *C. rodgersii*, *P. regularis* and *H. coccoradiata*). Interestingly, fertilization in one of the deeper water species, *H. tuberculata* indicated greater sensitivity to low pH/high P_{CO_2}, albeit not significant. A recent study of a subtidal echinoid indicated that fertilization was sensitive to acidification as sperm concentrations were lowered (Reuter et al. accepted manuscript).

Our multistressor study indicates that fertilization in six coastal Australian species is not significantly impaired by near-future warming, acidification and hypercapnia. Although retrospective (post hoc) power analysis has been evoked to have confidence in such non-significant results (Havenhand and Schlegel 2009), this approach is statistically fraught (Underwood 1999; Hoenig and Heisey 2001; Lenth 2001). The weight of evidence thus far from recent climate change and previous ecotoxicology studies (reviewed in Byrne 2010) indicates that fertilization in coastal echinoderms and molluscs may not be a suitable experimental model to discern impacts of ocean change on marine life histories. However, empirical data for a greater diversity of species from different habitats are required to assess the performance of marine gametes as the ocean continues to warm and acidify and this needs to be facilitated by comparable experimental conditions. In addition, marine gametes are exposed to a multitude of stressors, the effects of which may be exacerbated by ocean change (Mead and Epel 1995; Przeslawski et al. 2005, 2008; Crain et al. 2008). For instance decreased pH increases the bioavailability of a suite of toxicants lethal to fertilization and development (Knutzen 1981; Byrne et al. 1988). Increased temperature also exacerbates the negative effect of toxicants (McLusky et al. 1986). For marine invertebrates, future prospects for the early life history stages are of particular concern because their sensitivity to climate change stressors may be the bottleneck for persistence of species in a changing ocean (Byrne 2010; Dupont et al. 2010). Development can fail at any stage and determination of the comparative sensitivities of embryos, larvae and juveniles to climate change stressors are needed to determine where vulnerabilities lie. As a first approach to identify vulnerable life history stages, embryonic development would be an appropriate experimental endpoint. For instance if embryonic development fails due to warming then the question of comprised larval calcification due to ocean acidification may not be relevant. An initial focus on early development will assist identification of species for which longer term experiments are warranted.

In the single study of the effects of near-future ocean warming and acidification (using CO_2 gas) on marine invertebrate embryos, warming was the most serious contemporary climate change stressor to *H. erythrogramma* (Byrne et al. 2009). Embryos of *H. erythrogramma* may not reach the skeleton forming stage in a warm ocean regardless of pH. For those embryos that reach the larval stage, increased acidification and P_{CO_2} may have a negative effect on larval physiology due to hypercapnia and decreased availability of the carbonate ions needed to produce the skeleton (Kurihara 2008; Dupont et al. 2008, 2010; Pörtner 2008; Clark et al. 2009; O'Donnell et al. 2010).

Understanding the vulnerabilities of the developmental stages integral to the persistence of marine populations is crucial as we endeavour to predict how marine populations and ecosystems will fare in the face of climate change. Multifactorial experiments with an initial focus on early developmental endpoints (e.g. cleavage, gastrulation), are likely to be most efficient and informative with regard to assessment of the interactive effects of climate change stressors on marine life histories and identification of vulnerable species.

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