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# Comparing the effect of elevated $pCO_2$ and temperature on the fertilization and early development of two species of oysters

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**Abstract** This study compared the synergistic effects of elevated $pCO_2$ and temperature on the early life history stages of two ecologically and economically important oysters: the Sydney rock oyster, *Saccostrea glomerata* and the Pacific oyster, *Crassostrea gigas*. Gametes, embryos, larvae and spat were exposed to four $pCO_2$ (375, 600, 750, 1,000 µatm) and four temperature (18, 22, 26, 30°C) levels. At elevated $pCO_2$ and suboptimal temperatures, there was a reduction in the fertilization success of gametes, a reduction in the development of embryos and size of larvae and spat and an increase in abnormal morphology of larvae. These effects varied between species and fertilization treatments with *S. glomerata* having greater sensitivity than *C. gigas*. In the absence of adaptation, *C. gigas* may become the more dominant species along the south-eastern coast of Australia, recruiting into estuaries currently dominated by the native *S. glomerata*.

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## Introduction

Recent studies have found that the early life history stages of estuarine and marine organisms, including gametes, embryos and particularly larvae are generally more sensitive to elevated $CO_2$ stress than adults (Havenhand et al. 2008; Parker et al. 2009). It is during the critical embryonic and larval stages that estuarine and marine organisms are perhaps most susceptible to environmental change because they are developing their physiological capacities (Pörtner et al. 1998), and it is during this stage that they commence the development of calcium carbonate ($CaCO_3$) shells and skeletons (Kurihara et al. 2007).

Heightened interest in determining the effect of elevated $CO_2$ stress on larval stages has arisen because current models are based on only a handful of empirical studies (Kikkawa et al. 2003; Kurihara et al. 2004, 2007; Kurihara and Shirayama 2004; Dupont et al. 2008; Havenhand et al. 2008; Kurihara and Ishimatsu 2008; Byrne et al. 2009; Parker et al. 2009; Watson et al. 2009). These studies on invertebrates have found reduced sperm motility and sperm speed of the sea urchin, *Heliocidaris erythrogramma* (Havenhand et al. 2008, but not in Byrne et al. 2009), and reduced egg production rates in the copepod, *Acartia steuri* (Kurihara et al. 2004), but not *Acartia tsuensis* (Kurihara and Ishimatsu 2008). Decreased fertilization of gametes has also been found in the sea urchins, *Echinometra mathaei* and *Hemicentrotus pulcherrimus* (Kurihara et al. 2004; Kurihara and Shirayama 2004) in the brittlestar, *Ophiothrix fragilis* (Dupont et al. 2008), in the Sydney rock oyster, *Saccostrea glomerata* (Parker et al. 2009; Watson et al. 2009), but not in the Pacific oyster, *Crassostrea gigas* (Kurihara et al. 2007; Havenhand and Schlegel 2009) or the mussel, *Mytilus galloprovincialis* (Kurihara et al. 2008). Studies have also found larvae of

sea urchins, *E. mathaei*, *H. pulcherrimus* (Kurihara et al. 2004; Kurihara and Shirayama 2004), oysters, *S. glomerata* (Parker et al. 2009) and *C. gigas*, (Kurihara et al. 2007) and the mussel, *M. galloprovincialis* (Kurihara et al. 2008) to be smaller in size with a greater incidence of abnormality when reared at elevated $pCO_2$. In one of the few studies published on the effect of elevated $pCO_2$ on vertebrates, Kikkawa et al. (2003) found increased mortality of larvae of red bream, *Pagrus major*, Japanese whiting, *Sillago japonica*, Japanese flounder, *Paralichthys olivaceus* and the eastern little tuna, *Euthynnus affinis*. More recently, the embryonic development, egg survival and hatching size of the orange clown fish, *Amphiprion percula* was not significantly affected by elevated $CO_2$ of 1,030 ppm (Munday et al. 2009a); however, during settlement the larvae exposed to ocean acidification showed impaired olfactory discrimination and homing ability (Munday et al. 2009b). Further, evidence on the effect of elevated $pCO_2$ on adult and larval estuarine and marine organisms suggests that the effect of elevated $pCO_2$ may be even more severe when combined with elevated temperature (Reynaud et al. 2003; Metzger et al. 2007; Parker et al. 2009). Such findings are a concern because over the last century, there has been a 0.74°C rise in surface ocean temperatures (Hoegh-Guldberg et al. 2007), and it is anticipated that surface global temperatures will rise a further 1–4°C by 2100 (IPCC: Solomon et al. 2007; Gooding et al. 2009). In one of the few studies to examine the synergistic impacts of elevated $pCO_2$ and temperature on marine organisms, Reynaud et al. (2003) found no reduction in calcification in the scleractinian coral, *Stylophora pistallata*, when reared at elevated $pCO_2$ but a 50% reduction in calcification when reared at elevated $pCO_2$ and elevated temperature. Similarly, Parker et al. (2009) found synergistic effects of elevated $pCO_2$ and temperature on fertilization, development and size of embryos and larvae of *S. glomerata.*

We have virtually no studies, however, which quantify and compare the impact of elevated $pCO_2$ and temperature within and between closely related marine species although the exception to this is Havenhand et al. (2008) and Byrne et al. (2009), who found differences in responses of *H. erythrogramma* in populations with a wide geographic separation. What still remains unclear is how elevated $pCO_2$ and temperature will impact across species. Some studies have shown that the impacts of elevated $pCO_2$ are similar across species (for example larvae of sea urchins, *E. mathaei*, *H. pulcherrimus*; Kurihara et al. 2004), while others have shown markedly different responses. In a recent study, Clark et al. (2009) compared the sensitivity of sea urchin species from different regions to determine whether they differed in response to elevated concentrations of $pCO_2$. They found that calcification was reduced by 13.8–36.9% in tropical (*Tripneustes grantilla*) and temperate (*Pseudechinus huttoni* and *Evechinus chloroticus*) species, but not in the polar species (*Sterechinus huttoni*; Clark et al. 2009). In view of the paucity of studies comparing responses of elevated $pCO_2$ amongst near and closely related species, this study was done to compare the effect of elevated $pCO_2$ and temperature on the fertilization and early development and any variability between two major and closely related commercial species of oysters, the Sydney rock oyster, *S. glomerata* and the Pacific oyster, *C. gigas*. The Sydney rock oyster is native to Australia and has been farmed on the central east coast of the country since the 1870s (Nell 1993). In contrast, *C. gigas* is native to Japan and has a worldwide distribution with successful introductions into France, New Zealand, Holland, England, Ireland, USA, Germany and Spain (Mitchell et al. 2000). *C. gigas* was introduced to Australia in the early 1940's and today it is farmed mainly in the south to south-eastern parts of Australia (Mitchell et al. 2000). Occupying hard substratum in estuarine locations (Lamprell and Healy 1998; Ross et al. 2009), both species are broadcast spawners, reproduce in the warmer spring/summer months and share a similar embryonic and larval development phase (Mitchell et al. 2000). Generally, however, the growth rates of *C. gigas* are faster giving it potentially a competitive advantage over the native *S. glomerata* (Nell et al. 1996; Heasman et al. 2000).

The aim of this study is to compare the effects of elevated $pCO_2$ and temperature on different species within the same group to provide a better understanding of how climate change will impact on ecosystem processes, sustaining aquacultural industries and assist in maintaining biodiverse, healthy ecosystems.

## Materials and methods

### Organism and treatments

Gravid *S. glomerata* were collected randomly from the four major oyster growing estuaries in New South Wales (NSW), Wallis Lake (32°10′S, 152°29′E), Port Stephens (32°72′S, 152°07′E), the Hawkesbury (33°30′S, 151°15′E) and Georges Rivers (34°00′S, 151°10′E) and *C. gigas* were collected from an aquaculture farm at Port Stephens, NSW, Australia (32°72′S, 152°07′E). Spawning was temperature induced for *S. glomerata*, and gametes were stripped from *C. gigas* to obtain spermatozoa and eggs. Sperm and eggs were collected from 10 males and 10 females from each species and were filtered through a 45-μm (sperm) and 63-μm (eggs) nylon mesh and were pooled separately. Viable spermatozoa were identified under the microscope as those that had normal motility. The concentration of

gametes was determined using a haemocytometer and Sedgwick–Rafter slide under a microscope for spermatozoa and eggs, respectively.

Three elevated concentrations of $pCO_2$ (600, 750 and 1,000 µatm), one ambient concentration of $pCO_2$ (current concentration of $pCO_2$: 375 µatm), one elevated temperature (30°C) and three natural spawning temperatures (18, 22 and 26°C) were selected for the study, based on projections by the IPCC (Houghton et al. 1996, 2001; Solomon et al. 2007) for likely ambient $pCO_2$ and temperature outcomes for 2100 (low, intermediate and high $CO_2$ emission scenarios). For a description of seawater chemistry, refer to Feely et al. (2004) and Orr et al. (2005).

Seawater (35 ppt) was collected from Little Beach (32°72′S, 152°07′E), Nelson Bay, NSW, Australia. Once back at the hatchery, the seawater was filtered using 1-µm nominal sized filter cartridges, and 110 mL of FSW was transferred into 120-mL sterile containers, which were subsequently sealed with screw capped lids to minimize gas exchange. The four $pCO_2$ concentrations used in this study were obtained via manipulation of the pH by direct bubbling of $CO_2$ into seawater. To determine the pH value corresponding to the appropriate $pCO_2$ levels, the total alkalinity (TA) was quantified in triplicate by Gran-titration (Table 1; Gran 1952; Butler 1982). Following the titration, the TA and selected $pCO_2$ values were entered into a $CO_2$ system calculation programme developed by Lewis and Wallace (1998), using the dissociation constants of Mehrbach et al. (1973). A randomized split plot design was used (Winer et al. 1991). To ensure that temperature remained at the treatment level throughout the experiment, thermostatically controlled immersion heaters (±0.5°C) were placed in separate water baths, with each of the three replicates with its own independent water bath and heater (12 water baths altogether). The pH of the seawater was measured using a combined pH electrode calibrated daily using NBS buffers.

## The effect of $pCO_2$ and temperature on the fertilization of gametes

To determine the effect of $pCO_2$ and temperature on the fertilization of *S. glomerata* and *C. gigas*, eggs and spermatozoa, obtained from 10 females and 10 males from each species, were incubated separately in ambient seawater (375 µatm, 25°C, 35 ppt) for 30 min. Although some studies pre-treat sperm, eggs or both in experimental seawater prior to fertilization (Carr et al. 2006; Hagström and Hagström 1959; Fujisawa 1989; Havenhand and Schlegel 2009), Byrne et al. (2009) found general trends in the

**Table 1** Experimental seawater conditions

| Temperature (°C) | Fertilization and D-veliger TA = 2,287 ± 15 µmol kg$^{-1}$ SW | | Umbonate TA = 2,284 ± 21 µmol kg$^{-1}$ SW | | Pediveliger TA = 2,288 ± 16 µmol kg$^{-1}$ SW | | Spat TA = 2,282 ± 19 µmol kg$^{-1}$ SW | |
|---|---|---|---|---|---|---|---|---|
| | $pH_{(NBS)}$ | $pCO_2$ (µatm) | $pH_{(NBS)}$ | $pCO_2$ (µatm) | $pH_{(NBS)}$ | $pCO_2$ (µatm) | $pH_{(NBS)}$ | $pCO_2$ (µatm) |
| 18 | 8.19 | 375 ± 20 | 8.19 | 375 ± 23 | 8.19 | 375 ± 43 | 8.19 | 375 ± 31 |
| 18 | 8.01 | 600 ± 38 | 8.01 | 600 ± 41 | 8.01 | 600 ± 41 | 8.01 | 600 ± 43 |
| 18 | 7.93 | 750 ± 35 | 7.93 | 750 ± 37 | 7.93 | 750 ± 47 | 7.93 | 750 ± 38 |
| 18 | 7.82 | 1,000 ± 37 | 7.82 | 1,000 ± 48 | 7.82 | 1,000 ± 38 | 7.82 | 1,000 ± 52 |
| 22 | 8.20 | 375 ± 17 | 8.20 | 375 ± 25 | 8.20 | 375 ± 46 | 8.20 | 375 ± 19 |
| 22 | 8.02 | 600 ± 40 | 8.02 | 600 ± 43 | 8.02 | 600 ± 52 | 8.02 | 600 ± 39 |
| 22 | 7.94 | 750 ± 39 | 7.94 | 750 ± 52 | 7.94 | 750 ± 37 | 7.94 | 750 ± 50 |
| 22 | 7.83 | 1,000 ± 20 | 7.83 | 1,000 ± 49 | 7.83 | 1,000 ± 40 | 7.83 | 1,000 ± 55 |
| 26 | 8.20 | 375 ± 24 | 8.20 | 375 ± 34 | 8.20 | 375 ± 44 | 8.20 | 375 ± 38 |
| 26 | 8.03 | 600 ± 56 | 8.03 | 600 ± 48 | 8.03 | 600 ± 29 | 8.03 | 600 ± 54 |
| 26 | 7.95 | 750 ± 41 | 7.95 | 750 ± 37 | 7.95 | 750 ± 35 | 7.95 | 750 ± 42 |
| 26 | 7.84 | 1,000 ± 53 | 7.84 | 1,000 ± 35 | 7.84 | 1,000 ± 54 | 7.84 | 1,000 ± 47 |
| 30 | 8.21 | 375 ± 19 | 8.21 | 375 ± 41 | 8.21 | 375 ± 26 | 8.21 | 375 ± 41 |
| 30 | 8.04 | 600 ± 36 | 8.04 | 600 ± 39 | 8.04 | 600 ± 33 | 8.04 | 600 ± 48 |
| 30 | 7.96 | 750 ± 48 | 7.96 | 750 ± 28 | 7.96 | 750 ± 29 | 7.96 | 750 ± 53 |
| 30 | 7.85 | 1,000 ± 56 | 7.85 | 1,000 ± 39 | 7.85 | 1,000 ± 48 | 7.85 | 1,000 ± 46 |

Total alkalinity (TA) calculated from the mean values obtained at each water change

The $pCO_2$ concentrations were determined using the $CO_2$ System Calculation Programme ($CO_2$ sys; Lewis and Wallace 1998) (±SE); Salinity = 35 ppt

*SW* seawater

response of gametes and embryos to temperature and pH perturbations were similar amongst species, despite different methodologies.

Following incubation, the eggs and spermatozoa were transferred into the $pCO_2$ and temperature treatments in each replicate 120-mL container to give a concentration of 50 eggs $mL^{-1}$ and $5 \times 10^7$ sperm $mL^{-1}$, respectively. After 2 h, the containers were inverted several times to ensure the embryos were dispersed evenly throughout the containers following which a 10 mL sub sample was taken from each container, and fertilization was stopped by the addition of 1 mL of 5% buffered formalin. Fertilization was quantified by observing if there was a cleavage plane present in the eggs (a total of 30 eggs were observed) in each 10 mL sample using a compound light microscope (*Leica* 100×) and the percentage fertilized calculated.

### The effect of $pCO_2$ and temperature on embryonic development and abnormality of D-veliger larvae following optimal and suboptimal fertilization

The early life cycle of these broadcast spawners is comprised of three free swimming larval stages lasting in total between 3 and 4 weeks and is highly dependant on temperature. These include D-veliger larvae (16–40 h), umbonate larvae (approximately 13 and 9 days for *S. glomerata* and *C. gigas*, respectively) and pediveliger larvae (approximately 22 and 16 days for *S. glomerata* and *C. gigas*, respectively). Following this time, the oysters develop into a sedentary juvenile spat (approximately 28 and 21 days for *S. glomerata* and *C. gigas*, respectively, Dinamani 1973). The deposition of a calcium carbonate shell begins during the late trochophore stage approximately 8–24 h after fertilization (Waller 1981; Hayakaze and Tanabe 1999; Kurihara et al. 2007) and therefore, $CO_2$-induced effects on calcification should be evident at the subsequent D-veliger stage.

To determine differences in the sensitivity of embryos and D-veliger larvae of *S. glomerata* and *C. gigas* exposed to elevated $pCO_2$ and temperature when fertilization occurs in optimal and suboptimal conditions, a two part experiment was set up.

In the first part of the experiment, fertilization occurred in the 'treatments' as described earlier. The embryos in the remaining 100 mL from the previous fertilization experiment were then allowed to develop in the various control and elevated $pCO_2$ and temperature treatments for 48 h. At the completion of this time, the experiment was stopped by the addition of 10 mL of 5% buffered formalin. The number of embryos that reached the D-veliger stage, the number of abnormal D-veligers (as described by His et al. 1997) and the length of the shell of thirty D-veligers (including normal and abnormal veligers) (O'Connor and Lawler 2004) in each replicate sample were quantified, using a Sedgwick–Rafter slide under a compound light microscope (*Leica* 100×). This part of the experiment was referred to as 'treatment' fertilization as it investigated the effect of $pCO_2$ and temperature on fertilization and embryonic development to the D-veliger stage.

In the second part of the experiment, fertilization of eggs and sperm occurred in ambient seawater (375 µatm, 25°C, 35 ppt). The remaining gametes that were not used in the first part of the experiment were pooled in 1 L of ambient FSW for 30 min (375 µatm, 25°C) to allow fertilization to take place. The fertilized embryos were then transferred into each of the selected $pCO_2$ and temperature treatments at a concentration of 50 eggs $mL^{-1}$, where they were left for 48 h until embryos had developed to the D-veliger larval stage. At the completion of this time, development was stopped by the addition of 10 mL of 5% buffered formalin. The total number of embryos that reached the D-veliger stage, the number of abnormal D-veligers (as described by His et al. 1997) and the length of the shell of the D-veligers (including normal and abnormal) were quantified for each replicate as described previously. This part of the experiment was referred to as 'ambient' fertilization as it investigated the effect of $pCO_2$ and temperature on embryonic development to the D-veliger stage, following fertilization at 'ambient' levels.

### The effect of $pCO_2$ and temperature on the size of larvae and spat

In addition to D-veligers, the effect of $pCO_2$ and temperature on shell length of *S. glomerata* and *C. gigas* was also determined for three other major developmental oyster stages including umbonate larvae, pediveliger larvae and spat. Oysters were maintained in the hatchery from fertilization through to the spat stage so that they were readily available for use in the experiment. Larvae were maintained in 1,000-L fibreglass tanks of 1 µm FSW (25°C, 35 ppt) at a concentration of 8 larvae $mL^{-1}$. Tanks were changed every 2 days and were thoroughly washed with Virkon S solution, rinsed with freshwater and left to air dry for at least 24 h (O'Connor et al. 2008). The screen size, feed rates and stocking densities used throughout the larval run were continually adjusted to suit the stage of development and size of larvae. When the larvae were large enough to be retained on a 212-µm screen and showed signs of settling (see O'Connor et al. 2008), they were treated with epinephrine bitartrate to induce settlement. The resulting spat were retained on 265-µm screens and were transferred into a spat bubbler system (O'Connor et al. 2008).

Umbonate and pediveliger larvae were collected at the beginning of each stage and retained on a 90-µm (mean starting size: 141.50 µm *S. glomerata*, 136.69 µm *C. gigas*) and 130-µm (mean starting size: 264.10 µm *S. glomerata*,

246.16 µm *C. gigas*) sieve (mesh), respectively. The larvae were then transferred into each of the $pCO_2$ and temperature treatments at a concentration of 2.5 larvae $mL^{-1}$ for the umbonate stage and 2 larvae $mL^{-1}$ for pediveligers. The larvae were fed (daily) on a known algal diet (15–40 $\times$ $10^5$ algal cells per day) of *C. muelleri*, *P. lutheri* and T. *Isochrysis aff. Galbana*, and daily water changes were made for each replicate sample. After 4 days, the experiments were stopped by the addition of 10 mL of 5% buffered formalin and the lengths of the shell of thirty larvae in each replicate sample were measured, using a Sedgwick–Rafter slide under a compound light microscope (*Leica* 100×). For the spat stage, relatively newly metamorphosed spat (2–4 week after metamorphosis) were collected when they had a mean size of 1,998.52 µm (*S. glomerata*) and 5,506.29 µm (*C. gigas*). The spat were transferred into each of the $pCO_2$ and temperature treatments at a concentration of 15 spat per container and were fed (daily) on a known algal diet of *C. muelleri*, *P. lutheri* and *T. Isochrysis aff. galbana*, and there were daily water changes for each replicate sample. As with the umbonate and pediveliger stage, the experiments were stopped after 4 day by the addition of 10 mL of 5% buffered formalin and the lengths of the shell of fifteen spat in each replicate sample were measured under a dissecting microscope (*Leica*). Results were graphed as shell 'growth' during the experiment by subtracting the mean starting size from the mean size at the completion of the experiment.

### Statistical analysis

To determine any significant differences between fertilization treatments, species, the percentage of gametes fertilized, shell growth of umbonate and pediveliger larvae and shell growth of spat were analyzed using a three-way ANOVA, where species was a fixed factor and $pCO_2$ and temperature were fixed and orthogonal factors, using SPSS 17.0 for windows (SPSS Australasia Pty. Ltd Chatswood, NSW, Australia). To determine any significant differences between fertilization treatments and species at the D-veliger stage, the number of embryos that reached the D-veliger stage, the percentage of abnormal D-veligers and shell length of D-veligers were analyzed using a four-way ANOVA, where species and fertilization type were fixed factors and $pCO_2$ and temperature were fixed and orthogonal factors. A randomized split plot design was used to calculate mean square ratios (Winer et al. 1991). Cochran's test was used to determine any heterogeneity of variances and data were transformed if significant. In instances where transformation did not correct heterogeneity, interpretation of the data was conservative because of the increased likelihood of Type I error (Underwood 1997). An SNK test was used to detect differences amongst means (Sokal and Rohlf 1995).

## Results

### The effect of $pCO_2$ and temperature on the fertilization of gametes

There was a significant interaction between 'species × temperature' and '$pCO_2$ × temperature' on the fertilization success of *S. glomerata* and *C. gigas* (Fig. 1; Table 2). Generally, as $pCO_2$ increased, the percentage fertilization of gametes significantly decreased. The optimal temperature for fertilization was 18, 22 and 26°C for *C. gigas* and 26°C for *S. glomerata* at control $pCO_2$. As temperature differed from this optimal, fertilization success was much greater in

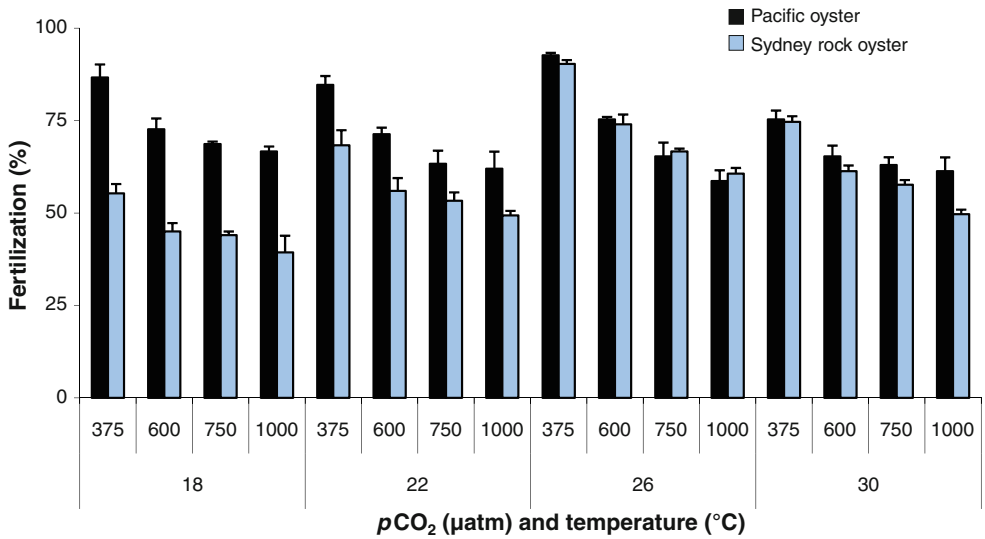


**Fig. 1** The percentage fertilization of gametes of *Crassostrea gigas* and *Saccostrea glomerata* reared at the $pCO_2$ (375, 600, 750, 1,000 µatm) and temperature (18, 22, 26, 30°C) treatments for 2 h; $n = 3$ (6/11/07)

**Table 2** Analysis of mean percentage fertilization of *Crassostrea gigas* and *Saccostrea glomerata* gametes reared at the $pCO_2$ (375, 600, 750, 1,000 µatm) and temperature (18, 22, 26, 30°C) treatments after 2 h; $n = 3$

| Source of variation | *df* | MS | *F* | *P* | SNK |
|---|---|---|---|---|---|
| Temperature (*T*) | 3 | 757.67 | 26.93 | *** | PO: 26 = 22 = 18 > 30°C |
| Bath (Temp) ($B_T$) | 8 | 28.14 | | | SRO: 26 > 30 > 22 > 18°C |
| Species (S) | 1 | 3,290.04 | 95.97 | *** | 18/22/30°C: PO > SRO |
| S × *T* | 3 | 871.15 | 25.41 | *** | 26°C: PO = SRO |
| $B_T$ × S | 8 | 34.28 | | | |
| $pCO_2$ | 3 | 2,292.58 | 124.79 | *** | |
| $pCO_2$ × *T* | 9 | 49.01 | 2.67 | * | |
| $B_T$ × $pCO_2$ | 24 | 18.37 | | | |
| S × $pCO_2$ | 3 | 11.46 | 0.57 | ns | |
| S × $pCO_2$ × *T* | 9 | 16.76 | 0.83 | ns | |
| $B_T$ × S × $pCO_2$ | 24 | 20.21 | | | |
| Total | 95 | | | | |

This was a three-way analysis with the species variable being fixed and $pCO_2$ and temperature being fixed and orthogonal

*PO* Pacific oyster, *SRO* Sydney rock oyster

Significance level indicated by asterisks, *ns* not significant, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. $C = 0.12$ ns

*C. gigas* than *S. glomerata* (Fig. 1; Table 2). After 2 h in the $pCO_2$ and temperature treatments, fertilization was reduced up to 26% for *C. gigas* and up to 51% for *S. glomerata* at elevated $pCO_2$ and suboptimal temperature (Fig. 1).

## The effect of $pCO_2$ and temperature on the development, abnormality and size of D-veliger larvae

### *Percentage development of D-veligers*

There was a four-way significant interaction between 'species × fertilization type × $pCO_2$ × temperature' on the percentage of embryos that developed to the D-veliger stage (Fig. 2; Table 3) with no effect of the waterbath in which replicates were immersed. Overall, at each temperature, the number of D-veligers decreased as $pCO_2$ increased (Fig. 2). In general, this decrease was greater for *S. glomerata* than *C. gigas*, particularly at the extremes of $pCO_2$ and temperature (Fig. 2, Table 3; SNK). The exception to this was 26°C at 375 µatm, where there was an equal percentage of D-veligers of *S. glomerata* and *C. gigas* which developed following 'ambient' and 'treatment' fertilization. The number of D-veligers was greatest at 26°C and 375 µatm for both species, with up to 100% (SE ± 0%) development. As temperature and $pCO_2$ deviated from this optimum the number of D-veligers was reduced. In general, the extent of this reduction was greatest following 'treatment' fertilization. The number of Pacific oyster embryos developing to the D-veliger stage was least at 18°C and 1,000 µatm with a 35% (SE ± 3%) and 44% (SE ± 4%) reduction after 48 h at 'ambient' and 'treatment' fertilization, respectively (Fig. 2). The number of Sydney rock oyster embryos developing to the D-veliger stage was least at 18°C and 1,000 µatm following 'ambient' fertilization and 30°C at 750–1,000 µatm following 'treatment' fertilization. The number of embryos developing to this stage was reduced by up to 58% (SE ± 3%) at 'ambient' fertilization and 100% at 'treatment' fertilization (Fig. 2), with the combined effects of elevated $pCO_2$ and temperature in the latter experiment having lethal effects on the larvae (Fig. 2).

### *Abnormality of D-veligers*

There were three-way and four-way significant interaction between 'fertilization × $pCO_2$ × temperature' and 'species × fertilization type × temperature' on the percentage of abnormal D-veligers after 24 h and between 'species × fertilization type × $pCO_2$ × temperature' at 48 h (Fig. 3; Table 3) with once again no effect of the waterbath in which replicates were immersed. Overall, the percentage of abnormal D-veligers of *S. glomerata* and *C. gigas* increased with increased $pCO_2$ across each of the experimental temperatures (Fig. 3). The number of abnormal D-veligers was lowest at both 22 and 26°C (Fig. 3). In general, the number of abnormal D-veligers was greater in the Sydney rock oyster compared to the Pacific oyster, particularly as temperature deviated from 26°C (Fig. 3; Table 3). In addition, embryos fertilized in the 'treatment' conditions were generally affected more than those fertilized in 'ambient' conditions. At the completion of the experiment (48 h), the number of abnormal D-veligers for the Pacific oyster was greatest at the extreme temperatures of 18 and 30°C and 1,000 µatm with up to a 53 and 40%

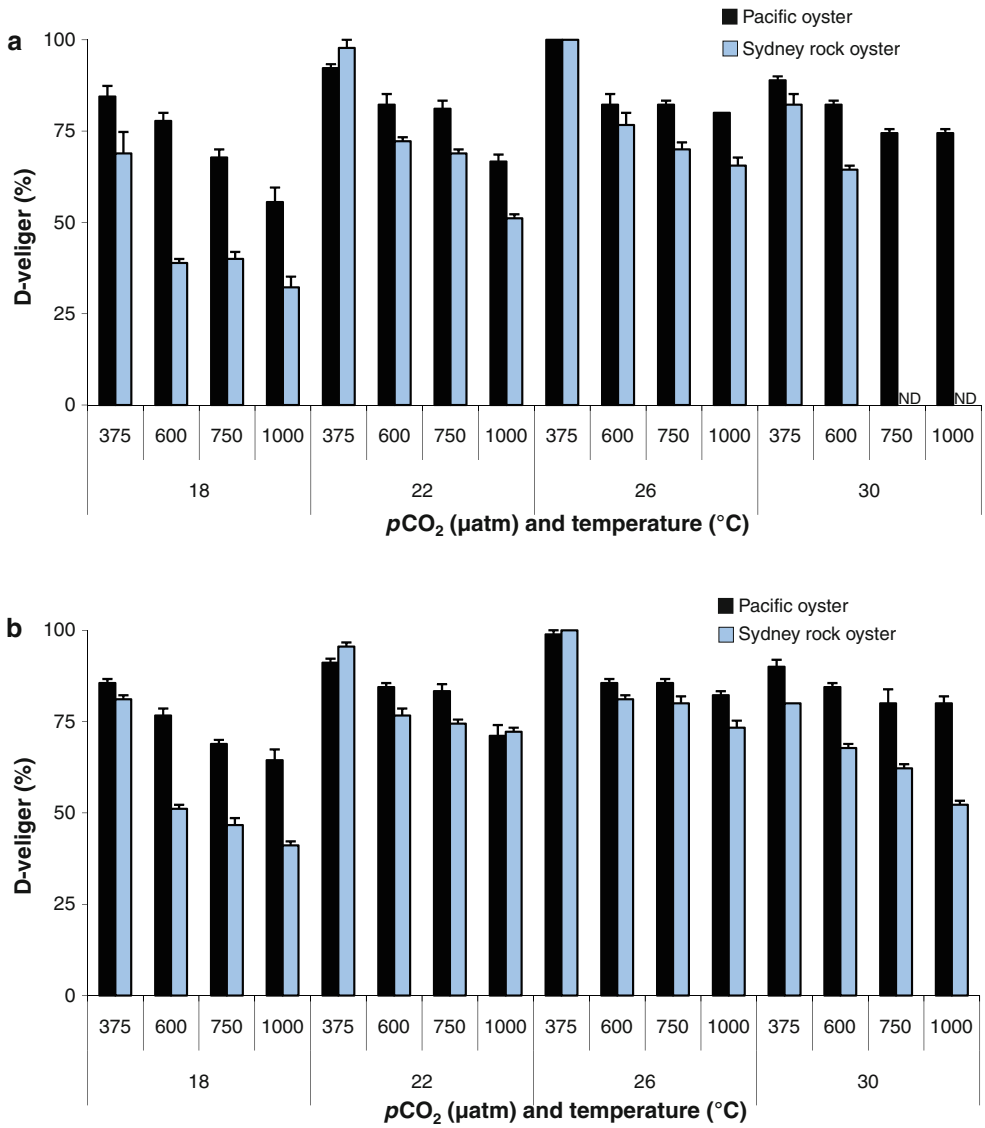


**Fig. 2** The percentage of *Crassostrea gigas* and *Saccostrea glomerata* embryos to reach the D-veliger stage after 48 h in the $pCO_2$ (375, 600, 750, 1,000 µatm) and temperature (18, 22, 26, 30°C) treatments following **a** 'treatment' fertilization and **b** 'ambient' fertilization. *ND* no development. Percentage of D-veligers includes those which were abnormal; $n = 3$ (6/11/07–8/11/07)

increase in abnormality following 'treatment' and 'ambient' fertilization, respectively, compared to the control (26°C, 375 µatm: 3% (SE ± 1%)). For the Sydney rock oyster, the number of abnormal D-veligers was greatest at 18°C and 600–1,000 µatm. In these treatments, there was up to 100% abnormality irrespective of whether fertilization occurred in the 'treatments' or in 'ambient' conditions (Fig. 3).

## The effect of $pCO_2$ and temperature on the size of larvae and spat

### *D-veligers*

The size of D-veligers of *S. glomerata* and *C. gigas* was also affected by elevated $pCO_2$, temperature and fertilization treatment. There was a significant three-way interaction between 'species × $pCO_2$ × fertilization type' on the shell length of D-veligers (Fig. 4; Table 3) with once again no effect of the waterbath in which replicates were immersed. Overall, as $pCO_2$ increased, the size of D-veligers decreased across each experimental temperature. Shell length was greatest at 26°C at 375 µatm for both species. At these optimal conditions, shell length was similar for *C. gigas* and *S. glomerata*. As temperatures deviated from optimal, however, *S. glomerata* D-veligers were smaller (Fig. 4; Table 3). For both species, D-veligers that were fertilized in the 'treatments' were generally smaller than those fertilized in 'ambient' conditions (Fig. 4). At the completion of the experiment (48 h), the size of D-veligers was least at 18°C at

**Table 3** Analysis of mean number of embryos to reach the D-veliger stage, mean number of D-veligers that were abnormal and mean shell length of D-veligers of *Crassostrea gigas* and *Saccostrea glomerata* when reared at the $pCO_2$ (375, 600, 750, 1,000 µatm) and temperature (18, 22, 26, 30°C) treatments following 'ambient' and 'treatment' fertilization for 48 h; $n = 3$

| Source of variation | *df* | % D-veliger ($C = 0.14$; $P < 0.01$) | | | % Abnormality ($C = 0.08$ ns) | | | Shell length ($C = 0.19$; $P < 0.05$) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | MS | *F* | *P* | MS | *F* | *P* | MS | *F* | *P* |
| Temperature (*T*) | 3 (2) | 5,322.54 | 234.61 | *** | 8,291.06 | 339.90 | *** | 1,332.15 | 1,909.67 | *** |
| Bath (Temp) ($B_T$) | 8 | 22.69 | | | 24.39 | | | 0.70 | | |
| Species (S) | 1 | 12,675.00 | 648.51 | *** | 17,438.96 | 1,147.60 | *** | 1,130.25 | 526.95 | *** |
| S × *T* | 3 | 1,867.15 | 95.48 | *** | 2,697.62 | 177.52 | *** | 262.32 | 122.30 | *** |
| $B_T$ × S | 8 | 19.56 | | | 15.20 | | | 2.15 | | |
| Fertilization (F) | 1 | 2,552.24 | 821.61 | *** | 2,033.41 | 218.59 | *** | 99.98 | 196.14 | *** |
| F × *T* | 3 | 402.06 | 115.82 | *** | 97.67 | 21.88 | ** | 1.19 | 2.34 | ns |
| $B_T$ × F | 8 | 3.47 | | | 9.30 | | | 0.51 | | |
| $pCO_2$ | 3 | 7,822.74 | 641.47 | *** | 15,362.14 | 1,199.48 | *** | 476.18 | 221.59 | *** |
| $pCO_2$ × *T* | 9 | 210.34 | 17.25 | *** | 60.37 | 4.71 | ns | 2.24 | 1.04 | ns |
| $B_T$ × $pCO_2$ | 24 | 12.20 | | | 12.81 | | | 2.15 | | |
| S × F | 1 | 1,301.77 | 96.14 | *** | 190.39 | 10.96 | * | 4.76 | 3.18 | ns |
| S × $pCO_2$ | 3 | 1,046.50 | 113.47 | *** | 1,724.10 | 135.37 | *** | 39.25 | 17.97 | *** |
| F × $pCO_2$ | 3 | 469.20 | 73.31 | *** | 117.66 | 9.81 | *** | 4.31 | 2.95 | ns |
| S × F × *T* | 3 | 302.08 | 22.31 | *** | 139.14 | 6.61 | * | 0.46 | 0.31 | ns |
| $B_T$ × S × F | 8 | 13.54 | | | 12.74 | | | 1.50 | | |
| S × $pCO_2$ × *T* | 9 | 260.57 | 28.25 | *** | 139.14 | 10.92 | *** | 4.78 | 2.19 | ns |
| $B_T$ × S × $pCO_2$ | 24 | 9.22 | | | 12.74 | | | 2.18 | | |
| F × $pCO_2$ × *T* | 9 | 205.20 | 32.06 | *** | 128.11 | 10.68 | *** | 1.24 | 0.85 | ns |
| $B_T$ × F × $pCO_2$ | 24 | 6.40 | | | 12.00 | | | 1.46 | | |
| S × F × $pCO_2$ | 3 | 212.06 | 17.23 | *** | 45.36 | 2.78 | ns | 2.42 | 3.04 | ns |
| S × F × $pCO_2$ × *T* | 9 | 192.74 | 15.66 | *** | 264.18 | 16.21 | *** | 1.42 | 1.79 | ns |
| $B_T$ × S × F × $pCO_2$ | 24 | 12.31 | | | 16.30 | | | 0.80 | | |
| Total | 191 | | | | | | | | | |
| SNK | Treatment fertilization | | Ambient fertilization | | Treatment fertilization | | Ambient fertilization | | Treatment fertilization and ambient fertilization | |
| | 375 µatm | | 375 µatm | | 375 µatm | | 375 µatm | | 375 µatm | |
| | (18/22/30°C): PO > SRO | | (18–26°C): PO = SRO | | (18°C): PO < SRO | | (18–26°C): PO = SRO | | (18/22°C): PO > SRO | |
| | (26°C): PO = SRO | | (30°C): PO > SRO | | (22/26°C): PO = SRO | | 600 µatm | | (26°C): PO = SRO | |
| | 600 µatm | | 600 µatm | | 600 µatm | | (18–26°C): PO < SRO | | 600 µatm | |
| | (18–30°C): PO > SRO | | (18/22/30°C): PO > SRO | | (18–22°C): PO < SRO | | 750 µatm | | (18–22°C): PO > SRO | |
| | 750 µatm | | (26°C): PO = SRO | | (26°C): PO = SRO | | (18–26°C): PO < SRO | | (26°C): PO = SRO | |
| | (18–30°C): PO > SRO | | 750 µatm | | 750 µatm | | 1,000 µatm | | 750 µatm | |
| | 1,000 µatm | | (18–30°C): PO > SRO | | (18–26°C): PO < SRO | | (18–26°C): PO < SRO | | (18–26°C): PO > SRO | |
| | (18–30°C): PO > SRO | | 1,000 µatm | | 1,000 µatm | | | | 1,000 µatm | |
| | | | (18/26/30°C): PO > SRO | | (18–26°C): PO < SRO | | | | (18–26°C): PO > SRO | |

This was a four-way analysis with the species variable being fixed and fertilization type, $pCO_2$ and temperature being fixed and orthogonal. Since no D-veligers developed at 30°C at 750–1,000 µatm for the SRO, this temperature was excluded from the analysis of abnormality and shell length

*PO* Pacific oyster, *SRO* Sydney rock oyster

Significance level indicated by asterisks, *ns* not significant, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

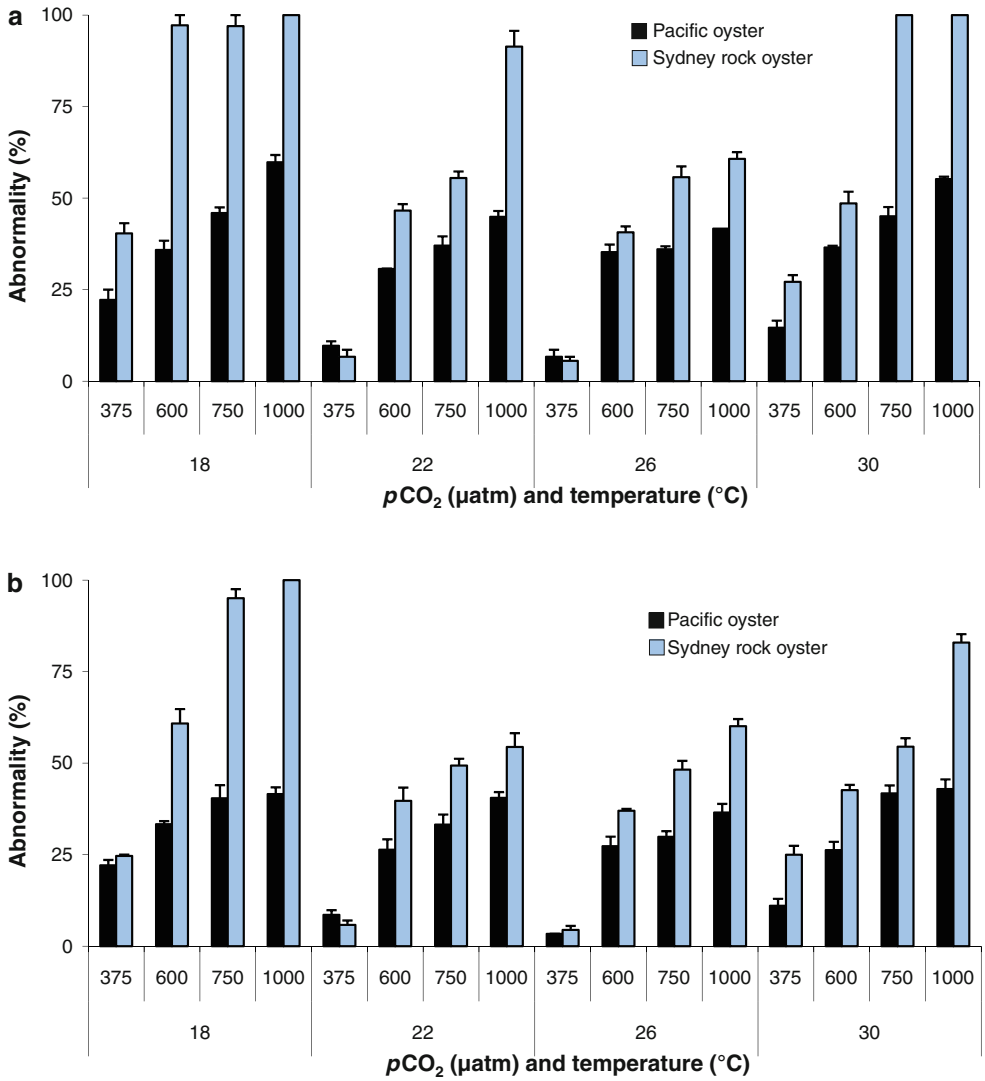


**Fig. 3** The percentage of *Crassostrea gigas* and *Saccostrea glomerata* embryos to reach the D-veliger stage that were abnormal after 48 h in the $pCO_2$ (375, 600, 750, 1,000 µatm) and temperature (18, 22, 26, 30°C) treatments following **a** 'treatment' fertilization and **b** 'ambient' fertilization. Percentage abnormality calculated by dividing the number of abnormal larvae with the number of larvae which developed in each replicate treatment; $n = 3$ (6/11/07–8/11/07)

750–1,000 µatm for the Pacific oyster and 18°C at 1,000 µatm for the Sydney rock oyster. At these combinations, there was up to a 12 (16%) and 26 µm (34%) reduction in size for *C. gigas* and *S. glomerata*, respectively (Fig. 4).

*Umbonate, pediveligers and spat*

There was significant interaction between 'species × temperature' and '$pCO_2$ × temperature' for the size of umbonate larvae (Fig. 5; Table 4). There was also a significant interaction between 'species × $pCO_2$ × temperature' for the size of pediveliger larvae and spat (Figs. 6, 7; Table 4). In general, at each temperature, shell growth decreased as $pCO_2$ increased. The exception to this was at 18°C for umbonate larvae of both species and '30°C' and '18 and 26°C' for pediveliger larvae of *C. gigas* and *S. glomerata*, respectively, where there was no difference in shell growth across each $pCO_2$ (Figs. 5, 6, 7, Table 4; SNK).

The growth of umbonate larvae was greatest at 30°C at 375 µatm and least at 18°C at 375–1,000 µatm for both species (Fig. 5). At this stage of development, growth decreased with decreasing temperature and, with the exception of 18°C, increasing $pCO_2$. This decrease was greater for *S. glomerata* compared to *C. gigas* at each treatment (Fig. 5, Table 4; SNK). At 18°C, umbonate larvae of *S. glomerata* put on very little shell growth during the 4-day experiment, especially at higher $pCO_2$. In fact at 18°C and 750 µatm, larvae reduced in size, being smaller than their initial size at the beginning of the experiment (Fig. 5).

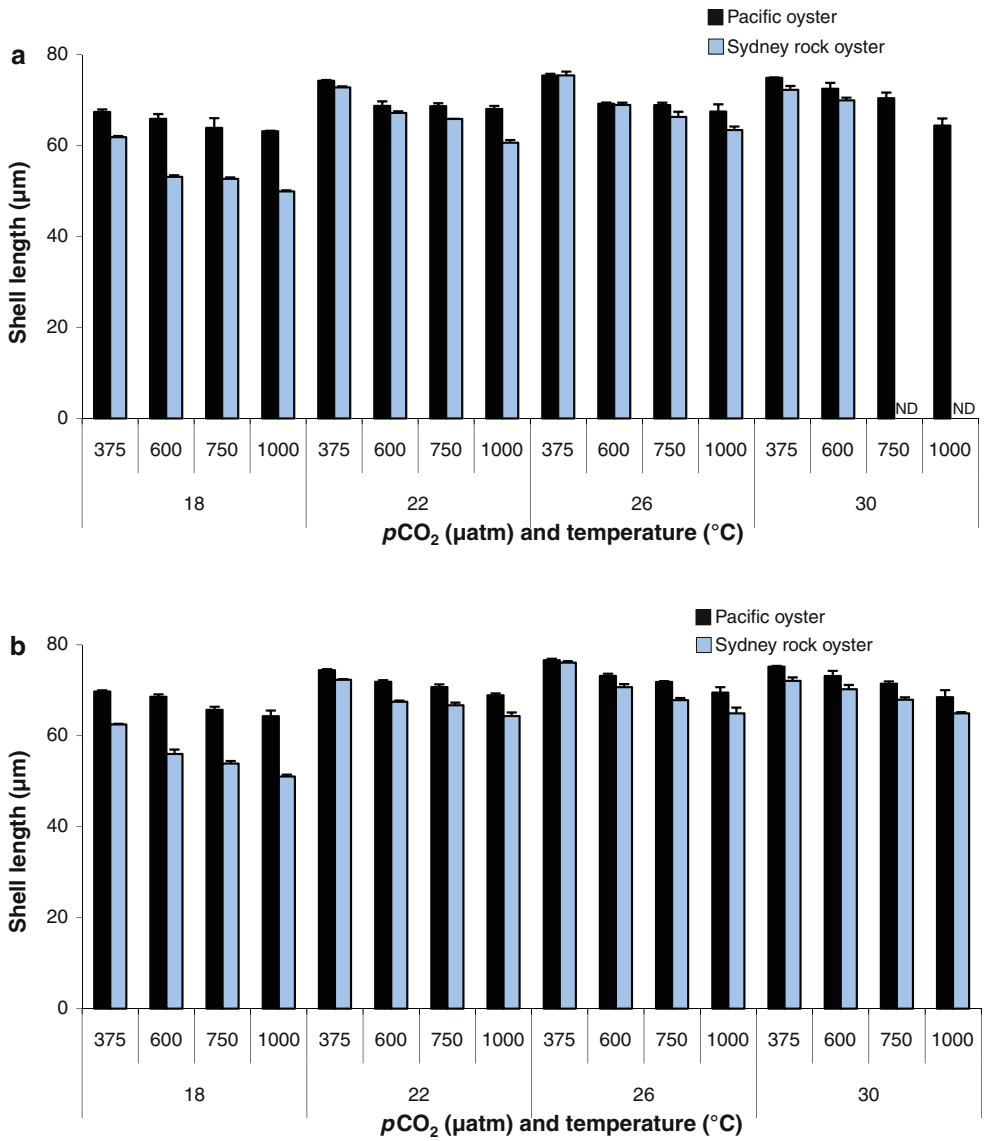


**Fig. 4** The mean shell length of D-veliger larvae of *Crassostrea gigas* and *Saccostrea glomerata* after 48 h in the $pCO_2$ (375, 600, 750, 1,000 µatm) and temperature (18, 22, 26, 30°C) treatments following **a** 'treatment' fertilization and **b** 'ambient' fertilization. *ND* no development; $n = 3$ (6/11/07–8/11/07)

The shell growth of pediveliger larvae was greatest at 30°C at 375 µatm for *S. glomerata* and 26°C at 375 µatm for *C. gigas* and shell growth was least at 18°C at 600–1,000 µatm and at 22°C at 750–1,000 µatm for both species (Fig. 6, Table 4). At this stage of development, the shell growth of both species was similar; however, at the $pCO_2$ and temperature extremes, *S. glomerata* was once again slightly smaller than *C. gigas* (Fig. 6, Table 4; SNK).

Finally, as with the larval stages, the shell growth of spat decreased significantly with increased $pCO_2$ and temperature. Shell growth of spat was greatest at 26°C at 375 µatm for both species (695 ± SE 70 µm *S. glomerata*; 949 ± SE 25 µm *C. gigas*) and smallest at 18°C at 600–1,000 µatm and at 22°C at 750–1,000 µatm for *S. glomerata* and 30°C at 750–1,000 µatm for *C. gigas*. At these $pCO_2$ and temperature combinations, shell growth was reduced by up to 556 µm (80%) and 898 µm (95%) for *S. glomerata* and *C. gigas*, respectively (Fig. 7). At optimal conditions, shell growth of *C. gigas* spat was greater than that of *S. glomerata*. At the elevated temperature of 30°C and 600–1,000 µatm, however, this trend was reversed as *C. gigas* were significantly smaller (Fig. 7, Table 4).

## Discussion

This study found that exposure of the early life history stages of the Pacific oyster, *C. gigas* and the Sydney rock

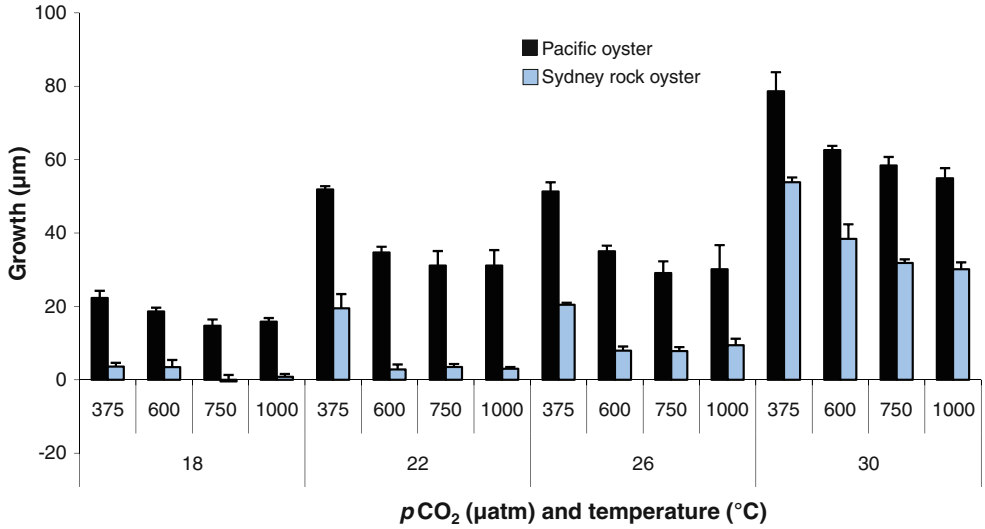


**Fig. 5** The mean shell growth of umbonate larvae of *Crassostrea gigas* and *Saccostrea glomerata* after 4 days in the $pCO_2$ (375, 600, 750, 1,000 µatm) and temperature (18, 22, 26, 30°C) treatments; $n = 3$ (6/01/08–10/01/08)

**Table 4** Analysis of mean shell growth of *Crassostrea gigas* and *Saccostrea glomerata* umbonate, pediveligers and spat after 4 days at the $pCO_2$ (375, 600, 750, 1,000 µatm) and temperature (18, 22, 26, 30°C) treatments; $n = 3$

| Source of variation | *df* | Umbonate ($C = 0.21$; $P < 0.05$) | | | Pediveliger ($C = 0.58$ ns) | | | Spat ($C = 0.16$ ns) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | MS | *F* | *P* | MS | *F* | *P* | MS | *F* | *P* |
| Temp (*T*) | 3 | 7,247.24 | 280.97 | *** | 808.13 | 78.60 | *** | 231,852.14 | 60.36 | *** |
| Bath (Temp) ($B_T$) | 8 | 25.79 | | | 10.28 | | | 3,841.11 | | |
| Species (S) | 1 | 13,842.24 | 712.69 | *** | 4,496.19 | 912.66 | *** | 43,748.71 | 17.11 | ** |
| S × T | 3 | 203.12 | 10.46 | ** | 20.51 | 4.16 | * | 61,317.66 | 23.99 | *** |
| $B_T$ × S | 8 | 19.42 | | | 4.93 | | | 2,556.34 | | |
| $pCO_2$ | 3 | 1,335.03 | 64.42 | *** | 316.55 | 42.76 | *** | 854,550.96 | 230.49 | *** |
| $pCO_2$ × *T* | 9 | 87.04 | 4.20 | ** | 16.55 | 2.24 | ns | 33,526.59 | 9.04 | *** |
| $B_T$ × $pCO_2$ | 24 | 20.73 | | | 7.40 | | | 3,707.53 | | |
| S × $pCO_2$ | 3 | 25.28 | 1.64 | ns | 11.83 | 1.65 | ns | 87,823.10 | 47.88 | *** |
| S × $pCO_2$ × *T* | 9 | 8.55 | 0.55 | ns | 23.32 | 3.25 | * | 18,488.67 | 10.08 | *** |
| $B_T$ × $pCO_2$ × *T* | 24 | 15.45 | | | 7.18 | | | 1,834.39 | | |
| Total | 95 | | | | | | | | | |
| SNK | | 18/22/26/30°C: PO > SRO | | | 375 µatm | | | 375 µatm | | |
| | | PO: 30 > 26 = 22 > 18°C | | | (18/26°C): PO > SRO | | | (18–30°C): PO > SRO | | |
| | | SRO: 30 > 26 > 22 > 18°C | | | (22/30°C): PO = SRO | | | 600 µatm | | |
| | | | | | 600 µatm | | | (18/22°C): PO = SRO | | |
| | | | | | (18/26°C): PO = SRO | | | (26/30°C): PO > SRO | | |
| | | | | | (22/30°C): PO > SRO | | | 750 µatm | | |
| | | | | | 750 µatm | | | (18/22°C): PO = SRO | | |
| | | | | | (18/30°C): PO > SRO | | | (22/26°C): PO > SRO | | |
| | | | | | (22/26°C): PO = SRO | | | 1,000 µatm | | |
| | | | | | 1,000 µatm | | | (18/22/26°C): PO = SRO | | |
| | | | | | (18/30°C): PO > SRO | | | (30°C): PO > SRO | | |
| | | | | | (22/26°C): PO = SRO | | | | | |

This was a three-way analysis with the species variable being fixed and $pCO_2$ and temperature being fixed and orthogonal

*PO* Pacific oyster, *SRO* Sydney rock oyster

Significance level indicated by *asterisks*, *ns* not significant, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

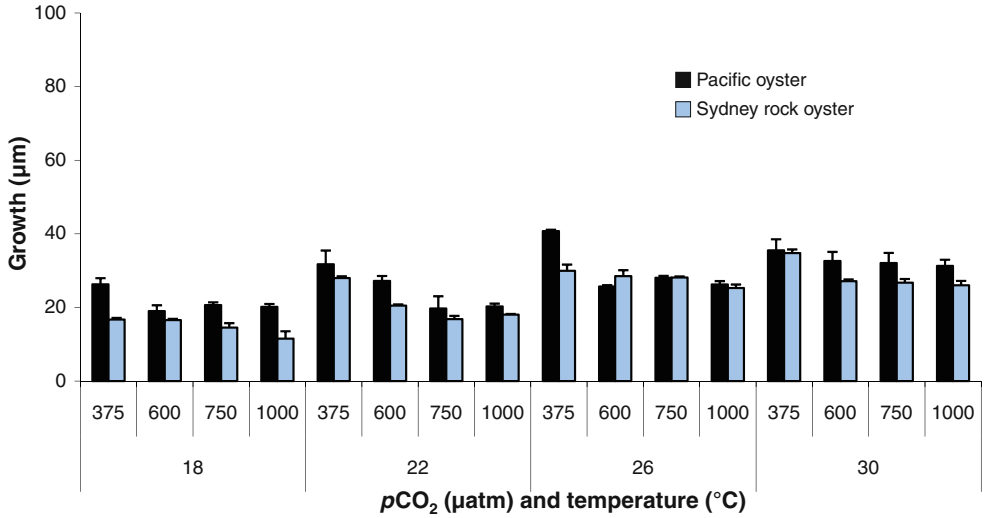


**Fig. 6** The mean shell growth of pediveliger larvae of *Crassostrea gigas* and *Saccostrea glomerata* after 4 days in the $pCO_2$ (375, 600, 750, 1,000 µatm) and temperature (18, 22, 26, 30°C) treatments; $n = 3$ (16/01/08–20/01/08)

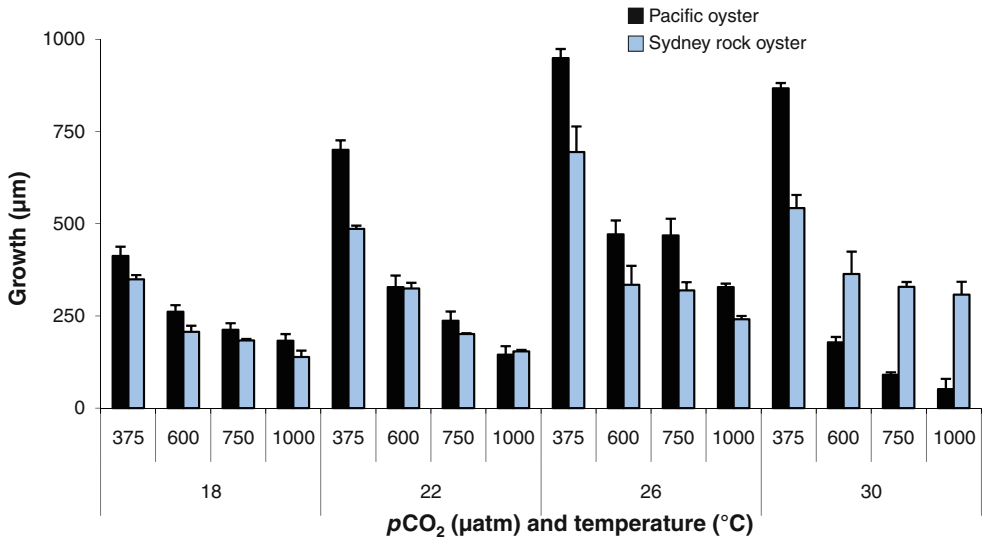


**Fig. 7** The mean shell growth of spat of *Crassostrea gigas* and *Saccostrea glomerata* after 4 days in the $pCO_2$ (375, 600, 750, 1,000 µatm) and temperature (18, 22, 26, 30°C) treatments; $n = 3$ (15/03/09–19/03/09)

oyster, *S. glomerata* to elevated $pCO_2$ and temperature has deleterious effects on fertilization, development and size with the extent of these effects varying between the two species.

## The effect of $pCO_2$ and temperature on the fertilization of gametes

Fertilization success of *C. gigas* and *S. glomerata* gametes was reduced by up to 26 and 51%, respectively, at elevated $pCO_2$ of 1,000 µatm and suboptimal temperature compared to the controls (90–92%: 375 µatm, 26°C). This result differs from those of previous studies on oysters which found no effect of elevated $pCO_2$ on the fertilization success of *C. gigas* (Kurihara et al. 2007; Havenhand and Schlegel 2009). The difference in response of *C. gigas* in this study may be the result of intraspecific differences amongst oyster populations. *C. gigas* is a widely farmed aquaculture species with a worldwide distribution. This study is the first to find a significant difference in the response of Australian populations of *C.gigas* to elevated $pCO_2$ compared to the non-significant responses of the Japanese (Kurihara et al. 2007; albeit at considerably greater $pCO_2$ level) and Swedish (Havenhand and Schlegel 2009 at comparable $pCO_2$ level) oyster populations. A similar difference in response amongst geographical

locations was found in the sea urchin, *H. erythrogramma* (Havenhand et al. 2008; Byrne et al. 2009). Elevated $pCO_2$ had no effect on the fertilization success of an Australian population of the sea urchin (Byrne et al. 2009), but had a significant reduction in the fertilization success of a Swedish population (Havenhand et al. 2008). The difference in results suggests that given no differences in methodology that responses may be due to adaptive capacity of a species to climatic conditions which differ between populations with a large geographic distribution (Jansen et al. 2007). Previously, it was believed that the major effects of elevated $pCO_2$ on oysters began during the late trochophore stage, coinciding with the onset of shell mineralization (calcification) (Kurihara et al. 2007). In contrast, this study shows that the effects of elevated $pCO_2$ occur much earlier and are thus not solely related to changes in calcification. The reduction of fertilization success shown in this study may have occurred because the speed of motile sperm, a pivotal factor controlling fertilization success in many marine organisms, may have been reduced (Christen et al. 1983; Havenhand et al. 2008). Havenhand et al. (2008) showed that the sperm motility, sperm speed and cleavage of the eggs of the sea urchin, *H. erythrogramma* were reduced upon exposure to elevated $pCO_2$. Reductions in fertilization success and cleavage speed have also been reported in the sea urchins, *H. pulcherrimus* and *E. mathaei* when exposed to elevated $CO_2$ between 500 and 2,000 ppm (Kurihara et al. 2004; Kurihara and Shirayama 2004). Reduced hatching success has also been documented for the copepods, *A. erythraea* (2,000–10,000 ppm, Kurihara et al. 2004) and *C. finmarchicus* (8,000 ppm, Mayor et al. 2007), but not for *A. tsuensis* (Kurihara and Ishimatsu 2008) in response to elevated $CO_2$. The level of $CO_2$ used in many of these studies is much higher than those used in this study. Reductions in the number of eggs that reach the cleavage stage has been attributed to a drop in protein synthesis and an increase in mitochondrial abnormalities induced by exposure to elevated $pCO_2$ (Grainger et al. 1979; Pagano et al. 1985a, b; Cipollaro et al. 1986; Kurihara and Shirayama 2004). While the fertilization success of both species in this study was significantly affected by elevated $pCO_2$, the reduction in fertilization success seen in *S. glomerata* was nearly double that of *C. gigas*.

Fertilization success of *C. gigas* and *S. glomerata* was significantly affected by temperature interacting with $pCO_2$. The optimal temperature for fertilization was 18–26°C for *C. gigas* and 26°C for *S. glomerata*. At temperatures above and below these levels, fertilization was significantly reduced. The exception to this was at 1,000 µatm, where the percentage fertilization was equal across each experimental temperature. Indeed, temperature is recognized as a limiting factor for marine organisms, particularly during the earliest developmental stages including eggs, zygotes and cleavage (Runnström 1927). Runnström (1927) suggested that the upper thermal limits of gametes and embryos may be as little as 1–3°C above an organism's natural temperature range. In this study, gametes of *S. glomerata* and *C. gigas* were successfully fertilized at 30°C, a temperature which is 4°C above their natural temperature range; however, the percentage of gametes fertilized at this temperature was significantly reduced. This suggests that fertilization success of both *C. gigas* and *S. glomerata* will likely decrease in a future warming ocean.

### The effect of $pCO_2$ and temperature on the percentage development and abnormality of D-veliger larvae

There was a significant negative effect of $pCO_2$ and suboptimal temperature on the number of embryos which developed to the D-veliger stage and this effect was generally greater for *S. glomerata*. Elevated $pCO_2$ and temperature (750–1,000 µatm, 30°C) had lethal effects on the larvae of *S. glomerata,* but not those of *C. gigas*. A previous study on the temperature and salinity tolerance of larvae of *S. glomerata* found that the optimum temperature for the survival of D-veligers was 26°C with survival compromised above and below this optimal level (Dove and O'Connor 2007). In contrast, a similar study on *C. gigas* found that larvae were able to survive over a wide range of temperatures (His et al. 1989).

Similar to Kurihara et al. (2004) and (2007), who found a similar increase in abnormal morphology of larvae in the sea urchins, *H. pulcherrimus* and *E. mathaei* and oyster larvae of *C. gigas* exposed to elevated $pCO_2$ many of the D-veligers in this study suffered significant abnormality. In this study, the percentage of abnormal larvae was similar for both species at the optimal temperature of 26°C, but as temperature deviated above or below the optimal, the percentage of abnormal larvae was greater in *S. glomerata* than in *C. gigas*. Oysters and other bivalves are believed to be particularly sensitive to $CO_2$-induced changes in pH mainly due to their inability to properly compensate for disturbances in extracellular ion and acid–base status (Michaelidis et al. 2005). Michaelidis et al. (2005) found that growth of juveniles and adults of the mussel, *M. galloprovincialis* was slowed during exposure to $CO_2$-acidified seawater (pH 7.3) which they attributed to the combination of a reduction in metabolic rate, net degradation of proteins and dissolution of the $CaCO_3$ shell to accumulate $HCO_3^-$ to compensate for acidosis. There have been limited studies which have considered the physiological effects of $CO_2$-induced hypercapnia on the early developmental stages of marine organisms, however, perhaps the negative effects of elevated $pCO_2$ on embryos,

larvae and spat in this study can be partially explained by similar disturbances in acid–base status.

Moreover, reduced development and growth of marine larvae at elevated $pCO_2$ could be attributed to a reduction in the rate of feeding. Dove and Sammut (2007a) found that the survival and growth of adult Sydney rock oysters was compromised during exposure to estuarine acidification caused by outflows from acid sulphate soils (ASS). In a similar study, they also found that exposure of adult Sydney rock oysters to ASS-affected waters (pH 5.5) caused a significant reduction in their filtration rate (Dove and Sammut 2007b). Similarly, Bamber (1987) found that feeding was inhibited, and tissue and shell growth was reduced in the clam, *Venerupis decussata* at pH $\leq$ 7.0. Further, feeding activity in *C. gigas* was suppressed at pH $<$ 7.0 and reduced in the oyster, *Ostrea edulis* and mussel, *Mytilus edulis* at pH $\leq$ 7.2 (Bamber 1990).

A reduction in the rate of feeding may also help to explain the difference in the response of *S. glomerata* and *C. gigas* to elevated $pCO_2$ and temperature. Despite sharing a similar biology, the sensitivity of *S. glomerata* and *C. gigas* to elevated $pCO_2$ and temperature in this study was markedly different with *S. glomerata* showing greater sensitivity. Already, *C. gigas* has a competitive advantage over *S. glomerata*. Bayne (2002) attributed this advantage to faster rates of feeding together with greater metabolic efficiencies of both feeding and growth. In addition, organisms with a broader global geographical distribution such as *C. gigas* typically have a wider temperature tolerance (Dove and O'Connor 2007).

### The effect of 'ambient' and 'treatment' fertilization on D-veligers

The results of this study indicate that acute exposure to near-future elevations of $pCO_2$ and temperature has deleterious effects on the early life history stages of both *C. gigas* and *S. glomerata.* There is now a need to determine the chronic effect of an acidifying, warming ocean to identify whether oysters have the potential to acclimate or adapt. Relatively few studies have investigated the long-term impacts of elevated $pCO_2$ on marine organisms (Langdon et al. 2000; Collins and Bell 2004; Langenbuch and Pörtner 2004; Michaelidis et al. 2005; Fine and Tchernov 2007; Jokiel et al. 2008; Melzner et al. 2009) and even fewer have investigated its long-term impacts in synergy with changes in temperature (Anthony et al. 2008; Gooding et al. 2009; Martin and Gattuso 2009). In species such as the marine worm, *S. nudus*, acute exposure to elevated $pCO_2$ was initially tolerated through a reduction in metabolic rate. Following a more chronic (7 week) exposure, however, elevated $pCO_2$ caused 100% mortality (Langenbuch and Pörtner 2004). In contrast, in species such as the red coralline alga, *L. cabiochae*, elevated $pCO_2$ caused a reduction in calcification during the first month of exposure, but no reduction in calcification in the following 11 months (at ambient temperature) (Martin and Gattuso 2009). In this study, a two part experiment was used to test the effects of elevated $pCO_2$ and temperature on embryonic development to the D-veliger stage. In the first part of the experiment known as 'treatment' fertilization, oysters were exposed to the $pCO_2$ and temperature treatments for both fertilization and development to the D-veliger stage. In the second part of the experiment known as 'ambient' fertilization, oysters were exposed to the $pCO_2$ and temperature treatments for development to the D-veliger stage only, with fertilization occurring in ambient seawater (25°C, 375 µatm). The results showed that in general, the effects of elevated $pCO_2$ and temperature on the D-veliger stage were greater following 'treatment' fertilization compared to 'ambient' fertilization, particularly for *S. glomerata* where the combination of elevated $pCO_2$ (750–1,000 µatm) and elevated temperature (30°C) had lethal effects on the larvae following 'treatment' fertilization only. These results suggest that chronic exposure of the early life history stages of *C. gigas* and *S. glomerata* to elevated $pCO_2$ and temperature may lead to even greater effects than those so far predicted.

### The effect of $pCO_2$ and temperature on the size of larvae and spat

The size of D-veliger, umbonate and pediveliger larvae and spat of *C. gigas* and *S. glomerata* were all significantly affected by elevated $pCO_2$ and temperature. In general, as $pCO_2$ increased and temperature deviated from optimal, the size of larvae and spat decreased. The severity of these effects differed significantly between species. In general, the larval stages (D-veliger, umbonate and pediveligers) of *S. glomerata* were more sensitive to $pCO_2$ and temperature with greater reductions in growth. At the spat stage, however, the combined effects of elevated $pCO_2$ and elevated temperature (30°C, 600–1,000 µatm) caused the least reduction in growth in *S. glomerata* rather than *C. gigas*. Typically, *C. gigas* is believed to be a more robust species than *S. glomerata* (White 2002) and indeed, this was evident in the response of the larval stages to elevated $pCO_2$ and temperature in this experiment. The reduced tolerance of *C. gigas* to elevated $pCO_2$ and temperature during the spat stage was unexpected. It may be that the natural upper thermal range of *S. glomerata* spat is higher than that of *C. gigas*.

The reduced growth experienced by the early developmental stages in this study was likely caused, at least in part, by a reduction in calcification. Reductions in calcification have been well documented for a number of adult

marine organisms in response to elevated $pCO_2$ (Gao et al. 1993; Bijma et al. 1999; Langdon et al. 2000; Leclercq et al. 2000; Riebesell et al. 2000; Reynaud et al. 2003; Orr et al. 2005) including bivalves (Gazeau et al. 2007). It has been suggested that calcification during the early development of oysters is one of the processes most sensitive to elevated $pCO_2$ (Kurihara et al. 2007). Reductions in the size of a single larval development stage have been documented for other marine organisms including the sea urchins, *H. pulcherrimus* and *E. mathaei* (≥2,000 ppm, Kurihara and Shirayama 2004) and in the oyster *C. gigas* (2,268 ppm, Kurihara et al. 2007) upon exposure to elevated $pCO_2$. In this study, the size of D-veliger, umbonate and pediveliger larvae as well as spat of each species was reduced at elevated $pCO_2$. In fact, during the umbonate stage, larvae experienced slight shell dissolution, reducing in size at elevated $pCO_2$ and suboptimal temperature compared to the beginning of the experiment. The greatest tolerance to elevated $pCO_2$ was during the pediveliger larval stage. During their life cycle, oysters experience marked differences in the deposition of their $CaCO_3$ shell. Initially, the first larval shell is deposited in the form of amorphous calcium carbonate (ACC) which is soon after transformed partially to aragonite (Carriker and Palmer 1979; Weiss et al. 2002). Following metamorphosis, during the juvenile and adult stage, calcite then becomes the more predominant form of $CaCO_3$ (Stenzel 1964). Both ACC and aragonite are less stable at elevated $pCO_2$ than calcite and are therefore more prone to dissolution (Kurihara et al. 2007). Given this, it was anticipated that metamorphosed spat, which secrete the most stable form of $CaCO_3$ (calcite), would be the most tolerant to elevations in $pCO_2$. The fact that spat were highly sensitive to elevated $pCO_2$ indicates that physiological processes may be affected in addition to calcification including metabolism and/or protein synthesis (Pörtner et al. 1998; Michaelidis et al. 2005; Langenbuch et al. 2006).

There was also an effect of temperature interacting with $pCO_2$ on the size of *C. gigas* and *S. glomerata* larvae and spat. Generally, for each stage of development, the size of larvae and spat was least at the lower temperature of 18°C. This result was not surprising given that many previous studies have found a correlation between reduced size and lower temperature (His et al. 1989; Dove and O'Connor 2007), mainly attributed to lower metabolic rate and poor assimilation of energy (Anil et al. 2001; Calcagno et al. 2005). The reductions in the size, particularly of larvae, in this study may have significant implications for oysters at the individual and population level. At the individual level, it has been suggested that the frequency of encountering and clearing food is lower in smaller than larger larvae, thereby increasing the chances of starvation due to lower feeding rates (Anger 1987; Strathmann 1987; Kurihara et al. 2007). At the population level, decreases in size during the early developmental stages of marine organisms can result in smaller settlers, reduced competitive ability, greater post-settlement mortality and less recruitment into the adult population (Connell 1961; Anil et al. 2001). Previous studies have found that even small changes in the number of bivalves which develop to settlement can have large consequences for the adult population (Gosselin and Qian 1997; Hunt and Scheibling 1997). The significant reductions in development of D-veliger larvae seen in this study may therefore lead to reductions in both natural and commercial oyster populations. Furthermore, reduced growth of oysters following settlement may not only reduce competitive ability and increase susceptibility to predation but may also increase the time taken for oysters to reach market size, thus impacting on the economic viability of important aquaculture industries.

In summary, in this study, elevated $pCO_2$ and temperature caused reduced fertilization of gametes, reduced development and increased abnormality of D-veliger larvae and reduced growth of larvae and spat of *C. gigas* and *S. glomerata*, with greater impacts on *S. glomerata*. If our oceans continue to acidify and warm, *C. gigas* may become the more dominant species along the south-eastern coast of Australia, further recruiting into estuaries currently dominated by the native *S. glomerata*. At a global level, this study has extended implications for the fertilization and embryonic development of estuarine species, which may not respond similarly, even if they are closely related and for marine and estuarine ecosystems and biodiversity over the next century.

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