Impact of increased seawater $pCO_2$ on the host and symbiotic algae of juvenile giant clam *Tridacna crocea*

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**Abstract** Increases in atmospheric CO$_2$ cause decreases in calcium carbonate saturation, which is predicted to affect the calcification process of most marine calcifiers. At the same time, the increase of seawater $pCO_2$ is also known to increase the productivity of primary producers. Giant clams host symbiotic dinoflagellates (‘zooxanthellae’: *Symbiodinium* spp.) that provide nutrition and use CO$_2$ as their primary source for photosynthesis. This leads to the hypothesis that increased seawater $pCO_2$ rise could positively affect the production of giant clam zooxanthellae, and dampen effects of CO$_2$ on host giant clams. To test this hypothesis, we measured the shell growth rate, photosynthesis rate, respiration rate and zooxanthellae density of the juvenile *Tridacna crocea* reared under three different $pCO_2$ conditions. Results revealed that negative shell growth of juvenile *Tridacna crocea* was observed once seawater Ω$_{arag}$ reached less than 2.33. Additionally, although zooxanthellae density in *T. crocea* increased with seawater $pCO_2$ rise, zooxanthellae productivity did not change, suggesting that the productivity per zooxanthella decreased in high $pCO_2$ seawater. Our findings suggest future seawater $pCO_2$ rise will not increase productivity of zooxanthellae, thus giant clam will be negatively impacted in the coming centuries.

**Introduction**

There is now a critical need to understand the effects of environmental change on marine organisms caused by increasing anthropogenic atmospheric CO$_2$. When oceans uptake atmospheric CO$_2$, seawater pH declines, a phenomenon which is called ocean acidification (OA, Caldeira and Wickett 2003). Because decreased seawater pH also reduces calcium carbonate saturation state (Ω, Orr et al. 2005), OA is projected to adversely impact the physiology and biology of principally marine calcifiers such as mollusks (excluding cephalopods), echinoderms, and corals (Doney et al. 2009).

The effect of OA on mollusks has garnered attention, not only for scientific interest, but also due to the economic value of mollusks (Narita et al. 2012; FAO 2016). A number of studies have evaluated the effect of OA on several commercially important mollusk and have illustrated negative impacts on their survival (Berge et al. 2006), growth (Michaelidis et al. 2005; Berge et al. 2006), calcification (Gazeau et al. 2007), shell hardness (Beniash et al. 2010), and metabolism (Michaelidis et al. 2005). Meanwhile, despite the socio-economic value of giant clams, in the Indo-Pacific region, only a few studies have evaluated their susceptibility to OA.

Giant clams are classified in the order Cardiida and the
subfamily Tridacninae and are the largest living bivalves. Within the group, there are 12 recognized living species, comprised of ten species of the genus *Tridacna* and two of the genus *Hippopus*, which all commonly inhabit in the coral-reef ecosystems of the Indo-Pacific Ocean (MolluscaBase 2018). Due to their economic value as a food source and for ornaments, they have been over-harvested for centuries, and, together with other human impacts on coral reef environments, this has led to giant clams being listed in the Red List of Threatened Species (Wells 1996; Neo et al. 2015). Giant clams are known to show the highest growth rate among mollusks, up to 16 times higher than oysters (Bonham 1965) and the shells of giant clams are mainly composed of aragonite calcium carbonate (Taylor et al. 1973). Watson et al. (2012) first reported effects of OA and indicated that both high seawater $p$CO$_2$ and temperature reduced the survival of the juvenile *Tridacna squamosa*. Another study indicated that the growth rate of the juvenile giant clams *T. squamosa*, *T. crocea* and *T. maxima* cultured for one year at Waikiki Aquarium, under conditions with seawater with high $p$CO$_2$ (700 to 1,400 μatm) and high nutrient concentrations, was slower compared to the previously reported values of the same species cultured under normal seawater conditions (Toonen et al. 2012).

Meanwhile, giant clams have the distinct characteristic of a symbiotic relationship with dinoflagellates (‘zooxanthellae’: *Symbiodinium* spp., Yonge 1953). Establishment of the symbioses is reported to occur at the juvenile stage just after metamorphosis from the veliger larval stage (Hirose et al. 2006). Similar to hermatypic corals, giant clams receive a significant amount of energy from photosynthetic products produced by zooxanthellae (Trench et al. 1981). The influence of OA on these symbiotic marine calcifiers is of great interest because while high seawater $p$CO$_2$ can decrease calcification rates, it may enhance the photosynthetic rates of symbiotic algae. Indeed, Watson (2015) demonstrated that although the growth rate of juvenile *Tridacna squamosa* was reduced when reared at low light intensity (35 μmol photons m$^{-2}$ s$^{-1}$) in high $p$CO$_2$ seawater, the impact was less pronounced when the clams were reared at high light intensity (304 μmol photons m$^{-2}$ s$^{-1}$) and high $p$CO$_2$. This result has been interpreted that the negative effect of OA on giant clam hosts was dampened by the increase photosynthetic rate of zooxanthellae. However, until now, there has been no study examining the direct effect on the net productivity of giant clams under high $p$CO$_2$ conditions. For corals, several studies have reported that elevated $p$CO$_2$ does not enhance the photosynthesis and productivity of zooxanthellae (Goiran et al. 1996; Schneider and Erez 2006; Marubini et al. 2008; Takahashi and Kurihara 2012). However, one study reported that photosynthetic rates increased in under high $p$CO$_2$ (Suggett et al. 2013), while another study reported a reduction in photosynthetic rate under high $p$CO$_2$ (Anthony et al. 2011). One reason for this lack of response, or conflicting responses, in coral photosynthetic rates may be because zooxanthellae of corals utilize mainly HCO$_3^-$ rather than CO$_2$ for photosynthesis (Al-Moghrabi et al. 1996; Allemand et al. 1998). In contrast to corals, the zooxanthellae of giant clams have been hypothesized to predominantly use CO$_2$ for photosynthesis (Yellowlees et al. 1993; Goiran et al. 1996; Leggat et al. 2000). Additionally, unlike corals where the zooxanthellae are intracellular and located at the oral endoderm (Yellowlees et al. 2008), the endosymbiotic zooxanthellae of giant clams are reported to be extracellular, living within the Z-tubes connected to the stomach (Norton et al. 1992). Therefore, it is possible that OA may have different effects on coral and giant clam physiologies.

Here, we examine the effect of OA on both shell growth and metabolism of giant clams, including respiration and photosynthetic rates to test the possibility that high $p$CO$_2$ will stimulate the net production of zooxanthellae. Juveniles of the giant clam *Tridacna crocea* were cultured at 3 different pH / $p$CO$_2$ conditions predicted to occur in future coral reef ecosystems.

**Material and methods**

**Organisms**

Juveniles of the giant clam *Tridacna crocea* were provided by the Okinawa Prefectural Fisheries Research and Extension Center, Okinawa Island, Japan. Individuals were artificially fertilized, infected by monoculture *Symbiodinium* taken by the giant clam *T. crocea* and cultured
for 1 y in a flow-through outdoor tank. Sixty one-year-old juveniles of similar sizes were selected from the cultured stock at the Center, and transported to Sesoko Station, University of the Ryukyus. All 60 juveniles were immediately moved to an outdoor tank (187 L) continuously supplied with seawater pumped (2.5 L min$^{-1}$) from 4–5 m depth in front of Sesoko Station and acclimatized for 3 weeks before starting the experiment.

**Experimental set-up**

Just before starting the experiment, 30 juvenile *T. crocea* of approximately the same size (7.2±0.65 mm) were selected and placed individually in 30 culture containers (130 ml in volume). Each of 10 culture containers were supplied with control (pCO$_2$/pH: 400 μatm/pH 8.2), Mid CO$_2$(1,000 μatm/pH 7.8), or High CO$_2$ (2,000 μatm/pH 7.6) seawater at the rate of 250 ml min$^{-1}$. *T. crocea* juveniles were cultured for 4 weeks under these conditions. Seawater pCO$_2$ was adjusted by continuously bubbling flowing pumped seawater (2.5 L min$^{-1}$) in the bubbling tank with air or air-CO$_2$ mixed gas at a rate of 3.0 L min$^{-1}$ made up by mixing air and pure CO$_2$ using a mass flow controller (HORIBASTEC, Japan). All 30 containers were placed in a water bath (120×77×22 cm; 187 L) receiving running seawater (3 L min$^{-1}$) pumped from the ocean. The experimental setup was placed outdoors under natural irradiance conditions. Every 10 min seawater temperature was logged using a data logger (Hobo U22-001, Onset Corp., USA), and light irradiance was logged using a quantum light meter (MDS-MkV/L, JFE, Japan), which was placed within the water bath. Seawater pH and temperature of the seawater running from the bubbling tank were measured every day using a temperature-compensated pH electrode (Mettler Toledo, MP125, USA), and the average pH (n=30) during the experiment was calculated for each condition (Table 1). Pre-measurement of pH and temperature at each container was conducted to verify that there was no variation among containers. Seawater volume and seawater exchange rate of the container was large enough so that the metabolism of the giant clam did not influence seawater pH within the container. The pH electrode was calibrated every day before measurement using NBS scale buffer solutions. Salinity were measured every week for all containers using a refractometer (Atago, 100-S, Japan). Total alkalinity (A$_T$) were measured every week for 6 randomly selected containers using an autoburette titrator (Kimoto, ATT-05, Japan). Seawater pCO$_2$, HCO$_3^-$, CO$_3^{2-}$ and Ω$_{arag}$ were calculated based on pH temperature, alkalinity and salinity using the CO2SYS program of Lewis and Wallace (1998), with dissociation constants $K_1$ and $K_2$ from Mehrbach et al. (1973) and the aragonite solubility ($K_{spa}$) of Mucci (1983) (Table 1).

**Measuring shell growth**

The growth rates of the clams were evaluated as the change in shell size during the 4 weeks culture period. Pictures of the clams were taken just before introducing the clams to the different pCO$_2$ conditions (time 0: all giant clams reared at control seawater) and 1, 2, 3 and 4 weeks after starting the experiment. Shell width and shell height were measured using Image Processing and Analysis in JAVA (ImageJ) software and the growth rates were analyzed as the weekly percentage (%) change of shell width and height size. Additionally, we evaluated the correlation between the mean seawater aragonite saturation during the culture and the mean shell growth rate calculated as the change in shell height normalized by wet weight. The wet weight was measured after culture by pat drying the clams with a paper towel and weighing them on an electronic balance (0.1 mg precision HR-200, A&D, Japan). For the individuals that died during the experiment, wet weight was measured just after the clams were observed to be dead.

| pH (NBS scale) | pCO$_2$ (μatm) | HCO$_3^-$ (μmol kgSW$^{-1}$) | CO$_3^{2-}$ (μmol kgSW$^{-1}$) | TCO$_3$ (μmol kgSW$^{-1}$) | alkalinity (μmol kgSW$^{-1}$) | Ω$_{arag}$ | salinity |
|----------------|----------------|-----------------|----------------|----------------|----------------|-----------|----------|
| control 8.16 ± 0 | 422 ± 48 | 1,740 ± 38 | 205 ± 17 | 1,957 ± 25 | 2,249 ± 25 | 3.3 ± 0.3 | 34.5 ± 0.8 |
| 1000 μatm 7.8 ± 0 | 1,153 ± 67 | 2,009 ± 48 | 100 ± 4 | 2,140 ± 49 | 2,258 ± 49 | 1.59 ± 0.1 | 34.3 ± 0.7 |
| 2000 μatm 7.54 ± 0 | 2,126 ± 215 | 2,120 ± 49 | 60 ± 3 | 2,237 ± 47 | 2,268 ± 47 | 0.97 ± 0.1 | 34.7 ± 0.7 |
Measuring photosynthesis and respiration rate

Photosynthesis and respiration rates were calculated by measuring the oxygen consumption under light and dark conditions the day before starting the experiment (day 0: all giant clams reared at control seawater) and 1, 2, 3 and 4 weeks after the start of the experimental culture. Each clam was set in an airtight chamber (20 ml volume) containing control, Mid CO\(_2\) or High CO\(_2\) seawater and a stir bar powered by a waterproof magnetic stirrer. Each chamber was set in a water bath in which the seawater temperature was adjusted to 27°C for 20 min prior to measurement. Oxygen concentrations of each chamber were measured at time 0, 30 and 60 min after incubation using O\(_2\) probes (FIBOX3, PreSens, Germany). Light conditions were set to 300 μmol photon cm\(^{-2}\) s\(^{-1}\) using two metal halide lamps. Net photosynthesis rates were calculated by the consumption of oxygen under light conditions, and the respiration rate under dark conditions during the 60 min incubation. The effect of seawater pCO\(_2\) on net photosynthesis and respiration rate were analyzed by determining the percentage (%) change compared to day 0.

Measurement of zooxanthellae density

After the 4 weeks culture experiment, all soft tissue of each clam was removed from the shell and homogenized within a centrifuge tube (5 ml volume). After diluting with seawater filtered using millipore filter (0.22 μm), the number of zooxanthellae was counted by a hemocytometer and normalized by the wet weight (g).

Statistical analyses

All statistical analyses including MANOVA, linear regression model, one-way ANOVA, and Dunnett post-hoc test were conducted using the statistical software JMP 7.

Results

Seawater carbonate chemistry

Seawater temperature during the one-month culture was 26.6±0.86°C, and light intensity fluctuated from 30 to 800 μmol photon cm\(^{-2}\) s\(^{-1}\) during the daytime (Fig. 1).

The seawater pH and pCO\(_2\) of control, Mid and High CO\(_2\) conditions were 8.16±0.04 (pCO\(_2\): 422±48 μatm), 7.8±0.01 (pCO\(_2\): 1,153±67 μatm), and 7.54±0.03 (pCO\(_2\): 2,126±215 μatm), respectively (Table 1).

Survival and growth rate

Three control individuals died (1 clam at 3\(^{rd}\) week and 2 clams at 4\(^{th}\) week) along with three Mid CO\(_2\) (2 clams at 3\(^{rd}\) week and 1 clam at 4\(^{th}\) week) and one High CO\(_2\) condition individual (4\(^{th}\) week). There was a significant difference in the weekly percentage (%) change in shell heights among pCO\(_2\) conditions (MANOVA, \(F_{(2,20)}=3.99, P=0.03\)), but not with time (MANOVA, \(F_{(3,18)}=0.19, P=0.89\), Fig. 2). The weekly percentage (%) change in shell length did not show significant differences among pCO\(_2\) conditions (MANOVA, \(F_{(2,20)}=0.86, P=0.43\)) and time (MANOVA, \(F_{(3,18)}=1.25, P=0.31\)). The growth rate of shell height normalized by the wet weight (mm g\(^{-1}\) d\(^{-1}\)) showed a significant correlation with the seawater aragonite saturation (\(\Omega_{arag}\), shell growth rate=0.03*\(\Omega_{arag}\)-0.07, \(r^2=0.35, F_{(1,27)}=14.6, P=0.0007\), Fig. 3), and the growth rate was calculated to become negative when \(\Omega_{arag}\) reached 2.33.

Photosynthesis and respiration

Net photosynthesis rate were significantly different over time (MANOVA, \(F_{(3,18)}=11.9, P<0.0002\)), but not among pCO\(_2\) conditions (\(F_{(2,20)}=1.07, P=0.36\), Fig. 4). Respiration rates were also significantly different over time (MANOVA, \(F_{(2,18)}=13.8, P=0.0002\)) but not among pCO\(_2\) conditions (MANOVA, \(F_{(2,20)}=1.58, P=0.22\), Fig. 4). Due
to a problem with the oxygen probes, we were not able to measure respiration rates at week 3. The absolute values for the measured photosynthesis and respiration rate at weeks 0, 1, 2, and 4 are shown in supplementary data (Fig. S1).

**Zooxanthellae density**

The zooxanthellae densities of the giant clam cultured for one month were significantly different between $pCO_2$ conditions (one-way ANOVA, $F_{(2,20)}=5.27, P=0.01$), and Mid and High CO$_2$ giant clams showed 1.7 and 2.1 times higher zooxanthellae density compared to the control, respectively (Dunnett post-hoc test, $P<0.05$, Fig. 5).

**Discussion**

Results of the present study revealed that the growth rate of juvenile *Tridacna crocea* were negatively affected by high seawater $pCO_2$, and first demonstrated that shell dissolution could start at seawater $\Omega_{arag}<2.33$. Additionally, although net photosynthesis was not affected by high seawater $pCO_2$, the productivity per zooxanthella of *T. crocea* decreased with seawater $pCO_2$. 

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**Fig. 2** The weekly percentage (%) changes of juvenile *Tridacna crocea* growth rate measured by shell height (A) and shell length (B) during the 4 weeks culture at 3 different $pCO_2$ conditions. Mean and S.E. are shown in the error bars. $n=7$, 7 and 9 for control, Mid and High CO$_2$.

**Fig. 3** Relationship between the growth rate measured by the mean increase in shell height per wet weight per day (mm g$^{-1}$d$^{-1}$) of the juvenile giant clam *Tridacna crocea* and seawater aragonite saturation ($\Omega_{arag}$). Growth rate was significantly correlated with seawater $\Omega_{arag}$ (growth rate$=0.03*\Omega_{arag}-0.07$, $r^2=0.35$, $F_{(1,27)}=14.6$, $P=0.0007$). Asterisks show the individuals that have died during the experiment.
The study by Watson et al. (2012) indicated that the survival rates of juvenile giant clams *Tridacna squamosa* were reduced when cultured longer than 40 days under high pCO$_2$ conditions (981 μatm/pH 7.84). More recently, however, they also reported that while *T. squamosa* juveniles were lethally affected when reared at 660–937 μatm pCO$_2$ under low light conditions (35 and 65 μmol photon m$^{-2}$s$^{-1}$), no lethal effects were found when reared at the same pCO$_2$ concentration but under high light conditions (304 μmol photon m$^{-2}$s$^{-1}$). Since in the present study the *T. crocea* juveniles were cultured under natural sunlight conditions (light conditions ranged from 30–800 μmol photon m$^{-2}$s$^{-1}$), light availability may be one of the reasons we did not see any lethal effects from high seawater pCO$_2$ treatment. These results also suggest that, at least in normal ambient coral reef conditions, OA will not lethally affect juvenile giant clams, although this could change with increasing turbidity or depth that causes lower light intensity, as discussed by Watson (2015).

Although no effect was detected on the growth in terms of shell length, in terms of shell height the growth of *T. crocea* juveniles was significantly different between pCO$_2$ conditions. Mid and High pCO$_2$ juveniles show negative percentage growth throughout the experiment, which may be due to the net dissolution of the shell. From the evaluation of shell growth rates and seawater Ω$_{arag}$, it was calculated that negative growth was observed from Ω$_{arag}$ = 2.33, which is equivalent to 715 μatm seawater pCO$_2$ in the studied seawater (temperature 27°C, salinity 34.5, $A_T$ = 2249 μmol kg$^{-1}$). Shell synthesis of larvae and juvenile stages of several shellfish including hard clams, oysters, and mussels has been indicated to be negatively impacted by a decrease in Ω$_{arag}$ (Green et al. 2004; Kurihara et al. 2007, 2008; Parker et al. 2009; Gazeau et al. 2010; 2013), and the threshold has been reported to be around Ω$_{arag}$ < 2.0 (Barton et al. 2012; Waldbusser et al. 2015). Additionally, growth rates of juvenile corals have been indicated to show non-linearly relations with Ω$_{arag}$ and negative growth was only observed when Ω$_{arag}$ reached 0.12 (Albright 2011). Although direct comparison is difficult due to the methodological differences between studies, juvenile giant clams are suggested to be highly sensitivity to OA compared to the other calcifiers. The mean Ω$_{arag}$ of most coral reefs has been reported to be higher than 3.0 (Sutton et al., 2016) and the field average Ω$_{arag}$ measured at the present studied site ranged from 3.24 (winter) to 3.54 (summer, Kurihara et al. unpubl data). However, it is also well known that most coral reefs show a very high Ω$_{arag}$/pCO$_2$ diurnal variation, according to the tidal range and metabolism of the benthic community, such that Ω$_{arag}$ can range from 1.86 to 6.36 in Okinawa (Ohde and van Woestik 1999), or even from 1.13 to 6.46 at some reef such as LEI reef in GBR (Shaw et al. 2012). Therefore, it is suggested that dissolution of wild giant clam shells may start within a few decades, principally at night time when Ω$_{arag}$ shows the lowest values. Furthermore, taking into account that giant clam juveniles in the wild, generally live buried in sediments, and the sediment pore water have been observed to show lower Ω$_{arag}$ due to microbial respiration (Andersson and Mackenzie 2011), it
is possible that small juvenile of giant clams will experience an even lower $\Omega_{arag}$ environment than in the overlying seawater.

Both giant clams and corals are known to host zooxanthellae, however, the main carbon source for the photosynthesis has been suggested to differ between giant clams (mainly CO$_2$) and corals (HCO$_3^-$ and CO$_2$, Yellowlees et al. 2008). The reason for these differences is still in question, however it has been proposed the sources of carbon reflect the different environment that the zooxanthellae are exposed to; giant clam: extracellular vs. corals: intracellular (Leggat et al. 2000). If the zooxanthellae of giant clams preferably use CO$_2$ as reported, it could be hypothesized that increases in seawater $p$CO$_2$ may increase their productivity. However, in contrast to our expectation we did not see any changes in the photosynthetic rate of giant clams reared at high $p$CO$_2$/low pH conditions. One reason could be that carbonic anhydrase activity, which has been suggested to supply CO$_2$ to zooxanthellae (Yellowlees et al. 1993), is high enough and the giant clam zooxanthellae are not under-saturated in terms of CO$_2$. In addition, since the zooxanthellae density increased with seawater $p$CO$_2$, it is suggested that the productivity per zooxanthella decreased in high $p$CO$_2$ seawater. The reason for this decrease is not clear, however, indirect negative impacts of high $p$CO$_2$ through the host can be supposed. Although we did not observe a decrease in respiration rates of juvenile giant clams, decreased ammonium excretion or clearance rates have been observed in juvenile clams of Ruditapes decussatus (Fernández-Reiriz et al. 2011). Because symbiotic zoo-

**Supplemental Fig. 1** Net photosynthesis (A) and respiration rate (B) of juvenile Tridacna crocea before and after 1, 2, 3 and 4 weeks exposure to 3 different $p$CO$_2$ conditions (Control, Mid $CO_2$ and High $CO_2$). Measurement for respiration was unable to conducted at week 3 due to malfunction of the O$_2$ sensor. Mean and S.E. are shown in the error bars. Number above each bar show number of replicates.
xanthellae are known to acquire nutrients from the host (Yellowless et al. 2008), depression of host activity by high $p$CO$_2$ could affect the zooxanthellae. From nutritive calculation, it has been suggested that about 95% of the carbon fixed by zooxanthellae is translocated to the giant clam host (Fitt et al. 1993). Meanwhile, giant clams are also known to be highly efficient filter feeders and that the more than half of the total carbon needed for respiration and growth is derived by feeding on particulate organic matters, principally at early juvenile stages (Klump et al. 1992). Hence, if the clearance rate of giant clams is affected by the high $p$CO$_2$, as observed in other juvenile clams (Fernández-Reiriz et al. 2011), this could be another negative impact to the giant clam host.

**Conclusions**

Preset results revealed that high seawater $p$CO$_2$ decreased shell growth rate and productivity per zooxanthella of juvenile *Tridacna crocea*. Hence, giant clams can be negatively impacted in the coming centuries. Taking into account the economic value of giant clams as a food source and for ornaments, principally in reef island countries, and being listed in the Red List of Threatened Species, further local management of these species is suggested be essential under future climate change.

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