Molluscan Research 30(3): 125–130
http://www.mapress.com/mr/

ISSN 1323-5818
Magnolia Press

The effects of exposure to near-future levels of ocean acidification on shell characteristics of *Pinctada fucata* (Bivalvia: Pteriidae)

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Abstract

Atmospheric carbon dioxide concentrations have greatly increased since the beginning of the industrial age. This has led to a decline in global ocean pH by 0.1 units, and continued decline of 0.3–0.5 units is predicted by the end of 2100. Acidification of the ocean has led to decreased calcification rates and dissolution of calcareous structures in a range of marine species. Shells of the pearl oyster *Pinctada fucata* exposed to acidified seawater (pH 7.8 and pH 7.6) for 28 days were 25.9% and 26.8% weaker than controls (pH 8.1–8.2), respectively, but there was no reduction in the organic content of shells exposed to acidified conditions. Scanning electron microscopy analysis of the growing edge of nacre lining the shells of *P. fucata* showed that shells exposed to acidified conditions (pH 7.6) showed signs of malformation and/or dissolution, when compared to controls. The reduction in shell strength and the possible nacre malformation could have broad impacts on the ecology of pearl oysters and consequences for the cultured pearl industry that relies on them.

Key words: Hypercapnia, tropical mollusc, shell strength, nacre deposition, climate change, pearl oyster

Introduction

Since the beginning of the industrial age, carbon dioxide (CO_2) has been accumulating in the atmosphere at a rate 100 times faster than has been inferred for the past 640,000 years (IPCC 2007). The oceans absorb 25–30% of the CO_2 released into the atmosphere (Feely *et al*. 2004; Fabry *et al*. 2008), where the natural carbonate buffering system should prevent changes in pH. However, this process is slow and cannot keep pace with the current rate of CO_2 increase (Hoegh-Guldberg *et al*. 2007). This has resulted in a decline in surface ocean pH of 0.1 units since the beginning of the industrial age (Hoegh-Guldberg *et al*. 2007), and this decline is predicted to continue, resulting in a decrease of 0.3–0.5 units by the end of 2100 (Caldeira and Wickett, 2003).

Acidification of the oceans has the potential to affect a wide variety of marine organisms. Calcifying organisms have received much attention in ocean acidification research, due to the potential for reduced calcification rates and dissolution of calcified structures when exposed to reduced pH (Fabry *et al*. 2008). Dissolution of calcareous structures and reduced calcification rates have been documented in many marine invertebrates, including scleractinian corals (Gattuso *et al*. 1998; Marubini *et al*. 2001; Schneider and Erez 2006; Anthony *et al*. 2008; Ries *et al*. 2009), coccolithophores (Sciandra *et al*. 2003), and temperate bivalve molluscs (Michaelidis *et al*. 2005; Gazeau *et al*. 2007; Ries *et al*. 2009). It has been shown that in molluscs, these processes can result in reduced shell mass, which compromises the structural integrity of their shells, causing a reduction in shell strength (Buschbaum *et al*. 2007; McClintock *et al*. 2009). This could have ecological implications for bivalves and potential economic impacts for industries that rely on them, such as the cultured pearl industry. No prior study has directly determined the strength of tropical bivalve shells following exposure to acidified conditions.

Pearl oyster shells are composed of three layers; the inner nacreous layer, the middle prismatic layer and the outer organic layer (Fougerouse *et al*. 2008). The outer layer, or periostracum, has been shown to play an important role in reducing fouling on the shells of bivalves including pearl oysters (Guenther and De Nys 2006) by protecting against settlement of encrusting epibionts (Scardino *et al*. 2003) and deterring boring predators (Harper and Skelton 1993). It has been suggested that the periostracum of mollusc shells may help protect the shells from dissolution caused by contact with acidified water (Ries *et al*. 2009), however no conclusive evidence of this has been found. As no maintenance is performed on the periostracum once it is formed, it naturally wears off with time and may be absent from older parts of the pearl oyster shell (Guenther and De Nys 2006). Exposure to acidified conditions may increase the rate of erosion of the periostracum, which would accelerate fouling of the shells. Such shells may be more prone to predation because of reduced structural integrity caused by encrusting and boring fouling organisms (Buschbaum *et al*. 2007). The nacreous and crystalline layers of the pearl oyster shell also contain organic components (Fougerouse *et al*. 2008) that could potentially be affected by ocean acidification. The nacre and calcite layers are composed of nacre tablets and calcite crystals, respectively. Each nacre tablet and calcite crystal is surrounded by an organic matrix which assists in their formation and holds them together, thereby providing structural support to the shell (Fougerouse *et al*. 2008). Despite being on the inside of

the shell, the growing ventral and lateral edges of the nacreous layer are exposed to seawater when the mantle retracts, making them prone to dissolution or malformation by ocean acidification. Reductions in the organic content of the shell may decrease the structural integrity and strength of the shell, which could have detrimental effects on the ecology and culture of pearl oysters.

The mantle of pearl oysters secretes new nacre over the prismatic layer, at the growth front of nacre. Nacre tablets originate as nano-sized aragonite crystals which form from 'dimples' in the organic matrix that covers the outer prismatic columns. The aragonite crystals grow, filling the dimples, and forming nacre tablets which coalesce to form the nacreous layer (Saruwatari *et al*. 2009). It has been suggested that this process occurs in a compartment which is isolated from the extrapallial fluid and mantle cells by a thin organic film which adheres to the growing mineralization front (Nudelman *et al*. 2008). This process may protect the developing nacre layer from erosion caused by contact with acidified water, however the degree of isolation from external conditions provided by this organic film has not been investigated. Pearl oysters are farmed for their nacre producing ability which is utilized by the cultured pearl industry. If the key physiological process of nacre deposition is affected by ocean acidification, the productivity of the cultured pearl industry could be greatly impacted. Nacre secretion and pearl formation has been shown to be adversely affected by exposure to low temperatures (<12°C), low salinities (≤ 19 ppt), pollutants and aerial exposure (Lucas 2008). However, no prior study has investigated the effect of ocean acidification on nacre in live pearl oysters.

This study aimed to determine the effects of predicted near-future levels of ocean acidification on the shell and nacre characteristics of the Akoya pearl oyster *Pinctada fucata* (Gould, 1850), sometimes referred to as *Pinctada imbricata* Röding, 1798. Specifically, the effect on shell strength, organic content of the shells and characteristics of nacre secretion were investigated.

Materials and Methods

Experimental Setup

This study was conducted at the Marine and Aquaculture Research Facilities Unit (MARFU), located at James Cook University, Townsville, Australia. Prior to use, water used in all experiments was filtered to 25 µm through a series of sand filters. The pH control system consisted of three 500 L header tanks. One functioned as the control and remained at ambient pH (pH 8.1–8.2) for the duration of the experiment, and the remaining two were used as treatments at reduced pH (pH 7.8 and pH 7.6). The pH in these tanks was constantly monitored and adjusted with gaseous CO_2 (Aqua Medic AT control) to maintain treatment levels of pH. The control (pH 8.1–8.2) and treatment (pH 7.8 and pH 7.6) levels of pH used in this study represent the current global average pH, and the upper and lower predicted pH values for the end of 2100, respectively (Watson *et al*. 2009). pH probes in each of the header tanks were calibrated with NBS buffers, and all pH values reported are on the NBS scale. Water was gravity fed from each of the header tanks at a rate of 0.7 L min^{-1} into replicate 19 L aquaria housed within water baths. The water baths and replicate aquaria were maintained at 26°C for the duration of the experiment.

Oysters

Adult *Pinctada fucata* used in this study originated from Hervey Bay, south eastern Queensland (25°0'0S, 153°0'0E), and had a mean (± S.D) dorso-ventral shell height of 53.7 ± 2.6 mm. Oysters were fed a micro-algae concentrate (Instant Algae®, Reed Mariculture Inc., Campbell, CA) at a ration of 30,000 cells mL^{-1} daily, which was added to the replicate tanks for the duration of the experiment. The algal paste was suspended in water from each of the header tanks, then added to the individual experimental tanks. The water flow was turned off for one hour to allow the oysters to feed before being turned back on, and the slow flow rate through the experimental tanks ensured the algal diet was not quickly flushed from the system. Water quality was maintained through daily siphoning of the aquaria to remove debris.

Shell Strength

Fifty adult *P. fucata* were used in this experiment. Five oysters were randomly selected prior to the start of the experiment and were used to determine baseline levels of shell strength. The remaining 45 oysters were divided equally between the three levels of pH, to give 15 oysters per treatment. After 28 days exposure to the treatments, the oysters were removed and the visceral mass discarded. The left shell valve of each oyster was used to measure shell strength, while the right valve was retained for subsequent analysis of nacre structure using scanning electron microscopy (SEM). The left shell valve was placed on the crushing plate of an Instron universal testing machine, which was pressed against a load cell at a rate of 4 mm min^{-1} to determine the load required to crush the shell (load refers to the amount of force pushing the shell into the load cell). A reading of load was recorded automatically every 0.1 seconds until completion of the test, and graphed. The force required to crack the shell initially was recorded, as was the maximum load required to crush the shell. Both these measurements were taken because the initial crack in the shell tended to be around the shell margin, which from a predation perspective may not compromise the oyster. However crushing of the shell resulted in a crack right through the shell, which would allow predators access to oyster tissue. Data were analysed using a Kruskal Wallis test, with three Mann-Whitney post-hoc tests and an adjusted alpha level of 0.0167 (SPSS v. 16, SPSS Inc).

Organic Content of Shells

Forty five adult *P. fucata* were divided equally between the three pH treatments, giving fifteen oysters per treatment. These oysters were exposed to the treatments for 28 days

before the visceral mass of each oyster was discarded and the shells dried to a constant weight. The organic content of shells was estimated as ash-free dry weight (AFDW) following heating of the dried shells at 500°C overnight. AFDW was determined by subtracting ash weight from previously determined dry weights. Data were analysed using one-way ANOVA (SPSS v. 16, SPSS Inc).

Nacre Characteristics

The right shell valves of oysters previously used to determine shell strength were used to investigate the nacre characteristics using SEM. Three shells from each pH treatment were randomly chosen for SEM analysis. An area of the ventral part of the right shell valve, representing the growing edge of the nacre (Saruwatari *et al*. 2009) (Figure 1), was removed using a Dremel™ tool fitted with a diamond cutting wheel. The resulting shell pieces were glued onto SEM stubs with plastic conductive carbon cement (Proscitech, Thuringowa, QLD, Australia), gold coated in a JEOL JUC-5000 Magnetron Sputtering Device and viewed at 10000 kV in a JEOL JSM-541OLV SEM. Scanning electron micrographs were taken from the section area to show both newly secreted nacre and the interface between the nacre and the prismatic layer of the shell.

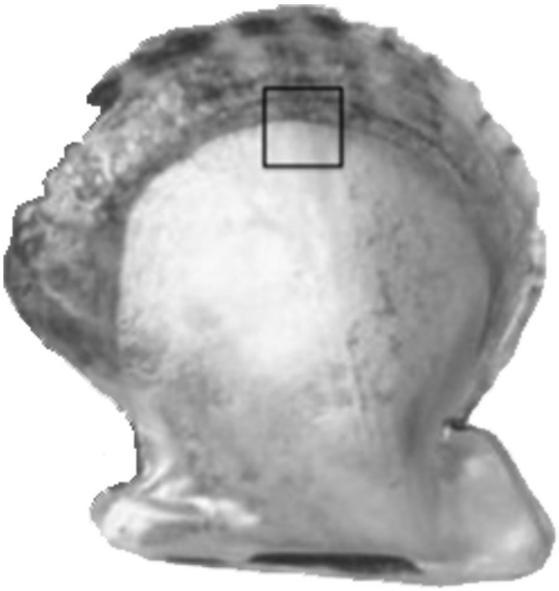

FIGURE 1. Inside surface of the shell of *Pinctada fucata*. The square indicates the border between nacre and prismatic shell layers subject to SEM analysis.

Results

Shell Strength

This experiment found a significant decline in shell strength as treatment pH decreased (Figure 2A). The maximum force that shells in the pH 7.6 treatment could withstand was significantly lower than that for shells held in the control pH (8.1–8.2) (Mann-Whitney U, 28.5, Z= -2.656, $p < 0.0167$). Shells from the control pH had a mean (± S.E) maximum load of 0.2635 ± 0.247 kN, while those from the treatment pH levels (pH 7.8 and pH 7.6) had maximum loads of 0.1953 ± 0.193 kN and 0.1756 ± 0.145 kN, respectively.

There was no significant difference in the force required to initially crack the shells exposed to the control (pH 8.1–8.2) and treatment (pH 7.8 and pH 7.6) levels of pH (Figure 2B) (ANOVA, $F= 0.581_{(2, 42)}$, $p > 0.05$). The mean force required to crack the shells initially was very similar across treatments, with the control shells initially cracking at a load of 0.1468 ± 0.302 kN, while those in the treatments (pH 7.8 and pH 7.6) cracked at loads of 0.1374 ± 0.099 kN and 0.141 ± 0.056 kN, respectively.

Organic Content of Shells

There were no significant differences between the organic content of *P. fucata* shells from the control pH (pH 8.1–8.2) and that of shells from treatment levels of pH (Figure 2C) (ANOVA, $F= 0.281_{(2, 42)}$, $p > 0.05$). The shells of oysters in the pH 7.6 treatment contained slightly less organic matter than those from the control pH, however this difference was minimal. Shells of oysters held in the control pH had a mean (± S.E) organic content of 0.33 ± 0.03 g, while those held in pH 7.8 and pH 7.6 contained 0.34 ± 0.02 g and 0.31 ± 0.02 g of organic matter, respectively.

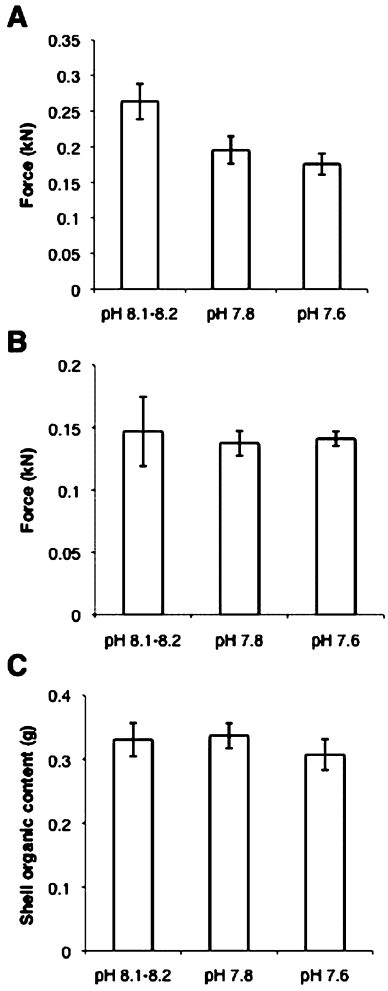

FIGURE 2. Mean (± S.E) maximum force required to crush (**A**), force required to initially crack (**B**) and organic content (**C**) of *Pinctada fucata* shells exposed to control (pH 8.1–8.2) and acidified seawater (pH 7.8 and 7.6) for 28 days.

Nacre Characteristics

This study found a notable difference in the appearance of the growing edge of the nacreous layer of *P. fucata* held in the extremes of the pH treatments. Oysters from the control (pH 8.1–8.2) had nacre that showed a distinct boundary between the fully formed and developing nacre tablets, as previously demonstrated for this species (Saruwatari *et al.* 2009) (Figure 3A). New growth of nacre also showed a clear wave-like pattern, consistent with the reported pattern for nacre growth in pearl oysters (Fougerouse *et al*. 2008), and nacre tablets forming within an extensive organic matrix. Inspection of nacre from oysters held in the pH 7.6 treatment showed no distinct boundary between the developing and fully formed nacre tablets, as seen in the control, with nacre tablets in oysters from the pH 7.6 treatment gradually becoming smaller in the direction of shell growth (Figure 3B). Closer inspection of the area of new nacre tablet formation in oysters from the control pH revealed straight-edged tablets developing in the organic matrix (Figure 3C), while the developing region of nacre from oysters in the pH 7.6 treatment was characterised by sparse, irregularly shaped tablets within a reduced organic matrix (Figure 3D).

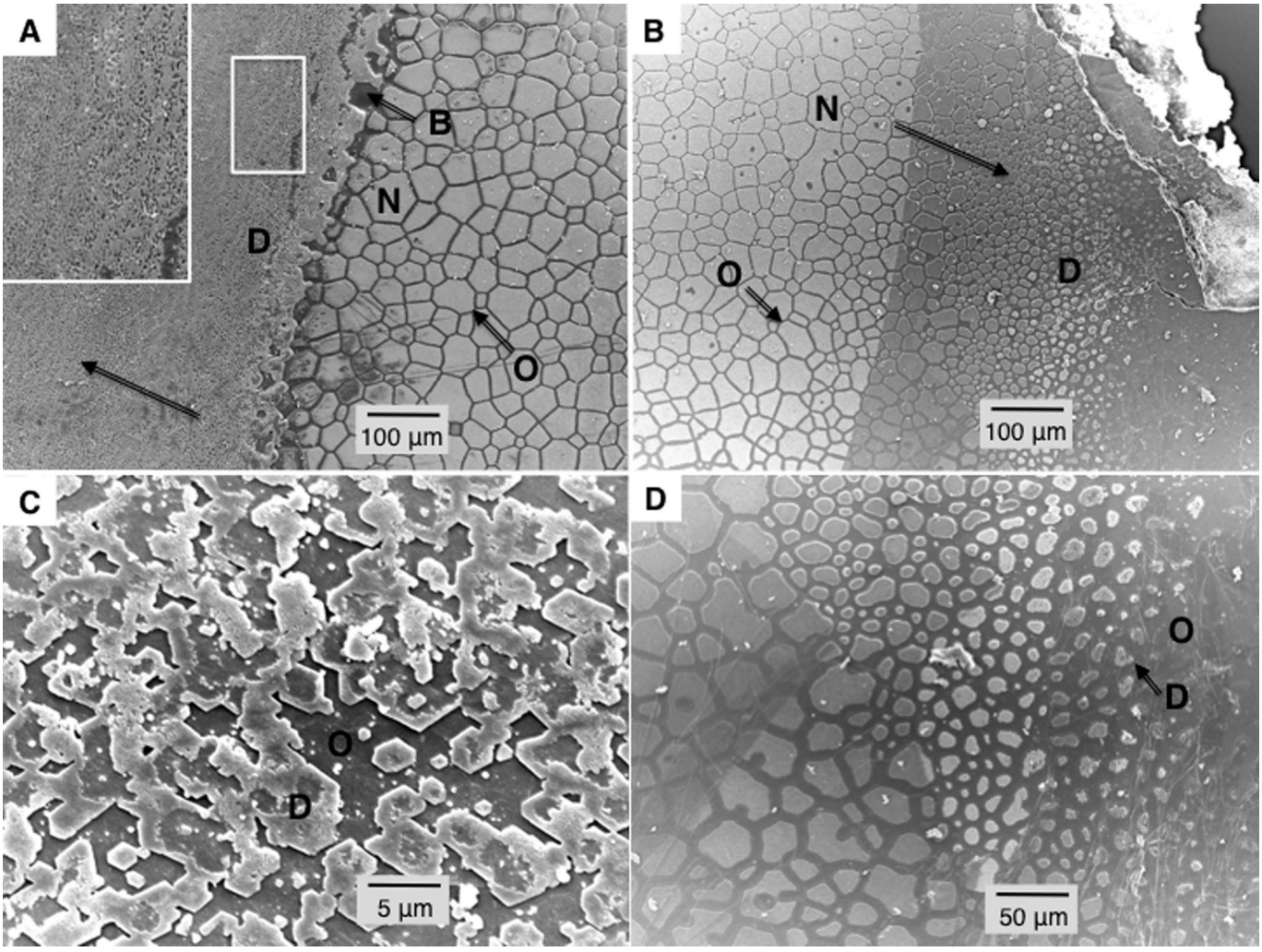

FIGURE 3. SEM micrographs of the growing edge of the nacre layer within shells of *Pinctada fucata* held for 28 days in control (pH 8.1–8.2) (**A**) and acidified (pH 7.6) (**B**) seawater, and a close-up of the region of new nacre development in oysters exposed to control (**C**) and acidified (**D**) seawater. N = fully formed nacre, O = organic matirx, D = developing nacre tablets, B = boundary between fully formed and developing nacre. Black arrows indicate direction of growth, inset shows characteristic wave-like growth pattern of developing nacre.

Discussion

When exposed to predicted near-future levels of ocean acidification, shells of *Pinctada fucata* were significantly weaker than those held at ambient pH even after only 28 days exposure. This suggests that the structural integrity of the shells was compromised through dissolution in the lower pH treatments. McClintock *et al*. (2009) made a similar observation when exposing Antarctic bivalves to CO_2-acidified seawater at pH 7.4. Their study found that bivalve shells dissolved under these conditions, revealing the calcite and aragonite prisms, which decreased the structural integrity of the shell. Although strength was not directly tested, McClintock *et al.* (2009) concluded that such structural degredation of the shells would reduce their integrity and strength. Although no other studies have directly investigated the effects of acidification on the strength of bivalve shells, research on temperate bivalves

exposed to acidified seawater has shown reduced calcification rates (Michaelidis *et al*. 2005; Gazeau *et al*. 2007; Ries *et al*. 2009), which would compromise the structural integrity of the shell. Recent research conducted with the same pH-control system used in this study found evidence of shell dissolution in larvae of the oyster, *Saccostrea glomerata*, exposed to acidified seawater (Watson *et al*. 2009). It was found that empty shells in the pH 7.6 treatment dissolved at a much greater rate than those in the control, and larvae demonstrated retarded shell deposition and/or dissolution when cultured at pH 7.6 (Watson *et al*. 2009). Although larval shells have compositional differences to adult shells that results in a lower saturation state, the findings of Watson *et al*. (2009) show that bivalve shells can dissolve and lose structural integrity when exposed to acidified seawater.

Despite evidence of dissolution of the calcareous components of *P. fucata* shells in this study, no difference between treatments was found in the organic content of shells, indicating that the predicted near-future levels of ocean acidification tested here did not cause degredation of the organic matrix or periostracum of the shells. Green *et al*. (2004) made a similar observation when the hard clam, *Mercenaria mercenaria*, was exposed to seawater with an aragonite saturation state of ~0.3, corresponding to a pH of ~7.1. At this level of undersaturation, the smallest size classes (0.2–0.3 mm) of clam showed complete dissolution of the calcareous component of the shell, leaving only the organic matrix, within 2 weeks exposure to the treatment (Green *et al*. 2004). These results support the findings of the present study, suggesting that the organic component of bivalve shells is resistant to levels of pH exceeding those predicted for the near-future, and those used in the present study.

Preliminary results on the effects of ocean acidification on the nacre characteristics of pearl oysters showed that the processes supporting nacre deposition may be affected by predicted near-future levels of ocean acidification. Previous research has shown that nacre deposition can be affected by a variety of environmental conditions such as water temperature, salinity, pollutants, and current (Lucas, 2008), but no research has been done on the effects of ocean acidification on nacre secretion and/or structure in pearl oysters. SEM conducted in this study indicated that growth of new nacre tablets was disorganised in oysters held at pH 7.6, as the developing tablets were misshapen and irregular. They contrasted clearly with developing nacre tablets of oysters held at ambient pH. There also appeared to be fewer developing nacre tablets at the growing edge of the nacre layer in the shells of oysters in the pH 7.6 treatment, and a lack of the characteristic wave-like growth pattern in the area of new nacre development compared to those held at pH 8.1–8.2. These results suggest that the physiological processes driving nacre deposition may be affected by ocean acidification, or that there may be some dissolution of the developing nacre tablets resulting from contact with acidified seawater which may have crossed the thin organic film enclosing the nacre tablets.

This is the first study to report on the effects of predicted near-future levels of ocean acidification on the strength and structural integrity of the shells of a tropical bivalve. The results have broad implications for both the ecology and culture of pearl oysters. For example, shells held for only 28 days at pH 7.6 showed significantly reduced shell strength which, presumably, would render oysters more susceptible to predation. Many of the predators of pearl oysters crush the shells of their prey (e.g., Pit and Southgate 2003; Humphrey 2008) and reduced shell strength may facilitate predation by this means. Pit and Southgate (2003) reported that crushing predators of the black-lip pearl oysters, *Pinctada margaritifiera* (Linnaeus, 1758), had an optimal size range for prey, and that this size range generally increased with increasing size of the predator. Pearl oysters reach their ‘escape size’ when they become too large for a predator of a given size to crush (Gervis and Sims 1992). Buschbaum *et al*. (2007) found that shells of the littorinid, *Littorina littorea* (Linnaeus, 1758), infected with boring polychaetes were not only weaker, but were preferentially selected by the predatory crab *Carcinus maenas* (Linnaeus, 1758). Although this crab typically preys upon smaller periwinkles (13–17 mm shell height), it selected large periwinkles infected with boring polychaetes in preference to non-infected periwinkles. This suggests that reduced shell strength made it easier for crabs to prey on the periwinkles (Buschbaum *et al*. 2007), and that reduced shell strength effectively increased the escape size of *L. littorea*. On this basis, it is reasonable to assume that reduced shell strength in pearl oysters, brought about by ocean acidification, would increase their effective escape size, and facilitate predation of a broader size range of pearl oysters which would have significant impacts on the culture of this species.

The potential effects of ocean acidification on nacre deposition may have devastating implications for the cultured pearl industry, as operations rely on production of high quality nacre from cultured oysters. The results of this study suggest that the physiological process of nacre deposition may be affected by ocean acidification, and developing nacre tablets may experience dissolution from contact with acidified water. While this process may impact the quality of nacre lining pearl oyster shells, it should be noted that the most economically important product of the cultured pearl industry is cultured ‘round’ pearls, not mother-of-pearl from the shells. Round pearls are cultured within the gonad of pearl oysters (Taylor and Strack, 2008) and therefore have no direct contact with the external environment. However, ocean acidification does reduce some physiological processes in *P. fucata* (Welladsen 2009). It may also influence the rate of nacre secretion and nacre quality of round pearls despite their isolation from the external environment. Such a scenario could be catastrophic for the cultured pearl industry. Further research into this area is being conducted to determine how the process of nacre deposition may be affected by ocean acidification, and possible ways to adapt husbandry practices to compensate for this. It should also be noted that this study was conducted over a 28 day period, and longer experimental periods that

allow for some acclimation of the oysters to acidified conditions would be needed in future research to indicate the likely long-term effects of ocean acidification on pearl oyster shell characteristics. In this study, oysters were not allowed to acclimate to the treatment conditions. It might therefore be possible that the results obtained include stress responses to the sudden change of pH. However, our additional research showed that pearl oysters acclimate to reduced pH within 72 hours of transfer, and recover from the acute stress of transfer (Welladsen 2009). The observed responses reported here therefore indicate that prolonged exposure to reduced ocean pH will lead to changes in the shell characteristics of pearl oysters.

Acknowledgments

This study was funded by AIMS@JCU and an internal research grant from James Cook University. The authors would like to thank Stan Hudson, Greg Duffin, James Galbraith, and Dr Dongchun Lou for their technical assistance, and the reviewers for their comments and suggestions.

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