2.4.2.2 Species

Calcification rates within each site did not vary as expected across species, despite large differences in morphology between species. Across all regions and time periods, the mean calcification rates were 1.15 mg cm\(^{-2}\) d\(^{-1}\) for *A. pulchra*, 0.66 mg cm\(^{-2}\) d\(^{-1}\) for *P. damicornis* and 0.70 mg cm\(^{-2}\) d\(^{-1}\) for *G. aspera*. Tukey’s HSD post-hoc comparisons showed that *A. pulchra* had significantly higher calcification rates than both *P. damicornis* (*p < 0.001*) and *G. aspera* (*p < 0.001*), but that *P. damicornis* and *G. aspera* were not significantly different from each other (*p = 0.765*). However these differences were not consistent across locations and seasons. For example, at Coral Bay in winter 2011, *G. aspera* had the highest growth rate, followed by *P. damicornis*, then *A. pulchra*, with the only significant difference between *G. aspera* and *A. pulchra* (*p = 0.023*). Then in summer 2012, growth rates showed no significant differences between species, while in winter 2012, *A. pulchra* grew significantly faster than *G. aspera* (*p = 0.007*), which grew significantly faster than *P. damicornis* (*p = 0.005*). In summer 2013, there was again no significant difference in growth rate between *A. pulchra* and *G. aspera*, however both grew significantly faster than *P. damicornis* (*p < 0.05*). At the Abrolhos, *A. pulchra* had significantly higher growth rates compared to the other species in both seasons (*p < 0.001*), however there were no significant differences between *G. aspera* and *P. damicornis* in both seasons (*p > 0.05, Figure 2.4*). At Marmion, there was very high mortality in *P. damicornis* in the first summer (2012) and consequently only one species comparison can be made for winter 2011, where *G. aspera*, again, grew faster than *P. damicornis*, but this difference was not significant (*p = 0.354*). It is important to note that while *A. pulchra* generally had higher growth rates than the other species, these differences were small compared to the differences between these morphologies that have been reported in the literature. Furthermore, the genus *Pocillopora* is commonly reported in the literature to grow faster than *Goniastrea* (see summary in Harriott, 1999).

For all species, AICc indicated that the best model for explaining variation in growth, contained latitude, temperature anomaly and season as variables (Table 2.4). Summed AICc weights (Figure 2.6) for *A. pulchra* indicated that latitude was the best explanatory variable for growth.
rate, followed by temperature anomaly, while season had little effect. Variation in growth rates for *P. damicornis*, were mostly attributed to temperature anomaly. Latitude had little effect, given that temperature anomalies were associated with dramatically reduced growth at all locations, which was also evident in the large contribution of season to the variation in growth, with an overall anti-seasonal effect. For *G. aspera*, both latitude and temperature anomaly contributed most to variation in growth, but with season also contributing given the reduced growth rates in summer.

As with calcification rates, there were not consistent trends in survival rates among species, however survival was generally lower in the branching species (*A. pulchra* and *P. damicornis*) compared to the massive species, *G. aspera*. *G. aspera* had the highest survival rates at all locations, except the Abrolhos, where *P. damicornis* had 100% survival in both seasons. At Coral Bay and Marmion however, *P. damicornis* experienced the lowest survival of all species (Figure 2.5).

Table 2.4. Best GLM models for predicting coral calcification within species.

<table>
<thead>
<tr>
<th>Model Terms</th>
<th>df</th>
<th>AICc</th>
<th>AICc weight</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. pulchra</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>latitude + temperature anomaly + season</td>
<td>6</td>
<td>23.648</td>
<td>1</td>
<td>0.559</td>
</tr>
<tr>
<td><em>P. damicornis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>latitude + temperature anomaly + season</td>
<td>5</td>
<td>47.916</td>
<td>1</td>
<td>0.374</td>
</tr>
<tr>
<td><em>G. aspera</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>latitude + temperature anomaly + season</td>
<td>6</td>
<td>122.515</td>
<td>1</td>
<td>0.455</td>
</tr>
</tbody>
</table>
Figure 2.6. Relative importance of variables contributing to variation in calcification rate within each species, as indicated by the sum of weighted AICc for each variable for all possible models.
2.4.2.3 *Season*

Mean calcification rates either showed little variation between seasons or were considerably lower in summer compared to winter, for all species and locations (*Figure 2.4*). Indeed, any seasonal effect was most strongly manifested as both reduced growth and survival during summer when temperature anomalies were highest; however the presence of this anti-seasonal effect varied among species and sites. Interestingly, the more temperate locations showed a stronger anti-seasonal effect than the tropical location in the first year of the study, with both the Abrolhos and Marmion having significantly slower growth rates in summer 2012 compared to winter 2011 (*p* > 0.001 for all species and both locations), while for Coral Bay there were no significant differences between seasons (*p* > 0.05 for all species). Incorporating all species and locations, calcification rates in the summer of 2012 were significantly (*p* < 0.001) lower than in the previous winter (*Figure 2.4*). Then in the summer of 2013, overall calcification rates at Coral Bay and Marmion showed no significant difference to winter 2012 rates (*p* = 0.989). The average rates of survival regardless of location were 91% in winter and 85% in summer for *A. pulchra* and 86.5% in winter and 59.5% in summer for *P. damicornis*. Rates of survival were higher in *G. aspera* (100% in winter and 93% in summer), but were still lower in summer compared to winter (*Figure 2.5*).

2.5 *Discussion*

Our results showed that summer calcification rates were either similar to, or slower than in winter over two years, with this trend being consistent across tropical and temperate locations. These findings are in strong contrast with the literature, which generally reports that coral growth rates and *in situ* rates of net community calcification are much higher in summer than in winter and that this seasonality is usually strongest at higher latitude locations (Shinn 1966; Crossland 1981; Isern et al. 1996).

Similar rates of winter and summer calcification have been observed in other reef systems before, including one of those studied here. Similar rates of net community calcification were
recorded in both summer and winter for a coral-dominated reef flat community at Ningaloo (~200 mmol m⁻² d⁻¹ in 2008 and 2009) (Falter et al. 2012). At the time those measurements were made, no evidence of bleaching or thermal stress had ever been recorded at Ningaloo. The authors suggested that the higher-than-expected observed rates of winter net calcification and their apparent decoupling from daytime net photosynthetic production, were the result of increased particle uptake during winter months (Wyatt et al. 2010; 2013). Similarly, rates of net community calcification at Kaneohe Bay barrier reef in Hawaii (21.5°N) were comparable in both winter and in summer (290 vs. 230 mmol m⁻² d⁻¹, respectively) (Shamberger et al. 2011), while rates of net community calcification at Davies Reef on the GBR (18.8°S), were only slightly higher in summer than in winter (Albright et al. 2013). Furthermore, at the organismal level, there have been some studies in which no seasonality was detected in linear extension of Acropora at the tropical locations of Jamaica (Lewis 1968; Tunnicliffe 1980) and Barbados (13°N, Lewis et al., 1968). Therefore, while it is more common for corals and entire reef communities to exhibit higher rates of calcification in summer than in winter (Shinn 1966; Crossland 1981; Isern et al. 1996), there are a limited but increasing number of examples where seasonal changes in calcification rates are far less pronounced, even in the absence of any notable signs of thermal stress.

We would have expected greater seasonality in calcification rates for the higher latitude sites, however; instead we observed the opposite trend. The most likely explanation for the observed lack of seasonality in coral calcification rates was the anomalously high water temperatures through consecutive summers from 2011 to 2013. The 2011 marine heat wave was responsible for widespread bleaching down the entire WA coast (Moore et al. 2012). This historic summer was followed by anomalously high summer temperatures in 2012 and 2013, which caused less severe patchy bleaching during both years at several reefs across WA, including some at which this study was conducted (Ningaloo, Ningaloo Atlas, 2013; Abrolhos, authors’ pers. obs. 2012, 2013). In 2012 and 2013, summer temperature anomalies were 1.5-3°C above average for sustained periods of time (2-5 months). Both in situ and laboratory studies have indicated that calcification rates are reduced under high temperature stress (Al-Horani 2005; Carilli et al.
A number of laboratory studies have shown optimum temperatures for calcification to be a few degrees below the summer maximum for a particular location (Clausen and Roth 1975; Jokiel and Coles 1977; Marshall and Clode 2004). Field studies in the Red Sea and GBR analyzing historical temperature records and growth bands of coral cores, have provided strong evidence that heat stress is responsible for reduced calcification rates in recent years (Cooper et al. 2008; De'ath et al. 2009; Cantin et al. 2010). However, a recent in situ study has demonstrated that these reductions in calcification may also be due to the changes in carbonate chemistry that have already occurred since the industrial revolution (Albright et al. 2016). Interestingly, prior to the 2011 heat wave event, calcification rates of Porites at Coral Bay and the Abrolhos were reported to have increased over the period of 1900-2010 in response to increasing water temperatures (Cooper et al. 2012). The authors did note however, that the increased rates of calcification may be unsustainable as ocean temperatures continue to rise. The findings of the present study align with previous works showing reduced calcification rates during periods of high temperature induced stress and importantly demonstrate the effects of prolonged, non-lethal exposure to elevated temperatures. However, as there is no in situ coral calcification data available using this method or these species to compare to (other studies have used growth banding of Porites colonies), we are unable to put these results into the context of a ‘normal’ year.

It is possible that our samples were also recovering from bleaching or sub-bleaching thermal stress experienced during the heat wave in 2011. Although no signs of bleaching were observed in the samples during each survey, bleaching may have preceded the mortalities of some samples. The colonies that were chosen for sampling showed no signs of stress at the time these experiments were established, although it is not known whether they had bleached during the summer heat wave in 2011, approximately 3-5 months earlier. Moore et al. (2012) reported 20 to 30% bleaching at our study sites, however the change in total coral cover was surprisingly low at Coral Bay and Marmion (2.2%, 23.3% and 7.7% for Coral Bay, the Abrolhos and Marmion respectively). Regardless of whether the parent colonies had bleached prior to when our experiments began, it is likely that most corals were at least stressed by the 2011 warming
and were still recovering during the study period. For example, while it can take a few months to a year for corals to recover their chl a levels and symbiont concentrations following a bleaching event, it may take more than a year to recover their initial tissue biomass and lipid reserves (Jokiel and Coles 1990; Fitt et al. 1993; 2000). Even in unbleached corals, lipid reserves are much lower in above average temperatures compared to normal years (Grottoli et al. 2004), indicating some level of sub-bleaching stress. Bleaching can also impair a coral’s ability to reproduce (Szmant and Gassman 1990; Baird and Marshall 2002), which has longer-term consequences for reef recovery following bleaching. Another longer-term effect of bleaching on coral physiology, is a reduction in growth rate between one and four years after the event (Goreau and Macfarlane 1990; Leder et al. 1991; Suzuki et al. 2003; Omata et al. 2006), while at locations subject to high levels of anthropogenic stress, recovery can take at least eight years (Carilli et al. 2009). Similarly, we found no evidence that corals became more resilient to future stress after being exposed to a severe thermal stress event, at least over a time scale of two years. Indeed, corals appeared to be more vulnerable to seasonal temperature stress in 2012 and 2013, following the extreme temperatures of 2011. This susceptibility was evident not only in the reduced summer growth rates but also in the lower survival rates in summer compared to winter across most species and latitudes. However, in the absence of growth data from years when thermal stress was not present, we recognize that some degree of caution is necessary in interpreting these results. Determining baseline in situ calcification rates in normal temperature years would be a valuable study for future research in WA corals.

Species differences in growth rate provided further evidence of the effects of sub-lethal temperature stress. Extension rates for Acropora and Pocillopora are usually ~10 and ~5 times faster than Goniastrea respectively (see Pratchett et al. 2015 for a summary of the literature). In contrast, calcification rates for G. aspera in this study were consistently higher than P. damicornis and sometimes even higher than A. pulchra. Given that faster growing corals (particularly acroporids and pocilloporids) are reported to be more severely impacted by bleaching, while slow-growing corals (e.g. faviids) are more resistant (Brown Suharsono 1990; Gleason 1993; Marshall and Baird 2000), it is likely that the branching species in our study
were more sensitive to the temperature stress, resulting in their reduced growth compared to the more resistant *G. aspera*. The patterns in the growth data were again reflected in the survival data, with generally lower survival in *A. pulchra* and *P. damicornis* compared to *G. aspera*. It is important to note however, that while extension rates of branching species are substantially higher than massive species, surface area-normalized calcification rates (as reported in this study) may be less dissimilar. That is, measuring extension rates may underestimate growth rates in massive corals, because it does not take in-filling and skeletal density into account. Massive corals such as *Goniastrea* form much more robust skeletons than *Acropora* species but their outward growth or extension rates are slower. Thus using techniques such as buoyant weight, which measure overall mineral deposition, are better indicators calcification than extension rates.

There is extensive evidence that coral growth rates are reduced at higher latitude sites (Crossland 1981; Grigg 1982; Stimson 1996; Harriott 1999). Harriott (1999) recorded extension rates for a range of species at the temperate Lord Howe and Solitary Islands (31.5°S and 30°S respectively). Growth for a variety of *Acropora* species ranged from 20 to 49 mm/y compared to ~80 to 120 mm/y in tropical locations (10°N) (Yap and Gomez 1984; Charuchinda and Hylleberg 1984), *Pocillopora damicornis* growth rates were 12 to 16 mm/y compared to 36 mm/y at Lizard Island (15°S) (Oliver 1985) and *Goniastrea australensis* had a growth rate of 2.8 mm/y, which was much slower than rates of 6-10 mm/y for Faviids in tropical locations (10°S) (Buddemeier et al. 1974; Highsmith 1979). We also found that calcification rates were highest in the tropical environment of Coral Bay, followed by the sub-tropical Abrolhos Islands and then the temperate environment of Marmion. Both Coral Bay and the Abrolhos had significantly higher calcification rates than Marmion, however there was comparatively little difference in growth between the Coral Bay and Abrolhos locations. Although the Abrolhos lie 5° of latitude to the south of Coral Bay, they are situated ~60 km offshore where they are continuously bathed in the warm waters of the Leeuwin Current (Pearce 1997). The average monthly temperature range at the Abrolhos and Coral Bay are relatively similar at ~ 20 to 24°C and ~22 to 27°C respectively. In contrast, at the temperate Marmion Reef water temperatures
range from ~17 to 23°C. Cooler water temperatures as well as reduced light availability at this high latitude location are the most likely reasons for Marmion G. aspera growing ~ 5 times slower than its tropical counterpart in Coral Bay.

Western Australian nearshore reefs have long been thought to be relatively protected from coral bleaching due to isolation from human impact factors and a lack of any historical evidence of mass coral bleaching (however, severe mass bleaching was recorded offshore at Scott Reef in 1998, Smith et al. 2008). There had been no large-scale mass bleaching events recorded in WA nearshore waters until the wide spread bleaching of reefs along the length of the coastline during the marine heat wave in 2011 (Moore et al. 2012; Speed et al. 2013). This study provides evidence of the on-going impacts of thermal stress on the life histories of WA corals for at least two years following the heat wave. This was apparent in the aseasonality of growth rates and reduced summer survival for a variety of coral morphologies at locations spanning ~10° of latitude. The lack of seasonality at the high latitude reefs was particularly unusual given the greater seasonal variation in light and temperature at these sites. We saw further indication of temperature stress in the form of reduced growth and survival in normally fast-growing but temperature-sensitive species. While no signs of bleaching were observed and corals appeared healthy during the study, suppressed growth may be a clear indication of sub-bleaching stress. Furthermore, sub-lethal stress associated with temperature anomalies are likely to also reduce reproduction and recruitment (Szmant and Gassman 1990; Heyward and Negri 2010), further compromising population maintenance and recovery from disturbances such as cyclones, predation and algal overgrowth (Knowlton and Jackson 2008). The susceptibility of the branching species to temperature stress could also have important and widespread implications for coral community dynamics in WA. With WA sea surface temperatures set to rise by more than 2°C by 2100 (Feng et al. 2009) and the SW Australian coast being identified as one of the three Indian Ocean “hot spots” with higher rates of warming (Pearce and Feng 2007), WA corals may be more vulnerable to the impacts of climate change than once thought.
Chapter 3: Effect of Ocean Warming and Acidification on the Early Life Stages of Sub-tropical Acropora spicifera


3.1 Abstract

This study investigated the impacts of acidified seawater (pCO₂~900 µatm) and elevated water temperature (+3°C) on the early life history stages of Acropora spicifera from the sub-tropical Houtman Abrolhos Islands (28°S), in Western Australia. Settlement rates were unaffected by high temperature (27°C, ~250 µatm), high pCO₂ (24°C, ~900 µatm), or a combination of both high temperature and high pCO₂ treatments (27°C, ~900 µatm). There were also no significant differences in rates of post-settlement survival after four weeks of exposure between any of the treatments, with survival ranging from 60% to 70% regardless of treatment. Similarly, calcification, as determined by the skeletal weight of recruits, was unaffected by an increase in water temperature under both ambient and high pCO₂ conditions. In contrast, high pCO₂ significantly reduced early skeletal development, with mean skeletal weight in the high pCO₂ and combined treatments reduced by 60% and 48% respectively, compared to control weights. Elevated temperature appeared to have a partially mitigative effect on calcification under high pCO₂, however this effect was not significant. Our results show that rates of settlement, post-settlement survival, and calcification in sub-tropical corals are relatively resilient to increases in temperature. This is in marked contrast to the sensitivity to temperature reported for the majority of tropical larvae and recruits in the literature. The sub-tropical corals in this study appear able to withstand an increase in temperature of 3°C above ambient, indicating that they may have a wider thermal tolerance range and may not be adversely affected by initial increases in water temperature from sub-tropical 24°C to 27°C. However the reduction in skeletal weight with high pCO₂ indicates that early skeletal formation will be highly vulnerable to the changes in ocean pCO₂ expected to occur over the 21st century, with implications for their longer-term growth and resilience.
3.2 Introduction

Increases in atmospheric CO₂ are driving the two major global threats to coral reefs: ocean warming and acidification (Hoegh-Guldberg et al. 2007; Veron et al. 2009). Consequently, coral reefs are currently facing unprecedented rates of change in both seawater temperature and carbonate chemistry. Many coral reefs around the world have already shown declines in overall coral cover and species diversity in response to a combination of local and climatic disturbances (Baker et al. 2008; Hughes et al. 2010; De'ath et al. 2012; McClanahan et al. 2014). Successful sexual reproduction is critical to sustaining reefs and replenishing damaged areas as well as maintaining genetic diversity (Richmond 1997; Ayre and Hughes 2000) and therefore the potential for coral to adapt to changing environmental conditions (Palumbi et al. 2014).

Additionally, the success of early life processes is critical to the dispersal of corals into refugia at higher latitude locations, as occurred, for example, during the Last Interglacial period (Greenstein and Pandolfi 2008). These potential strategies for coping with rapid environmental change require successful sexual reproduction and recruitment as well as high rates of juvenile growth and post-settlement survival (Ritson-Williams et al. 2009).

Although reproduction and the early life stages are critical for coping with environmental change, it has been shown that corals in these early stages can be particularly sensitive to local and climatic pressures (Gilmour 1999; Kurihara 2008; Putnam et al. 2010; Albright and Langdon 2011; Olsen et al. 2014). For example, elevated water temperature can cause embryonic aberrations (Bassim et al. 2002), reduced larval motility (Bassim and Sammarco 2003), reduced larval survivorship and settlement (Randall and Szmant 2009), and reduced pre-competency periods (Heyward and Negri 2010). However, the responses of larvae to elevated temperature are varied, with other studies showing no effect on larval survival and settlement (Ross et al. 2013), or higher settlement rates under elevated temperature (Nozawa and Harrison 2000; 2007) as well as varied susceptibility to temperature with differing exposure duration (Cumbo et al. 2013b) and day of release (Cumbo et al. 2013a). There are comparatively few studies investigating the effects of acidified seawater on the early life histories of corals; however, some of the deleterious effects of acidified treatments include: reduced sperm motility.
(Morita et al. 2010), reduced fertilization under low sperm concentrations (Albright et al. 2010), and reduced post-settlement calcification and growth (Albright et al. 2008; Cohen et al. 2009; Suwa et al. 2009; Albright et al. 2010; de Putron et al. 2011). Other studies have reported no effect of acidification on fertilization (at optimal sperm concentrations), metamorphosis, larval survival (Chua et al. 2013b), or even growth (under ambient temperature conditions; Anlauf et al. 2011). Similarly, larval settlement appears to be unaffected by acidified treatments (Albright et al. 2008; Anlauf et al. 2011) unless the Crustose Coralline Algae (CCA) settlement substrate has been pre-treated under acidified conditions. In this case settlement is reduced (Albright et al. 2010; Doropoulos et al. 2012a; Webster et al. 2013b) as chemical settlement cues are disrupted due to shifts in the microbial community associated with the CCA (Webster et al. 2013a; 2013b).

While both elevated temperature and acidification have been shown to have negative impacts on adult corals and corals in the early life history stages, there have been relatively few studies investigating the combined impact of these two stressors. Since increases in ocean $p$CO$_2$ and temperature are predicted to occur simultaneously, it is important to investigate the potential for synergistic effects. In adult corals, the interactive effect of these stressors is generally cumulative, with elevated $p$CO$_2$ and temperature conditions reducing bleaching thresholds (Anthony et al. 2008) and calcification (Reynaud et al. 2003). In the early life history stages the combination of elevated $p$CO$_2$ and temperature does not appear to have any impact on metamorphosis, settlement, larval survival (Anlauf et al. 2011; Chua et al. 2013a), larval photophysiology, or larval respiration (Putnam et al. 2013) but has a strong synergistic effect on early skeletal growth (Anlauf et al. 2011). Also, while a study found that fertilization success was not affected by elevated $p$CO$_2$ and temperature at optimal sperm concentrations (Chua et al. 2013a), other work reported significantly reduced fertilization under high $p$CO$_2$ and temperature at low sperm concentrations (Albright and Mason 2013). Existing studies on multiple stressors have been under tropical conditions, with temperature treatments set near the upper thermal limits of tropical adult corals. It is unknown how elevated temperature, elevated $p$CO$_2$, or a combination of these, will affect the early life history stages in cooler sub-tropical regions,
where oceanic surface water uptake of CO₂ is greater (Feely et al. 2001), and the interactive effects of these two stressors, particularly on calcification rates, may be different to the tropics.

Coral spawning at the Abrolhos Islands occurs in the months of February and March, coinciding with warmest water temperatures for the year, with monthly averages of ~23 to 24°C. During the marine heat wave of 2011, prolonged exposure to water temperatures ~2-4°C above normal (~26-28°C), from January to April, resulted in mass bleaching of adult corals at the Abrolhos (Moore et al. 2012; Abdo et al. 2012). While adult corals have well defined thermal thresholds that are relative to the long-term average temperature conditions at their particular geographical location (Coles et al. 1976; Coles and Jokiel 1977; Jokiel and Coles 1977), there is little in situ information on the thermal tolerance of new recruits and juveniles. Interestingly, a number of post-bleaching surveys have reported that juvenile corals appeared to be more resistant than adults of the same species to bleaching stress, with smaller size classes (> 10 cm) having lower rates of both bleaching and mortality (Mumby 1999; Loya et al. 2001; Depczynski et al. 2013). This could suggest that juveniles can tolerate a broad range of temperature conditions, in contrast with laboratory studies that report reduced survival of juveniles under elevated temperature conditions (Edmunds et al. 2001; Bassim and Sammarco 2003; Randall and Szmant 2009). However, to date both in situ and laboratory studies have almost exclusively focused on corals in the tropics and therefore it is unknown whether sub-tropical recruits, like adult corals, will have thermal thresholds that are relative to their geographical location and normal sub-tropical temperature regimes. Examining how sub-tropical recruits will respond to projected changes in ocean temperature and chemistry will help to determine whether these reefs have the potential to act as refugia or as ‘stepping-stones’ to facilitate the expansion of corals into formerly temperate regions under future emissions scenarios. Therefore the aims of this study were to investigate the impacts of elevated water temperatures and acidified conditions on the early life history stages, including settlement, post-settlement survival, and early skeletal growth of a ubiquitous coral species at the sub-tropical Houtman Abrolhos Islands.
3.3 Methods

3.3.1 Collection Methods

The plate coral *Acropora spicifera* is one of the most abundant and widespread species at the Houtman Abrolhos Islands and on tropical reefs throughout WA (Veron and Marsh 1988). Prior to spawning, 15 gravid adult (~30 x 30 cm) *A. spicifera* colonies were collected off Basile Island in the Southern Group of the Abrolhos Islands (28°52’S, Figure 1.4). Colonies were maintained in flow-through outdoor aquaria exposed to natural lighting, which received seawater filtered to 20 µm. Gametes were collected from six colonies (the greatest number of colonies that spawned synchronously) and pooled, representing equal contributions from all six genotypes. Larvae were cultured (Heyward and Negri 1999) and maintained in ambient conditions (~24°C and ~pH 8.1) until they reached the planulae stage. The planulae stage of development occurred at 6 d post-fertilization, at which point larvae were introduced to the treatment tanks.

3.3.2 Experimental Set-up

All seawater entering the aquarium system was foam fractionated and UV-sterilized to remove solids and bacteria. Each of the four treatments consisted of a single sump tank (160 L), which fed into four replicate tanks (25 L) that contained the coral larvae. Seawater was first treated in the sump tanks, and then flowed into the replicate tanks every 3 h for an almost complete water change (~90%). Using a sump tank to treat the seawater resulted in the four replicate tanks being pseudoreplicated within each treatment system. The seawater carbonate chemistry was adjusted using an automated CO₂ bubbling system (CO₂ Set Professional, AquaMedic, Germany), which controlled the pH to ±0.01 units via a solenoid system. The pH was checked manually in all tanks every second day using a Schott Handylab pH 12 pH meter. The pH varied from set values by only ±0.05 to ±0.08 over the course of the experiment, across all treatments (Table 3.1). Total Alkalinity (TA) samples were taken from each tank once per week and analysed using the spectrophotometric method (Yao and Byrne 1998). Aragonite saturation state (Ω<sub>ar</sub>) and partial pressure of carbon dioxide (pCO₂) were calculated from pH, TA, and
temperature using the program CO2SYS (Lewis et al. 1998) using a salinity of 35.5 (Lourey et al. 2006). Salinity was checked twice a week using a handheld refractometer. Water temperature was maintained by circulating seawater through heater chillers (Resun, CL 150) and continuously monitored using Hobo Pendant temperature loggers in both sump and replicate tanks (±0.5 to ±0.9°C). The four temperature-pCO₂ conditions (Table 3.1, Figure 3.1) were: ambient temperature with ambient pCO₂ (Control: 24°C, ~250 µatm), high temperature with ambient pCO₂ (High T: 27°C, ~250 µatm), ambient temperature with high pCO₂ (High pCO₂: 24°C, ~900 µatm), and high temperature with high pCO₂ (High T + pCO₂: 27°C, ~900 µatm).

While the ambient temperatures in the controls represented average present-day temperatures around the Abrolhos during and after coral spawning (Foster et al. 2014), ‘ambient’ pCO₂ levels in the present experiments are closer to that of a pre-industrial (~250 µatm) rather than present-day atmosphere (~390 µatm). This was likely the result of seawater being pumped into the experimental facility in the afternoon when net photosynthesis by adjacent nearshore communities of benthic primary producers were driving pCO₂ below atmospheric levels in waters around the intake. In contrast, our ‘high’ pCO₂ levels (~900 µatm) are similar to projected atmospheric levels by the year 2100 under the RCP 8.5 scenario (~930 µatm) (Meinshausen et al. 2011). Fluorescent aquarium lights (Aqua One Fluroglow reflectors with 30-w marine blue and tropical fluorescent tubes), maintained at a 12 h: 12 h light/dark cycle, were set up 15 cm above the replicate tanks to provide a mean (± SE) light intensity of 212 ± 8 µmol photons m⁻² s⁻¹ (Biospherical Instruments, QSL-2100).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Temperature (°C)</th>
<th>pH_f (µmol kg⁻¹)</th>
<th>TA (µmol kg⁻¹)</th>
<th>pCO₂ (µatm)</th>
<th>Ω_ar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
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<td>8.22 ± 0.05</td>
<td>2308 ± 40</td>
<td>242 ± 22</td>
<td>4.51 ± 0.14</td>
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<tr>
<td>High T</td>
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<td>8.18 ± 0.05</td>
<td>2312 ± 26</td>
<td>275 ± 24</td>
<td>4.68 ± 0.17</td>
</tr>
<tr>
<td>High pCO₂</td>
<td>24.1 ± 0.6</td>
<td>7.77 ± 0.06</td>
<td>2307 ± 30</td>
<td>872 ± 58</td>
<td>1.93 ± 0.08</td>
</tr>
<tr>
<td>High T + pCO₂</td>
<td>27.4 ± 0.9</td>
<td>7.75 ± 0.08</td>
<td>2309 ± 32</td>
<td>976 ± 103</td>
<td>2.03 ± 0.12</td>
</tr>
</tbody>
</table>

TA: total alkalinity; pCO₂: partial pressure of carbon dioxide; Ω_ar: aragonite saturation state.
3.3.3 Larval Settlement and Post-settlement Survival

For settlement assays, 40 motile larvae were transferred into 50-mL clear acrylic tubes with 100-µm mesh covering both ends to allow for water exchange. Plastic transparency paper, washed and soaked in ambient seawater for one week, lined the inside of the tubes. The transparency paper lining was used to facilitate easy removal of the recruit skeletons at the end of the experiment and allow for subsequent analyses (3D X-ray microscopy, SEM, X-ray Diffraction and Raman Spectroscopy) of the skeleton discussed in later chapters. However, it should be noted that plastic is not the natural settlement substrate for larvae. Three 0.5-cm X 0.5-cm crustose coralline algae (CCA) chips of the genus Hydrolithon, collected from the same site as adult A. spicifera colonies, were added to each tube. Tubes were then transferred into the replicate treatment tanks. Settlement was counted daily in three tubes per replicate tank (n = 4, as tubes were pooled within each of the four tanks per treatment). Larvae were counted as ‘settled’ when they had metamorphosed (flattened disc-shape). Due to foam fractionation and UV-sterilising of the seawater, all naturally occurring microalgae and bacteria (including free Symbiodinium), were effectively removed. Polyps were therefore inoculated with cultured clade C1 zooxanthellae (cultured by V. Beltran, AIMS) at 7 d post-settlement. At four weeks post-
settlement, the surviving juveniles were counted \((i.e.\) from the number of larvae that had settled) to determine percent post-settlement survival \((n = 4\) per treatment).

3.3.4  **Skeletal Weight**

To remove organics from the samples, polyps were immersed in 3-7% sodium hypochlorite \((\text{NaClO})\), then rinsed three times in deionized water and left to air dry. Five primary polyp \((i.e. \) single mouth) skeletons from each treatment were removed from the paper using a scalpel and individually weighed to an accuracy of 1 µg on a Mettler-Toledo microbalance to determine mean skeletal mass. Individual skeletons were randomly selected across tubes and tanks. The average calcification rate over the course of the experiment was determined by dividing these weights by the number of days between when the majority of larvae settled and when they were removed from the tanks at the end of the experiment \((31\) d).

3.3.5  **Data Analysis**

For settlement and post-settlement survival, data from each of the four tanks per treatment were pooled prior to analyses \((i.e.\) averages of the three tubes per tank were first calculated). All data were checked for homogeneity of variance using Levene’s Test for equality of variance and checked for normality using the Shapiro-Wilk test. All data met assumptions of equality of variance and normality. Two-way ANOVA’s were used to analyze differences in settlement rates, post-settlement survival, and skeletal weight. Fixed factors were temperature \((24\) and \(27^\circ\)C\)) and \(p\text{CO}_2\) \((250\) and \(900\) µatm). Since the same divisor \((31\) d\)) was applied to all skeletal weights, it was not necessary to conduct statistical tests on calcification rate data. All statistical analyses were conducted in SPSS version 21.

3.4  **Results**

Spawning occurred \(9\) d after the February full moon, on the \(6^{th}\) of March 2013 at \(\approx 21:00\). Spawning during 2013 was likely split, with the full moon falling at the end of February and the potential for a second spawning after the March full moon in early April. In 2012, mass spawning at the Abrolhos occurred \(6\) d after the March full moon on the \(14^{th}\) of March (authors
pers obs). At 6 d post-fertilization, larvae were competent to settle (planulae stage) and were swimming down through the water column. The majority of the larval settlement (~80-85%) occurred 7 d after fertilization (the day after being introduced to the treatments and settlement substrata). By 10 d, all competent larvae across all treatments had settled (i.e. all larvae had either settled or died, as swimming larvae were no longer evident). Larval settlement occurred on both the CCA and the transparency paper, with the majority of settlement occurring on the CCA chips or on the transparency paper immediately adjacent to the CCA.

There was no significant effect of temperature, $p$CO$_2$, or their interaction on larval settlement (Table 3.2) with settlement rates being approximately 35 to 45% regardless of treatment (Figure 3.2). There was also no significant effect of temperature, $p$CO$_2$, or their interaction on post-settlement survival (Table 3.2), after being maintained under treatment conditions for four weeks. Post-settlement survival ranged from ~60 to 70% across all treatments (Figure 3.3).

Table 3.2. Two-way analysis of variance (ANOVA) of settlement, post-settlement survival, and skeletal weight of Acropora spicifera recruits under four temperature-$p$CO$_2$ conditions.

<table>
<thead>
<tr>
<th>Two-way ANOVA</th>
<th>n</th>
<th>df</th>
<th>F-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Settlement</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature</td>
<td>16</td>
<td>1</td>
<td>0.649</td>
<td>0.436</td>
</tr>
<tr>
<td>$p$CO$_2$</td>
<td>16</td>
<td>1</td>
<td>2.261</td>
<td>0.159</td>
</tr>
<tr>
<td>Temperature * $p$CO$_2$</td>
<td>16</td>
<td>1</td>
<td>0.942</td>
<td>0.351</td>
</tr>
<tr>
<td><strong>Post-settlement survival</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature</td>
<td>16</td>
<td>1</td>
<td>0.068</td>
<td>0.799</td>
</tr>
<tr>
<td>$p$CO$_2$</td>
<td>16</td>
<td>1</td>
<td>0.102</td>
<td>0.755</td>
</tr>
<tr>
<td>Temperature * $p$CO$_2$</td>
<td>16</td>
<td>1</td>
<td>0.156</td>
<td>0.700</td>
</tr>
<tr>
<td><strong>Skeletal weight</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temperature</td>
<td>20</td>
<td>1</td>
<td>0.046</td>
<td>0.833</td>
</tr>
<tr>
<td>$p$CO$_2$</td>
<td>20</td>
<td>1</td>
<td>32.193</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Temperature * $p$CO$_2$</td>
<td>20</td>
<td>1</td>
<td>1.541</td>
<td>0.234</td>
</tr>
</tbody>
</table>
**Figure 3.2.** Mean (±SE) percent settlement of *Acropora spicifera* under treatment conditions at one week post-fertilization (n = 4 per treatment).

**Figure 3.3.** Mean (±SE) percent post-settlement survival of *Acropora spicifera* after being maintained under treatment conditions for 1 month (n = 4 per treatment).
In contrast to rates of settlement and post-settlement survival, elevated \( pCO_2 \) reduced skeletal development at both ambient and elevated temperatures, while temperature alone had no effect (Figure 3.4). Under ambient \( pCO_2 \), skeletal weights were \(~250 \mu g\) (at both temperatures), while under elevated \( pCO_2 \), skeletal weights were \(~100 \mu g\) (60% lower than the control) at ambient temperature and \(~150 \mu g\) (48% lower than the control) at elevated temperature (Figure 3.4). Higher \( pCO_2 \) significantly reduced skeletal weight; however, neither temperature nor the interaction of temperature and \( pCO_2 \) had a significant effect on skeletal weight (Table 3.2).

**Figure 3.4.** Mean (±SE) skeletal weight (bars on the left y axis) and calcification rates (circles on the right y axis) of Acropora spicifera after 1 month under treatment conditions (\( n = 5 \) per treatment).
3.5 Discussion

Our study applied quite extreme $p$CO$_2$ and temperature treatments ($p$CO$_2$ $\sim$900 µatm and $+3^\circ$C) relative to ambient conditions at the Abrolhos Islands ($p$CO$_2$ $\sim$300-500 µatm: Foster et al. 2014; 19-24°C: Pearce et al. 1999). Yet there was no effect of temperature, $p$CO$_2$, or a combination of both factors on coral larval settlement or post-settlement survival. There have been numerous studies reporting increased settlement and rates of development under elevated temperature treatments (Nozawa and Harrison 2007; Heyward and Negri 2010; Woolsey et al. 2013; Chua et al. 2013a). Under short-term exposure (hours) settlement generally increases in response to elevated temperature, even when extreme treatments (4-7°C above ambient) have been applied (Edmunds et al. 2001; Nozawa and Harrison 2007). However, under longer-term exposure (days to weeks), the effect of temperature on settlement appears to be dependent on the severity of the treatment, with slightly elevated temperatures (2°C above ambient) in sub-tropical conditions enhancing settlement (Nozawa and Harrison 2000), and more extreme elevations in temperature (3-4°C above ambient) greatly reducing it (Bassim and Sammarco 2003; Randall and Szmant 2009). Similarly, significant declines in survivorship are reported in larval and post-settlement survival experiments with comparable temperature elevations (Edmunds et al. 2001; Bassim and Sammarco 2003; Nozawa and Harrison 2007; Randall and Szmant 2009; Schnitzler et al. 2012; Woolsey et al. 2013; Figueiredo et al. 2014). Conversely, Olsen et al. (2013, 2014) saw no effect of temperature ($+3.5^\circ$C) on settlement, larval survival, or post-settlement survival, but did report that elevated temperature caused sub-lethal stress. Baird et al. (2006) and Baria et al. (2015) also recorded no effect on larval survival at $+4^\circ$C and $+3^\circ$C respectively, however in both of these studies, more extreme temperature ($+8^\circ$C and $+6^\circ$C) resulted in significantly reduced survival rates. Recent work has recorded differences in thermal tolerance between coral larvae from different latitudes; with high latitude (31°S) larvae better able to cope with elevated temperature than low latitude larvae (Woolsey et al. 2015).
Our temperature treatment was relatively extreme, with an elevation of ~3°C above ambient for a prolonged period of time (weeks). Therefore, we would have expected both reduced settlement and survival in response to temperature. However, unlike most other studies investigating coral settlement and survival under future climate regimes, our study was conducted at a sub-tropical reef, where the mean ambient temperature at spawning time is ~24°C (corresponding with the warmest monthly temperatures for the year at the Abrolhos).

The lack of a temperature effect may have been due to this experiment taking place under cooler temperature conditions, with the elevated temperature treatment being ~27°C compared to the 30-34°C treatments in tropical experiments, even though it was still ~3°C above normal ambient temperatures at this site. This suggests that larvae and recruits in the sub-tropics may have a wider thermal tolerance range than those in the tropics. This broader thermal tolerance may arise for several reasons. Sub-tropical reefs experience a wider annual range in temperatures than tropical reefs, which could enable greater tolerance to temperature extremes, albeit at lower temperatures. Additionally, some coral species at the Abrolhos likely originated from tropical reefs following poleward dispersal via the Indonesian Throughflow and Leeuwin Current (Cresswell 1996; Domingues et al. 2007) and therefore may be ‘pre-equipped’ with a physiology that is capable of withstanding tropical temperatures. *Acropora spicifera* in particular has a wide geographic range and is commonly found in both tropical and sub-tropical locations. However, *A. spicifera* has also been recognized as one the most abundant reef-building coral species at the Abrolhos for at least ~30 yr (Veron and Marsh 1988), suggesting that it has had a major presence in these islands for at least half a century if not longer. Thus, we expect that most of the recruitment of *A. spicifera* at the Abrolhos is from local parental colonies and any such ‘pre-adaption’ to tropical temperatures evident in the larvae we studied would have been retained over many generations. This ability to acclimate to a broad range of temperatures may be a key factor in the on-going resilience and survival of this species in the face of climate change at the Abrolhos and could inform us on how other more circum-global reef-building species will adjust to regional changes in climate over the coming century.

Interestingly, the only other study recording post-settlement survival at a high latitude location (Lord Howe Island, ~31°S), reported increased post-settlement survival at 3°C above ambient.
(26°C) compared to the control at 23°C, then drastic declines in survival when the temperature was increased an additional 3°C (29°C; Nozawa and Harrison 2007). Their results, in conjunction with our own, could indicate that the survival rates of coral recruits in sub-tropical and even temperate environments may not be as vulnerable to initial ocean warming as they are in more tropical environments. While the settlement and survival of *A. spicifera* appears to be less sensitive to increases in temperature than reef-building coral from more tropical environments, caution should be exercised in extrapolating these results to all sub-tropical species, particularly those species not commonly found in the tropics. In order to test this hypothesis thoroughly, the responses of a range of species, as well as recruits of the same species from a range of latitudes, need to be determined and would be a key area for future research.

Our findings align with the majority of the acidification research, which report that high $p$CO$_2$ has no direct effect on coral settlement (Albright et al. 2008; Albright 2011; Anlauf et al. 2011), or larval and post-settlement survival (Suwa et al. 2009; Anlauf et al. 2011; Nakamura et al. 2011; Chua et al. 2013b). The only other study testing the combined impact of acidification and elevated temperature on settlement similarly found no effect of $p$CO$_2$, temperature, or a combination of both stressors (Anlauf et al. 2011), however the high temperature treatment in that experiment was only elevated to 1°C above ambient.

While there appeared to be little effect of acidification on settlement and survival, there was a significant reduction in mean skeletal weight in both high $p$CO$_2$ treatments compared to ambient $p$CO$_2$ treatments. The high $p$CO$_2$ with ambient temperature treatment had skeletal weights that were on average 60% lower than the control, and the high $p$CO$_2$ with high temperature treatment had weights that were 48% lower than the control, while elevated temperature alone had no effect. Certainly the findings of the present study and the majority of the literature show significant impacts of acidification on juvenile skeletal growth. Other studies report a ~20-35% decline in skeletal mass for tropical coral recruits at similar pH treatments (Cohen et al. 2009; de Putron et al. 2011; Drenkard et al. 2013). While calcification in this study appears to be more
severely impacted by acidification, there are a number of key differences that may account for this. Firstly, the main difference between the present study and other experiments was its longer exposure time; five weeks compared to eight days, (Cohen et al. 2009), two weeks (de Putron et al. 2011) and three weeks (Drenkard et al. 2013). A longer exposure time, as well as more mature juvenile skeletons, could explain some differences. Secondly, while the acidified conditions in the present study were similar to prior studies, the pH of ambient treatments in our study (~8.2) was closer to pre-industrial conditions than other studies (~8.03 to 8.17). This larger difference between treatment conditions may have contributed to the more severe impact on skeletal formation. Additionally, the temperature applied as the ambient condition in this study was 24°C, compared to control temperatures ranging from 25 to 29°C in tropical experiments (Cohen et al. 2009; de Putron et al. 2011; Drenkard et al. 2013).

Our results suggest that warmer water temperatures, at least in sub-tropical locations, may initially mitigate the effects of high $pCO_2$ on calcification. In this study, the mean skeletal weight in the elevated temperature treatment was ~25% heavier than the ambient temperature treatment under elevated $pCO_2$ conditions. This difference in weight was not statistically significant and therefore further work is required to provide substantial evidence for this hypothesis. Nevertheless, the 25% increase with higher temperatures suggests that warmer water temperatures in sub-tropical locations could initially aid in calcification under high $pCO_2$ conditions. A similar enhancing effect of temperature on calcification has been predicted due to pH up-regulation (McCulloch et al. 2012) below physiological thresholds. As with settlement and survival, it appears that initial temperature increases will not have a negative effect on calcification in coral recruits in the sub-tropics, however the impact of high $pCO_2$, regardless of temperature, is likely to be severe.

It has been widely reported that corals at high latitudes have lower thermal tolerance limits than tropical corals and that thermal tolerance is relative to the ambient temperature at a particular location (Coles et al. 1976; Coles and Jokiel 1977; Jokiel and Coles 1977). Indeed, adult corals at the sub-tropical Abrolhos Islands appear to be susceptible to thermal stress when
temperatures exceed ~26°C for prolonged periods of time. This was evident from widespread coral bleaching during the 2011 heat wave event (Moore et al. 2012; Abdo et al. 2012) and the reduced summer growth rates observed the following year when temperatures were again anomalously high (Foster et al. 2014). However, there is very little information on the thermal tolerance limits of sub-tropical coral recruits and juveniles. The lack of response to a relatively severe temperature treatment in the present study could suggest that sub-tropical coral larvae and recruits may have some flexibility when it comes to thermal tolerance limits. It is interesting that they appear to be better able to tolerate increases in water temperature than adult corals at the Abrolhos, which bleached at a similar ~3°C increase in water temperature (Moore et al. 2012; Abdo et al. 2012). However, the impacts of sub-lethal temperature stress cannot be discounted, with a number of studies reporting sub-lethal cellular responses in coral larvae (Olsen et al. 2013; Ross et al. 2013; Olsen et al. 2014). While our study does not directly compare adult and juvenile responses, we note that the Abrolhos experienced mass coral bleaching at +3°C, while the larvae and recruits in our study were not negatively impacted by the same temperature elevation. Below we discuss the possibility that corals in the early life stages are pre-equipped to deal with a wide range of temperatures.

Numerous field and laboratory studies have reported that upper thermal tolerance limits are not fixed (Coles 2003; Oliver and Palumbi 2009; 2011), and that acclimation (over less than 2 yr) can achieve the same heat tolerance in corals as long-term adaptation (Palumbi et al. 2014). Similar phenotypic plasticity has been recorded in corals exposed to a 6°C change in temperature over the course of a single day (Mayfield et al. 2012). Indeed corals in the Arabian Gulf are able to withstand a 20°C annual range in temperatures (~15 to 35°C), compared to the 5-7°C range tolerated in most tropical reef systems (Coles and Jokiel 1977; Jokiel and Coles 1977; Coles 2003). Perhaps the plasticity in thermal tolerance limits observed geographically and temporally can be extended to different stages of the lifecycle. Evidence of such plasticity can be seen in the shuffling of Symbiodinium clades with age. Little et al. (2004) reported that ~33% of four-week old juveniles of Acropora tenuis harbored the thermally resilient
*Symbiodinium* D compared to ~50% with the growth enhancing *Symbiodinium* C1. At four months old, the frequency of *Symbiodinium* D occurrence had increased to ~90% while *Symbiodinium* C1 frequency had been reduced to 0%. The authors suggested that corals change their symbiont associations depending on their energetic needs, with greater energy demands during certain stages of the lifecycle requiring the C1 strain (Little et al. 2004). Conversely, the low frequency of strain D in 4-week old juveniles could indicate that at this stage of the lifecycle corals are more resilient to thermal stress and do not require high levels of strain D. Similarly in the present study, recruits were inoculated with *Symbiodinium* C1 only, and yet still showed no negative responses to elevated temperature, which could further indicate some resistance to thermal stress in these early stages.

Putnam et al. (2010) demonstrated that coral larvae are well suited to fluctuating temperature environments, with greater photosynthetic efficiency recorded under fluctuating, compared to single, temperature treatments. The hypothesis that coral larvae and new recruits have a wide thermal tolerance range and thrive in a fluctuating thermal environment makes evolutionary sense. Coral larvae are able to disperse to locations hundreds of kilometers away from parental colonies depending on current speeds and larval duration (Treml et al. 2008; Graham et al. 2008; van Oppen et al. 2008). New recruits could end up in thermal environments vastly different from their source location. Similarly, in the first few days post-fertilization, larvae are positively buoyant (Harrison and Wallace 1990) and are thus confined to the top 0.5-1 m of the water column (Wellington and Fitt 2003), where there are large natural daily fluctuations in temperature, light, salinity, and carbonate chemistry (Gilmour et al. 2009; Teneva et al. 2013). Perhaps exposure to large daily fluctuations or regional differences in environmental parameters require larvae and newly settled recruits to be pre-equipped to deal with a wide range of conditions. If juveniles in sub-tropical and temperate locations do exhibit a wide thermal tolerance range, then this could indicate some resilience to future increases in water temperature, which could potentially continue into the adult phase. Certainly the potential for high latitude recruits and juveniles to absorb small increases in temperature warrants further investigation.
In contrast, the severe impact of high $p$CO$_2$ on calcification, regardless of temperature, is concerning. While post-settlement survival was not negatively impacted by acidification in this study, the reduced ability to grow leaves juveniles much more susceptible to size-specific mortality, which results from algal overgrowth, predation, and storm damage (Knowlton and Jackson 2008; Osborne et al. 2011; De'ath et al. 2012; Doropoulos et al. 2012b). Thus, the survival rates reported in this study are likely to be an overestimation for sub-tropical coral recruits under high $p$CO$_2$ conditions. Interestingly, the increase in temperature had little effect on calcification rates, even appearing to aid with calcification under acidified conditions, rather than reducing it further. This interactive effect between temperature and acidification contradicts what has been observed in early tropical coral calcification (Anlauf et al. 2011), and further indicates that sub-tropical recruits may not be adversely affected by initial increases in water temperature.

Experiments investigating the effects of temperature and $p$CO$_2$ on corals and other marine organisms yield varying results. While it is clear that increased water temperatures and $p$CO$_2$ have a largely negative impact on corals, the magnitude of the impact is likely to vary not only with the magnitude and duration of the increase, but also among life history stages and between reefs at different geographical locations. The persistence of coral reefs in the face of changing environmental conditions, both locally and globally, depends strongly on their adaptive capacity in the early life history stages. Additionally, larval dispersal and recruitment provide the only means by which coral can shift to more favorable habitats, where successful, early, post-recruitment processes will be critical. Our data provide insights into how predicted increases in ocean temperatures and $p$CO$_2$ can have varying effects on life history stages. In particular, the resilience of sub-tropical larvae and recruits to increases in temperature, as well as their connectivity to tropical and temperate reefs, may be critical to the persistence of many coral species through range expansion and should be key areas for future research.
Chapter 4: Seeing red: Coral larvae are attracted to a healthy looking reefs

Foster T, Gilmour JP (in review) Seeing red: Coral larvae are attracted to a healthy looking reefs. Marine Ecology Progress Series.

4.1 Abstract

Settlement cues play an essential role in larval habitat selection and influence post-settlement survival. Recent studies have investigated the impacts of elevated temperature and $p$CO$_2$ on the ability of marine larvae to locate the reef. In coral larvae, there has been a focus on chemical settlement cues, which are critical to successful habitat selection, but less is known about the role of spectral cues. In this study, we provided larvae with crustose coralline algae (CCA), their preferred settlement surface (and chemical cue), and either a white or a red synthetic settlement surface, that simulated the wavelengths emitted by bleached and unbleached CCA respectively. We performed these experiments under four temperature-$p$CO$_2$ regimes to determine whether elevated temperature (+3°C) or $p$CO$_2$ (900 µatm) affected how larvae respond to settlement cues. Settlement rates increased by ~85% on the synthetic surface if the background colour was red compared to white. Larvae preferentially settled on the CCA chips since these provided both a chemical and spectral cue. However, once all the space on the CCA was occupied, larval settlement on the synthetic surface only occurred if it appeared red. Settlement on the red synthetic substrate also occurred around the CCA chips, further suggesting that coral larvae rely on both chemical and spectral settlement cues to choose a site for attachment. Neither elevated temperature nor elevated $p$CO$_2$ directly affected larval settlement rates or substrate preference, but our findings indicate that larvae could be indirectly affected by these stressors; high water temperatures and acidification may disrupt not only chemical cues, but also spectral cues by causing CCA bleaching, thereby inhibiting the ability of larvae to either ‘smell’ or ‘see’ the reef.
4.2 Introduction

Many coral reef organisms have a pelagic stage in their life history during which they disperse for hours to months, before recruiting to a suitable reef habitat. Once thought to be passive particles, it is now known that coral larvae actively distinguish and select habitat types (Babcock and Mundy 1996; Raimondi and Morse 2000; Baird et al. 2003; Harrington et al. 2004). Habitat selection affects early post-recruitment survival (Harrington et al. 2004) and also influences the likelihood of surviving to the adult (reproductive) stages, as once the coral larva has metamorphosed it will usually remain attached to the substrate for the duration of its life, able to alter its habitat conditions only through asexual growth and propagation. Thus, it is not surprising that the larvae of corals and other sessile organisms have well developed sensory abilities.

Once coral larvae are competent to settle, they begin to actively seek a suitable settlement substrate (Harrison and Wallace 1990). The surface irregularity and angle of the substrate have been shown to affect settlement (Carleton and Sammarco 1987). In addition to these physical attributes, chemical cues associated with reef substrata, such as crustose coralline algae (CCA) and microbial biofilms, are known to induce settlement (Morse et al. 1988; 1996; Heyward and Negri 1999; Negri et al. 2001). The ability to detect favoured settlement surfaces highlights that coral larvae have a relatively advanced chemical sensory system. Some coral larvae have settlement preferences that are specific to certain CCA species (Morse et al. 1988; Raimondi and Morse 2000), while others have been shown to prefer substrate communities specific to the depth occupied by adult corals of the same species (Baird et al. 2003). More recently, larvae have been shown to detect chemical signals associated with ‘healthy’ coral reef substrates, in preference to those associated with degraded, seaweed-dominated substrates (Dixson et al. 2014). This has led to concerns about the potential for recovery in overfished and locally degraded areas, where recruitment is inhibited by chemical signals (Dixson et al. 2014). In addition to local stressors, global changes in oceanic carbonate chemistry and temperature can also impede coral larval settlement by driving shifts in the microbial communities associated with CCA (Webster et al. 2011; 2013a; 2013b).
While chemical cues clearly influence coral larval settlement, less is known about the other sensory capabilities larvae use to identify a suitable settlement substrate. Both coral and fish larvae respond to acoustic cues and are attracted to ‘reef sounds’ produced by fish and crustaceans (Simpson et al. 2005; Vermeij et al. 2009). Coral larvae are also sensitive to light, displaying positive phototaxis (Szmant and Meadows 2006) and preference for certain light intensities at settlement (Mundy and Babcock 1998). More recently, larvae have been found to exhibit colour preferences during settlement, with a remarkable preference for red settlement substrates (Mason et al. 2011). In those experiments, coral larvae were offered a variety of different coloured, unconditioned plastic surfaces as settlement substrates, with no chemical settlement cue (such as CCA) present. Regardless of coral species or shape of the plastic settlement surface, larvae consistently preferred red settlement substrates, which the authors suggested was an adaptation for identifying coralline algae (Mason et al. 2011). We tested the importance of photosensory and chemosensory cues in influencing settlement rates under four temperature-pCO₂ scenarios. In particular, our study further investigates the preference coral larvae have for red settlement substrates and determines whether this colour preference is altered under elevated temperature and pCO₂.

4.3 Methods

4.3.1 Experimental Set-up

See Chapter 3 for full details on the experimental set-up and larval culturing. Larvae of the plate coral Acropora spicifera from the sub-tropical Houtman Abrolhos Islands in Western Australia were cultured in aquaria and maintained under ambient conditions (~24°C and ~pH 8.1) until they reached the planulae stage of development. Once larvae reached competency, they were transferred into the temperature-pCO₂ treatment aquaria (Table 3.1). Forty larvae were added to 50 mL clear acrylic tubes, lined with transparency paper (washed and soaked in filtered seawater), with 100 µm mesh on either end to allow for water exchange, and three chips (~0.5 - 1 cm²) of Hydrolithon CCA added to all tubes. CCA was collected from the same site as the
parent colonies. To preserve their chemical cues, CCA chips were maintained in filtered seawater in outdoor aquaria under ambient conditions until the commencement of the experiment. The clear settlement tubes were given either a white or a red background (n = 6 per temperature-\(p\)CO\(_2\)-background colour treatment), which simulated the wavelengths emitted by healthy (red) and bleached (white) CCA. This assay aimed to investigate settlement surface preference based on the chemosensory (synthetic vs natural CCA substrate) and photosensory (red vs white background colour) abilities of larvae, and the effects of elevated temperature and/or \(p\)CO\(_2\) on these sensory systems.

### 4.3.2 Fluorescence Emission Spectra

To determine whether the red and white synthetic backgrounds simulated the spectral emission of healthy and bleached CCA respectively, fluorescent emission spectra were obtained for the red and white plastic backgrounds as well as for bleached and unbleached CCA. These spectra were measured using a Leica TCS SP2 multiphoton confocal microscope. Samples were scanned in multiphoton mode using an excitation wavelength of 488 nm. Scans were from 490 to 720 nm, using \(xy\lambda\) mode with 10 nm windows. Spectra were peak normalized (the peak emission value was set to 100) in order to compare peak locations between samples.

### 4.3.3 Data Analysis

Binomial logistic regression analysis was used to predict the probability of settlement surface preference as a function of background colour and temperature- \(p\)CO\(_2\) treatment. Student’s t test was used to test for a difference in overall settlement with changes in background colour. All statistical analyses were conducted in SPSS version 22.

### 4.4 Results

#### 4.4.1 Fluorescence Emission Spectra

The fluorescence emission spectra of the red and white synthetic surfaces had similar spectral properties to unbleached and bleached CCA respectively (Figure 4.1). The red synthetic surface
peaked at 600 nm, while the unbleached CCA peaked at 580 nm. The white synthetic surface and bleached CCA followed similar spectral trends with both exhibiting maximum emission at the UV end of the spectrum (< 400 nm), and both clearly differing from the spectra emitted by the red surfaces.

**Figure 4.1.** Fluorescence emission spectra of the synthetic settlement surfaces (red and white) and bleached and unbleached CCA.

### 4.4.2 Settlement

The temperature and pCO₂ treatments did not significantly affect larval settlement rates, regardless of the background colour ($p = 0.470$). In contrast, rates of larval settlement varied considerably with the colour of the synthetic substrate (**Figure 4.2**). There was significantly ($p = 0.001$) higher settlement overall when the background colour was red (43%) compared to white (22%). Settlement on the CCA was ~10-20% regardless of background colour, but settlement on the synthetic substrate was ~30% when the background appeared red and ~5% when it appeared white. When the background colour was white, larvae were ~5 times more likely to settle on the CCA compared to the synthetic substrate (Odds ratio = 4.86, 95% CI: 3.8 to 6.2), but when the background colour was red, larvae were twice as likely to settle on the synthetic substrate compared to the CCA (Odds ratio = 1.78, 95% CI: 1.34 to 2.35). However, larvae had far more available space on the synthetic surfaces than on the CCA chips. Thus in reality, larvae consistently preferred to settle on the CCA, with most of the surface area of the
small CCA chips occupied by recruits in all treatments (Figure 4.3). Settlement on the synthetic substrate then occurred at much higher rates if the substrate appeared red. Settlement on the red synthetic substrate also occurred adjacent to the CCA chips, generally forming a ring or cluster of settled larvae around the CCA (Figure 4.3).

**Figure 4.2.** Coral settlement (% mean ± SE) on Crustose Coralline Algae (CCA) versus the synthetic substrate when the background colour was either red or white, under control, elevated temperature (High T), elevated $pCO_2$ (High $pCO_2$) and elevated temperature and $pCO_2$ (High T + $pCO_2$) conditions.

**Figure 4.3.** Settlement on the red synthetic surface typically occurred near or surrounding the CCA chip. A: Planulae ‘searching’ the surface of a CCA chip; B: settlement in the red background treatment, with newly settled larvae on and adjacent to the CCA chip; C: CCA chip with much of the surface occupied by 1-month old recruits; D: 1-month old recruits settled around CCA chips on the synthetic substrate when it appeared red; E: the CCA chip and background colour have been removed to show a typical ring formation of 1-month old recruits surrounding the CCA chip when the background colour was red (dotted line indicates where CCA used to be).
4.5 Discussion

Background colour had a remarkable effect on the settlement of coral larvae regardless of temperature-\(p\text{CO}_2\) treatment. When the background colour was red, there was an increase in overall settlement, particularly due to much higher settlement rates on the red synthetic surface. These observations suggest that settlement and selectivity are influenced by more than just chemical cues and certainly indicate that larvae display photosensitivity. These findings are consistent with those of Mason et al. (2011), who first suggested that coral larvae use spectral cues for habitat selection during settlement, with a strong preference for red settlement surfaces. The fluorescent emission spectra of CCA and the red plastic cable ties in Mason et al. (2011) exhibited peaks at 580 and 590 nm respectively, suggesting that the red preference may be an adaptation to aid in the location of CCA for settlement. Subsequent work has shown that coral larvae show photosensitivity to long wavelengths (Mason and Cohen 2012) and may have photoreceptor-like cells at the aboral end of the larvae to allow light sensing during settlement (Mason et al. 2012). Similarly, in the present study the red synthetic substrate and unbleached CCA had spectral peaks at 600 nm and 580 nm respectively, highlighting that the red synthetic substrate simulated the spectral properties of healthy CCA.

In our study, the small CCA chip size (and therefore limited availability of settlement space) as well as the similar settlement rates on the CCA (~10-20%) regardless of background colour, suggests that larvae did not prefer the red synthetic surfaces to the CCA, but would settle on them if better options (i.e. CCA) were not available. That is, providing a red background appeared to facilitate settlement on available space on the synthetic surface, when the preferred surface (CCA) was already occupied. This was further highlighted by the tendency to settle in a ring around the CCA chip. These findings indicate that coral larvae respond to both spectral and chemical settlement cues, but ultimately prefer substrata that satisfy both criteria.

Crustose coralline algae and associated microbial communities are clearly the preferred settlement substrata for coral larvae, but with differences among species (Harrington et al. 2004). A high cover of consolidated CCA, as opposed to other substrate types, (e.g. sediment and coral
rubble) (Sheppard et al. 2002; Schuhmacher et al. 2005), or benthic organisms more typical of degraded reefs (e.g. macroalgae and sponges) (Aronson et al. 2002; Dixson et al. 2014), may be fundamental to the resilience of the reef following severe disturbances (Sheppard et al. 2008; Gilmour et al. 2013). High CCA cover may also distinguish optimal settlement substrates from those with a high abundance of coral competitors. When distinguishing optimal substrata for settlement and metamorphosis, larvae probably respond to a complex combination of chemical, spectral and textural cues. Surface texture and structure are important, as larvae routinely settle in cryptic microhabitats (Harrison and Wallace 1990; Edmunds et al. 2004; Vermeij 2005). This is likely another reason they were attracted to the structure provided by the CCA. Microcrevices and ledges help to protect recruits and maximize survival in the smallest and most vulnerable size classes by minimizing interactions with large competitive dominants (Vermeij 2006). However as corals grow into larger and less vulnerable size classes, exposure to light for autotrophic nutrition becomes a more important requirement. Therefore larvae must discern conditions that are not only suitable for initial attachment but also for later growth and survival.

Climate change has the potential to detrimentally alter the important cues on which coral larvae rely to find suitable a settlement substrate. Warmer water temperatures and acidification change microbial biofilms on the CCA surface, thereby disrupting chemical settlement cues and inhibiting larval settlement (Albright and Langdon 2011; Webster et al. 2011; Doropoulos et al. 2012a; Webster et al. 2013a; 2013b). Furthermore, elevated temperature and pCO$_2$ can also cause bleaching in CCA (Anthony et al. 2008; Webster et al. 2011), changing the colour of CCA from red or dark pink to pale pink or white. In our study the CCA chips were not pre-treated in manipulated temperature-pCO$_2$ tanks, therefore the chemical settlement cues presumably remained intact and the CCA were not bleached while larval substrate selection and settlement were occurring. Thus the larvae settled preferentially on the healthy CCA chips provided. However once the CCA chips were occupied, larvae deemed the synthetic substrate adequate for settlement only if they perceived it as red i.e. larvae only settled on the synthetic substrate with the spectral properties similar to that of healthy CCA. The white synthetic substrate provided neither a chemical nor a spectral cue and consequently settlement was
severely reduced, with larvae tending to continue to swim and search, using up their lipid reserves, until they eventually died. Consequently, the effects of increased water temperature and $pCO_2$ could be additive, with the disruption of settlement by inhibiting both chemical and spectral cues. Although these impacts of warmer water temperature and acidification on settlement are indirect, if larvae can neither ‘smell’ nor ‘see’ the reef, they may be unable to locate it, which could in turn lead to significant reductions in recruitment success.
Chapter 5: Ocean Acidification Causes Structural Deformities in Juvenile Coral Skeletons


5.1 Abstract

Rising atmospheric CO$_2$ is causing the oceans to both warm and acidify, reducing calcification rates of corals globally. Successful coral recruitment and high rates of juvenile calcification are critical to the replenishment and ultimate viability of coral reef ecosystems. While elevated $p$CO$_2$ has been shown to reduce the skeletal weight of coral recruits, the structural changes caused by acidification during initial skeletal deposition are unknown. Here we show, using high resolution 3-dimensional X-ray microscopy, that ocean acidification ($p$CO$_2$ ~ 900 µatm) causes not only reduced overall mineral deposition, but also a deformed and porous skeletal structure in newly settled coral recruits. In contrast, elevated temperature (+3°C) had little effect on skeletal formation except to partially mitigate the effects of elevated $p$CO$_2$. The striking structural deformities we observed show that new recruits are at significant risk, being unable to effectively build their skeletons in the $p$CO$_2$ conditions predicted to occur under a ‘business as usual’ emissions scenario (RCP 8.5) by the year 2100.

5.2 Introduction

Atmospheric CO$_2$ is set to rise to >900 ppm by the end of the century under a ‘business as usual’ scenario (RCP 8.5), with corresponding elevations in both oceanic temperature (+ ~3°C) and $p$CO$_2$ (+ ~500 µatm) (IPCC 2013). Both ocean temperature and $p$CO$_2$ are key environmental factors affecting coral calcification rates (Reynaud et al. 2003; De'ath et al. 2009; Manzello 2010). There is usually a parabolic relationship between temperature and calcification, with optimal temperatures for calcification normally relative to local conditions (Coles and Jokiel 1977; Marshall and Clode 2004), and a negative correlation between calcification and $p$CO$_2$ (Langdon et al. 2000; Marubini et al. 2001). However the interactive effect of elevated...
temperature and $pCO_2$ on calcification appears to be variable, with both positive (Langdon and Atkinson 2005; Muehllehner and Edmunds 2008) and negative (Reynaud et al. 2003; Rodolfo-Metalpa et al. 2011) interactions reported in adult corals. In situ, adult coral calcification is already thought to have declined by 14-30% worldwide in recent years, with elevated temperature and ocean acidification thought to be the major causes (De'ath et al. 2009; Manzello 2010).

Corals in the early life stages have also been shown to be sensitive to changes in temperature and $pCO_2$ (Edmunds et al. 2001; Negri et al. 2007; Albright et al. 2008; 2010; Albright and Langdon 2011; Webster et al. 2013b). Successful reproduction and post-recruitment processes (particularly growth) are essential to maintaining coral reef health (Richmond 1997). Therefore it is particularly important to know how these environmental changes will affect skeletal growth in the early stages of development, when tiny recruits (~1 mm) are most vulnerable to mechanical damage, overgrowth, and predation. Experiments examining the impact of ocean acidification on calcification of newly settled recruits have reported strong effects, with 20-60% reductions in skeletal mass under acidified conditions (Cohen et al. 2009; de Putron et al. 2011; Drenkard et al. 2013; Foster et al. 2015). The combined effect of elevated temperature and $pCO_2$ on skeletal mass however, may be dependent on geographical location, with a negative impact in the tropics (Anlauf et al. 2011) and a positive impact in the sub-tropics (Foster et al. 2015). Importantly, these studies have highlighted the vulnerability of new recruits to ocean acidification, and in particular, their limited ability to calcify under high $pCO_2$ conditions. However to date, such research has relied solely on bulk measurements of skeletal mass to assess these impacts. There have been no analyses of the specific structural changes to the juvenile skeleton under acidification or warming.

In this study we used high resolution 3-dimensional (3D) X-ray microscopy and scanning electron microscopy (SEM) to examine the skeletons of newly settled coral recruits of an abundant plate coral species, from the sub-tropical Houtman Abrolhos Islands in Western Australia. Coral recruits were grown for one month under four temperature-$pCO_2$ regimes.
ray microscopy was then used to generate 3D images and data sets of the skeletons to discern both visual and quantitative differences in skeletal structure between the four regimes. The 3D reconstructions enabled the extraction of bulk measurements (surface area and volume), cross-sectional measurements (height and basal plate thickness), and internal measurements (corallite wall thickness and tertiary septa width); structural information that would be impossible to obtain using conventional two-dimensional imaging methods. SEM images of the same individuals were also acquired to more closely examine abnormalities in the skeletal microstructure.

5.3 Methods

5.3.1 Experimental Set-up

For a detailed description of the experimental set-up and larval culturing see Chapter 3. Gravid adult colonies of Acropora spicifera were collected off Basile Island in the Pelsaert group of the Houtman Abrolhos Islands (28°52’S, 113°57’E, Figure 1.4) in Western Australia, prior to the 2013 March mass-spawning event. On the night of spawning, fertilized eggs (cross-fertilized from 6 spawning colonies, representing equal contributions from all 6 genotypes) were transferred to larval rearing tanks and maintained at ambient conditions until larvae reached the planula stage of development at 6 days post-fertilization. Larvae were then transferred to treatment tanks (four replicate tanks per treatment). Seawater was treated in four separate sump tanks (one sump per treatment) that flowed into the replicate tanks every three hours for an ~90% water change. The carbonate chemistry was adjusted using a pH-dependent feedback system, which bubbled pure CO₂ into the seawater when pH deviated 0.01 pH units from set values (CO₂ Set Professional, AquaMedic, Germany). The pH was checked manually in all tanks every second day (Schott handlylab pH 12 pH meter). Total Alkalinity (TA) samples were taken from each tank once a week and processed using linear array spectrometers (Yao and Byrne 1998) and a titrator (Metrohm 865 Dosimat Plus). Salinity was checked weekly using a refractometer (35.5). Aragonite saturation state ($\Omega_{ar}$) and $p$CO₂ were calculated from pH, TA, salinity and temperature using the program CO2SYS (Lewis et al. 1998). Water temperature
was controlled with heater chillers (Resun, CL 150) and monitored using Hobo Pendant temperature loggers. The four temperature-$p$CO$_2$ conditions were: 1) ‘Control’ (24°C and 250 µatm), 2) elevated temperature (‘High T’; 27°C and 250 µatm), 3) elevated $p$CO$_2$ (‘High $p$CO$_2$’; 24°C and 900 µatm), and 4) elevated temperature and elevated $p$CO$_2$ (‘High T + $p$CO$_2$’; 27°C and 900 µatm) (Table 3.1, Figure 5.1 and Figure 5.2). The annual temperature range at the Abrolhos is ~19-24°C (Pearce et al. 1999). Thus the high temperature treatment (27°C) represents an elevation of 3°C above the mean monthly water temperature at the Abrolhos around spawning time (24°C). The high $p$CO$_2$ treatment ($p$CO$_2$ ~ 900 µatm) is similar to projections for the year 2100 under the RCP 8.5 scenario (~930 µatm) (Meinshausen et al. 2011). The ‘ambient’ $p$CO$_2$ levels are closer to pre-industrial (250 µatm) than present-day atmosphere (~390 µatm). This was likely due to seawater being pumped into the experimental facility in the afternoon when photosynthesis of benthic primary producers in the region was driving $p$CO$_2$ below atmospheric levels. A rigorous study of the variation in carbonate chemistry at the Abrolhos is yet to be undertaken, however spot measurements taken over 2011 to 2013 show the range for $p$CO$_2$ and pH to be ~300-500 µatm and ~7.9-8.1 respectively (Foster et al. 2014). Light levels were maintained at a 12 h: 12 h light/dark cycle, with a mean (± SE) irradiance of 212 ± 8 µmol photons m$^{-2}$ s$^{-1}$ (Biospherical Instruments, QSL-2100). The light levels applied in this (212 µmol photons m$^{-2}$ s$^{-1}$) and other juvenile calcification studies (62-135 µmol photons m$^{-2}$ s$^{-1}$) (Cohen et al. 2009; Anlauf et al. 2011; de Putron et al. 2011; Ohki et al. 2013; Drenkard et al. 2013) are relatively low compared to natural light levels experienced on a shallow reef. Higher light intensities experienced in situ may increase calcification rates relative to those recorded in laboratory experiments, however, larvae often show a preference during settlement for micro-crevices and ledges that offer protection from predators but are presumably exposed to lower light intensities. Thus the lower light levels we used may be more representative of their environment in the early post-recruitment phase.
Figure 5.1. Variation in seawater pH (A), total alkalinity (B), $pCO_2$ (C) and aragonite saturation state (D) in each treatment for the duration of the experiment. Measurements of pH were taken every second day and TA was measured every week. Mean weekly TA measurements were used to determine high frequency measures of $pCO_2$ and aragonite saturation state.

Figure 5.2. Variation in pH with time of day for each treatment.
5.3.2 Larval Settlement

Larvae \((n = 40)\) were transferred into 50 mL clear acrylic tubes, with 100 µm mesh at the ends to allow for water exchange. Plastic transparency paper lined the inside of the tubes and crustose coralline algae (CCA) chips \((Hydrolithon)\) were added to each tube to induce settlement. The transparency paper lining was used for both easy removal of the recruit skeletons at the end of the experiment and also as a low-density substrate, easily excluded from 3D X-ray microscopy measurements of the skeleton. The tubes were transferred into replicate treatment tanks. At 7 days post-settlement recruits were inoculated with cultured clade C1 zooxanthellae \((V. \text{ Beltran, AIMS})\), a common clade in this region \((\text{Silverstein et al. 2011})\). Settled juveniles were grown for 4 weeks post-settlement under treatment conditions. Before termination of the experiment, each individual was examined and photographed using both a stereo microscope and a fluorescence microscope to verify that recruits were alive until the endpoint of the experiment. To remove organic material, recruits were immersed in 3-7% sodium hypochlorite and rinsed three times in deionized water.

5.3.3 3D X-ray Microscopy

Five primary polyps were randomly selected across tubes and tanks from each treatment for X-ray microscopy analyses. Recruits needed to meet four selection criteria in order to be usable for scanning: 1) be in the primary polyp stage of development, 2) have settled on the plastic transparency paper, 3) have settled a suitable distance from other recruits, and 4) remained attached to the paper and undamaged throughout processing. All X-ray scans were conducted using an Xradia Versa XRM520 X-ray microscope. Scanning was undertaken under the following conditions to produce data with \(~1.3 \mu \text{m pixel resolution}: \text{voltage} = 50 \text{ kV}, \text{power} = 4 \text{ W}, \text{exposure time} = 20 \text{ seconds}, \text{binning} = 2 \text{ and angle} = \text{full 360°}, \text{with a total of 3201 projections per sample. Reconstruction of the projections was conducted using the Xradia software XMReconstructor.}
5.3.4 Quantitative Data Extraction

The software Avizo Fire was used to process the reconstructed projections and extract quantitative data. The Edit New Label Field tool was used to set threshold radiodensity values, which removed the transparency paper, leaving only the CaCO$_3$ skeleton. The samples were then volume rendered to create a 3D image (Figure 5.3). A Label Analysis was conducted on the segmented dataset to determine the surface area and volume of the sample. All other measurements were made manually, using the Measure tool (Figure 5.4). For height, corallite wall thickness and basal plate thickness a mean of three measurements per sample was determined, while the mean diameter was calculated from four measurements per sample. For height and basal plate thickness the three highest or thickest points in the sample were selected by scrolling through the slices of the sample. For diameter and corallite wall thickness, a single slice near the base showing the whole diameter was selected and measurements were taken at the same points and along the same axis for each sample. The length and width were measured for all tertiary septa.

Figure 5.3. Top-down and side view 3D X-ray images, from reconstructed scans, identifying the key skeletal structures discussed in this study. Scale bar is 500 µm.
Figure 5.4. Examples of manual measurements of diameter, corallite wall thickness (A, B), tertiary septa length : width (C, D), height and basal plate thickness (E, F). A, C and E show 3D reconstructions of skeletons and the location of the single ortho slice depicted in B, D and F. Scale bars are 500 µm.

5.3.5 Scanning Electron Microscopy

After X-ray scanning, the same individuals were mounted on stubs with carbon tabs, while still attached to the transparency paper. The sample was surrounded with carbon tape and the whole tab was then coated with 10 nm of platinum and 10 nm of carbon. Images from the tops of the corallite wall and tips of the tertiary septa were acquired at 5 kV using a Zeiss 55 field emission SEM. The two areas of interest were selected based upon their rough or porous appearance in the 3D reconstructions of high pCO₂ skeletons.
5.3.6 Data Analysis

Two-way analyses of variance were conducted on the X-ray microscopy measurements to test for significant effects of elevated temperature, $\rho$CO$_2$ and their interaction. All data were checked for equality of variance with Levene’s test for homogeneity. The Shapiro-Wilk test was used to check for normality and data were log transformed if normality assumptions were not met. All statistical analyses were conducted in SPSS version 21.

5.4 Results

Coral skeletons from low $\rho$CO$_2$ treatments (Control and High T) were typically radially symmetric and had six tertiary septa of similar size (Figure 5.5 A, E, Figure 5.7, Figure 5.8 and Movie 5.1). Skeletal surfaces of the low $\rho$CO$_2$ corals were also smooth and appeared solid at both the corallite wall and tertiary septa (Figure 5.5 C, D, G, H). In contrast, high $\rho$CO$_2$ corals had a variety of deformities disrupting their symmetry. Most high $\rho$CO$_2$ corals had missing or unevenly sized tertiary septa, with recruits commonly having overgrown septa on one side of the mouth and missing or stunted septa on the other side (Figure 5.5 I, M). Perhaps the most striking difference between high and low $\rho$CO$_2$ corals was the deep pitting and porous microstructure of the corallite wall (Figure 5.5 K, O), a feature that was consistent across all individuals grown in high $\rho$CO$_2$ treatments (Figure 5.9 and Figure 5.10). Though not as severe, shallow pitting was also observed in the tertiary septa of the high $\rho$CO$_2$ corals (Figure 5.5 L, P, Figure 5.9 and Figure 5.10). Other deformities observed only under high $\rho$CO$_2$ included small gaps in the lattice-like structure of septa and synapaticulae (horizontal connecting structures) (Figure 5.6 E-F) and more severe deformities, such as large sections of the skeleton being completely absent (Figure 5.6 G-H and Movie 5.2). Fractures were also observed in the septa and corallite walls of 50% of the high $\rho$CO$_2$ corals (Figure 5.6 A-D), while no fractures were recorded in the low $\rho$CO$_2$ corals.