Effects of diel CO_2 cycles on the early development and behaviour of coral reef fishes under ocean acidification

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Statement of the contribution of others

This thesis includes collaborative work with my advisors Prof. Philip Munday and Prof. Mark McCormick, as well as Craig Humphrey. While undertaking these collaborations I was responsible for experimental design, animal collection and care, data collection, analysis, and interpretation of my results. My co-authors provided intellectual guidance, editorial assistance, statistical assistance, financial support, and technical assistance.

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General Abstract

Increasing atmospheric CO_2 levels are causing a reduction of ocean surface water pH and shift in carbonate chemistry, a process termed ocean acidification. Ocean acidification poses a major threat to marine ecosystems, with a large body of work documenting negative effects of elevated CO_2 on a diverse range of shallow water coastal marine species. However, most studies to date have used stable CO_2 treatments, not considering the substantial diel CO_2 variation that occurs in many shallow water marine habitats. This thesis investigates how the presence of diel CO_2 cycles modifies the early development and behaviour of coral reef fishes under elevated CO_2, and how the effects of diel CO_2 cycles are further modified by elevated temperature and parental effects.

In **chapter 2** I investigate the interactive effects of elevated CO_2 and diel CO_2 cycles on juvenile survival, growth and otolith development in two species of coral reef fish. There was no effect of CO_2 treatment on survival and otolith development of *Acanthochromis polyacanthus* or *Amphiprion percula*. While growth was not significantly affected by CO_2 treatment in either species, there was a trend for fish reared under diel-cycling elevated CO_2 to be more similar in size to control fish, compared to those reared under stable elevated CO_2. These results suggest that the early development of juvenile coral reef fishes under future ocean acidification conditions is unlikely to be affected by diel cycles in CO_2.

The behavioural alterations that have been observed in coral reef fishes under elevated CO_2 are likely to interact with diel CO_2 cycles, due to the concentration-dependent effects that stable elevated CO_2 has on onset times and the magnitude of behavioural impairments.

Therefore, **chapter 3** investigates the effects that diel CO_2 cycles have on behavioural lateralization in *A. polyacanthus* and response to a predator cue in *Am. percula* under elevated CO_2. As expected, exposure to stable elevated CO_2 caused behavioural impairments in both species. However, diel CO_2 cycles substantially reduced the severity of behavioural abnormalities caused by elevated CO_2 and in some cases fully alleviated the negative effects. These results highlight that past studies may have over-estimated the impacts of ocean acidification on the behavioural performance of coral reef fishes, because they did not include natural CO_2 cycles in the ocean acidification treatments.

The responses of shallow water marine organisms to ocean acidification can be altered by elevated temperature. In **chapter 4** I tested whether the interaction between elevated CO_2 and diel CO_2 cycles is further modified by elevated temperature. Survival, growth and behavioural traits were measured in juvenile *A. polyacanthus.* A significant interaction between CO_2 treatments and temperature was only detected for survivorship. Survival was lower in the two diel-cycling elevated CO_2 treatments, but only when temperature was elevated. In other traits, independent effects of elevated CO_2, and interactions between elevated CO_2 and diel CO_2 cycles were detected, but these effects were not influenced by temperature. Elevated temperature had significant, negative effects on most traits measured. Overall, the results of this chapter demonstrate that while elevated temperature had a stronger effect on most traits it did not alter the interactive effects elevated CO_2 and diel CO_2 cycles had on growth and behavioural traits.

Previous studies have shown that parental exposure to elevated CO_2 can mitigate negative effects of elevated CO_2 on juvenile growth and survival. However, the outcome of parental

effects in the presence of diel CO_2 cycles may differ from those expressed in a stable CO_2 environment. To test this, **chapter 5** investigates the effects that parental exposure to stable elevated and diel-cycling elevated CO_2 had on the survival and growth of juvenile anemonefish, *Am. melanopus*. Within-generation exposure to stable elevated CO_2 caused a significant reduction in juvenile growth; however, there was no effect of elevated CO_2 on juvenile growth when diel CO_2 cycles were present in the juvenile elevated CO_2 treatment, or when parents had experienced the same conditions as the juveniles (either stable elevated and diel-cycling elevated CO_2). Additionally, parental exposure to diel CO_2 cycles did not alter the effects of diel CO_2 in juveniles. These results illustrate the importance of considering natural CO_2 cycles when predicting the long-term impacts of OA on marine ecosystems.

The research presented here is among the first to test how natural CO_2 cycles in shallow water habitats affect the performance of marine fishes under ocean acidification. The results show that the performance of coral reef fishes under elevated CO_2 is enhanced when a diel-cycling CO_2 regime is present. Future studies should investigate the underlying mechanisms responsible for the observed improvements under diel-cycling elevated CO_2. Additionally, CO_2 is the not the only environmental parameter to fluctuate daily in shallow coral reef habitats. Diel cycles in temperature also exist and thus future work should also incorporate fluctuating temperature treatments. Overall, this body of research highlights the critical importance of incorporating natural environmental variability into experiments to accurately assess the responses of shallow water coastal marine organisms to future ocean conditions.

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Chapter 1: General Introduction

Atmospheric carbon dioxide (CO_2) levels are rising at a rate unprecedented in recent geological time, as a result of increased human activity (Doney and Schimel 2007; Pelejero et al. 2010). Since the industrial revolution, atmospheric carbon dioxide (CO_2) levels have increased by over 40%, from 280 parts per million (ppm) to a present-day value of over 405ppm (www.esrl.noaa.gov/gmd/ccgg/trends/). Rising atmospheric CO_2 levels are causing surface ocean waters to become more acidic and warmer (Feely et al. 2004; Collins et al. 2013). The partial pressure of CO_2 (pCO_2) in surface oceans is in approximate gas equilibrium with atmospheric CO_2 and thus rises at the same rate as the atmosphere (Doney et al. 2009). Approximately one third of anthropogenic CO_2 released into the atmosphere is absorbed by the oceans (Sabine et al. 2004). Increased uptake of CO_2 by the oceans is causing a shift in the relative concentrations of carbonate and bicarbonate ions and a reduction in pH, a process that has been termed 'ocean acidification' (Feely et al. 2004; Orr et al. 2005; Doney et al. 2009). Furthermore, increasing concentrations of atmospheric CO_2, in addition to other greenhouse gases, result in more heat being retained within the atmosphere, most of which is ultimately absorbed by the oceans. Based on the worst-case RCP8.5 emissions scenario, atmospheric CO_2 levels could reach 1000 ppm by the year 2100 (Meinshausen *et al.*, 2011). This is expected to result in the simultaneous reduction of mean surface water pH by 0.3-0.4 units and increase in sea surface temperature by 2-3 °C compared to pre-industrial values (Caldeira and Wickett 2003; Collins et al. 2013).

Ocean acidification poses a major threat to marine ecosystems (Fabry et al. 2008; Hoegh-Guldberg and Bruno 2010; Fabricius et al. 2011; Doney et al. 2012; Kroeker et al. 2012, 2013a).

Over a decade's worth of laboratory experiments have documented the potential impacts of reduced seawater pH and elevated pCO_2 on marine organisms, with most studies carried out on shallow water coastal species due to their ecological and economic importance. Additionally, most studies have focussed on early life stages as they are predicted to be more sensitive to environmental change than older life stages. Pioneering ocean acidification research focussed on calcifying species, such as molluscs and corals, due to concern that the reduction in carbonate saturation state associated with ocean acidification would affect their ability to form skeletons (Feely et al. 2004; Orr et al. 2005; Fabry et al. 2008). However, it is now clear that ocean acidification can negatively affect numerous traits (e.g. growth, survival, behaviour, metabolism and reproduction) across a wide range of shallow water taxa (Kroeker et al. 2013b; Wittmann and Pörtner 2013; Nagelkerken and Munday 2016; Cattano et al. 2018). Furthermore, the responses of early life stages of shallow water marine organisms to ocean acidification can be significantly altered if temperature is also elevated (Pörtner and Knust 2007; Byrne 2011; Harvey et al. 2013; Kroeker et al. 2013b; Przeslawski et al. 2015) and if multiple generations experience similar conditions (Munday 2014; Ross et al. 2016; Donelson et al. 2018).

While ocean acidification experiments have revealed largely negative impacts in general, the responses of shallow water coastal marine organisms to elevated CO_2 have also been highly species-specific (Harvey et al. 2013; Kroeker et al. 2013b; Wittmann and Pörtner 2013). These species-specific responses may, in part, be related to the fact that most ocean acidification experiments to date have not considered the CO_2 conditions an organism naturally experiences, instead using CO_2 treatments consistent with open ocean environments (McElhany and Busch 2013; Gunderson et al. 2016; Wahl et al. 2016). Indeed, empirical

studies testing populations across their geographic range, have shown that sensitivity to future ocean acidification conditions is linked to the local pCO_2 conditions experienced (Kelly et al. 2013; Pespeni et al. 2013; Thomsen et al. 2017; Vargas et al. 2017). Laboratory experiments are a crucial tool for understanding the potential impacts of ocean acidification on shallow water marine species. However, for studies to be ecologically relevant, experiments must incorporate an understanding of the CO_2 environment that is naturally experienced by the study organism (McElhany and Busch 2013).

Ocean acidification projections are based on data from open ocean environments that have relatively stable pCO_2 through time (Doney et al. 2009; Hofmann et al. 2011). In fact, the current annual cycle in surface open ocean pCO_2 is only between 60-98 µatm (McNeil and Sasse 2016; Gallego et al. 2018). However, this cycle is predicted to increase to between 200-400 µatm by the year 2100 due to the reduced buffering capacity of acidified seawater and the effects of increased temperature on biological activity and CO_2 solubility (McNeil and Sasse 2016; Gallego et al. 2018; Kwiatkowski and Orr 2018). This will result in surface open ocean pCO_2 levels of 1000 µatm becoming a reality decades early than previously thought on a seasonal basis (McNeil and Sasse 2016; Gallego et al. 2018; Kwiatkowski and Orr 2018). When open ocean waters flush onto shallow water coastal habitats their carbonate chemistry can be substantially modified by a range of biological and physical processes, resulting in significant fluctuations in CO_2 on a variety of temporal scales (Hofmann et al. 2011; Duarte et al. 2013; Waldbusser and Salisbury 2014; Hendriks et al. 2015). Continuous time series data has revealed that pCO_2 fluctuations in such habitats often exceed those in the open ocean by an order of magnitude (Hofmann et al. 2011; Shaw et al. 2012). In some instances, these fluctuations can even exceed mean CO_2 levels projected to occur over the next century (Shaw

et al. 2012; Duarte et al. 2013; Baumann et al. 2015). Furthermore, these natural pCO_2 fluctuations are also expected to increase in magnitude throughout the century (Schulz and Riebesell 2013; Shaw et al. 2013a; Pacella et al. 2018). Due to all mentioned above, as mean oceanic pCO_2 levels rise, shallow water marine organisms will be exposed to higher pCO_2 levels for longer periods of time in addition to experiencing a greater range of pCO_2 levels.

Perhaps the most well-known natural pCO_2 fluctuations in marine ecosystems are those that occur across a 24 hour period in a range of shallow water coastal habitats, such as coral reefs (Kayanne et al. 1995; Shaw et al. 2012; Kline et al. 2015; Page et al. 2017; Takeshita et al. 2018), macroalgal beds (Frieder et al. 2012; Wahl et al. 2018), seagrass meadows (Challener et al. 2016; Cyronak et al. 2018; Pacella et al. 2018) and salt marshes (Baumann et al. 2015). These diel CO_2 cycles are driven primarily by the processes of photosynthesis and respiration across a day-night cycle, but are modified by physical properties such as flow trajectory, flow rates and residence time, which ultimately alters the contact time between the seawater and the benthic community (Falter et al. 2013; Cyronak et al. 2018; Wahl et al. 2018). Consequently, the magnitude of diel CO_2 cycles is usually positively correlated with biomass and residence time and negatively correlated with water flow (Page et al. 2017; Takeshita et al. 2018; Wahl et al. 2018). Diel CO_2 cycles are also influenced by the seasons and are greater in the warmer months due to increased solubility of CO_2 and community production (Shaw et al. 2012; Albright et al. 2013; Kline et al. 2015).

It has been proposed that shallow water coastal habitats may mitigate the impacts of ocean acidification on the marine organisms that live in them by lowering pCO_2 and providing temporal refuge from the stresses of elevated CO_2 (Hendriks et al. 2014, 2015; Bracken et al.

2018). Indeed, several studies accounting for diel CO_2 variability have shown that they can significantly alleviate the negative effects of ocean acidification (Dufault et al. 2012; Comeau et al. 2014; Frieder et al. 2014; Ou et al. 2015; Chan and Eggins 2017; Enochs et al. 2018; Wahl et al. 2018; White et al. 2018). However, other studies have reported negligible (Clark and Gobler 2016; Gobler et al. 2017; Kwan et al. 2017) and negative effects (Cornwall et al. 2013; Mangan et al. 2017; Onitsuka et al. 2018). Collectively, these works highlight the vital importance of incorporating natural CO_2 variability in experiments to accurately assess the responses of shallow water marine organisms to ocean acidification. Consistent with previous ocean acidification research there has been a focus on the responses of calcifying species. How diel CO_2 cycles will interact with elevated pCO_2 to affect the performance of non-calcifying shallow water marine organisms, such as fish, is less well known.

Effects of ocean acidification on marine fishes

Teleost fishes maintain a relatively constant alkaline pH (7.7-8.1) and a relatively low pCO_2 (<3000 µatm) in their extracellular fluids (Heuer and Grosell 2014). Consequently, there is a relatively small gradient in pCO_2 between the plasma and external environment, which makes fish sensitive to increases in external CO_2. Fish acutely exposed to elevated CO_2 levels in seawater experience an increase in plasma pCO_2 and a decrease in pH (Wood et al. 1990; Baker et al. 2009; Esbaugh et al. 2012). However, fish have a high capacity for acid-base regulation which enables them to defend against intra- and extracellular acidosis under elevated CO_2 conditions. Fishes experiencing acidosis actively accumulate HCO_3^- ions, in exchange for Cl^- ions, to buffer changes in pH, with most studies showing that full pH compensation is achieved within hours to days (Baker et al. 2009; Esbaugh et al. 2012; Heuer

and Grosell 2014; Heuer et al. 2016). However, this compensatory mechanism is predicted to be costly (Pörtner and Knust 2007; Ishimatsu et al. 2008; Melzner et al. 2009) and thus could have negative consequences for other biological processes.

Survival and growth

Adult fish have been shown to tolerate CO_2 levels far greater than those projected to occur by the end of the century (Ishimatsu et al. 2008; Melzner et al. 2009). However, early life stages of marine fishes are expected to be more sensitive to ocean acidification because: (1) their homeostatic mechanism may not be fully developed and (2) their smaller size increases the cost of homeostasis (Brauner 2009; Melzner et al. 2009). In contrast to expectation, the effects of ocean acidification on the early life stages of marine fishes have been highly variable (Heuer and Grosell 2014). While some species exhibit a reduction in survival and growth and increase in tissue deformities under elevated pCO_2 (Baumann et al. 2012; Frommel et al. 2012, 2014; Miller et al. 2012; Stiasny et al. 2016) others appear resilient with negligible effects observed (Munday et al. 2011b; Bignami et al. 2013b; Frommel et al. 2013; Hurst et al. 2013; Perry et al. 2015). Furthermore, for some species, exposure to elevated pCO_2 has been shown to have affects that are contrary to predictions, such as increased growth rate (Munday et al. 2009a; Schade et al. 2014; Cattano et al. 2017). To date, only one study has investigated how diel CO_2 cycles will interact with elevated CO_2 to affect the survival and growth of marine fishes under ocean acidification conditions. Diel CO_2 cycles (450-2000 µatm) were shown to alleviate the negative effects of elevated CO_2 (1000 µatm) on growth of larval pink salmon, *Oncorhynchus gorbuscha* (Ou et al. 2015). Mores studies are needed to determine how common this response may be.

Otolith development

While not considered a calcifying group, fish do possess calcium carbonate structures, most notably in the form of aragonite ear bones (otoliths) (Payan et al. 2004). Otoliths are paired, calcified structures, located within the inner ear and surround by a saccular epithelium filled with endolymph. Otoliths are much denser than water, and therefore move at different amplitudes to the body (Popper and Lu 2000). The position of otoliths in the inner ear and their movement over sensory hair cells enables fish to detect sound, body orientation and acceleration (Popper and Lu 2000). Changes in otolith morphology can therefore result in altered auditory capacity and swimming performance, with potential ecological consequences (Gagliano et al. 2008; Bignami et al. 2013a). For example, larval fish rely on sound cues to orientate themselves to find a suitable settlement habitat (Simpson et al. 2004; Tolimieri et al. 2004; Radford et al. 2011). Thus, altered auditory capacity caused by changes in otolith morphology could affect recruitment and population sustainability. In general, exposure to elevated CO_2 tends to have a positive effect on otolith size; larval and juveniles fishes exposed to elevated CO_2 often have larger otoliths compared with similar aged and sized fish from current-day ambient conditions (Checkley et al. 2009; Munday et al. 2011a; Bignami et al. 2013a). However, the effects are also dependant on CO_2 concentrations, with greater effects on otolith size at higher CO_2 levels, and no apparent effect of moderate CO_2 levels (~1000 μatm) in some species (Munday et al. 2011a). Otolith growth is proportional to the concentration of HCO_3^- ions within the endolymph surrounding them (Payan et al. 2004). Consequently, increased otolith growth under elevated CO_2 is most likely caused by elevated plasma HCO_3^- associated with compensatory acid-base regulation (Heuer and Grosell 2014). It is currently unknown how diel CO_2 cycles will affect otolith development under elevated CO_2.

Behaviour

Some of the most notable effects of ocean acidification on marine fishes observed to date are impaired sensory ability and alterations of ecologically important behaviours, with most studies conducted on coral reef fishes (Clements and Hunt 2015; Nagelkerken and Munday 2016; Cattano et al. 2018). Several studies have shown that a range of behavioural responses, in both tropical and temperate species, are altered under elevated CO_2 levels including: predator avoidance behaviours, lateralization, the ability to learn, escape performance and activity/anxiety (Clements and Hunt 2015; Nagelkerken and Munday 2016; Cattano et al. 2018). Behavioural abnormalities caused by elevated CO_2 are expected to have significant ecological consequences for fish populations through effects on recruitment, dispersal, predator-prey/competitive interactions and habitat preference (Munday et al. 2010; Allan et al. 2013; McCormick et al. 2013; Nagelkerken and Munday 2016).

Behavioural abnormalities in fish under elevated CO_2 conditions are thought to be linked to the effects of acid-base regulation on the functioning of type A γ-aminobutyric acid ($GABA_A$) neurotransmitter receptors (Nilsson et al. 2012; Hamilton et al. 2014; Heuer et al. 2016). $GABA_A$ receptors are gated ion channels with specific conductance for HCO_3^- and Cl^-. As previously mentioned, under elevated CO_2 fish increase intra- and extracellular HCO_3^- concentrations to prevent plasma and tissue acidosis (Heuer and Grosell 2014). This altered ion gradient is thought to turn some $GABA_A$ receptors from inhibitory to excitatory, ultimately leading to behavioural impairments (Heuer et al. 2016). The role of $GABA_A$ in behavioural alterations is supported by experiments showing that gabazine, a $GABA_A$ antagonist, reverses the behavioural abnormalities that occur under elevated CO_2 (Nilsson et al. 2012; Hamilton et al. 2014; Ou et al. 2015).

The behavioural impairments and potential ecological consequences listed above could manifest before the end of the century, as some have been observed at CO_2 levels around 700 µatm (Munday et al. 2010; Simpson et al. 2011; Welch et al. 2014). This is a major concern, as present evidence suggests that there is limited capacity for behavioural impairments to be mediated by long-term acclimation (Allan et al. 2014; Munday et al. 2014; Welch et al. 2014). It is possible that adaptation through the selection of tolerant genotypes could act as a rescue mechanism. Indeed, individual variation (the raw material upon which natural selection acts) in behavioural tolerance has been observed at CO_2 levels around 700 µatm (Munday et al. 2010; Ferrari et al. 2011a; Welch et al. 2014). If such phenotypic variation is heritable it could potentially lead to the rapid selection of tolerant genotypes. Unfortunately, a recent study has shown that while behavioural tolerance to elevated CO_2 in a coral reef fish has a genetic basis, and is heritable in the short-term, it is masked by non-adaptive phenotypic plasticity over the long-term (Welch and Munday 2017), inferring that there could be a limited capacity for adaptation of behavioural traits under elevated CO_2.

While evidence for acclimation or adaptation of behavioural traits to ocean acidification is scarce, it is possible that diel CO_2 cycles may provide some beneficial effects to behavioural impairments under elevated CO_2 in marine fishes. However, to date, only one study has investigated how diel CO_2 cycles will interact with elevated CO_2 levels to affect the behaviour of marine fishes under ocean acidification conditions. Diel CO_2 cycles had no significant effect on behavioural responses of juvenile blacksmith, *Chromis punctipinnis* (Kwan et al. 2017), although behavioural responses were also unaffected by exposure to stable elevated CO_2, so this species may simply be tolerant of projected future CO_2 levels. It remains unknown how

the presence of diel CO_2 cycles will affect behaviours of marine fish which are negatively impacted by stable elevated CO_2.

Effects of ocean warming on marine fishes

For ectothermic animals, such as fishes, temperature dictates metabolic performance and thus ultimately individual fitness (Clarke and Johnston 1999; Pörtner and Knust 2007; Pörtner and Farrell 2008). Consequently, marine fishes are potentially vulnerable to rising temperatures driven by climate change. For most species in their normal temperature range (i.e. thermal window), metabolic performance is enhanced towards the thermal optimum as temperatures increase from the thermal minimum (Pörtner and Knust 2007; Pörtner and Farrell 2008). However, the ability to maintain metabolic function is reduced at temperatures above the optimum as physiological systems cannot keep pace with oxygen demands to the tissues (Pörtner and Knust 2007; Pörtner and Farrell 2008; Nilsson et al. 2009; Donelson et al. 2012). Furthermore, the energetic costs of maintaining cellular function rises at temperatures above the optimum, increasing the basic costs of living (Clarke and Johnston 1999; Pörtner and Knust 2007; Sokolova et al. 2012). Due to its tight control on metabolic performance and bioenergetics, temperature can affect a range of traits in marine fishes, including; growth and survival (McCormick and Molony 1995; Green and Fisher 2004; Gagliano et al. 2007; Munday et al. 2008; Todd et al. 2008; Neuheimer et al. 2011; McLeod et al. 2015; Watson et al. 2018), swimming performance (Brett 1964; Lee et al. 2003; Green and Fisher 2004; Johansen and Jones 2011) and reproduction (Pankhurst et al. 1996; Hilder and Pankhurst 2003; King et al. 2003; Donelson et al. 2014). Changes in temperature can also affect behaviours that are

linked to aerobic capacity such as foraging behaviour, fast starts and activity (Biro et al. 2010; Nowicki et al. 2012; Johansen et al. 2014; Allan et al. 2015; Scott et al. 2017).

Tropical species are considered to be among the most vulnerable to increasing temperatures because they have evolved in a relatively stable thermal environment and thus are expected to exhibit narrow thermal tolerance ranges (Deutsch et al. 2008; Tewksbury et al. 2008; Wright et al. 2009). For example, in coral reef ecosystems seasonal temperatures may only differ by up to 5–6˚C annually (Albright et al. 2013; Shaw and McNeil 2014), whereas in temperate systems annually cycles greater than 15°C are observed (Baumann et al. 2015; Challener et al. 2016). Additionally, it is thought that many tropical species are already living close to their thermal optimum at current-day summer temperatures (Deutsch et al. 2008; Tewksbury et al. 2008; Wright et al. 2009). Experimental work on coral reef fishes supports this by demonstrating that an increase of only 2°C above summer temperatures can negatively affect a range of traits, including; survival, growth, reproduction, aerobic scope, fast starts and activity (Munday et al. 2008; Nilsson et al. 2009; Donelson et al. 2012, 2014; Rummer et al. 2014; Warren et al. 2016, 2017; Scott et al. 2017; Rodgers et al. 2018).

Combined effects of ocean acidification and warming

While there has been extensive research into the potential effects of ocean acidification and ocean warming on shallow water coastal marine organisms (Harvey et al. 2013; Kroeker et al. 2013b; Przeslawski et al. 2015; Cattano et al. 2018), most studies have tested the effects of these climate change drivers in isolation. However, multiple drivers can interact in ways to affect organisms differently than expected, based on responses to the drivers in isolation (Pörtner and Farrell 2008). Interactions between stressors can be broadly classified into three

types: (1) additive effects in which stressors independently affect an organism such that their combined effects are simply the sum of the individual effects, (2) antagonistic effects in which one stressor offsets the effect of the other and (3) synergistic effects in which stressors interact such that their combined effects are greater than the sum of their individual effects (Przeslawski et al. 2015; Gunderson et al. 2016). Meta-analyses have revealed that synergistic effects appear to be the most common outcome of the combined effects of ocean acidification and warming (Harvey et al. 2013; McBryan et al. 2013; Przeslawski et al. 2015). Thus, understating how multiple climate change drivers affect shallow water marine organisms is vital to accurately predict future impacts, as extrapolations based on a single driver response could lead to incorrect conclusions (Gaylord et al. 2015; Riebesell and Gattuso 2015; Gunderson et al. 2016).

The number of studies investigating the potential interactive effects of ocean acidification and warming on marine fishes has grown in recent years. These works have shown that there are interactive effects on growth and survival (Miller et al. 2012; Pimentel et al. 2014; Flynn et al. 2015; Gobler et al. 2018), metabolic performance (Munday et al. 2009b; Enzor et al. 2013; Grans et al. 2014) and a number of behavioural traits (Nowicki et al. 2012; Domenici et al. 2014; Ferrari et al. 2015). As with most ocean acidification research all studies to date have been conducted using stable elevated CO_2. Therefore, it is currently unknown if elevated temperature will modify any interactive effects elevated CO_2 and diel CO_2 cycles have on the performance of marine fishes.

Parental effects and ocean acidification

Most studies examining the effects of ocean acidification on shallow water coastal marine organisms have been short-term (i.e. days to weeks) and have focused on a single life-stage within a generation (Kroeker et al. 2013b; Calosi et al. 2016). While informative, these studies do not include the complexity of organisms' responses to a changing environment, such as that the conditions experienced in one life-stage can influence the performance of later life-stages (Marshall and Morgan 2011; Burton and Metcalfe 2014). For example, juvenile Olympia oysters performed worse under ocean acidification conditions if the larval stage had also experienced elevated CO_2 (Hettinger et al. 2012). Studies such as this highlight that by not considering multiple life stages the impacts of ocean acidification may be over- or underestimated (Munday et al. 2013; Sunday et al. 2014; Ross et al. 2016).

Parents can significantly alter the phenotype of their offspring based on the conditions they experience – termed parental effects (Badyaev and Uller 2009; Burgess and Marshall 2014). Thus, parental effects can be an important source of phenotypic variation in performances between individuals, ultimately influencing short-term selection and the evolutionary trajectories of populations (Mousseau and Fox 1998; Badyaev and Uller 2009; Chevin et al. 2010; Bonduriansky et al. 2012). The outcome of parental effects on offspring fitness is dependent on how predictable the conditions offspring will encounter are from the parental environment (Marshall and Uller 2007; Burgess and Marshall 2014; Guillaume et al. 2016). If conditions are sufficiently predictable, parental effects can act to increase offspring fitness ('anticipatory parental effects' sensu Burgess and Marshall 2014). Parental effects occur through a variety of non-genetic mechanisms (Bonduriansky et al. 2012). One area that is

gaining interest is epigenetic effects, whereby gene expression is altered without changes in the underlying DNA sequence (e.g. DNA methylation) (Bonduriansky et al. 2012). Epigenetic effects therefore have the capacity to prime physiological processes so that offspring can better cope with environment conditions similar to those experienced by the parents (Ho and Burggren 2010; Ryu et al. 2018).

Due to the potential for parental effects to influence the performance of offspring under altered environmental conditions, they have gained interest as a potential mechanism that may enable marine organisms to persist in the face of ongoing ocean acidification and warming (Munday 2014; Ross et al. 2016; Donelson et al. 2018). Indeed, several studies have shown that parental effects can alleviate the negative effects of stable elevated CO_2 on the early life stages of shallow water coastal marine organisms, including fishes (Miller et al. 2012; Allan et al. 2014; Murray et al. 2014) and a range of invertebrates (Dupont et al. 2013; Pedersen et al. 2014; Suckling et al. 2014; Parker et al. 2015; Rodríguez-Romero et al. 2016; Chakravarti et al. 2016). For example, the negative effects of stable elevated CO_2 on metabolic rates, survival and growth in juvenile cinnamon anemonefish were absent if parents experienced similar elevated CO_2 condition (Miller et al. 2012). In contrast, parental exposure produced offspring more vulnerable to ocean acidification in two species of bivalve shellfish (Griffith and Gobler 2017). It is currently unknown if the presence of diel CO_2 cycles will alter the outcome of parental effects on offspring traits, which limits our ability to accurately predict how shallow water marine organisms will respond to ocean acidification in the long-term.

Thesis outline and aims

This thesis examines the effects that diel CO_2 cycles have on the early development and behaviour of coral reef fishes under ocean acidification. Furthermore, it examines how such effects are modified by the presence of elevated temperature and by parental effects. While the potential impacts of stable elevated CO_2 on marine organisms are well documented, very little is known about how diel CO_2 cycles will affect the sensitivity of marine organisms to ocean acidification.

In **chapter 2**, I investigate the impacts that diel CO_2 cycles have on the early development of two coral reef fish (the spiny damselfish *Acanthochromis polyacanthus* and orange clownfish *Amphiprion percula*) under ocean acidification. Specifically, juvenile survival, growth and otolith development were measured and compared between control, stable elevated and two diel-cycling elevated CO_2 treatments. The study allowed me to determine how diel CO_2 cycles may affect the early life-history performance of marine fishes under elevated CO_2.

In **chapter 3**, I investigate the impacts diel CO_2 cycles have on behavioural performance under ocean acidification in both *A. polyacanthus* and *Am percula*. The aims of this chapter were twofold. Firstly, I wanted to determine if the magnitude of diel pCO_2 cycles affects the behavioural performance of coral reef fishes under ocean acidification conditions. Secondly, I wanted to test if the presence of diel pCO_2 cycles affects the mean CO_2 level at which behavioural abnormalities occur (i.e. the onset of behavioural abnormalities). To achieve this, two experiments were conducted using a series of stable and diel cycling pCO_2 treatments. I

measured behavioural lateralization in *A. polyacanthus* and response to a predator cue in *Am percula*. The result of this chapter shed light on how diel CO_2 cycles affect the behavioural sensitivity of marine fishes to ocean acidification.

After assessing how diel CO_2 cycles affect the early development and behaviour of coral reef fish under elevated CO_2, **chapter 4** tests if these effects are modified by the presence of elevated temperature. In this chapter I focus on one species, *A. polyacanthus.* Fish were reared under control, stable elevated and two diel-cycling elevated CO_2 treatments at both current-day and end of century temperatures. I measured the effects on survival, growth, behavioural lateralization, escape performance and activity/anxiety and compared among the treatments. This study expands our knowledge on the interactive effects between elevated CO_2 and temperature.

Finally, in **chapter 5** I investigate if diel CO_2 cycles alter the outcome of parental effects on the early development of the coral reef anemonefish *Am. melanopus*. Here, breeding pairs were conditioned to control, stable elevated and diel-cycling elevated CO_2 treatments. Juveniles from control pairs were reared in all CO_2 treatments, whereas juveniles from the two elevated CO_2 treatments were only reared in the same conditions as their parents. Comparisons between treatments allowed me to determine both the within-generation effects of stable elevated and diel-cycling elevated CO_2 on juvenile growth and survival, and how these responses were modified by parental exposure. This study illustrates the importance of considering diel CO_2 cycles when predicting the long-term impacts of ocean acidification on shallow water coastal marine organisms.

Chapter 2: Diel CO_2 cycles do not modify juvenile survival, growth and otolith development in two coral reef fish under ocean acidification

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2.1 Summary

Recent studies show that daily variation in pCO_2 levels can modify the life-history and calcification responses of marine organisms to ocean acidification. The early life stages of coral reef fish exhibit varied growth, survival and otolith development responses to elevated pCO_2, yet no studies to date have considered the substantial diel pCO_2 cycles that occur in shallow reef habitats. Here, I reared three clutches of juvenile *Acanthochromis polyacanthus* and *Amphiprion percula* under control (500 µatm), stable elevated (1000 µatm) and diel-cycling elevated (1000 ± 300 and 1000 ± 500 µatm) pCO_2 for 11 and 6 weeks respectively. Survival was unaffected by exposure to either elevated stable or diel-cycling pCO_2 conditions in both species. For *A. polyacanthus* there was a non-significant trend of decreased standard length and wet weight under stable elevated pCO_2 conditions, whereas values in both the diel-cycling treatments were closer to those observed under control conditions. A similar non-significant trend was observed for *Am. percula*, except that exposure to stable elevated pCO_2 conditions resulted in slightly longer and heavier fish. Finally, otolith size, shape and symmetry in both species were unaffected by exposure to either elevated stable or diel-cycling pCO_2 conditions. Overall, the results suggest that the growth, survival and otolith

development of juvenile coral reef fishes under ocean acidification is unlikely to be affected, in isolation, by diel cycles in pCO_2.

2.2 Introduction

Increased uptake of CO_2 by the oceans is causing a reduction in pH and shift in the relative concentrations of carbonate and bicarbonate ions, a process referred to as ocean acidification (OA) (Doney et al. 2009). Based on current emission trajectories, atmospheric CO_2 levels could reach 1000 ppm by the year 2100 (Meinshausen et al. 2011), which would result in a reduction of mean surface water pH of 0.3-0.4 units compared to pre-industrial values (Caldeira and Wickett 2003). A large body of experimental research has shown that such dramatic changes in ocean chemistry can affect growth and survival of many shallow water marine organisms (Kroeker et al. 2013b; Wittmann and Pörtner 2013; Przeslawski et al. 2015). Furthermore, the capacity of calcifying species to produce their shells and skeletons will be retarded by the reduction in availability of carbonate ions and carbonate saturation state that accompanies OA (Doney et al. 2009). These impacts are expected to have far-reaching implications for community dynamics and ecosystem functioning (Doney et al. 2012; Kroeker et al. 2013a; Nagelkerken and Connell 2015).

Most of our understanding on how OA will impact shallow water marine organisms is based on experiments that have used stable levels of elevated pCO_2 consistent with atmospheric projections (McElhany and Busch 2013; Gunderson et al. 2016; Wahl et al. 2016). However, unlike open oceans, CO_2 levels in shallow water habitats are not in equilibrium with the atmosphere over short time scales, experiencing fluctuations that, in some cases, can exceed the average CO_2 projected to occur by the year 2100 (Hofmann et al. 2011; Duarte et al. 2013).

Among the most conspicuous CO_2 fluctuations are those that occur over a 24h period. These diel CO_2 cycles, driven primarily by the processes of photosynthesis and respiration across a day-night cycle, occur in a range of shallow water habitats including coral reefs, kelp forests, seagrass meadows and salt marshes (Frieder et al. 2012; Shaw et al. 2012; Baumann et al. 2015; Challener et al. 2016). In such habitats, the pCO_2 of seawater decreases during the day due to the drawdown of CO_2 from net photosynthesis and increases at night due to the release of CO_2 from net respiration. Importantly, a number of studies have shown that diel pCO_2 cycles can modify organismal responses to OA, questioning the ecological relevance of using stable CO_2 treatments (e.g. Alenius & Munguia, 2012; Dufault *et al.*, 2012; Cornwall *et al.*, 2013; Frieder *et al.*, 2014; Clark & Gobler, 2016; Chan & Eggins, 2017; Mangan *et al.*, 2017). Consistent with previous OA research these experiments have largely focussed on the responses of calcifying species. How diel pCO_2 cycles will interact with OA to affect the performance of non-calcifying shallow water marine organisms, such as fish, is less well known (but see Ou *et al.* 2015; Davidson *et al.* 2016).

Marine fish were expected to be one of the most resilient groups to OA based on their high capacity for acid-base regulation (Baker et al. 2009; Melzner et al. 2009; Heuer and Grosell 2014). To prevent plasma and tissue acidosis under elevated pCO_2, fish actively increase their intracellular and extracellular HCO_3^- concentrations to buffer changes in pH (Baker *et al.*, 2009; Esbaugh *et al.*, 2012; Heuer & Grosell, 2014). Consistent with their capacity for acid-base regulation, adult fish can usually tolerate CO_2 levels far greater than those projected to occur by the year 2100 (Ishimatsu et al. 2008; Melzner et al. 2009). However, early life stages of marine organisms are often less tolerant to environmental change than adults, because their smaller size increases the cost of homeostasis (Brauner 2009; Melzner et al. 2009).

Indeed, early life stages of some marine fish appear more sensitive to elevated pCO_2. For example, inland silverside (*Menidia beryllina*) larvae exposed to 1100 µatm CO_2 for one week exhibited reduced growth and survival (Baumann et al. 2012). Similarly, larval and juvenile Atlantic cod (*Gadus morhua*) exhibit reduced growth, increased tissue deformities and reduced survivorship at elevated pCO_2 (Moran and Støttrup 2011; Frommel et al. 2012; Stiasny et al. 2016). In contrast, elevated pCO_2 had limited effects on growth rate and survival in walleye pollock (*Theragra chalcogramma*), cobia (*Rachycentron canadum*), Atlantic herring (*Clupea harengus*), Baltic cod (*G. morhua)* and scup (*Stenotomus chrysops*) (Franke and Clemmesen 2011; Bignami et al. 2013b; Frommel et al. 2013; Hurst et al. 2013; Perry et al. 2015), possibly associated with the variable pCO_2 environments these species often inhabit.

Another concern for early life-stages of marine fish is how OA might impact the development of otoliths (ear bones), which are mainly comprised of aragonite calcium carbonate (Payan et al. 2004). The position of otoliths in the inner ear and their movement over sensory hair cells enables fish to detect sound, body orientation and acceleration (Popper and Lu 2000). Changes to the size, shape or symmetry of otoliths as a result of altered calcification could result in reduced auditory performance and individual fitness (Gagliano et al. 2008; Bignami et al. 2013a). Otolith calcification is dependent on the chemical composition of the endolymph within the inner ear (Payan et al. 2004). As such, otolith development could be impacted by the increases in extracellular HCO_3^- concentrations associated with acid-base regulation under elevated pCO_2 conditions. Indeed, increased otolith size at elevated pCO_2 has been observed in the larvae of sea bass (*Atractoscion nobilis*), orange clownfish (*Amphiprion percula*), cobia (*R. canadum*) and Atlantic cod (*G. morhua)* (Checkley et al. 2009; Munday et al. 2011a; Bignami et al. 2013a; Maneja et al. 2013). In contrast, otolith size in

Atlantic herring (*C. harengus*), Baltic Cod (*G. morhua)* and spiny damselfish (*Acanthochromis polyacanthus*) was unaffected by elevated pCO_2 levels (Franke and Clemmesen 2011; Munday et al. 2011b; Frommel et al. 2013).

Diel pCO_2 cycles have been measured on coral reefs more than any other shallow water habitat (Wahl et al. 2016), and can range anywhere from ± 50 to 600 µatm around the mean (Shaw et al. 2012; Albright et al. 2013; Kline et al. 2015). Importantly, recent studies have shown that calcification in corals (Dufault et al. 2012; Comeau et al. 2014; Chan and Eggins 2017) and the behavioural performance of coral reef fish (**Chapter 3**) under OA conditions is improved by the presence of diel pCO_2 cycles. It is currently unknown how diel pCO_2 cycles will affect the development of juvenile coral reef fishes under OA. Here, I investigated the effects of stable vs. diel-cycling elevated pCO_2 on the growth, survival and otolith development in juveniles of two coral reef fish, the spiny damselfish (*A. polyacanthus*) (Bleeker, 1855), and orange clownfish (*Am. percula*) (Lacepède, 1802). Both species are models for investigating the potential impacts of OA on coral reef fishes (Munday et al. 2009a, 2011b, a; Dixson et al. 2010; Welch et al. 2014; Heuer et al. 2016). Juveniles were reared at 500, 1000, 1000 ± 300 and 1000 ± 500 µatm. The stable 1000 µatm pCO_2 treatment represented the open ocean projection for the end of this century typically used in past OA experiments (Kroeker et al., 2013). The cycling pCO_2 treatments represented high levels of variation currently observed in shallow tidal lagoons (Santos *et al.*, 2011; Shaw *et al.*, 2012). In other reef areas smaller diel pCO_2 cycles are more typical (Albright *et al.*, 2013; Kline *et al.*, 2015). However, a threefold amplification in diel pCO_2 cycles on coral reefs is predicted to occur over the next century (Shaw et al. 2013a), and thus the magnitude of fluctuations seen in tidal lagoons today may become more common in other reef areas by the year 2100.

2.3 Materials and Methods

Brood-stock and clownfish larval rearing

Adult *A. polyacanthus* were collected using hand nets from the Bramble Reef area (site 1: 18°22'S, 146°40'E; site 2: 18°25'S, 146°40'E) of the Great Barrier Reef in July 2015. Fish were transported to an environmentally controlled aquarium research facility at James Cook University (JCU) (Townsville, Australia) where they were housed as breeding pairs in 60 L aquaria at temperature conditions matching the collection location. An existing brood-stock of *Am. percula* at JCU was used. These pairs had been collected from the Cairns region of the Great Barrier Reef and housed at JCU for four years. Adult *A. polyacanthus* and *Am. percula* pairs were maintained under ambient pCO_2. Temperatures were increased at a rate of 0.5°C *per* week until the summer breeding temperature of 29°C was reach in the first week of November 2015. Adult pairs were provided with half a terracotta pot to act as a shelter and spawning site. Aquaria were checked each morning for the presence of newly laid clutches. Breeding pairs were fed *ad libitum* on commercial fish feed pellets (INVE Aquaculture Nutrition NRD 12/20) once *per* day outside the breeding season and twice *per* day during the breeding season (November–May).

A. polyacanthus develop directly into benthic juveniles and do not have a pelagic larval phase. By contrast, *Am. percula* have a pelagic larval phase lasting 11-12 days before they are competent to settle to reef habitat. Rearing of larval *Am. percula* was performed using methods similar to those described by Munday *et al*. (2009). Briefly, on the night of hatching (6–8 d) the pot was removed from the parental aquarium and transferred to an aerated 100 L larval rearing aquarium. Larvae were reared in a semi-closed system under ambient pCO_2, where aquariums had no water flow during the day and were then slowly flushed with filtered

seawater each night. This daily cycle ensured that larvae could feed *ad libitum* throughout daylight hours and that any unconsumed food was removed each night. Larvae were fed rotifers (*Brachionus* sp.) at 20 individuals' mL^{-1} each morning for the first 5 days. On these days 3 mL of non-viable *Nannochloropsis* algal paste was also added to the tanks to feed the rotifers. Freshly hatched *Artemia* naupli were added *ad libitum* from days 3-11. A commercial weaning fish feed (INVE Aquaculture Nutrition Wean-S 200-400 µm) was added on days 10 and 11. A summer light cycle of 13 h of light/11 h of dark was simulated with fluorescent lights.

Experimental design and data collection

Three offspring clutches were used per species, each from a different parental pair. *A. polyacanthus* and *Am. percula* clutches were transferred to the experimental system (see below) one-day post hatch (dph) and at the end of their larval phase (12 dph) respectively. Clutches were split between pCO_2 treatments in duplicate tanks *per* treatment (12-15 *A. polyacanthus* per tank and 10 *Am. percula* per tank). Clutches of the two species were kept in separate tanks.

A. polyacanthus and *Am. percula* were reared for 11 and 6 weeks respectively. *A. polyacanthus* juveniles were fed a combination of freshly hatched *Artemia* naupli and weaning fish feed daily for the first four dph. From 5-21 dph they were fed daily on the weaning feed and the switched to a small pellet fish feed (INVE Aquaculture Nutrition NRD 5/8) at 22 dph. *Am. percula* juveniles were fed on the weaning fish feed throughout the experiment.

Survival and growth: At the end of the rearing period clutches were euthanised with clove oil anaesthetic (James Cook University animal ethics committee - permit A2210). Individuals were then blotted dry, weighed (nearest mg) on an analytical balance (AX224, Sartorius, Bradford, USA) and photographed in a lateral position on a gridded sheet. Once photographed, the fish were stored in 70% ethanol for otolith extraction. Standard length (SL) to the nearest 0.1 mm was estimated for each fish from the digital photographs using ImageJ software (http://rsb.info.nih.gov/ij/).

Otolith morphometries: Left and right sagittal otoliths were removed from each individual, cleaned, and stored dry in well-plates. Otoliths were then photographed from the distal side using a 17.28 MP digital camera (DP73, Olympus, Macquarie Park, Australia) attached to a light microscope (BX53, Olympus, Macquarie Park, Australia) to produce a calibrated grey-scale image. Morphometric measurements (otolith area, perimeter, rectangularity and circularity) were obtained from the images using ImageJ software.

Experimental system, CO_2 manipulation and seawater parameters

Experiments were conducted in an 11,000 L re-circulating seawater system. Briefly, the system consisted of a large external 3,700 L sump tank connected to a bio-filter, protein skimmer, UV steriliser and a 1000 L algal bio-remediation tank. The external sump supplied water to four separate 1,600 L re-circulating systems that comprised of one 1000 L sump tank and fifteen 40 L holding tanks, contained within a temperature-controlled room. Water was supplied at a rate of approximately 1,600 L *per* day allowing for a complete exchange with the external sump. Holding tanks were supplied with water at a rate of 1 L min^{-1}. Both the internal sumps and holding tanks were aerated with ambient air.

Elevated pCO_2 treatments were achieved by dosing the 1000 L internal sumps with CO_2. This was controlled by solenoid valves (M-Ventil Standard, Aqua Medic, Germany) connected to a pH control system (Aqua Medic AT Control System, Aqua Medic, Germany) with laboratory grade pH electrodes (Neptune Systems, USA). The Aqua Medic AT Control System has a curve function which allowed us to create fluctuating pCO_2 profiles. pH profiles in the fluctuating pCO_2 treatments were recorded every other day using a pH meter (InLab Expert Pro electrode and Seven2Go Pro meter, Mettler Toledo, Switzerland) set to take a reading every 15 min. For the stable pCO_2 treatments pH_{NBS} was measured twice daily using the same model of pH meter. Seawater pH on the total hydrogen ion concentration scale (total scale, pH_T) was measured each week with a spectrophotometer following standard operating procedures (Dickson et al. 2007) using the indicator dye meta/*m*-cresol purple (mCP) (*m*-cresol purple sodium salt 99%, non-purified, Acros Organic). Daily and fluctuating pH_{NBS} measurements were converted to pH_T based on the offset between weekly pH_T and pH_{NBS} measurements. Temperature was recorded daily with a digital thermometer (Comark C26, Norfolk, UK). Salinity readings were taken weekly using a conductivity sensor (HQ15d; Hach, USA). Total alkalinity was measured weekly using Gran Titration (Metrohm 888 Titrando Titrator Metrohm AG, Switzerland) and certified reference material from Dr. A. G. Dickson (Scripps Institution of Oceanography). All seawater parameters were measured in randomly chosen holding tanks. pCO_2, HCO_3^-, CO_3^{2-} and ΩAr values were calculated as a function of pH_T, temperature and salinity using CO_2SYS (Pierrot et al. 2006) employing constants from Mehrbach et al. (1973) refit by Dickson and Millero (1987) and the $KHSO_4$ dissociation constant from Dickson (1990). Mean values for each of these seawater parameters are presented in **Table 2.1**.

Table 2.1 Seawater parameters. Values are means ± 1 SD for daily average, minimum, maximum and range of pH_T and pCO_2. Mean ± 1 SD for total alkalinity (TA), bicarbonate (HCO_3^-), carbonate (CO_3^{2-}), aragonite saturation (ΩAr) temperature (°C) and salinity over the experiment are also shown.

Parameter	pCO_2 treatment (µatm)			
	500	1000	1000 ± 300	1000 ± 500
pH	8.01 ± 0.01	7.75 ± 0.02	7.77 ± 0.08	7.80 ± 0.16
Min. pH	-	-	7.64 ± 0.03	7.57 ± 0.02
Max. pH	-	-	7.89 ± 0.01	8.01 ± 0.01
pH range	-	-	0.24 ± 0.03	0.44 ± 0.02
pCO_2 (µatm)	480 ± 20	990 ± 46	961 ± 195	934 ± 389
Min. pCO_2 (µatm)	-	-	681 ± 21	482 ± 19
Max pCO_2 (µatm)	-	-	1304 ± 102	1591 ± 98
pCO_2 range (µatm)	-	-	623 ± 96	1109 ± 95
TA (µmol kg^{-1})	2570 ± 54	2574 ± 42	2582 ± 43	2583 ± 43
HCO_3^- (µmol kg^{-1})	1956 ± 54	2201 ± 49	2183 ± 60	2145 ± 129
CO_3^{2-} (µmol kg^{-1})	259 ± 10	160 ± 7	167 ± 25	183 ± 53
Aragonite (ΩAr)	4.13 ± 0.16	2.56 ± 0.11	2.70 ± 0.40	2.92 ± 0.85
Temperature (° C)	28.7 ± 0.3	28.9 ± 0.3	28.9 ± 0.3	29.0 ± 0.2
Salinity	36.7 ± 0.4	36.7 ± 0.4	36.7 ± 0.4	36.7 ± 0.4

Statistical analyses

Growth and survival: The effects of CO_2 treatment on juvenile survival were tested using a generalised linear model fitted with a binomial distribution. The effects of CO_2 treatment on fish standard length (SL) and wet weight were assessed using linear mixed effects models (LMM's). Parental pair and tank were included as random factors, with tank nested within parental pair.

Otolith development: Otolith shape and size are dependent on fish size. Therefore, the effects of CO_2 treatment on otolith morphometric traits were tested using LMM's with SL as a

covariate. Once again, parental pair and tank were included as random factors, with tank nested within parental pair. Prior to running the models, the relationship between each morphometric trait and SL was tested at the level of treatment. If all treatments exhibited the same relationship the effects of SL were examined in isolation. However, if the relationship between a morphometric trait and SL differed between treatments the interaction between treatment and SL was tested. Directional asymmetry of the otoliths (i.e. is the right or left otolith usually larger) was assessed using signed differences. Signed differences were determined by subtracting the value for the left otolith from that of the right otolith (R-L) for otolith area and perimeter only, as rectangularity and circularity are absolute values which have no meaning. The frequency of positive (right otolith larger) versus negative (left otolith larger) scores among the CO_2 treatments was then compared with a chi-square test of independence. The effects of CO_2 treatment on the magnitude of otolith asymmetry with respects to otolith area and perimeter were tested using LMM's as described above using unsigned differences between left and right otoliths. Analyses were conducted in R version 3.4.0 (R Core Team 2017) using the 'nlme' (Pinheiro et al. 2017), 'lme4' (Bates et al. 2015) and 'lsmeans' (Lenth 2016) packages.

2.4 Results

Survival and growth

Mean survival of juvenile *A. polyacanthus* was unaffected by CO_2 treatment, ranging from 93.5% to 89.3% (**Figure 2.1a**, χ^2 = 0.09, df = 3, *P* = 0.993). CO_2 treatment also had no significant effect on mean survival of juvenile *Am. percula* (**Figure 2.1b**, χ^2 = 0.98, df = 3, *P* = 0.807), ranging from 96.7% to 81.7%.

There was no significant effect of CO_2 treatment on mean SL and weight of juvenile *A. polyacanthus* (**Figure 2.1c, e**, max. *F* (3,18) = 1.95, *P* = 0.157). However, there was a trend of reduced SL (3.5%) and weight (6.7%) in the stable, elevated CO_2 treatment compared with control conditions. Mean SL and weight of fish reared at diel-cycling elevated CO_2 (1000 ± 300 and 1000 ± 500 μatm) were more comparable to fish reared at control conditions. Overall, there was no significant effect of CO_2 treatment on mean SL and weight of juvenile *Am. percula* (**Figure 2.1d, f**, max. *F* (3,18) = 1.86, *P* = 0.173). However, there was a trend of increased SL (8.1%) and weight (11.6%) in the stable, elevated CO_2 treatment compared to control condition. Once again, mean SL and weight of fish reared at diel-cycling elevated CO_2 (1000 ± 300 and 1000 ± 500 μatm) were similar to fish reared at control levels.

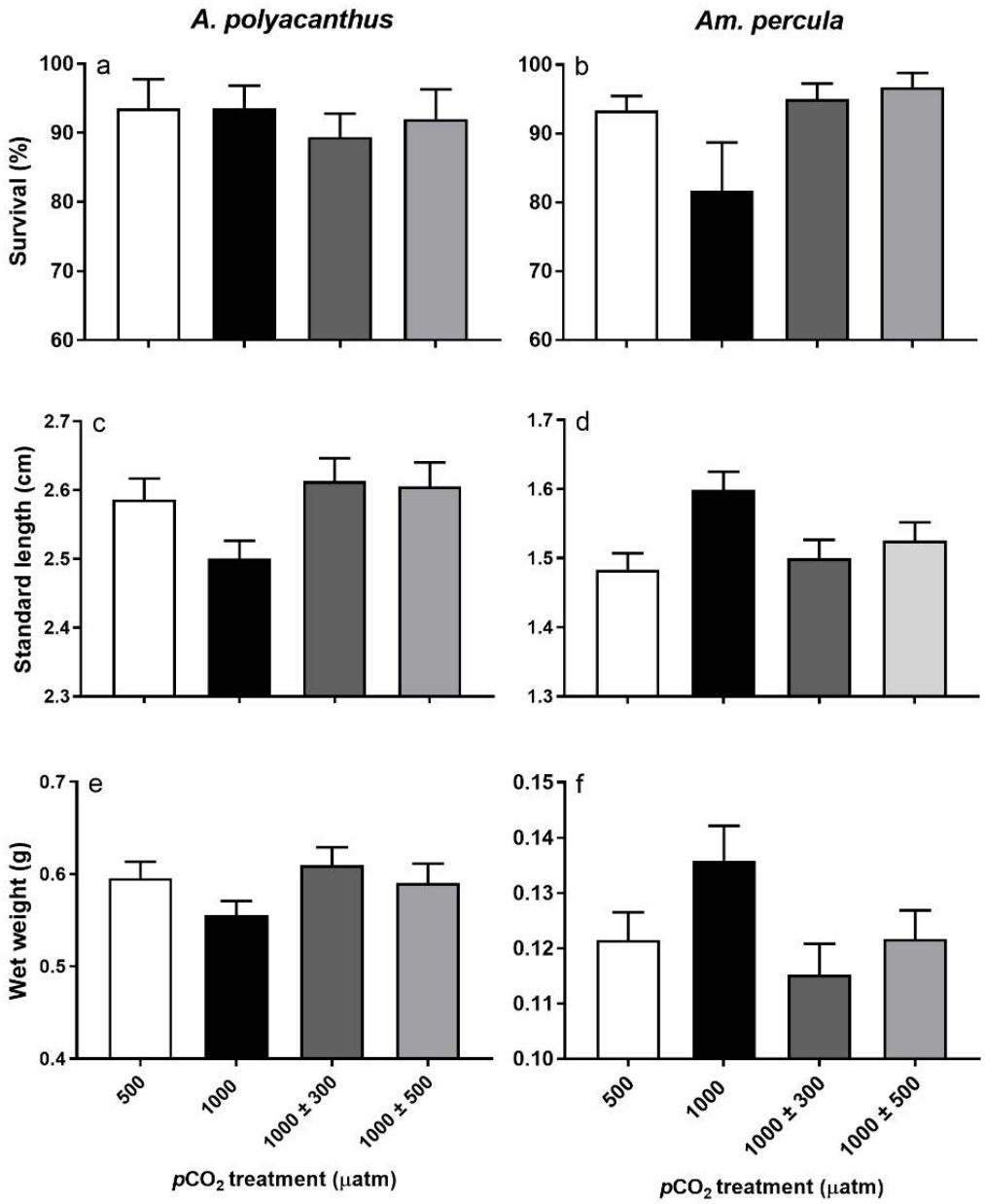

Figure 2.1 Survival (a, b), standard length (c, d) and weight wet (e, f) of juvenile *Acanthochromis polyacanthus* and *Amphiprion percula* reared in stable and diel-cycling elevated pCO_2. Bars represent means ± SE.

Otolith development

Otolith size and shape

Otolith area and perimeter (left and right otoliths) in juvenile *A. polyacanthus* and *Am. percula* were unaffected by CO_2 treatment (**Figure 2.2a-d**, max. F (3,18) = 2.00, P = 0.150). As expected, there was a significant effect of SL on otolith area and perimeter in both species (min. F (1,159) = 580.55, P < 0.001). The effect of SL on otolith area and perimeter matched the direction of trends in body size, with area and perimeter smaller in *A. polyacanthus* at 1000 µatm pCO_2 compared with the control and two diel-cycling elevated CO_2 treatments. By contrast, otolith area and perimeter were larger in *Am. percula* at 1000 µatm pCO_2 compared with the control and two diel-cycling elevated CO_2 treatments. Otolith rectangularity and circularity (left and right otoliths) were also unaffected by CO_2 treatment in both species (**Figure 2.2e-h**, max. F (3,17) = 1.39, P = 0.281). SL only had a significant effect on otolith circularity of left and right otoliths in juvenile *Am. percula* (F (1,173) = 12.75, P < 0.001).

Size difference within otolith pairs

There was no evidence of directional asymmetry in juvenile *A. polyacanthus* and *Am. percula*, with no difference in the distribution of positive and negative scores among CO_2 treatments for either otolith area (**Figure 2.3a, b**) or perimeter (**Figure 2.3c, d**) (all P > 0.160). Furthermore, the magnitude of otolith asymmetry with respects to otolith area (**Figure 2.4 a, b**) and perimeter (**Figure 2.4 c, d**) was unaffected by CO_2 treatment in both species (max. F (3,17) = 1.07, P = 0.389).

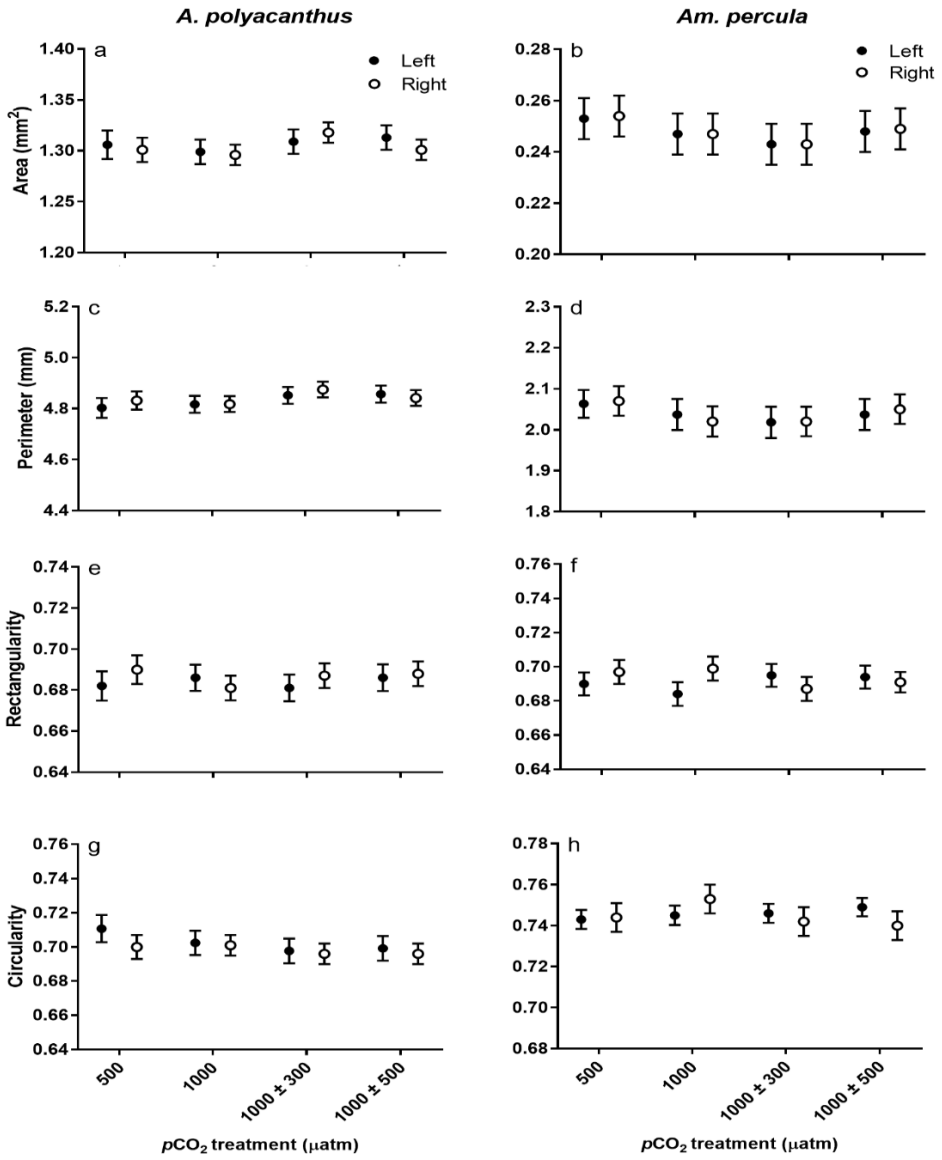

Figure 2.2 Otolith area (a, b), perimeter (c, d), rectangularity (e, f) and circularity (g, h) in juvenile *A. polyacanthus* and *Am. percula* reared in stable and diel-cycling elevated pCO_2. Points represent least-square means ± SE.

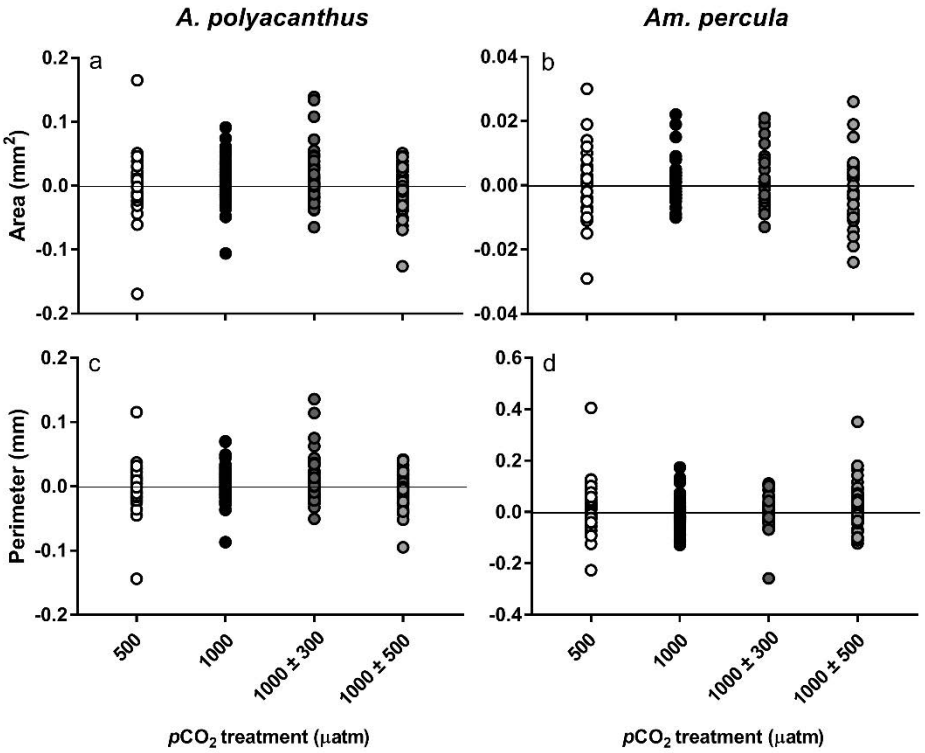

Figure 2.3 Signed differences of otolith area (a, b) and perimeter (c, d) in juvenile in juvenile *A. polyacanthus* and *Am. percula* reared in stable and diel cycling-elevated pCO_2. Positive values represent cases were the right otolith was larger and negative values represent cases were the left otolith was larger.

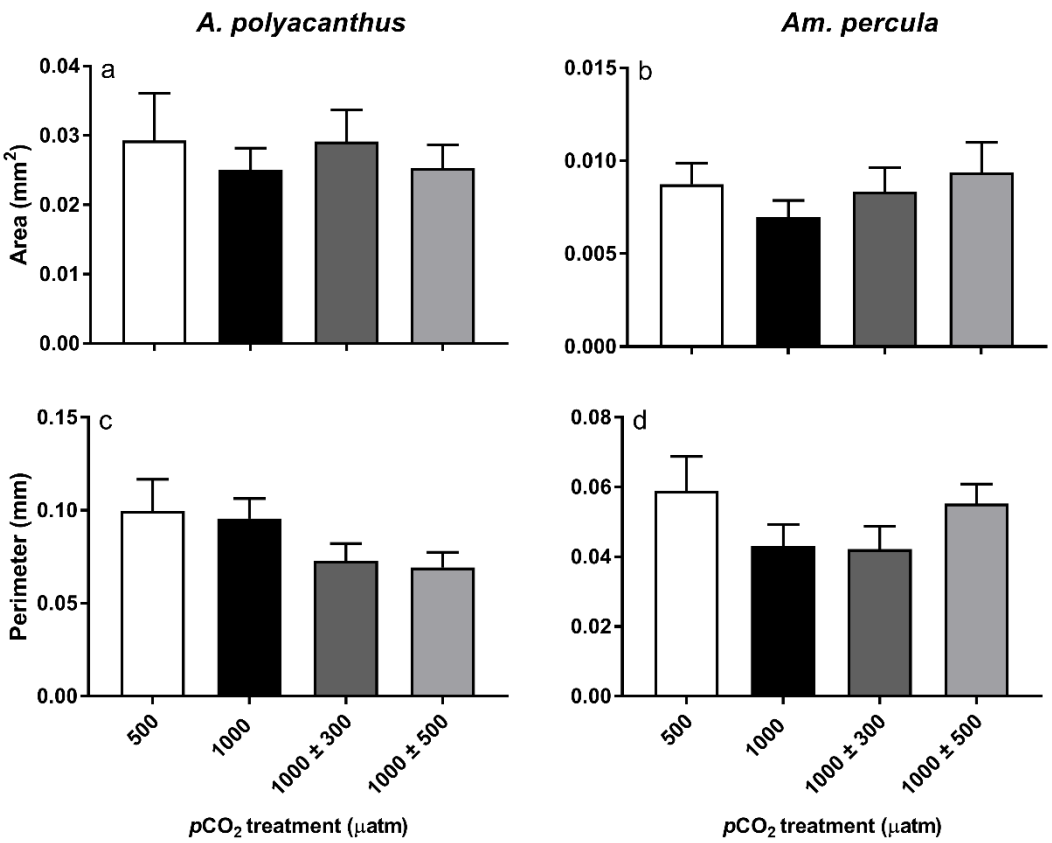

Figure 2.4 Unsigned differences of otolith area (a, b) and perimeter (c, d) in juvenile in juvenile *A. polyacanthus* and *Am. Percula* reared in stable and diel-cycling elevated pCO_2. Bars represent means ± SE.

2.5 Discussion

The early life stages of marine fish have displayed varied growth, survival and otolith calcification responses to elevated pCO_2, with positive, negative and no effects all being observed across a range of tropical and temperate species (Heuer and Grosell 2014). In general, however, most species appear to be relatively resilient. Yet, most species have also been tested in stable high pCO_2 conditions, even though many species experience substantial daily or seasonal pCO_2 cycles in the shallow coastal habitats they occupy. Here I show that the growth, survival and otolith development responses in two coral reef fish that have been commonly tested under OA conditions are not significantly affected by the presence of diel CO_2 cycles.

I detected no significant effect of stable elevated pCO_2 (1000 µatm) on the survival, growth and otolith development of juvenile *A. polyacanthus*, although there was a trend of decreased standard length (SL) and weight compared to the control and diel-cycling CO_2 treatments. This finding is consistent with a previous study in which juvenile *A. polyacanthus* were reared at 850 µatm pCO_2 for three weeks (Munday et al. 2011b). Similar results were observed in juvenile scup (*S. chrysops*) exposed to 1200-1600 µatm pCO_2 for eight weeks (Perry et al. 2015), and in larval Baltic cod (*G. morhua*) exposed up to 3200 µatm pCO_2 (Frommel et al. 2013). Similarly, there was no significant effects of stable elevated pCO_2 on survival and growth of juvenile *Am. percula*. However, there was a trend of increased SL and weight compared to control conditions. This trend for increased growth is consistent with a previous study where larval *Am. percula* reared under similar levels of pCO_2 to those used here tended to exhibit significantly increased growth (Munday et al. 2009a). The lack of a statistically significant difference in growth between treatments in this study could be due to a couple of

reasons. Firstly, Munday et al. (2009) found that the effects of elevated pCO_2 on growth were family dependent, being either positively affected or unaffected. Thus, it is possible that the families used in this study were all less sensitive to elevated pCO_2. Secondly, in this study, we tested fewer clutches than the previous study by Munday *et al.* 2009. Thus, the lack of statistical significance may have risen from lower statistical power. Indeed, power analysis revealed that one more clutch with a similar variance structure would have resulted in a significant difference in growth between the control and stable 1000 µatm pCO_2. Importantly, growth in both the diel-cycling elevated CO_2 treatments was very similar to the control, demonstrating that elevated CO_2 does not increase growth when combined with daily variation. Finally, otolith development of juvenile *Am. percula* was unaffected by exposure to 1000 µatm pCO_2, either stable or with daily variation. This observation is in agreement with previous work on larval *Am. percula* which showed that otolith development was only impacted at much higher CO_2 levels (1700 µatm) (Munday et al. 2011a).

Diel pCO_2 cycles (± 300 and 500 µatm) did not significantly modify the growth, survival and otolith development of juvenile *A. polyacanthus* and *Am. percula.* However, in both species there was a trend for SL and weight in the diel-cycling treatments to be more comparable to control conditions. In contrast, behavioural responses in both species were significantly modified by the presence of diel pCO_2 cycles under OA conditions (**Chapter 3**). To my knowledge only two other studies to date have investigated the effects of stable vs cycling pCO_2 on the growth and survival in fish. Davidson et al. (2016) showed that the growth and survival responses of juvenile summer flounder (*Paralichthys dentatus*) did not differ between static and diel-cycling pH treatments. However, all treatments were based around a present day mean. More comparable to this study, Ou et al. (2015) reared larval pink salmon

(*Oncorhynchus gorbuscha*) under a series of constant pCO_2 treatments (450, 1000 and 2000 µatm) and one diel-cycling pCO_2 treatment (450-2000 µatm). They found that wet weight was unaffected by exposure to 1000 µatm and the 450-2000 µatm pCO_2 cycling treatment, as was observed in this study. However, they also showed the negative effect 1000 µatm pCO_2 had on length and growth rate was absent in the cycling treatment, demonstrating that diel pCO_2 cycles can modify the growth of fish. Finally, they also showed that the negative effects that exposure to 2000 µatm pCO_2 had on all growth traits they measured were absent in the 450-2000 µatm cycling treatment. However, in this case it is unclear whether these mediating effects were driven entirely by the effects of fluctuations in pCO_2 or also influenced by a change in mean pCO_2. This highlights the importance of using fully factorial experiments when investigating the impacts of diel pCO_2 cycles on the sensitivity of marine organisms to OA conditions (Boyd et al. 2016).

While studies investigating the interactive effects of elevated pCO_2 and diel pCO_2 cycles on the growth, survival and calcification responses of marine fish are scarce, several have been carried out on calcifying organisms. For example, Frieder et al. (2014) showed that mussel larvae (*Mytilus californianus*) reared under variable low pH conditions (7.51 ± 0.15) suffered less negative developmental effects compared to those reared at stable low pH conditions (7.51), although this positive effect was not observed for shell length. Similarly, calcification rates of coral branches (*Acropora hyacinthus*) reared under elevated diel-cycling pCO_2 conditions (400-2000 µatm) were >21% greater compared to those reared at a 1000 µatm pCO_2 constant treatment (Comeau *et al.*, 2014; see also Dufault *et al.*, 2012 and Chan *et al*., 2017). In contrast to these studies, exposure to low variable pH conditions (7.65 ± 0.4) reduced growth rates of a coralline macroalga (*Arthrocardia corymbosa*) more than exposure

to low stable pH conditions (7.65); this negative variable pH effect was also observed at control pH levels (Cornwall et al. 2013). In a similar study, Britton *et al.* (2016) showed that the positive effects variable pH had on the growth of a canopy-forming kelp (*Ecklonia radiata*) at present day pH levels were absent under OA conditions. Finally, the survival, growth and development of two bivalve species under elevated pCO_2 were largely unaffected by the presence of diel pCO_2 cycles (Clark & Gobler, 2016; Gobler *et al.*, 2017). My results are consistent with no negative effects of daily pCO_2 cycles and indicate possible positive effects. The range of responses among species highlights the importance of future OA studies using pCO_2 treatments that are naturally relevant to the study organism in order to accurately predict the response of marine organisms to future elevated CO_2 levels (McElhany and Busch 2013; Boyd et al. 2016; Wahl et al. 2016; Vargas et al. 2017).

In conclusion, I detected no significant effects of either stable or diel-cycling elevated CO_2 conditions on the growth, survival and otolith development in juveniles of two coral reef fish species. However, I did observe trends in length and weight data in both species which we believe warrant further investigation. This study focussed on the effects of elevated CO_2 in isolation. However, coral reef fish, including *A. polyacanthus,* are known to be highly sensitive to small increases in summer temperature, exhibiting reduced growth and survival (Munday et al. 2008) and numerous studies have demonstrated that elevated temperatures can modify the responses of marine organisms to elevated CO_2 (Kroeker et al. 2013b). Therefore, future studies should consider the potential interactive effects of elevated pCO_2, diel pCO_2 cycles and elevated temperature. Such experiments will be critical for accurately assessing the responses marine organisms to future climate change, especially those inhabiting dynamic CO_2 environments like coral reefs.

Chapter 3: Diel CO_2 cycles reduce severity of behavioural abnormalities in coral reef fish under ocean acidification

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3.1 Summary

Elevated CO_2 levels associated with ocean acidification (OA) have been shown to alter behavioural responses in coral reef fishes. However, all studies to date have used stable pCO_2 treatments, not considering the substantial diel pCO_2 variation that occurs in shallow reef habitats. Here, I reared juvenile damselfish, *Acanthochromis polyacanthus*, and clownfish, *Amphiprion percula*, at stable and diel cycling pCO_2 treatments in two experiments. As expected, absolute lateralization of *A. polyacanthus* and response to predator cue of *Am. percula* were negatively affected in fish reared at stable elevated pCO_2 in both experiments. However, diel pCO_2 fluctuations reduced the negative effects of OA on behaviour. Importantly, in experiment two, behavioral abnormalities that were present in fish reared at stable 750 µatm CO_2 were largely absent in fish reared at 750 ± 300 µatm CO_2. Overall, I show that diel pCO_2 cycles can substantially reduce the severity of behavioural abnormalities caused by elevated CO_2. Thus, past studies may have over-estimated the impacts of OA on the behavioural performance of coral reef fishes. Furthermore, the results suggest that diel pCO_2 cycles will delay the onset of behavioural abnormalities in natural populations.

3.2. Introduction

Increasing atmospheric CO_2 levels are expected to cause a reduction of ocean surface water pH by 0.3-0.4 of a unit by the year 2100, a process commonly referred to as ocean acidification (OA) (Doney et al. 2009). Ocean acidification projections are based on open ocean environments that are relatively stable over time (Doney et al. 2009). In contrast, coastal and shallow water habitats can experience substantial natural fluctuations in pCO_2 on a variety of temporal scales (Hofmann et al. 2011; Duarte et al. 2013). These fluctuations are driven by a range of biological and physical processes (Waldbusser and Salisbury 2014), and in some instances their magnitude can exceed mean CO_2 levels projected to occur over the next century (Hofmann et al. 2011; Duarte et al. 2013). Furthermore, natural pCO_2 fluctuations are expected to increase in size throughout the century, as increased CO_2 uptake by the oceans leads to reduced seawater buffering capacity (Shaw et al. 2013a; McNeil and Sasse 2016). Consequently, as mean oceanic pCO_2 levels rise, shallow water marine organisms will be exposed to higher pCO_2 levels for longer periods of time in addition to experiencing a greater range of pCO_2 levels.

Our current understanding of how natural pCO_2 fluctuations will interact with rising mean oceanic pCO_2 levels to affect the performance of shallow water marine organisms under future OA is limited. This is because most OA experiments have used stable pCO_2 levels consistent with open ocean projections, instead of pCO_2 levels naturally relevant to the study organism (McElhany and Busch 2013; Wahl et al. 2016) . While such experiments have demonstrated a range of impacts on traits across various taxa (Kroeker et al. 2013b; Wittmann and Pörtner 2013; Nagelkerken and Munday 2016), their ecological relevance is uncertain. Indeed, a handful of studies have shown that natural pCO_2 fluctuations can

significantly modify the biological responses of shallow water marine organisms to OA (Alenius and Munguia 2012; Dufault et al. 2012; Cornwall et al. 2013; Comeau et al. 2014; Frieder et al. 2014; Eriander et al. 2015; Ou et al. 2015; Clark and Gobler 2016). Consequently, there has been a call for experiments on shallow water marine organisms that include pCO_2 treatments representative of their natural habitats (McElhany and Busch 2013; Boyd et al. 2016; Wahl et al. 2016; Vargas et al. 2017). Results from such experiments will be vital for improving predictions of when the negative effects caused by elevated pCO_2 will become evident in natural populations (Shaw et al. 2013b).

Some of the most notable effects of stable elevated pCO_2 levels have been observed in coral reef fishes. Specifically, exposure to pCO_2 levels between 700-1000 µatm have been shown to impair a range of sensory systems and alter ecologically important behaviours (Heuer and Grosell 2014; Clements and Hunt 2015; Nagelkerken and Munday 2016). Alterations include, impaired anti-predator responses (Dixson et al. 2010; Munday et al. 2010, 2016; Ferrari et al. 2011a; Allan et al. 2013), loss of lateralization (Domenici et al. 2012; Welch et al. 2014), loss of learning (Ferrari et al. 2012; Chivers et al. 2014) and increased activity/boldness (Munday et al. 2010). Such behavioural abnormalities are expected to have significant ecological consequences for fish populations. For example, as a consequence of exhibiting riskier behaviour, predation-related mortality was significantly higher when settlement stage damselfish were exposed to elevated pCO_2 in the laboratory and released into their native habitat, inferring that recruitment and population sustainability will be threatened by projected future CO_2 levels in the ocean (Munday et al. 2010). Furthermore, the impacts that behavioural abnormalities have on predator-prey dynamics (Ferrari et al. 2011b; Allan et al.

2013) and competitive interactions (McCormick et al. 2013) will likely cause shifts in community structure with unknown consequences for ecosystem functioning.

Coral reefs are highly dynamic shallow water habitats that experience diel cycles in pCO_2. These daily CO_2 cycles are driven by the processes of photosynthesis/respiration and calcification/dissolution over a day-night cycle, but are also influenced by physical controls such as water flow and residence time (Anthony et al. 2011; Shaw et al. 2012; Falter et al. 2013). In shallow reef areas, diel variation in pCO_2 can range anywhere from ± 50 to 600 µatm around the mean (Kayanne et al. 1995; Shaw et al. 2012; Albright et al. 2013; Kline et al. 2015). Although the pCO_2 of coral reef waters is not in perfect equilibrium with the atmosphere over a daily timescale, the carbonate system is still heavily influenced by flushing with offshore waters and thus the mean pCO_2 of reef waters will rise in line with rising atmospheric CO_2 (Falter et al. 2013). To our knowledge, only three studies (all on calcifying corals) have explicitly considered diel pCO_2 variation when investigating the potential impacts of OA on coral reef organisms. Importantly, they found that the negative impacts of OA on growth and calcification were buffered by the presence of a diel cycling pCO_2 regime (Dufault et al. 2012; Comeau et al. 2014; Chan and Eggins 2017). The behavioural alterations that have been observed in coral reef fishes are likely to be sensitive to the interactive effects of diel pCO_2 cycles and rising mean pCO_2 levels for two reasons. Firstly, previous work has shown that it takes between 24-96 h of exposure to stable elevated pCO_2 levels for behavioural abnormalities to manifest, with shorter onset times at higher pCO_2 levels (Munday et al. 2010). Secondly, the negative effects of elevated pCO_2 on behavioural responses are concentration-dependent (Munday et al. 2010; Ferrari et al. 2011a; Welch et al. 2014). Consequently, diel pCO_2 cycles could reduce the severity of behavioural abnormalities, or

prevent them from manifesting, by providing fish with a recovery period, especially if pCO_2 levels drop below the onset threshold (600-700 µatm). Alternatively, experiencing higher maximum pCO_2 levels daily may lead to more severe behavioural abnormalities.

To determine how diel pCO_2 fluctuations affect the behavioural responses of coral reef fishes under OA I reared juvenile damselfish, *Acanthochromis polyacanthus* (Bleeker, 1855), and clownfish, *Amphiprion percula* (Lacepède, 1802) under a series of stable and diel cycling pCO_2 treatments in two different experiments. The aim of the first experiment was to determine if the magnitude of diel pCO_2 cycles affects the behavioural performance of coral reef fishes under OA. The aim of the second experiment was to determine if the presence of diel pCO_2 cycles affects the mean CO_2 level at which behavioural abnormalities occur (i.e. the onset of behavioural abnormalities). Specifically, in experiment one, the behaviour of fish reared at two stable CO_2 levels (480 and 1000 µatm) was compared with the behaviour of fish reared in two cycling CO_2 treatments of different magnitude (1000 ± 300 and 1000 ± 500 µatm). Therefore, this experiment enabled us to test if the magnitude of diel pCO_2 fluctuations affected the behaviour of fish under OA. In experiment two, the behaviour of fish reared at three stable CO_2 levels (460, 750 and 1000 µatm) was compared with the behaviour of fish reared in diel cycling CO_2 treatments at two different mean CO_2 levels (750 ± 300 and 1000 ± 300 µatm). Therefore, this experiment enabled us to test if the effect of diel pCO_2 cycles was dependent on the mean CO_2 level experienced by the fish. In both experiments, I measured behavioural lateralization in *A. polyacanthus* and the response to a predator cue by *Am. percula*. These traits were chosen for each species as previous studies have demonstrated clear negative impacts of exposure to stable, elevated pCO_2 conditions (Dixson et al. 2010; Munday et al. 2010; Nilsson et al. 2012; Welch et al. 2014). It was predicted that diel pCO_2

fluctuations could reduce the overall severity and delay the onset of behavioural abnormalities under OA conditions.

3.3 Materials and Methods

Study species

A. polyacanthus and *Am. percula* are common throughout the Indo-Pacific region. Both species are demersal spawners, laying their eggs within small caves and crevices in the reef matrix. In *A. polyacanthus*, eggs hatch into small juveniles, with both parents providing care to the eggs and offspring for up to 45 d post-hatching (Kavanagh 2000). In contrast, *Am. percula* has a relatively short larval phase of approximately 11 d before settling on the reef (Bay et al. 2006). Both species can be bred and reared in captivity with high success, which has led to their establishment as models for investigating the potential impacts of OA on coral reef fishes (Dixson et al. 2010; Munday et al. 2010; Welch et al. 2014; Heuer et al. 2016; Schunter et al. 2016).

Brood-stock and general rearing protocol

Adult *A. polyacanthus* were collected using hand nets from the Bramble Reef area (site 1: 18°22'S, 146°40'E; site 2: 18°25'S, 146°40'E) of the Great Barrier Reef in July 2015. Fish were transported to an environmentally controlled aquarium research facility at James Cook University (JCU) (Townsville, Australia) where they were housed as breeding pairs in 60 L aquaria at temperature conditions matching the collection location. An existing brood-stock of *Am. percula* at JCU was used. These pairs had been collected from the Cairns Region of the Great Barrier Reef and housed at JCU for four years. Adult *A. polyacanthus* and *Am. percula* pairs were maintained under stable, ambient pCO_2 (~490 µatm). Temperatures were

increased at a rate of 0.5°C *per* week until the summer breeding temperature of 29°C was reached in the first week of November 2015. Adult pairs were provided with half a terracotta pot to act as a shelter and spawning site. Aquaria were checked each morning for the presence of newly laid clutches. Pairs were fed *ad libitum* on commercial fish feed pellets (INVE Aquaculture Nutrition NRD 12/20) once daily outside the breeding season and twice daily during the breeding season (November–May).

Acanthochromis polyacanthus juveniles were fed a combination of freshly hatched *Artemia* naupli and weaning fish feed (INVE Aquaculture Nutrition Wean-S 200-400 µm) daily for the first four days post hatch (dph). 5-21 dph they were fed daily on the weaning feed and then switched to a small pellet fish feed (INVE Aquaculture Nutrition NRD 5/8) at 22 dph. Rearing of larval *Am. percula* was performed using methods described by Munday *et al*. (28) and in **Chapter 2**. Settled juveniles were fed daily on the weaning fish feed.

Experimental design

Experiment one

Experiment one was carried out at the aquarium research facility at JCU. Fish were reared at two stable (480 and 1000 µatm) and two cycling (1000 ± 300 and 1000 ± 500 µatm) CO_2 treatments (**Table 3.1** and **Figure 3.1**). The stable 1000 µatm pCO_2 treatment represented the open ocean projection for the end of this century typically used in many OA experiments (Kroeker et al. 2013b). The cycling pCO_2 treatments matched levels that have been observed in some tidal lagoons (Shaw et al. 2012). Diel pCO_2 fluctuations of between ± 50-150 µatm are more typical in other reef areas (Albright et al. 2013; Kline et al. 2015), however, the magnitude of fluctuations seen in tidal lagoons today may become more common in other

reef areas by the year 2100 as a amplification in diel pCO_2 fluctuations is predicted to occur over this time period (Shaw et al. 2013a).

Table 3.1 Seawater parameters for experiment one. Values are means ± 1 SD for daily average, minimum, maximum and range of pH_T and pCO_2. Mean ± 1 SD for total alkalinity (TA), temperature (°C) and salinity over the experiment are also shown.

Parameter	pCO_2 treatment (µatm)			
	480	1000	1000 ± 300	1000 ± 500
Average pH_T	8.01 ± 0.01	7.75 ± 0.02	7.77 ± 0.08	7.80 ± 0.16
Min. pH_T	-	-	7.64 ± 0.03	7.57 ± 0.02
Max. pH_T	-	-	7.89 ± 0.01	8.01 ± 0.01
pH_T range	-	-	0.24 ± 0.03	0.44 ± 0.02
Average pCO_2 (µatm)	480 ± 20	990 ± 46	961 ± 195	934 ± 389
Min. pCO_2 (µatm)	-	-	681 ± 21	482 ± 19
Max pCO_2 (µatm)	-	-	1304 ± 102	1591 ± 98
pCO_2 range (µatm)	-	-	623 ± 96	1109 ± 95
TA (µmol kg^{-1})	2570 ± 54	2574 ± 42	2582 ± 43	2583 ± 43
Temperature (° C)	28.7 ± 0.3	28.9 ± 0.3	28.9 ± 0.3	29.0 ± 0.2
Salinity	36.7 ± 0.4	36.7 ± 0.4	36.7 ± 0.4	36.7 ± 0.4

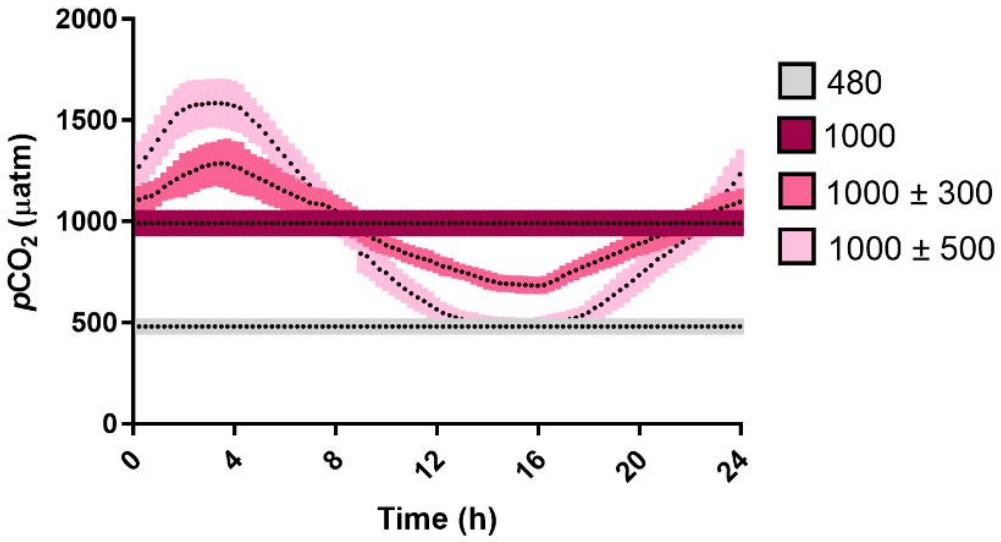

Figure 3.1 Mean daily pCO_2 profiles for experiment one. Coloured sections are ± 1 SD. Profiles for the stable pCO_2 treatments were based on measurements taken twice per day. In reality some minor daily variation would have likely occurred.

Experiment two

Experiment two was carried at the National Sea Simulator (SeaSim) facility at the Australian Institute of Marine Science (AIMS) (Cape Cleveland, Australia). Fish were reared at three stable (460, 750 and 1000 µatm) and two cycling (750 ± 300 and 1000 ± 300 µatm) CO_2 treatments (**Table 3.2** and **Figure 3.2**). Previous experiments indicate that behavioural abnormalities are first evident at around 700 µatm CO_2, although the magnitude of effect is often not as large as observed at higher CO_2 levels (Munday et al. 2010; Ferrari et al. 2011a; Welch et al. 2014). Therefore, the inclusion of the 750 and 750 ± 300 µatm CO_2 treatments enabled us to determine how diel pCO_2 cycles may affect the onset threshold of behavioural abnormalities.

Table 3.2 Seawater parameters for experiment two. Values are means ± 1 SD for daily average, minimum, maximum and range of pCO_2. Mean ± 1 SD for total alkalinity (TA), temperature (°C) and salinity over the experiment are also shown.

Parameter	pCO_2 treatment (µatm)				
	460	750	750 ± 300	1000	1000 ± 300
Average pCO_2 (µatm)	458 ± 7	748 ± 9	788 ± 203	994 ± 23	1042 ± 256
Min. pCO_2 (µatm)	443 ± 26	721 ± 29	527 ± 27	926 ± 121	667 ± 33
Max pCO_2 (µatm)	477 ± 42	773 ± 28	1025 ± 86	1060 ± 128	1328 ± 78
pCO_2 range (µatm)	49 ± 101	59 ± 112	498 ± 90	130 ± 216	661 ± 94
TA (µmol kg^{-1})	2322 ± 20	2325 ± 23	2326 ± 21	2330 ± 22	2330 ± 23
Temperature (°C)	28.5 ± 0.1	28.5 ± 0.1	28.5 ± 0.1	28.5 ± 0.1	28.5 ± 0.1
Salinity	35.4 ± 0.4	35.4 ± 0.4	35.4 ± 0.4	35.4 ± 0.4	35.4 ± 0.4

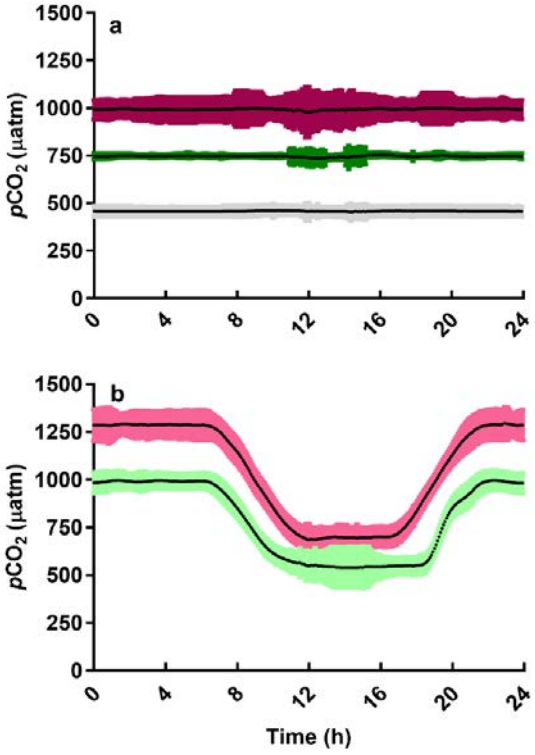

Figure 3.2 Mean daily (a) stable and (b) cycling pCO_2 profiles for experiment two. Coloured sections are ± 1 SD.

A similar protocol was employed in both experiments. Three offspring clutches were used per species, each from a different parental pair. In experiment one, *A. polyacanthus* and *Am. percula* clutches were transferred to the experimental system and split between pCO_2 treatments in duplicate tanks (12-15 *A. polyacanthus* per tank and 10 *Am. percula* per tank) at 1 and 12 dph respectively. In experiment two, offspring clutches were transferred to the experimental system and split between pCO_2 treatments in duplicate tanks (one tank per line; 15 *A. polyacanthus* per tank and 13-15 *Am. percula* per tank) at 14 and 12 dph respectively. *A. polyacanthus* clutches were transferred at 14 dph in experiment two, compared with 1 dph in experiments one, due to logistical reasons.

Behavioural lateralization trials on *A. polyacanthus* were performed 40-42 dph that equated to approximately six and four weeks of exposure to pCO_2 treatments in experiments one and two respectively. Predator cue trials on *Am. percula* were performed 18-20 dph that equated to approximately 1 week of exposure to pCO_2 treatments in both experiments. All behavioural trials were performed between 09:00 and 17:00. Fish were gently transferred to the behavioural arenas using a glass beaker to minimise handling stress. Fish from each pCO_2 treatment were tested at random times throughout the day to account for any possible time of day effects in the fluctuating treatments. Each fish was tested once, being placed in an isolation chamber within their experimental tank after a trial for the rest of the day. Research was carried out under approval of the James Cook University animal ethics committee (permit: A2210) and according to the University's animal ethics guidelines.

Experimental systems and CO_2 manipulation

Experiment one

The experimental system used at JCU was an 11,000 L re-circulating system. Briefly, the system consisted of a large external 3,700 L sump tank connected to a bio-filter, protein skimmer, UV steriliser and a 1000 L algal bio-remediation tank. The external sump supplied water to four separate 1,600 L re-circulating systems (one *per* pCO_2 treatment) made up from one 1000 L sump tank and fifteen 40 L holding tanks, contained within a temperature-controlled room. Water was supplied at a rate of approximately 1,600 L *per* day allowing for a complete exchange with the external sump. Holding tanks were supplied with water at a rate of 1 L min^{-1}. Both the internal sumps and holding tanks were aerated with ambient air.

Elevated pCO_2 treatments were achieved by dosing the 1000 L internal sumps with CO_2. This was controlled by solenoid valves (M-Ventil Standard, Aqua Medic, Germany) connected to a pH control system (Aqua Medic AT Control System, Aqua Medic, Germany) with laboratory grade pH electrodes (Neptune Systems, USA). The Aqua Medic AT Control System has a curve function that allowed us to create fluctuating pCO_2 profiles. pH profiles in the fluctuating pCO_2 treatments were recorded every other day using a pH meter (InLab Expert Pro electrode and Seven2Go Pro meter, Mettler Toledo, Switzerland) set to take a reading every 15 min. For the stable pCO_2 treatments pH_{NBS} was measured twice daily using the same model of pH meter. Seawater pH on the total hydrogen ion concentration scale (total scale, pH_T) was measured each week with a spectrophotometer following standard operating procedures (Dickson et al. 2007) using the indicator dye meta/*m*-cresol purple (mCP) (*m*-cresol purple sodium salt 99%, non-purified, Acros Organic). Daily and fluctuating pH_{NBS} measurements were converted to pH_T based on the offset between weekly pH_T and pH_{NBS} measurements. Temperature was

recorded daily with a digital thermometer (Comark C26, Norfolk, UK). Salinity readings were taken weekly using a conductivity sensor (HQ15d; Hach, USA). Total alkalinity was also measured weekly using Gran Titration (Metrohm 888 Titrando Titrator Metrohm AG, Switzerland) and certified reference material from Dr. A. G. Dickson (Scripps Institution of Oceanography). All seawater parameters were measured in randomly chosen holding tanks. pCO_2 values were calculated as a function of pH_T, temperature and salinity using CO_2SYS (Pierrot et al. 2006) employing constants from Mehrbach et al. (1973) refit by Dickson and Millero (1987) and the $KHSO_4$ dissociation constant from (Dickson 1990). Mean values for each of these seawater parameters are presented in **Table 3.1**.

Experiment two

The experimental system used at SeaSim was a flow-through system that comprised of 12 independent lines (duplicate independent lines per pCO_2 treatment). The system used ultra-filtered seawater (0.04 μm), temperature controlled to 28.5°C. Each seawater line supplied three custom made 50 L tanks at the rate of 50 L h^{-1}. The experimental tanks were placed in individual temperature-controlled water baths to ensure temperature stability (± 0.1˚C). Treatments and tank replicates were randomly positioned in the experimental room.

The management of pCO_2 and temperature was achieved through the design and implementation of a custom Model Predictive Control logic running on a micro-programmable logic controller (PLC) (Series S7-1500, Siemens, Australia). The micro-PLC was integrated with the general SeaSim control system, to provided SCADA (Siemens WinCC) accessibility and data archiving. The pCO_2 feedback for each of the replication lines was provided via non-dispersive infrared (NDIR) measurements (Hobbs et al. 2004). Tank water

was delivered to the equilibrator (Seasim, AIMS design, custom built) by an in-tank submersible pump (Universal Pump 1260, EHEIM, Deizisau, Germany) where the pCO_2 of the air in the chamber reaches and maintains equilibrium with the pCO_2 of the experimental water. The air was constantly delivered to a NDIR CO_2 analyser (Telaire T6613, Amphenol, Australia) that provided live feedback to the PLC. The CO_2 analysers were calibrated monthly using certified calibration gas mixtures at 0, 600 and 2000 ppm. The control system delivered CO_2 though Gas Mass Flow Controllers (GFC17 series, Aalborg, Orangeburg, USA) according to the profiling schedule designed for the pCO_2 treatment and the feedback signal coming from the experimental tanks. CO_2 was dissolved in the flow-through water by mean of membrane contactors (Membrana Liqui-Cel 2.5x8 Extra-Flow. 3M, USA). Total alkalinity was also measured weekly as described above. Mean values for seawater parameters are presented in **Table 3.2**.

Throughout the experiment incoming coastal water had a pCO_2 ranging between 500-550 µatm. Thus, to achieve a control pCO_2 level closer to 460 µatm, membrane contactors (Membrana Liqui-Cel 4x28 Extra-Flow) were used to remove CO_2, using CO_2 – depleted air as sweep gas. This was only possible for the control treatments and consequently the lower pCO_2 levels in the 750 ± 300 µatm treatment matched the pCO_2 of the incoming seawater (500-550 µatm).

Behavioural assays

Behavioural lateralization trials

Behavioral lateralization (i.e., favoring the left or right side during behavioral activities) is an expression of brain functional asymmetry and a strong determinant of fish behaviour.

Lateralized individuals show higher performance in cognitive tasks (Dadda and Bisazza 2006), schooling behaviour (Bisazza and Dadda 2005) and escape reactivity (Dadda et al. 2010). Lateralization in juvenile *A. polyacanthus* was determined using a detour test in a two-way T-maze using methods similar to those described by (Welch et al. 2014). The two-way T-maze consisted of an experimental arena (60 cm x 30 cm x 20 cm), with a runway in the middle (25 cm x 2 cm, length x width), and at both ends of the runway (2 cm ahead of the runway) an opaque barrier (12 cm x 12 cm x 1 cm) was positioned perpendicular to the runway. The maze was filled to a depth of 4 cm with the respective treatment water of the fish being tested, being changed after each trial. A single fish was placed at one end of the T-maze and given a 3 min habituation period, during which time it could explore the apparatus. At the end of the habituation period the fish was gently guided into the runway using a plastic rod with the observer standing directly behind the fish (the plastic rod was never placed closer than approximately twice the body length of the fish). At this point to minimise human interference affecting direction turned the observer slowly stepped back from the maze and the fish was allowed to swim to the end of the runway. In instances when a fish did not swim to the end, encouragement was provided by gently moving the plastic rod around at the beginning of the runway. Direction choice was recorded as the first direction turned when the fish exited the runway. Ten consecutive runs were recorded per fish. Twenty fish from each clutch (ten per tank) were tested per CO_2 treatment. To account for any possible asymmetry in the maze, turns were recorded alternately on the two ends of the runway. Turning preference (i.e. bias in left or right turns) at the population level was assessed using the relative lateralization index (L_R, from -100 to +100, indicating complete preference for left and right turning, respectively) according to the following formula: L_R = [(Turn to the right - Turn to the left) / (Turn to the right + Turn to the left)] * 100. The strength of lateralization (irrespective of its

direction) was also assessed at the individual-level using the absolute lateralization index L_A (ranging from 0 (an individual that turned in equal proportion to the right and to the left) to 100 (an individual that turned right or left on all 10 trials)). Lateralization trials in experiment two were performed with the observer blinded to the experimental treatments.

Predator cue trials

The ability to detect and elicit appropriate antipredator behaviour is critical for survival, especially in early life-stages that experience a greater predation threat (Almany and Webster 2006). The response of juvenile *Am. percula* to a predator cue was tested in a two-channel choice flume using methods similar to those described by (Munday et al. 2016). The flume combination was predator cue water *versus* untreated water. Water at the same pCO_2 level from two different sources (9 L buckets) was gravity fed into the choice flume, which is divided down half of its length. A constant flow rate of 100 ml min^{-1} was maintained and monitored using a flow meter and dye test after every water change. Water was changed after each trial. Fish were tested under the mean pCO_2 level of their respective treatments (i.e. fish reared under both 1000 and 1000 ± 300 µatm were tested at 1000 µatm), due to the logistical difficulties involved in manipulating predator cue water pH across a daily cycle. While this resulted in fish from cycling treatments experiencing a change in pCO_2 between experimental and test water, recent work has shown this has no effect on the response of *Am. percula* to a predator cue at far greater changes than experienced in this study (Munday et al. 2016). For each trial, a single test fish was placed in the centre of the downstream end of the choice flume and given a 2 min acclimation period. The position of the fish was then recorded every five seconds for a total of 2 min. A rest period of 4 min followed, during which time the water sources were switched to eliminate potential side preferences. The position of the fish was

then once again recorded every five seconds for a total of 2 min. Fish were not disturbed during the trial. Temperatures during the trials were kept within 1°C of the temperature in the rearing tanks. Eight fish from each clutch were tested per pCO_2 treatment (4 per tank). Predator cues were obtained from three common coral-cod, *Cephalopholis miniatus* as described by (Munday et al. 2016). Response to predator cue was assessed as the percentage of time spent in the cue water. In experiment one, the control fish from one clutch exhibited no response to the predator cue (i.e. did not avoid the predator cue) and so this clutch was excluded from data analysis.

Statistical analyses

The effects of pCO_2 treatment on absolute lateralization (L_A), relative lateralization (L_R) and percentage time spent in cue water were tested using mixed-effects logistic regressions (Warton and Hui 2011). Models for L_A data from experiment one and predator cue data from experiments one and two were over dispersed and so were re-run using a penalized quasi-likelihood. In all models, parental pair and tank were included as random factors, with tank nested within parental pair. Pairwise comparisons were performed using Tukey's post hoc tests. To determine if a treatment group demonstrated a turning direction preference Pearson's Chi-square tests were used, where we expected a 50:50 ratio for left/right turning preference. Finally, differences in the relative frequency distribution of L_R between treatments were tested using Kolmogorov-smirnov tests. Mixed-effects logistic regressions were conducted in R version 3.3.2 (R Core Team 2017) using the 'lme4' (Bates et al. 2015) and MASS (Venables and Ripley 2012) packages respectively. Pairwise comparisons were conducted using the 'multcomp' (Hothorn et al. 2008) package. Pearson's Chi-square tests were performed using Minitab 17.

3.4 Results

Experiment one

Absolute lateralization (L_A) was significantly influenced by CO_2 treatment (**Figure 3.3a**, χ^2 = 15.75, df = 3, P = 0.001). As expected, juveniles reared under stable elevated pCO_2 were less lateralized compared to those reared at control levels (P = 0.001). However, diel pCO_2 cycles significantly increased how lateralized juvenile *A. polyacanthus* were at 1000 µatm. L_A of juveniles reared under small fluctuations (± 300 µatm) was intermediate, but not significantly different, to those reared at control and stable elevated pCO_2 (min. P = 0.214). L_A of juveniles reared under large fluctuations (± 500 µatm) was fully restored to control levels being significantly greater than those reared at stable elevated pCO_2 (P = 0.01). Mean relative lateralization (L_R) in juvenile *A. polyacanthus* was unaffected by CO_2 treatment (**Figure 3.3b**, χ^2 = 0.52, df = 3, P = 0.914). Furthermore, no group exhibited a preference for left or right turning (**Figure 3.4**, max. χ^2 = 0.84, P = 0.358). Juveniles reared under stable elevated pCO_2 tended to have a narrower L_R distribution compared to the other treatments (**Figure 3.4**), although these differences were not significant (Max. KS = 0.15, P = 0.510).

Mean percentage time that juvenile *Am. percula* spent in predator cue water was significantly affected by CO_2 treatment (**Figure 3.3c**, χ^2 = 51.45, df = 3, P < 0.001). As expected, juveniles reared at stable elevated pCO_2 spent a greater amount of time in predator cue water compared to those reared at control levels (P < 0.001). However, diel pCO_2 cycles significantly reduced the amount of time that juvenile *Am. percula* spent in predator cue water at 1000 µatm. Juveniles reared under both small (± 300 µatm) and large (± 500 µatm) fluctuations demonstrated partial restoration of antipredator behaviour spending an amount of time in

predator cue water that was intermediate, and significantly different, to juveniles reared at control and stable elevated pCO_2 (max. $P < 0.001$).

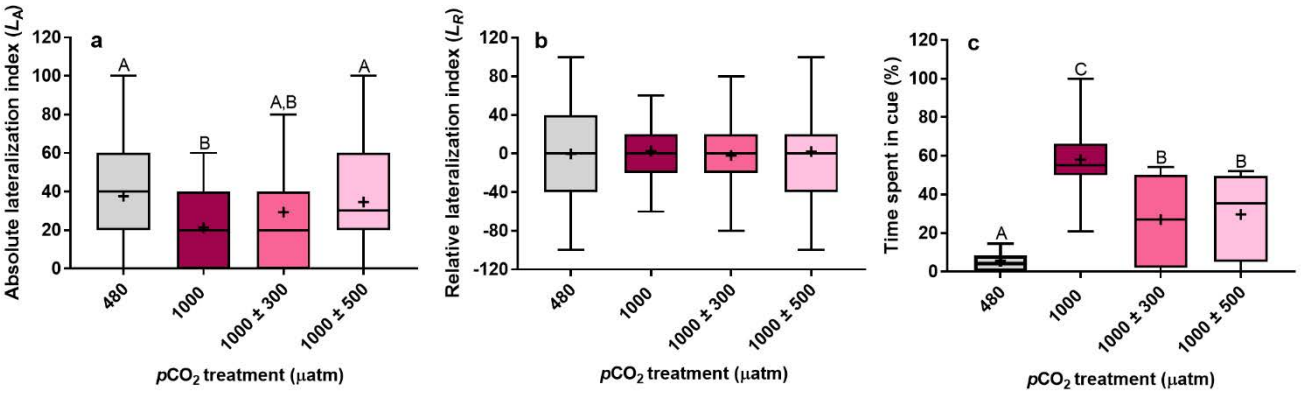

Figure 3.3 Effects of stable vs diel-cycling elevated pCO_2 on behavioural responses in experiment one: (a) absolute lateralization and (b) relative lateralization in juvenile *Acanthochromis polyacanthus* (n = 60 per treatment) were determined using a two-way T-maze. (c) response to predator cue of juvenile *Amphiprion percula* (n = 16 per treatment) was determined using a two-choice flume. Different letters represent significant differences between treatments (Tukey, $P < 0.05$). Boxplots are sized according to the 25th and 75th quartiles, where the line identifies the median and the whiskers indicate the minimum and maximum values. + signs represent means.

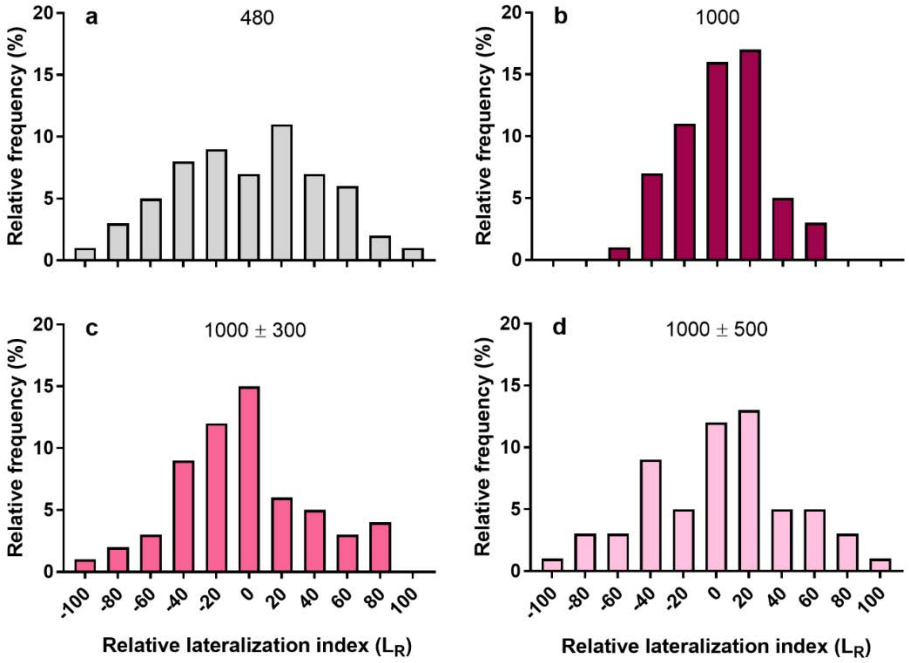

Figure 3.4 Relative lateralization (L_R) for juvenile *A. polyacanthus* presented with a T-maze choice chamber in experiment one. Juvenile fish from each pCO_2 treatment (n = 60 per treatment) were allowed to choose to turn left or right for a total of 10 turns. Graphs show L_R with positive and negative values indicating right and left turns, respectively. The extreme values of |100| indicate fish that turned in the same direction for all 10 turns.

Experiment two

As was observed in experiment one, mean L_A was significantly affected by CO_2 treatment (**Figure 3.5a**, $\chi^2 = 75.25$, df = 4, $P < 0.001$), with juveniles reared under stable elevated pCO_2 (750 and 1000 µatm) being less lateralized compared to those reared at control levels (max. $P < 0.001$). Diel pCO_2 cycles did not affect how lateralized juvenile *A. polyacanthus* were at mean pCO_2 level of 1000 µatm ($P = 0.986$). In contrast, diel pCO_2 cycles fully restored lateralization in juveniles reared at a mean CO_2 of 750 µatm, being similar to those reared at control levels ($P = 0.710$) and significantly greater than both the stable elevated CO_2 treatments (max. $P < 0.001$). Also, as observed in experiment one, mean L_R in juvenile *A.*

polyacanthus was unaffected by CO_2 treatment (**Figure 3.5b**, $\chi^2 = 4.86$, df = 4, $P = 0.302$), and no group exhibited a preference for left or right turning (**Figure 3.6**, max. $\chi^2 = 3.43$, $P = 0.064$). However, there were more individuals that were less lateralized in the 750, 1000 and 1000 ± 300 µatm CO_2 treatments. (**Figure 3.6**).

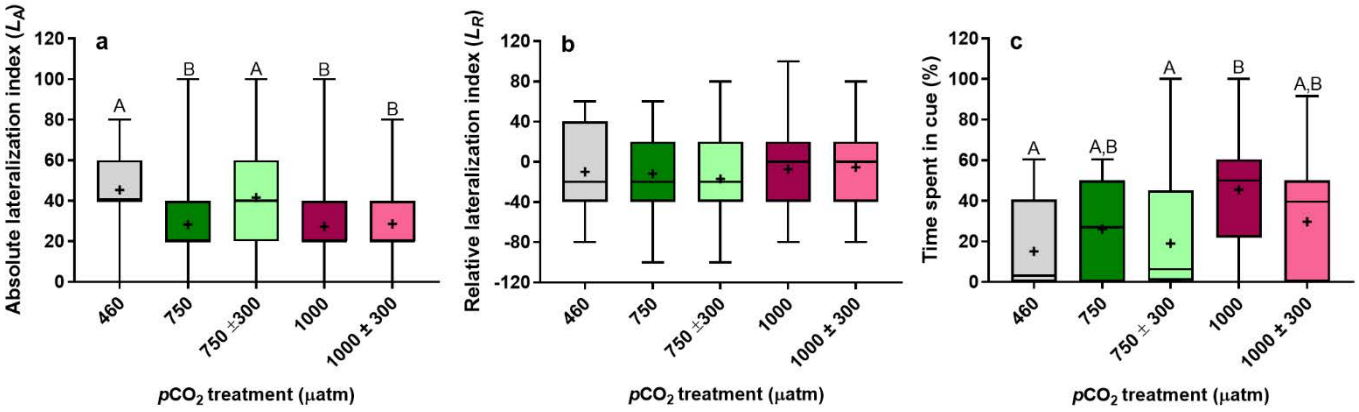

Figure 3.5 Effects of stable vs diel-cycling elevated pCO_2 on behavioural responses in experiment two: (a) absolute lateralization and (b) relative lateralization in juvenile *A. polyacanthus* (n = 60 per treatment) were determined using a two-way T-maze. (c) response to predator cue of juvenile *Am. percula* (n = 24 per treatment) was determined using a two-choice flume. Different letters represent significant differences between treatments (Tukey, $P < 0.05$). Boxplots are sized according to the 25th and 75th quartiles, where the line identifies the median and the whiskers indicate the minimum and maximum values. + signs represent means.

Similar to experiment one, CO_2 treatment significantly affected the mean percentage time that juvenile *Am. percula* spent in predator cue water (**Figure 3.5c**, $\chi^2 = 15.95$, df = 4, $P = 0.003$). As expected, juveniles reared at 1000 µatm CO_2 spent a greater amount of time in predator cue water compared to those reared at control levels ($P = 0.004$). The percentage time juveniles reared at 750 µatm CO_2 spent in predator cue water was intermediate, but not

significantly different, to those reared at control and 1000 µatm pCO_2 (min. P = 0.194). Diel pCO_2 cycles influenced the predator cue response of juvenile *Am. percula* at mean pCO_2 levels of 750 and 1000 µatm. Juveniles reared at 750 ± 300 µatm CO_2 spent a percentage of time in predator cue water that was more similar to those reared at control levels compared to those reared at 750 µatm CO_2. Finally, juveniles reared at 1000 ± 300 µatm CO_2 demonstrated partial restoration of antipredator behaviour, with juveniles spending a percentage of time in predator cue water that was intermediate to those reared at 460 and 1000 µatm CO_2 (min. P = 0.309).

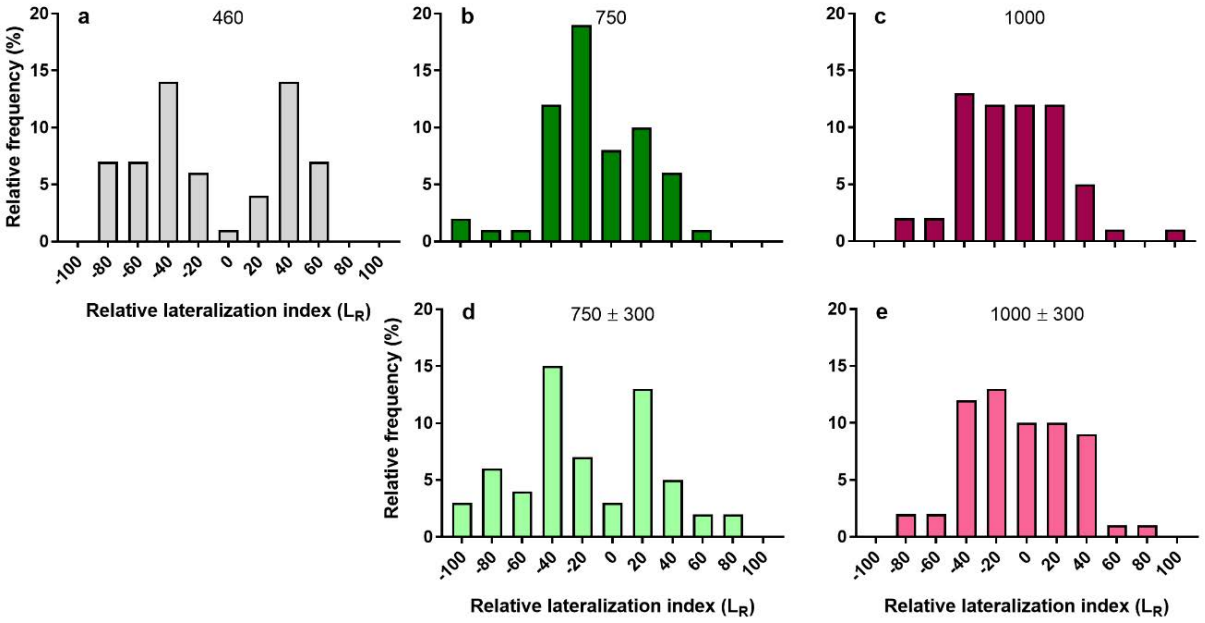

Figure 3.6 Relative lateralization (L_R) for juvenile *A. polyacanthus* presented with a T-maze choice chamber in experiment two. Juvenile fish from each pCO_2 treatment (n = 60 per treatment) were allowed to choose to turn left or right for a total of 10 turns. Graphs show L_R with positive and negative values indicating right and left turns, respectively. The extreme values of |100| indicate fish that turned in the same direction for all 10 turns.

3.5 Discussion

This study demonstrates for the first time that diel pCO_2 cycles can significantly modify the behavioural responses of fishes under OA. The negative impacts of elevated CO_2 on coral reef fish behaviour have been well documented and are expected to have significant ecological consequences for reef fish populations through effects on recruitment, predator-prey interactions, competition and habitat preference (Heuer and Grosell 2014; Clements and Hunt 2015; Nagelkerken and Munday 2016). However, all studies to date have exposed fish to stable levels of elevated CO_2, not considering the natural diel pCO_2 cycles that occur on coral reefs. Here I show that the severity of two behavioural abnormalities commonly observed under elevated CO_2 are reduced when fish experience a diel cycling pCO_2 regime. The extent of reduction was influenced by both the magnitude of fluctuation and mean pCO_2 level experienced, as well as the behavioural trait. Overall my results indicate that previous studies have probably over-estimated the behavioural impacts of OA on coral reef fishes once they have settled to reef habitats where diel CO_2 cycles are prevalent.

Previous research using stable pCO_2 treatments has found that behavioural abnormalities start to manifest in coral reef fish between 600-700 µatm. My results indicate that diel pCO_2 cycles will delay the onset of behavioural abnormalities. In experiment two, I show that behavioral abnormalities present in fish reared at a stable level of 750 µatm CO_2 were absent in fish reared at 750 ± 300 µatm CO_2. However, in both experiments, although less severe, behavioral abnormalities were still present in the fluctuating 1000 µatm CO_2 treatments. Thus, it appears that mean oceanic pCO_2 levels closer to 1000 µatm will need to be reached before behavioural abnormalities could manifest in natural populations of reef fishes. Furthermore, I observed full restoration of behavioural lateralization in juvenile *A.*

polyacanthus reared under 1000 ± 500 µatm CO_2, inferring that some behavioural abnormalities may not manifest at all for populations living in habitats with large CO_2 fluctuations, such as shallow reef flats and closed lagoons, even when average oceanic conditions reach 1000 µatm CO_2. The observation that diel pCO_2 variation can reduce and/or delay the onset of behavioural abnormalities in juvenile coral reef fish under OA is particularly important given the ecological consequences of behavioural abnormalities and that past research has shown a limited capacity for acclimation of behavioural traits to stable elevated pCO_2 (Allan et al. 2014; Welch et al. 2014). However, it is important to mention that behavioural abnormalities are still likely to occur in the pelagic larval phase of coral reef fish as they occupy a more stable CO_2 environment in the open ocean. Consequently, population replenishment and sustainability of reef fish populations could still be threatened by near-future OA due to impaired behaviour in the larval phase (Munday et al. 2009c, 2010; Devine et al. 2012b), even if behavioural effects are less severe in juveniles that have already settled to reef habitats. Finally, in experiment one, and to a lesser extent in experiment two, I observed more individual variation in predator cue responses of *Am. percula* at 1000 µatm CO_2 if fish were reared under cycling conditions. This level of individual variation has previously been observed only at a mean stable CO_2 of 700 µatm (Munday et al. 2010). Thus, in addition to potentially providing more time for reef fish populations to adapt to future OA conditions by delaying the onset of behavioural abnormalities, diel pCO_2 cycles may also increase the adaptive potential of fish populations at higher CO_2 levels by increasing the range of individual variation upon which selection can act.

The underlying mechanism of behavioural abnormalities in fish under OA conditions are linked to be the effects of acid-base regulation on the function of type A γ-aminobutyric acid

($GABA_A$) neurotransmitter receptors (Nilsson et al. 2012; Heuer and Grosell 2014). $GABA_A$ receptors are gated ion channels with specific conductance for HCO_3^- and Cl^-. Under elevated pCO_2 fish increase intracellular and extracellular HCO_3^- concentrations to prevent plasma and tissue acidosis (Baker et al. 2009; Esbaugh et al. 2012; Heuer and Grosell 2014). In a recent study on *A. polyacanthus* this compensatory mechanism was shown to be sufficient to reverse the transmembrane gradients of HCO_3^- in brain tissue, which could interfere with $GABA_A$ receptor function and cause behavioural alterations (Heuer et al. 2016). For coral reef fish it appears that complete acid-base regulation in the brain under stable elevated pCO_2 levels may take between 24-96 h, as this is the exposure period required before behavioural abnormalities manifest (Munday et al. 2010). My results suggest that, for fish reared under diel pCO_2 cycles exposure to lower CO_2 levels for several hours each day is enough to prevent the physiological changes that would normally occur at a stable high CO_2. Extracellular and intracellular pH regulation take place at different rates, occurring more quickly in the former. For example, in gulf toadfish (*Opsanus beta*) exposed to 1900 µatm CO_2, complete blood pH compensation was achieved after 2h, whereas muscle intracellular pH was not adjusted until after 24h (Esbaugh et al. 2012). Thus, based on the onset times under stable elevated pCO_2 (24-96 h), it appears that behavioural abnormalities do not manifest in coral reef fish until brain intracellular pH compensation is complete, although further testing is required to confirm this. This could explain why diel pCO_2 cycles alleviated the negative impacts of OA. Although no data is available, I assume coral reef fish would achieve pH compensation as fast, or faster, then toadfish in the example above, due to their higher metabolic rates and more active lifestyle. Therefore, I hypothesise that fish reared under diel pCO_2 cycles could track disturbances in extracellular pH but were not exposed to higher pCO_2 levels long enough for full brain intracellular pH compensation to occur.

It has been suggested that behavioural abnormalities may also be influenced by alterations in gene expression related to ion regulation (Chivers et al. 2014; Lai et al. 2015). Ion-regulation in blood and tissues are under circadian control in fishes (Peterson and Gilmore 1988; Dmitriev and Mangel 2000). In a recent study on *A. polyacanthus*, variation in behavioural tolerance to stable elevated pCO_2 (754 µatm) was linked to the differential expression of genes related to circadian rhythm control (Schunter et al. 2016). For example, offspring of CO_2 sensitive parents (i.e., those that exhibited behavioural abnormalities) upregulated the enzyme that catalyses the final reaction in the synthesis of melatonin, a key regulator of the circadian rhythm, which plays an important role in controlling ion-regulation (López-patiño et al. 2011). This indicates that CO_2 sensitive individuals might display more pronounced acid-base compensation if exposed to a sustained elevation of CO_2 due to a stronger influence of circadian rhythm control, leading to larger changes of the neuronal ion gradients that determine $GABA_A$ receptor function. My observations that diel pCO_2 cycles can alleviate the negative behavioural effects of OA suggests that fish were displaying normal or less circadian control over acid-base regulation and thus did not respond so strongly to internal pH changes caused by elevated CO_2 therefore avoiding altered brain ion gradients. Consequently, it appears that internal circadian rhythm control of acid-base regulation in coral reef fish is disrupted under stable elevated pCO_2, indicating that this process may be linked to the natural diel pCO_2 cycles occurring in shallow reef habitats.

In this study I repeated the control, stable 1000 µatm CO_2 and 1000 ± 300 µatm CO_2 treatments in two different experiments. Although I observed similar responses to predator cue in *Am. percula* in both experiments, there were some differences in the effects of CO_2

cycles on behavioural lateralization. In experiment one behavioural lateralization was partially restored in juvenile *A. polyacanthus* reared at 1000 ± 300 µatm CO_2, whereas no restoration was observed in experiment two. The reason for the different results between experiments is unclear, but one possible reason is differences in the duration that the high CO_2 peaks lasted. In experiment one the high peaks lasted approximately three hours whereas in experiment two they last close to eight hours. Consequently, fish in experiment one had less time to adjust their acid-base status during the high peak, which may have resulted in them exhibiting less severe behavioural impairments. The reason I did not observe a similar difference between experiments for response to predator cue in juvenile *Am. percula* may be because the effect of elevated pCO_2 on this trait was concentration dependent, as seen in experiment two. Due to logistical constraints, it was not possible to have duplicated experimental systems in experiment one. By contrast, experiment two had duplicate systems for each pCO_2 treatment. As similar results were observed in each experiment, I am confident that the pseudo-replication in experiment one did not affect the results. In general, the effects of stable elevated pCO_2 on lateralization and response to a predator cue observed in this study are consistent with previous work on the same species (Dixson et al. 2010; Munday et al. 2010, 2016; Welch et al. 2014), with one exception. Previous studies have reported a clear attraction of *Am. percula* to a predator cue (> 80% of time in predator cue water) at 1000 µatm CO_2, whereas *Am percula* in the current experiments exhibited neither attraction of avoidance of the predator cue (45-58% of time in predator cue water) at this CO_2 level. The same observation was also reported in adult goldskinny wrasse, *Ctenolabrus rupestris* (Sundin and Jutfelt 2015). Why fish in the current experiments exhibited a less dramatic change in antipredator behaviour at high CO_2 compared with previous experiments is unknown but could be related to some differences in protocol. In contrast to past studies, which reset the

fish to the starting position when the direction of the water sources was switched, fish were not disturbed during a trial in this study. Another potential factor is the life-stage that was tested. Previous studies tested settlement stage larvae, whereas settled juveniles were used in this study. Age-specific responses to predator cues, as well as expression of odorant receptor genes, have been observed in other species of fish (Hawkins et al. 2008; Johnstone et al. 2011).

In this study, I show that a diel pCO_2 cycle can substantially reduce the severity of behavioural abnormalities caused by elevated CO_2 in coral reef fishes. In contrast, behavioural impairments where still present in a temperate shark species reared under elevated CO_2 in a mesocosm that experienced diel CO_2 variation, although there was no stable elevated CO_2 treatment to compare against (Pistevos et al. 2015). A handful of other studies have also shown that daily pCO_2 fluctuations can significantly modify the biological responses of shallow water marine organisms to OA (Dufault et al. 2012; Cornwall et al. 2013; Comeau et al. 2014; Frieder et al. 2014; Ou et al. 2015; Clark and Gobler 2016). This highlights the importance of considering natural pCO_2 variability when trying to determine the response of shallow water marine organisms to OA. While our understanding of the magnitude and frequency of pCO_2 fluctuations *in situ* is growing, many shallow water habitats remain under or un-sampled (Wahl et al. 2016). Consequently, there is a need for more high resolution *in situ* studies that characterize natural CO_2 variability both spatially and temporally. Such data will establish ecologically relevant pCO_2 treatments to be used in laboratory experiments and allow us to better interpret results from past OA studies that have employed stable pCO_2 levels (McElhany and Busch 2013; Challener et al. 2016). This will be critical for accurately assessing

the likely effects of OA on shallow water marine organisms, and which species and ecosystem may be at greatest risk.

Chapter 4: Elevated temperature does not substantially modify the interactive effects between elevated CO_2 and diel CO_2 cycles on the survival, growth and behaviour of a coral reef fish

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4.1 Summary

Recent studies demonstrate that diel CO_2 cycles, such as those prevalent in many shallow water habitats, can potentially modify the effects of ocean acidification conditions on marine organisms. However, whether the interaction between elevated CO_2 and diel CO_2 cycles is further modified by elevated temperature is unknown. To test this, I reared juvenile spiny damselfish, *Acanthochromis polyacanthus*, for 11 weeks in two stable (450 and 1000 µatm) and two diel- cycling elevated CO_2 treatments (1000 ± 300 and 1000 ± 500 µatm) at both current-day (29°C) and projected future temperature (31°C). I measured the effects on survivorship, growth, behavioural lateralization, activity, boldness and escape performance (fast starts). A significant interaction between CO_2 and temperature was only detected for survivorship. Survival was lower in the two cycling CO_2 treatments at 31°C compared with 29°C but did not differ between temperatures in the two stable CO_2 treatments. In other traits I observed independent effects of elevated CO_2, and interactions between elevated CO_2 and diel CO_2 cycles, but these effects were not influenced by temperature. There was a trend towards decreased growth in fish reared under stable elevated CO_2 that was counteracted by diel CO_2 cycles, with fish reared under cycling CO_2 being significantly larger than fish reared under stable elevated CO_2. Diel CO_2 cycles also mediated the negative effect of elevated CO_2

on behavioural lateralization, as previously reported. Routine activity was reduced in the 1000 ± 500 µatm CO_2 treatment compared to control fish. In contrast, neither boldness nor fast-starts were affected by any of the CO_2 treatments. Elevated temperature had significant independent effects on growth, routine activity and fast start performance. The results demonstrate that diel CO_2 cycles can significantly modify the growth and behavioural responses of fish under elevated CO_2 and that these effects are not altered by elevated temperature, at least in this species. My findings add to a growing body of work that highlights the critical importance of incorporating natural CO_2 variability in ocean acidification experiments to more accurately assess the effects of ocean climate change on marine ecosystems.

4.2 Introduction

Increasing atmospheric carbon dioxide (CO_2) levels are driving ocean warming (OW) and a decline in seawater pH, a process referred to as ocean acidification (OA) (Collins et al. 2013). Both OA and OW are expected to significantly impact the performance of marine organisms, with severe consequences for community dynamics and ecosystem functioning (Schiel et al. 2004; Doney et al. 2012; Kroeker et al. 2012; Cheung et al. 2013; Wittmann and Pörtner 2013; Nagelkerken and Connell 2015). However, while there has been extensive research on the effects of OA and OW in isolation, comparatively less in known about their combined effects. Understating how multiple climate change drivers affect marine organisms is vital to accurately predict future impacts (Riebesell and Gattuso 2015).

To date, most OA experiments have tested shallow water coastal species, yet have been conducted using stable elevated CO_2 levels consistent with open ocean projections (McElhany

and Busch 2013; Wahl et al. 2016). Unlike the open ocean, CO_2 levels in shallow water habitats are not in equilibrium with the atmosphere over short time scales, with many habitats experiencing substantial fluctuations in CO_2 on a daily basis (Hofmann et al. 2011; Shaw et al. 2012; Duarte et al. 2013; Baumann et al. 2015; Challener et al. 2016). These diel CO_2 cycles are driven primarily by the processes of photosynthesis and respiration across a day–night cycle, but are also influenced by a variety of local hydrodynamic factors (Falter et al. 2013; Waldbusser and Salisbury 2014). Importantly, a number of studies have demonstrated that diel CO_2 cycles can significantly modify organismal responses to OA, highlighting the critical importance or incorporating natural CO_2 variability in OA experiments (e.g. Cornwall et al. 2013; Frieder et al. 2014; Ou et al. 2015; Enochs et al. 2018; Wahl et al. 2018; **Chapter 3**). However, it is currently unknown if the interaction between elevated CO_2 and diel CO_2 cycles will be modified by elevated temperature.

Marine fish were initially expected to be resilient to OA as they can tightly defend their internal pH under elevated CO_2 conditions *via* active control of acid-base relevant ions (Wood et al. 1990; Baker et al. 2009; Heuer and Grosell 2014). However, the increased energetic costs associated with this compensatory acid-base regulation could have detrimental effects on growth and survival, particularly in early life stages where the cost of homeostasis is greater (Brauner 2009). While some studies have indeed reported negative effects of OA on growth and survival in the early life stages of marine fish (e.g. Gobler et al., 2018; Miller et al., 2012; Murray et al., 2014; Stiasny et al., 2016), others have observed neutral (e.g. Bignami et al., 2013; Frommel et al., 2013; Munday et al., 2011; Watson et al., 2018) and even positive effects (e.g. Cattano et al., 2017; Munday et al., 2009). To date, only two studies have investigated how diel CO_2 cycles will interact with elevated CO_2 levels to affect the survival

and growth of marine fishes under OA conditions. Diel CO_2 cycles (450-2000 µatm) were shown to alleviate the negative effects of elevated CO_2 (1000 µatm) on growth of larval pink salmon, *Oncorhynchus gorbuscha* (Ou et al. 2015). In contrast, diel CO_2 cycles (1000 ± 300 and 500 µatm) had no significant effect on growth and survival in two species of coral reef fish (**Chapter 2**).

Some of the most notable effects of elevated CO_2 on marine fish have been the impacts on ecologically important behaviours (Nagelkerken and Munday 2016; Cattano et al. 2018). A wide array of behavioural responses, across a range of species, have now been shown to be altered under elevated CO_2, including: predator avoidance/prey detection behaviour (e.g. McMahon et al., 2018; Munday et al., 2016; Pistevos et al., 2015; Sundin and Jutfelt, 2015) escape responses (e.g. Allan et al., 2013; Munday et al., 2016), activity/boldness levels (e.g. Hamilton et al., 2014; Jutfelt et al., 2013; Munday et al., 2014) and lateralization (e.g. Jutfelt et al. 2013; Welch et al. 2014; Lopes et al. 2016; Schmidt et al. 2017). Nevertheless, species/trait-specific responses are also evident, with other studies reporting no impacts of elevated CO_2 on fish behaviour (e.g. Cattano et al., 2017; Heinrich et al., 2016; Jutfelt and Hedgärde, 2013; Laubenstein et al., 2018; Schmidt et al., 2017; Sundin et al., 2017). The changes in behavioural responses under elevated CO_2 observed in laboratory experiments are expected to have significant ecological consequences for fish populations through effects on recruitment, dispersal, predator-prey/competitive interactions and habitat preference (Nagelkerken and Munday 2016), although such effects may also be offset by compensatory and indirect effects in more diverse communities (Munday et al. 2014; Goldenberg et al. 2018). Only two studies have investigated how diel CO_2 cycles will interact with elevated CO_2 levels to affect the behavioural performance of marine fishes under OA conditions.

Behavioural impairments in two species of coral reef fish under elevated CO_2 were less severe, or absent, in the presence of diel-cycling CO_2 regime (1000 ± 300 and 500 µatm) (**Chapter 3**). In contrast, diel CO_2 cycles (587-1066 µatm) had no significant effect on behavioural responses of juvenile blacksmith, *Chromis punctipinnis*, (Kwan et al. 2017).

In contrast to elevated CO_2, the effects of elevated temperature on the growth and survival of marine fish are more consistent and predictable. Increases in temperature can result in increased growth if the species is living below its thermal optimum (Green & Fisher, 2004; McLeod *et al.*, 2015; Moyano *et al.*, 2016; Gobler *et al.*, 2018). By contrast, growth and survivorship are often reduced at temperatures above the thermal optimum, as metabolic demands exceed capacity (Pörtner and Knust 2007; Munday et al. 2008; Todd et al. 2008; Neuheimer et al. 2011). Elevated temperatures have also been shown to impact a range of behavioural responses, in some cases having stronger effects than elevated CO_2, particularly behaviours which are closely linked to physiological condition such as routine activity and escape performance (Biro et al. 2010; Allan et al. 2017; Schmidt et al. 2017; Laubenstein et al. 2018; Watson et al. 2018).

Considerably less is known about how OA and OW will interact to affect the performance of marine fish. However, a few studies have demonstrated that responses to one stressor can be modified by the presence of another. For example, the positive effects of elevated temperature on growth of larval flat fish, *Solea senegalensis,* were reduced when CO_2 was also elevated (Pimentel et al. 2014). Furthermore, while elevated temperature was shown to reduce growth and survival in larval Atlantic herring, *Clupea harengus*, the effects of elevated CO_2 were shown to be temperature dependent (Sswat *et al.*, 2018; see also Gobler *et al*.,

2018). From a behavioural perspective, elevated CO_2 and temperature interacted synergistically on predation rate, but antagonistically on predator selectivity of the dottyback, *Pseudochromis fuscus*, (Ferrari et al. 2015). Furthermore, elevated temperature reduced the effects of elevated CO_2 on relative laterization in the damselfish *Pomacentrus wardi* (Domenici et al. 2014). Collectively, these studies highlight the importance of studying the combined effects of OA and OW if we are to accurately predict the impacts of climate change on marine fish and the associated ecological consequences. It is currently unknown how elevated temperature will modify the interaction between elevated CO_2 and diel CO_2 cycles to affect the performance of marine fishes.

Here, I investigated the effects of elevated CO_2, diel CO_2 cycles and elevated temperature on the survival, growth and behaviour of a coral reef fish, the spiny damselfish, *Acanthochromis polyacanthus*. Diel CO_2 cycles in some shallow coral reef habitats have been shown to range up to ± 600 µatm around the mean (Shaw et al. 2012), although smaller ranges (< ± 200 µatm) are more common (Kayanne et al. 1995; Manzello 2010; Albright et al. 2013; Kline et al. 2015). Importantly, diel CO_2 cycles on coral reefs are predicted to be amplified up to threefold over the next century (Shaw et al. 2013a), and thus cycles with greater magnitude may become more common by the year 2100. To test if elevated temperature alters the effects of elevated CO_2 and diel CO_2 cycles on reef fishes, I reared juvenile damselfish for 11 weeks at control (450 µatm), stable elevated (1000 µatm) and two diel-cycling elevated (1000 ± 300 µatm and 1000 ± 500 µatm) CO_2 treatments at both 29 °C (current-day average summer temperature) and 31°C (year 2100 prediction; Collins et al., 2013) (**Table 4.1**). I compared survivorship and growth among treatments. I also tested behavioural lateralization, routine activity, boldness

and escape performance (i.e. fast starts) of fish from each treatment, as past research has shown that these traits can be affected by both elevated CO_2 and temperature.

4.3 Materials and Methods

Study species and brood-stock maintenance

A. polyacanthus is common on coral reefs in the Indo-west Pacific. They are demersal spawners, laying clutches of eggs within small caves and crevices in the reef matrix. Eggs hatch into small juveniles, with both parents providing care to the eggs and offspring for up to 45 d post-hatching (Kavanagh 2000). *A. polyacanthus* can be bred and reared in captivity with high success, which has led to their establishment as a model for investigating the potential impacts of OA and OW on coral reef fishes (Munday et al. 2008, 2011b; Donelson et al. 2012; Welch et al. 2014; Heuer et al. 2016).

Adult *A. polyacanthus* were collected using hand nets from the Bramble Reef area (site 1: 18°22'S, 146°40'E; site 2: 18°25'S, 146°40'E) of the Great Barrier Reef in July 2015. Fish were transported to an environmentally controlled aquarium research facility at James Cook University (JCU) (Townsville, Australia) where they were housed as breeding pairs in 60 L aquaria at temperature conditions matching the collection location. Breeding pairs were maintained under stable, ambient CO_2 (~490 µatm). Temperatures were increased at a rate of 0.5°C *per* week until the summer breeding temperature of 29°C was reached in the first week of December 2016. Breeding pairs were provided with half a terracotta pot to act as a shelter and spawning site. Aquaria were checked each morning for the presence of newly laid clutches of eggs. Pairs were fed *ad libitum* on commercial fish feed pellets (INVE Aquaculture

Nutrition NRD 12/20) once daily outside the breeding season and twice daily during the breeding season.

Experimental design

The experimental part of the study was carried out at the National Sea Simulator (SeaSim) facility at the Australian Institute of Marine Science (AIMS) (Cape Cleveland, Australia). Clutches of juveniles were transferred from JCU to the experimental system at AIMS at one day post hatch (dph) where they were reared for 11 weeks at two stable (450 and 1000 µatm) and two cycling (1000 ± 300 and 1000 ± 500 µatm) CO_2 treatments at both 29 and 31°C. A total of four offspring clutches were used in the experiment, sourced from two breeding pairs (two clutches per pair). Each clutch was split between the CO_2 and temperature treatments in duplicate tanks (15-25 fish per tank with tanks on different systems). Juvenile *A. polyacanthus* were fed 6mL of freshly hatched *Artemia* naupli (approx. 4000mL^{-1}) for the first four days post hatch (dph). From 5–28 dph they were fed daily (0.12g) on a weaning fish feed (INVE Aquaculture Nutrition Wean-S 200–400 µm) and from 29-42 dph they were fed daily (0.15g) on a small pellet fish feed (INVE Aquaculture Nutrition NRD 5/8). Feeding was increased to 0.2g after 42 dph and to 0.35g after 70 dph.

Fast start trials were performed 28-35 dph, lateralization trials were performed 42-49 dph and activity/boldness trials were performed 63-70 dph. Fast start and lateralization trials were only performed on three clutches due to logistic constraints. Behavioural trials took place across four consecutive days with the temperature treatment tested alternating between days. This allowed the room temperature to be adjusted accordingly, thus ensuring a stable testing temperature was maintained. Fish were randomly selected for each behavioural trial,

which means that some fish were likely tested for more than one behavioural trial, and the time between trials was to ensure that they recovered from any stress between trials. To ensure a fish was not tested twice within a trial, fish were placed in an isolation chamber in their experimental tank after testing for the rest of the day. All behavioural trials were performed between 09:00 and 17:00. Fish were gently transferred to the behavioural arenas using a glass beaker to minimise handling stress. Fish from each CO_2 treatment were tested at random times throughout the day to account for any possible time of day effects in the fluctuating treatments. At the end of the rearing period all fish were euthanised with clove oil anaesthetic. Each fish was blotted dry, weighed (nearest mg) on an analytical balance (AX224, Sartorius, Bradford, USA) and photographed in a lateral position next to a ruler. Standard length (SL) to the nearest 0.1 mm was estimated for each fish from the digital photographs using ImageJ software (http://rsb.info.nih.gov/ij/). Research was carried out under approval of the James Cook University animal ethics committee (permit: A2210) and according to the University's animal ethics guidelines.

Experimental system

The experimental setup used at SeaSim comprised of 16 independent flow-through systems (4 systems *per* CO_2 treatment). Each system supplied ultra-filtered seawater (0.04 µm), at either 29 or 31˚C, to three custom-made 50 L experimental tanks at the rate of 50 L h^{-1}. Thus, there was total of 48 tanks (6 tanks *per* CO_2/temp treatment). The experimental tanks were placed in individual temperature-controlled water baths to ensure temperature stability (± 0.1˚C). Treatments and tank replicates were randomly positioned in the experimental room.

pCO_2 and temperature levels were controlled through a custom designed Model Predictive Control running on a micro-programmable logic controller (PLC) (Series S7-1500, Siemens, Australia). The micro-PLC was integrated with the general SeaSim control system, to provided SCADA (Siemens WinCC) accessibility and data archiving. The CO_2 feedback for each of the replication lines was provided via non-dispersive infrared measurements. On each system, water from one tank was delivered to an equilibrator (Seasim, AIMS design, custom built) *via* an in-tank submersible pump (Universal Pump 1260, EHEIM, Deizisau, Germany) where the air space in the chamber maintains an equilibrium with the CO_2 of the experimental water. The air was constantly delivered to a nondispersive infrared CO_2 analyser (Telaire T6613, Amphenol, Australia) that provided live feedback to the PLC. The CO_2 analysers were calibrated monthly using certified calibration gas mixtures at 0, 600 and 2000 ppm. The control system delivered pure CO_2 though Gas Mass Flow Controllers (GFC17 series, Aalborg, Orangeburg, USA) according to the profiling schedule designed for the pCO_2 treatment and the feedback signal coming from the experimental tanks. CO_2 was dissolved in the flow-through water by membrane contactors (Membrana Liqui-Cel 2.5x8 Extra-Flow. 3M, USA). Throughout the experiment incoming coastal water had a pCO_2 ranging between 500-550 µatm. Thus, to achieve a control pCO_2 level closer to 450 µatm, membrane contactors (Membrana Liqui-Cel 4x28 Extra-Flow) were used to remove CO_2, using CO_2 – depleted air as sweep gas. Total alkalinity was measured from a random tank on half of the systems each week (the systems measured alternated each week) using Gran Titration (Metrohm 888 Titrando Titrator Metrohm AG, Switzerland) and certified reference material from Dr. A. G. Dickson (Scripps Institution of Oceanography). Mean values for seawater parameters are presented in **Table 4.1**.

Table 4.1 Experimental Seawater parameters. Values are means ± 1 SD for mean, min and max pCO_2, temperature, salinity and total alkalinity (TA), pCO_2 data is based on one reading every ten minutes. Temperature data is based on one reading per hour. Salinity and TA data are based on weekly measurements.

CO_2 treatment	Temperature treatment	Mean pCO_2 (µatm)	Min pCO_2 (µatm)	Max pCO_2 (µatm)	Temperature (°C)	Salinity	TA (µmol kg^{-1})
450	29	435 ± 38	425 ± 36	477 ± 163	28.8 ± 0.2	35.4 ± 0.4	2325 ± 33
450	31	475 ± 44	459 ± 31	527 ± 224	31.1 ± 0.3	35.4 ± 0.4	2306 ± 51
1000	29	992 ± 19	956 ± 74	1035 ± 95	28.8 ± 0.2	35.4 ± 0.4	2325 ± 32
1000	31	981 ± 8	958 ± 42	1002 ± 23	31.1 ± 0.3	35.4 ± 0.4	2310 ± 48
1000 ± 300	29	1009 ± 25	653 ± 25	1329 ± 89	28.8 ± 0.2	35.4 ± 0.4	2318 ± 38
1000 ± 300	31	1034 ± 33	668 ± 27	1349 ± 97	31.1 ± 0.3	35.4 ± 0.4	2318 ± 36
1000 ± 500	29	1060 ± 34	521 ± 43	1544 ± 80	28.8 ± 0.2	35.4 ± 0.4	2323 ± 32
1000 ± 500	31	1079 ± 49	556 ± 64	1570 ± 118	31.1 ± 0.3	35.4 ± 0.4	2314 ± 39

Behavioural assays

Fast starts

Fast start trials were performed using methods similar to those described by Munday et al. (2016). The setup consisted of a circular experimental arena (diameter 210 mm) placed inside an opaque rectangular container (550 x 410 x 320mm). A polystyrene lid was placed on top of the container with a white PVC tube (40mm x 300mm) inserted through the middle. An electromagnet was attached to the top of the PVC tube to which a weighted stimulus was attached via a metal disc. The arena was illuminated by an LED strip placed around the outside of the container. The container was filled with water from the treatment fish were reared in to a depth of 50mm to reduce movement in the vertical plane. Once introduced into the arena fish were given a 3 min habituation period. After this period, a fast start was elicited by turning off the electromagnet, thus releasing the stimulus. A length of fishing line attached to the weight caused it to stop when the tip of the weight only just touched the surface of the water after release from the magnet. To provide a sudden stimulation and allow calculation of the response latency the bottom edge of the PVC tube was placed just above the water level. This way there was no visual stimulation before mechanical stimulation. Fish were only startled when they were more than two body lengths from the arena's edge to minimize edge effects on escape responses. Responses to the stimulus were filmed as a silhouette from below with a high-speed camera (Casio EX-ZR2000; 480 fps). To do this, the experimental setup was placed on a wooden frame with a mirror placed inside at 45° degrees. The front of the frame was covered with black plastic sheet, to minimize external disturbance, with a small hole cut out in the middle for the camera lens. Five to seven fish per tank were tested. Video recordings were analysed with the observer blind to treatment using IMAGEJ software and calibrated against the base of the PVC tube. The very front of the fish was used as the reference point

for tracking. From the videos, we quantified non-locomotor traits (response latency and directionality) and locomotor traits (turning rate, mean escape speed, maximum escape speed and escape distance). The moment the stimulus weight hit the water was benchmarked by the first detectable water disturbance in the video. Fish escape variables were only measured if a C-start was initiated.

Non-locomotor variables

1. Response latency (in ms) was measured as the time interval between the stimulus onset and the first detectable movement leading to the escape of the animal.
2. Directionality: escape responses were divided into 'away' and 'towards' responses when the first detectable movement of the head was oriented away and towards the stimulus, respectively

Locomotor variables

1. Turning rate (degrees ms^{-1}) was measured as the angle turned before the fish swam away after the onset of the response.
2. Mean response speed (m s^{-1}) was measured as the distance covered within a fixed time (24ms) which corresponds to the average duration of the first two tail flips of the tail (the first two axial bends, i.e. stages 1 and 2 defined based on Domenici and Blake, (1997), which is the period considered crucial for avoiding predator ambush attacks)
3. Maximum speed (m s^{-1}) was measured as the maximum speed reached at any time during the response.
4. Escape distance was measured as the straight-line distance between the position of the fish at the onset of a C-start and the position of the fish after 24ms.

Behavioural lateralization

Lateralization in juvenile *A. polyacanthus* was determined using a detour test in a two-way T-maze using methods described in **Chapter 3**. The two-way T-maze consisted of an experimental arena (60 cm x 30 cm x 20 cm), with a runway in the middle (25 cm x 2 cm, length x width), and at both ends of the runway (2 cm ahead of the runway) an opaque barrier (12 cm x 12 cm x 1 cm) was positioned perpendicular to the runway. The maze was filled to a depth of 4 cm with the respective treatment water of the fish being tested, being changed after each trial. A single fish was placed at one end of the T-maze and given a 3 min habituation period, during which time it could explore the apparatus. At the end of the habituation period the fish was gently guided into the runway using a plastic rod with the observer standing directly behind the fish (the plastic rod was never placed closer than approximately twice the body length of the fish). At this point to minimise human interference affecting direction turned the observer slowly stepped back from the maze and the fish was allowed to swim to the end of the runway. In instances when a fish did not swim to the end, encouragement was provided by gently moving the plastic rod around at the beginning of the runway. Consecutive runs were carried out until 10 successful runs per fish had been recorded. Six to eight fish were tested per tank. To account for any possible asymmetry in the maze, turns were recorded alternately on the two ends of the runway. Turning preference (i.e. bias in left or right turns) at the population level was assessed using the relative lateralization index (L_R, from -100 to +100, indicating complete preference for left and right turning, respectively) according to the following formula: L_R = [(Turn to the right - Turn to the left) / (Turn to the right + Turn to the left)] * 100. The strength of lateralization (irrespective of its direction) was also assessed at the individual-level using the absolute lateralization index L_A (ranging from 0 (an individual that turned in equal proportion to the right and to the left)

to 100 (an individual that turned right or left on all 10 trials)). Trials were filmed, and fish were scored from video analysis with the observer blind to treatment. Direction choice was recorded as the first direction turned when the fish exited the runway.

Routine activity and boldness

Routine activity and boldness were determined using an open field test with methods similar to those described by Laubenstein et al. (2018). The setup consisted of a round, white plastic arena (26cm diameter, 6cm height) placed inside a white plastic bin (52 cm length, 32 cm width, 34cm height), which was opaque to minimize visual disturbance for the fish but allowed light through for filming. Two wooden planks (L = 60cm, H= 9cm, W=2cm) were placed across the top of each plastic bin with a sheet of white corflute on top which had a small circular hole cut into its centre, where a video camera (Casio EX-ZR2000) was placed. This was done so that the whole arena fitted into the camera's field of view. Fish were tested in their treatment water at a depth of 5cm. To begin a trial, a fish was transferred into the centre of the arena by gently transferring it with a beaker to minimize stress. The camera was turned on, and the fish was filmed for 15 min. Six to nine fish per tank were tested. Activity and space use in the tank were determined from the video using motion-tracking software (Lolitrack v4.1.0, Loligo Systems, Tjele, Denmark). Before each video analysis, a circular arena was drawn within the test arena, with the same central point, but which was 18 cm in diameter, or approximately 1.5 standard body lengths away from the edges of the test arena. This "centre zone" was used to quantify boldness, based on the idea that an open field is considered dangerous, and that venturing into the inner zone represents boldness, or the willingness to undertake risk. Therefore, we quantified boldness by measuring the number of visits made to the centre zone and time spent in the centre zone. The routine activity

parameters quantified by the software were: time active (%) and average swimming velocity (cm s^{-1}). All videos were analysed blind to treatment. The first 5 mins of each video were discarded to allow some habituation time.

Data accessibility

The datasets generated during the current study are available from the corresponding author on request or via the Tropical Research Data Hub (doi: 10.25903/5bd7c7f552897).

Statistical analysis

All analyses were conducted using R version 3.4.0. (R Core Team 2017) using the following packages: nlme, 'lme4 and glmmTBM. In all cases, except for survival data, mixed effects models were used so that random effects could be incorporated. In all mixed effects models, pCO_2 and temperature treatment were fixed factors, with clutch and tank as random factors with tank nested within clutch. Initially, fully interactive models were run. However, non-significant interaction terms were gradually removed. Final model choice was confirmed based on Akaike information criterion (AIC). When a CO_2 effect was observed pair-wise comparisons were conducted using Tukey's tests using the 'multcomp' package. When a significant CO_2 * temperature interaction was found (survival data only) planned contrasts were performed. For planned comparison we compared pCO_2 treatments across each temperature treatment separately and temperature treatments within a pCO_2 treatment.

Survival and growth

The effects of CO_2 and temperature on juvenile survival were tested using a generalized linear model (GLM) with a binomial distribution (due to the data being proportional), weighted to

the starting number of fish in each tank. The effects of CO_2 and temperature on wet weight and standard length were analysed using linear mixed effects models (LMEs). The number of fish remaining in each tank at the end of the experiment was included as a covariate. Additive models were used (number + CO_2 + temperature).

Fast starts

The effects of CO_2 and temperature on latency to respond to the stimulus were tested using an LME with distance of the fish from the centre of the experimental arena when the stimulus hit the water as a covariate. The effects of CO_2 and temperature on direction turned were analysed with a generalized linear mixed model (GLMM) with a binomial distribution. The effects of CO_2 and temperature on turning rate, mean escape speed, maximum escape speed and escape distance were tested using LMEs with total length included as a covariate for mean and maximum escape speed and escape distance. Additive models were used for latency, direction turned, turning rate and escape distance. For mean and maximum escape speed the model tested the interactive effects between total length and temperature and the additive effect of CO_2 (body length * temperature + CO_2)

Behavioural lateralization

GLMM's with binomial distributions and weighted to the number of runs (10) were used to test the effects of CO_2 and temperature on absolute lateralization (L_A) and relative lateralization (L_R) due to the proportional nature of the data. Additive models were used (CO_2 + temp).

Routine activity and boldness

A GLMM with a binominal distribution and weighted to the length of time of the trial (10mins) was used to test the effects of CO_2 and temperature on percentage time active. The effects of CO_2 and temperature on velocity and time spent in the centre zone (fish that did not visit the centre zone were excluded from analysis) were tested using LMEs. Finally, a mixed-effects hurdles model was used to test the effects of CO_2 and temperature on number of visits to the centre zone due to the high number of zeros present in the dataset. Total length was included as a covariate for all traits. Additive models were used for time active, velocity and number of visits to the centre zone. An interactive model was used for time spent in centre zone (body length * temperature * CO_2).

4.4 Results

Survival and growth

Elevated temperature (**Figure 4.1a**, $\chi2 = 29.59$, df = 1, $P < 0.001$) but not elevated CO_2 ($\chi2 = 0.74$, df = 3, $P = 0.863$) had an overall negative effect on survival of juvenile *A. polyacanthus.* Survivorship from hatching to 11 weeks post hatch decreased from 89.9% at 29°C to 78.2% at 31°C. In addition, a significant interactive effect between CO_2 and temperature was observed ($\chi2 = 7.85$, df = 3, $P = 0.049$). Specifically, elevated temperature had a negative effect on survival in the two diel-cycling CO_2 treatments (max. $P = 0.047$), but not under control or stable elevated CO_2 conditions (max. $P = 0.194$).

Wet weight of juvenile *A. polyacanthus* was significantly affected by CO_2 (**Figure. 4.1b**, $F_{3,54} = 6.32$, $P < 0.001$) and temperature (**Figure 4.1b**, $F_{1,54} = 58.90$, $P < 0.0001$) treatment. There was a trend of decreased (-4.7%) wet weight of fish reared under stable elevated CO_2

compared to those reared under control conditions, although this was non-significant (P = 0.506). However, fish reared under diel-cycling elevated CO_2 conditions were significantly heavier (+11.2% for ± 300 µatm and +6.6% ± 500 µatm CO_2 respectively) compared to those reared under stable elevated CO_2 conditions (min. P = 0.035). Fish reared under the smaller CO_2 fluctuations were also significantly heavier (+5.9%) compared to fish reared under control conditions (P = 0.033). Elevated temperature had a negative effect on wet weight of juvenile *A. polyacanthus*, decreasing it by 7.8% under 31°C. Finally, the number of fish remaining in the tank at the end of the experiment had a significant effect on wet weight, with heavier fish in tanks with fewer fish ($F_{1,54}$ = 42.10 P < 0.0001).

Standard length of juvenile *A. polyacanthus* was significantly affected by CO_2 (**Figure 4.1c**, $F_{3,54}$ = 3.46, P = 0.023) and temperature (**Figure 4.1c**, $F_{1,54}$ = 69.33, P < 0.0001) treatment. Fish reared at 1000 ± 300 µatm CO_2 were significantly longer (+3.2 %) compared to those reared under stable elevated CO_2 (P = 0.022). However, neither were significantly different compared to control fish (min. P = 0.144). Finally, fish reared under 1000 ± 500 µatm CO_2 were of comparable length to fish from all other CO_2 treatments (min. P = 0.133). Elevated temperature had a significant negative effect on length of juvenile *A. polyacanthus*, decreasing it by 4.5% under 31°C. The number of fish remaining in the tank at the end of the experiment had a significant effect on standard length, with fish reaching a longer size in tanks with fewer fish ($F_{1,54}$ = 18.08, P = 0.0001).

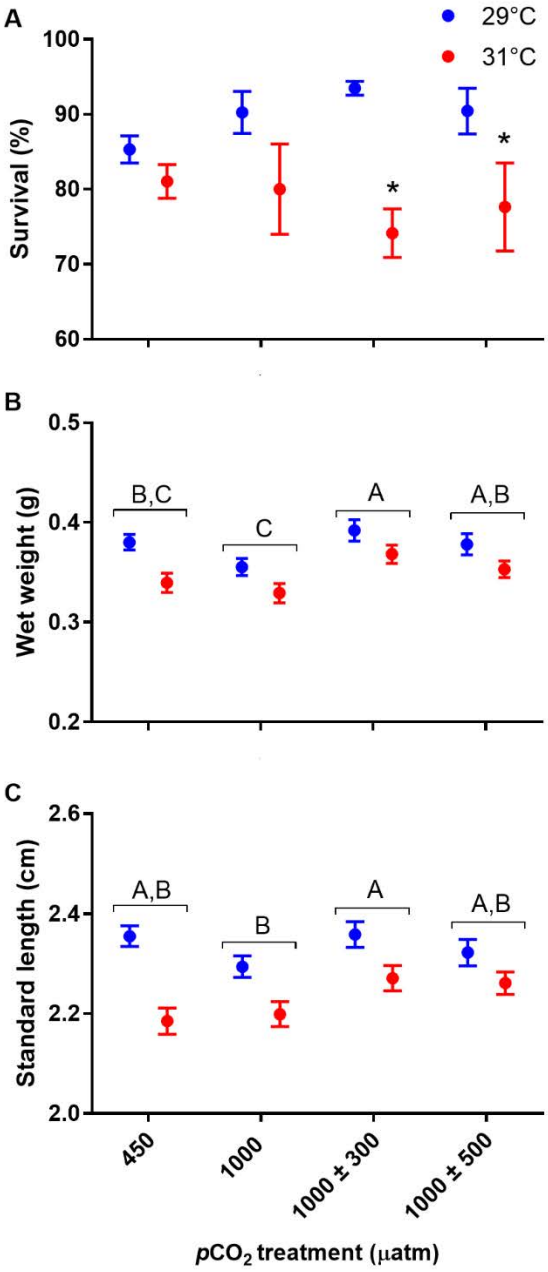

Figure 4.1 Effects of elevated CO_2 and temperature on survival and growth of juvenile *Acanthochromis polyacanthus.* Survival (A), wet weight (B) and standard length (C). Different letters represent significant differences between pCO_2 treatments (Tukey's, $P < 0.05$). Points represent means ± SE. Asterix (*) represent a significant difference between temperature treatments at the same pCO_2 level.

Behavioural lateralization

Absolute lateralization (L_A) was significantly influenced by CO_2 treatment (**Figure 4.2**, $\chi 2$ = 17.57, df = 3, $P < 0.001$) but not temperature ($\chi 2$ = 0.37, df = 1, P = 0.541). Juveniles reared under stable elevated CO_2 were less lateralized compared to those reared at control levels ($P < 0.001$). L_A of juveniles reared at 1000 ± 300 µatm CO_2 was partially restored being intermediate to fish reared under control and stable elevated CO_2 (min. P = 0.060). L_A of juveniles reared at 1000 ± 500 µatm CO_2 was fully restored to control levels being significantly greater than those reared at stable elevated CO_2 (P = 0.021). Relative lateralization (L_R) was unaffected by CO_2 ($\chi 2$ = 1.67, df = 3, P = 0.644) or temperature ($\chi 2$ = 0.15, df = 1, P = 0.697).

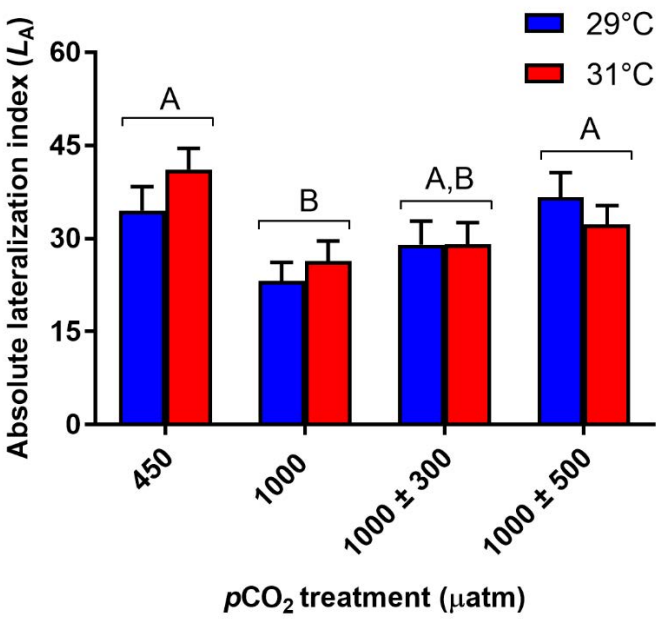

Figure 4.2 Effects of elevated CO_2 on absolute lateralization (L_A) in juvenile *A. polyacanthus.* Different letters represent significant differences between pCO_2 treatments (Tukey, $P < 0.05$). Bars represent means ± SE.

Routine activity and boldness

Percentage time active was significantly influenced by CO_2 (**Figure 4.3a**, $\chi 2 = 11.99$, df = 3, $P = 0.007$) and temperature (**Figure 4.3a**, $\chi 2 = 25.51$, df = 1, $P < 0.001$) treatments. Fish reared at 1000 ± 500 µatm CO_2 were significantly less active (-10.2%) than fish reared under control conditions ($P = 0.005$). No other significant differences between pCO_2 treatments were observed (min. $P = 0.059$). Elevated temperature had a negative effect on percentage time active, decreasing from 74.4% at 29°C to 64.9% at 31°C. Average velocity was also significantly affected by CO_2 (**Figure 4.3b**, $F_{3,441} = 5.15$, $P = 0.002$) and temperature (**Figure 4.3b**, $F_{1,441} = 16.66$, $P < 0.001$) treatment. Fish reared at 1000 ± 500 µatm CO_2 swam at lower speeds (-15.9%) compared to control fish ($P = 0.001$). Average velocity was negatively impacted by elevated temperature, decreasing by 14.4% at 31°C compared to fish reared at 29°C. Finally, total body length had a significant effect on average velocity ($F_{1,441} = 12.46$, $P < 0.001$), with longer fish swimming faster.

Number of visits to the centre zone was not significantly influenced by CO_2 treatment (**Figure 4.4a**, min. $P = 0.130$), but was affected by temperature (**Figure 4.4a**, $P = 0.010$). Fish reared at 31°C made fewer visits to the centre zone. There was no effect of CO_2 (**Figure 4.4b**, $F_{3,58} = 0.07$, $P = 0.974$) or temperature (**Figure 4.4b**, $F_{1,336} = 0.001$, $P = 0.974$) treatment on time spent in the centre zone.

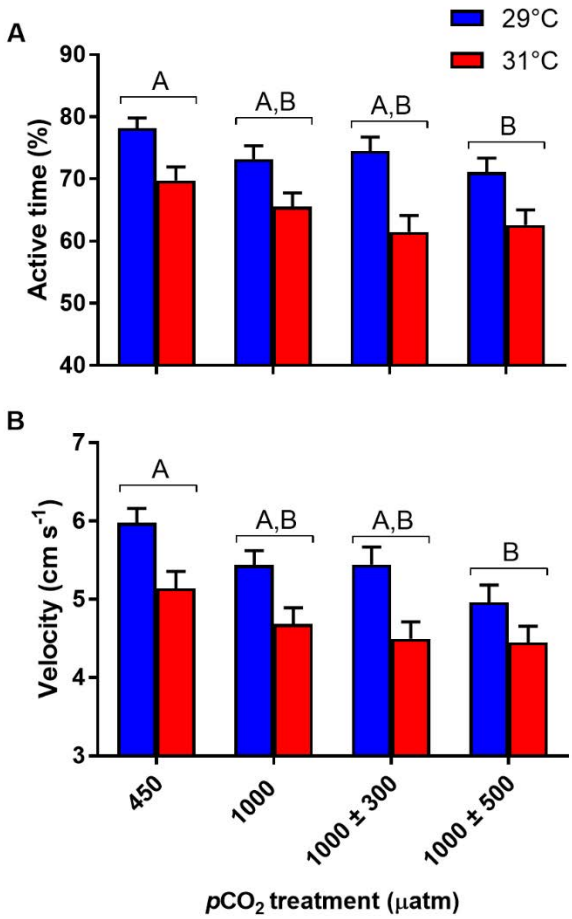

Figure 4.3 Effects of elevated CO_2 and temperature on routine activity traits of juvenile *A. polyacanthus.* % time active (A) and average velocity (B). Different letters represent significant differences between pCO_2 treatments (Tukey's, $P < 0.05$). Bars represent means ± SE.

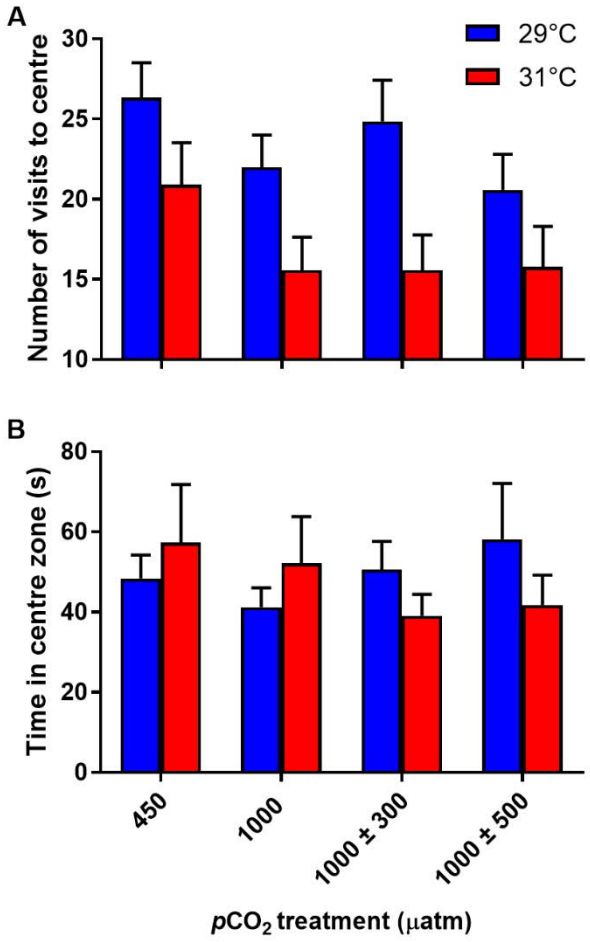

Figure 4.4 Effects of elevated CO_2 and temperature on the boldness traits of juvenile *A. polyacanthus*. Number of visits to centre zone (A) and time spent in centre zone (B). Bars represent means ± SE.

Fast starts

Latency in response to the stimulus was unaffected by CO_2 (**Figure 4.5a**, $F_{3,42} = 1.48$, $P = 0.234$) or temperature (**Figure 4.5a**, $F_{1,238} = 0.58$, $P = 0.448$) treatment. The distance fish were from the centre of the arena had a significant effect on escape latency with fish closer the centre responding more quickly to the stimulus ($F_{1,238} = 9.68$, $P = 0.002$). Direction of response was also unaffected by CO_2 (**Figure 4.5b**, $\chi 2 = 4.08$, df = 3, $P = 0.252$) or temperature (**Figure 4.5b**, $\chi 2 = 0.00$, df = 1, $P = 0.988$) treatment.

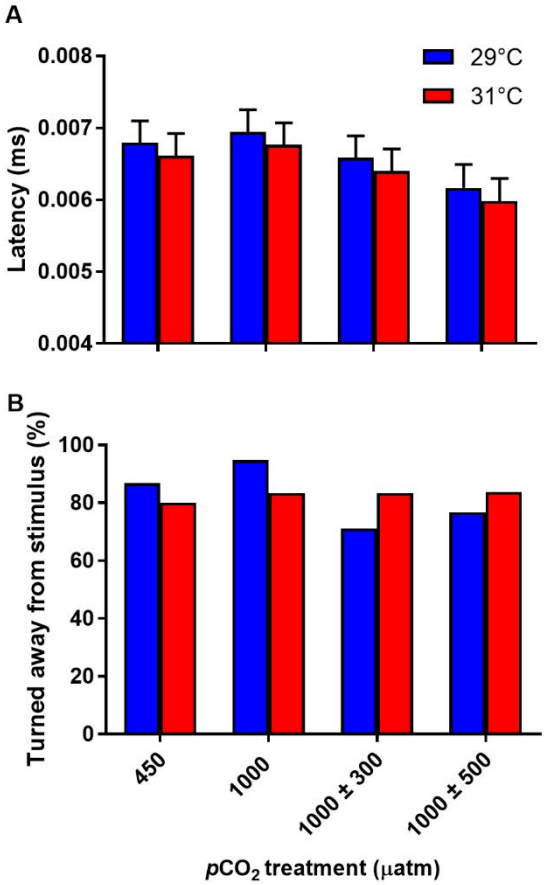

Figure 4.5 Effects of elevated CO_2 and temperature on non-locomotor fast start traits of juvenile *A. polyacanthus*. Response latency (A) and direction turned (B). Bars represent means ± SE.

All locomotor traits (turning rate, mean escape speed, maximum escape speed and escape distance) were unaffected by CO_2 treatment (**Figure 4.6 a-d**, max. $F_{3,42} = 1.54$, $P = 0.217$) but significantly affected by temperature (**Figure 4.6 a-d**, min. $F_{1,237} = 4.09$, $P = 0.044$). Fish reared at 31°C turned at a faster rate (+8.1%), had a greater mean (+4.2%) and maximum (+5.2%) escape speed and a greater escape distance (+6.9%) compared to fish reared at 29°C. Total body length had a significant positive effect on mean escape speed, maximum escape speed and escape distance (min. $F_{1,237} = 11.51$, $P < 0.001$).

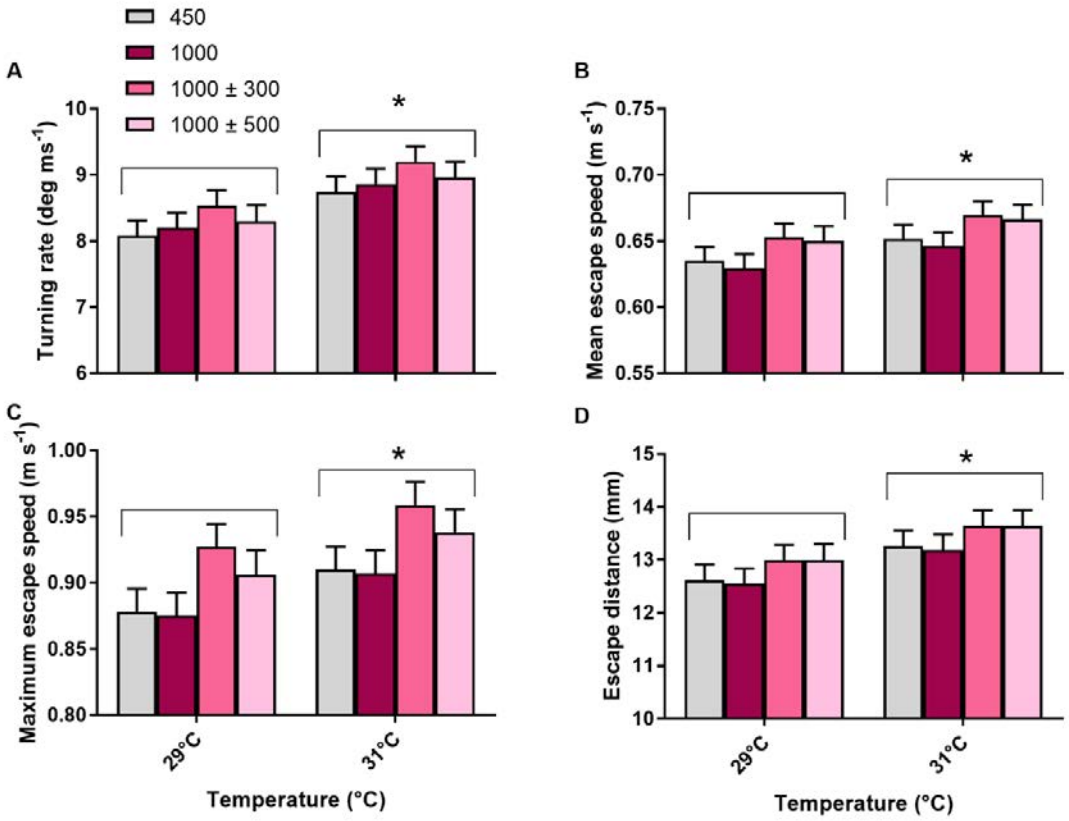

Figure 4.6 Effects of elevated CO_2 and temperature on locomotor fast start traits of juvenile *A. polyacanthus*. Turning rate (A), mean escape speed (B), maximum escape speed (C) and escape distance (D). Bars represent means ± SE. * indicates a significant difference between temperature treatments ($P < 0.05$).

4.5 Discussion

Recent studies have shown that diel CO_2 cycles can significantly modify the effects of ocean acidification conditions on marine organisms. However, whether the interaction between elevated CO_2 and diel CO_2 cycles is further modified by elevated temperature is unknown. Here, I report several independent effects of elevated CO_2, and interactions between elevated CO_2 and diel CO_2 cycles, that were mostly unaffected by elevated temperature. My results show that diel CO_2 cycles alleviated the negative effects elevated CO_2 had on growth and absolute lateralization but had a negative impact on routine activity. A significant interaction between CO_2 and temperature was only observed for survivorship, with lower survival in the two diel-cycling elevated CO_2 treatments observed at elevated temperature (31 °C). Finally, elevated temperature had significant independent effects of growth, routine activity and fast start performance.

Survival and growth

CO_2 effects

Survival and growth of juvenile *A. polyacanthus* were not significantly affected by stable elevated CO_2 conditions, although there was a trend of decreased growth compared with control fish. Previous experiments testing the effects of stable elevated CO_2 on fish early life stages have produced highly variable and species-specific results, with negligible, negative and positive effects on survival and growth all reported (Heuer and Grosell 2014). The sensitivity of marine fish to OA conditions has often been linked to the environmental CO_2 levels they experience in natural habitats, with tolerant species generally occurring in areas that have daily and seasonal CO_2 cycles, and thus may already be physiologically adapted to elevated CO_2 (e.g. Bignami et al., 2013; Frommel et al., 2013; Munday et al., 2011; Perry et

al., 2015). A recent meta-analysis lends some support to this theory by showing that mortality increased in pelagic species under elevated CO_2, whereas mortality often decreased in benthic species, which experience higher and more variable CO_2 compared with pelagic species (Cattano et al. 2018). My results support this as *A. polyacanthus* is a benthic reef species that shelters within the reef-matrix at night where it is likely exposed to CO_2 levels considerably higher than in the surrounding water column (e.g. Albright et al., 2013; Kline et al., 2015).

While natural CO_2 cycles have been used to explain tolerance to future OA conditions in marine fish, very few studies have incorporated them in their experimental designs (but see Ou et al. 2015; **Chapter 2**). Here, I show that diel CO_2 cycles had a significant positive effect on growth of juvenile *A. polyacanthus* under elevated CO_2. These beneficial effects were most apparent for weight wet, with fish reared under both the ± 300 and ± 500 µatm diel-cycling CO_2 regimes being significantly heavier compared to fish reared under stable elevated CO_2. I observed a similar positive effect of diel CO_2 cycles on standard length, although there was only a significant difference between the ± 300 µatm diel-cycling and the stable elevated CO_2 treatments. These results suggest that the positive effects of diel CO_2 cycles are magnitude dependent, with greater effects observed under the smaller ± 300 µatm diel-cycling CO_2 regime compared with ± 500 µatm CO_2. This may reflect the closer match of the ± 300 µatm CO_2 treatment to the diel fluctuations these fish experience on the reef. Similar trends to those described above were observed in a previous study (**Chapter 2**) carried out the same species and in which the same pCO_2 treatments were used, although no significant differences were detected in that study, most likely due to the lower levels of replication (three clutches compared to the four in this study). Positive effects of diel CO_2 cycles on growth under elevated CO_2 were also reported in the freshwater stage of larval pink salmon, *O. gorbuscha*,

where the negative effect that 1000 µatm CO_2 had on length was absent in the diel-cycling treatment (450-2000 µatm) (Ou et al. 2015).

The underlying physiological mechanism responsible for the observed positive effect of diel CO_2 cycles on growth under elevated CO_2 is unknown. However, my results and those of Ou et al. (2015) suggest that it is energetically less expensive for fish to live in a diel-cycling elevated CO_2 environment compared to a stable elevated CO_2 environment. Aerobic scope (the difference between resting metabolic rates (RMR) and maximal metabolic rates (MMR)) represents the energy budget available for an organism to undertake aerobic tasks (e.g. growth). Previous work has shown that exposure to stable elevated CO_2 can alter metabolic rates in some fish, highlighting an increased cost of living (Munday et al. 2009b; Enzor et al. 2013; Strobel et al. 2013; Ou et al. 2015; Cattano et al. 2018; Laubenstein et al. 2018). Thus, I suggest that the positive effects of diel CO_2 cycles on growth could be driven by increased aerobic scope under such conditions. In line with this, Ou et al. (2015) reported that maximal metabolic rates (MMR) of larval pink salmon after transition to saltwater were negatively impacted by stable elevated CO_2 (1600 µatm), but not a by diel-cycling conditions (450-1600 µatm), with resting metabolic rates (RMR) being similar in both treatments. However, because the mean CO_2 also differed between the stable and fluctuating treatments in that experiment it is not possible to be certain whether the changes in MMR were being driven primarily by the cycling CO_2 regime or change in mean CO_2. Alterations in aerobic capacity under elevated CO_2 may be linked to the increased metabolic costs associated with defending acid-base status (Perry and Gilmour 2006; Baker and Brauner 2012). Thus, I hypothesise that the cost of acid-base regulation under elevated CO_2 is reduced by the presence of diel CO_2 cycles, ultimately resulting in more energy available for life-history processes such as growth.

Temperature effects

Elevated temperature (31°C) negatively impacted both survival and growth of juvenile *A. polyacanthus*. This demonstrates that 31°C is above the optimal temperature for this species and is consistent with previous findings (Munday et al. 2008; Rodgers et al. 2018). The negative impacts of elevated temperature on growth and survival were most likely correlated to increased energetic demands associated with increased metabolic costs. Indeed, previous work on coral reef fish, including *A. polyacanthus*, has shown that exposure to 31°C causes a significant increase in RMR and subsequent decrease in aerobic scope (Munday et al. 2009b; Nilsson et al. 2009; Donelson et al. 2012). While elevated temperature (31°C) had an overall negative effect on survival of juvenile *A. polyacanthus*, CO_2 treatment dependent effects were present. There was a trend of decreased survival in all the CO_2 treatments at 31°C; however, significant differences were only observed in the two diel-cycling elevated CO_2 treatments. Most mortality was observed in the mornings, indicating that deaths were occurring at night during the high CO_2 peaks, within the first week of the experiment. These observations suggest that for newly hatched fish, which might not have fully developed acid-base regulation capacity, and for which the cost of homeostasis is greater (Brauner 2009), the metabolic demands to tolerate the higher CO_2 levels caused by diel cycles become too great under elevated temperature. The effects of elevated temperature on fish early life growth have been varied. For example, temperatures above the natural preference range were shown to enhance growth of larval yellowtail kingfish, *Seriola lalandi* (Watson et al. 2018). In contrast, elevated temperature negatively impacted the growth and survival of larval Atlantic herring, *Clupea harengus*, despite the elevated temperature still being within the species preferred thermal range (Sswat et al. 2018). Differences between studies could potentially be

linked to food availability, with studies reporting positive effects usually providing a continuous supply of high-density food that enabled energetic demands to be met. In this study, juvenile *A. polyacanthus* were fed a fixed ration of food daily, however, previous work on this species has shown that the effects of 31°C on growth were no different depending on whether fish were fed a high or low quality diet (Munday et al. 2008). Thus, I am confident that the feeding regime had little impact on the observed response.

Behaviour

CO_2 effects

Behavioural abnormalities in fish under OA conditions are thought to be linked to the effects of acid-base regulation on the function of type A γ-aminobutyric acid ($GABA_A$) neurotransmitter receptors which have specific conductance for HCO_3^- and Cl^- (Nilsson et al. 2012; Hamilton et al. 2014; Heuer et al. 2016). Under elevated CO_2 fishes increase intracellular and extracellular HCO_3^- concentrations, with a corresponding decrease in Cl^-, to prevent plasma and tissue acidosis (Wood et al. 1990; Baker et al. 2009; Heuer and Grosell 2014). This altered ion gradient is thought to turn some $GABA_A$ receptors from inhibitory to excitatory, ultimately leading to behavioural impairments (Heuer et al. 2016). Importantly, acid-base regulation in fish is under circadian control (Peterson and Gilmore 1988). In a recent study on *A. polyacanthus*, variation in behavioural tolerance to stable elevated CO_2 was linked to the differential expression of genes related to circadian rhythm control and indicated that behaviourally sensitive fish may have been displaying more pronounced acid-base regulation (Schunter et al. 2016). This suggests that diel CO_2 cycles, and associated circadian rhythms, are likely to have an important influence on the sensitivity of reef fishes to elevated CO_2.

Consistent with previous work, I observed that absolute lateralization of juvenile *A. polyacanthus* was significantly reduced under stable elevated CO_2 (Welch et al. 2014; **Chapter 3**). Similar results have been reported in a range of other species (Domenici et al. 2012; Jutfelt et al. 2013; Green and Jutfelt 2014; Lopes et al. 2016; Schmidt et al. 2017). Furthermore, in agreement with a past study, I show that the severity of behavioural impairment under elevated CO_2 is reduced by the presence of diel CO_2 cycles, with partial restoration in the smaller diel-cycling treatment and full restoration in the larger diel-cycling treatment. This is an important finding given the ecological consequences of behavioural abnormalities and that past research has shown a limited capacity for acclimation/adaptation of behavioural traits to stable elevated CO_2 (Welch et al. 2014; Welch and Munday 2017).. The lateralization results suggest that $GABA_A$ receptors were functioning more normally under elevated CO_2 when a diel cycling CO_2 regime was present. This could indicate that fish kept under diel CO_2 cycles underwent less acid-base compensation compared to those reared under stable elevated CO_2. For coral reef fish it appears that complete acid-base regulation in the brain under stable elevated CO_2 levels takes between 24–96 h, as this is the exposure period required before behavioural abnormalities manifest (Munday et al., 2010). My results suggest that for fish reared under diel-cycling CO_2, exposure to lower CO_2 levels for several hours each day is enough to prevent the physiological changes that would normally occur at a stable high CO_2 (see discussion in Chapter 2 for more detail). It is possible that changes in acid-base regulation under diel-cycling elevated CO_2 is linked to altered expression of circadian rhythm genes. Further work is needed to determine if and how acid-base regulation is altered under elevated CO_2 when diel CO_2 cycles are present.

Routine activity and boldness of juvenile *A. polyacanthus* were unaffected by exposure to stable elevated CO_2, which is consistent with some previous work (Nowicki et al. 2012; Heinrich et al. 2016; Sundin et al. 2017). Yet, these observations contrast with other studies on coral reef fish where significant effects of elevated CO_2 on routine activity have been documented (Munday et al. 2010, 2014; Cripps et al. 2011; Devine et al. 2012a; McCormick et al. 2013). Instead, my findings are more consistent with what has been observed in non-tropical species (Maneja et al. 2015; Sundin and Jutfelt 2015; Duteil et al. 2016; Schmidt et al. 2017). The underlying explanation for discrepancies between studies is unclear but may be related to methodological differences. In general, the studies on coral reef fish which have reported significant effects of stable elevated CO_2 on activity/boldness tested fish in the field on natural reef structures, or in an artificial tank environment which had a shelter. In contrast, many of the studies reporting no affects, including this one, conducted open field tests in an artificial environment, generally, without any shelter and/or with minimal acclimation time. Thus, it could be that without the option of a 'safe place' and/or insufficient acclimation time the impacts of elevated CO_2 are masked by the effects of a general stress response.

While routine activity was unaffected by stable elevated CO_2, it was altered by the presence of a ± 500 µatm CO_2 diel cycle, with fish in the cycling elevated CO_2 treatment being less active (-10.2%) and swimming slower (-15.9%) compared to controls. Impaired metabolic performance can likely be ruled out a as a potential underlying mechanism for the observed decrease in routine activity because increased growth was observed under these conditions. I propose that this decrease in routine activity was associated with altered anxiety levels. Activity in a novel environment can be an indicator of anxiety, whereby reduced activity is usually associated with increased anxiety (Egan et al. 2009; Maximino et al. 2010). Increased

anxiety/reduced boldness in stable elevated CO_2 conditions has been observed in juvenile Californian rockfish, *Sebastes diploproa,* (Hamilton et al. 2014) and three-spined stickleback, *Gasterosteus aculeatus*, (Jutfelt et al. 2013), but why I only observed altered anxiety in the largest diel-cycling elevated CO_2 treatment is unclear. Anxiety in fish is linked to $GABA_A$ receptor functioning, with normal function acting to reduce anxiety (Stewart et al. 2011; Hamilton et al. 2014). The behavioural laterization data and previous work (**Chapter 3**), where diel CO_2 cycles had a positive effect on behaviour, suggests that $GABA_A$ receptor functioning is closer to normal under elevated CO_2 when diel CO_2 cycles are present. Thus, it appears that another physiological mechanism, other than the $GABA_A$ pathway, may be responsible for the reduced activity observed in the 1000 ± 500 µatm CO_2 treatment. One possible explanation is that the higher CO_2 peaks may have altered stress hormone levels, such as cortisol, which are known to directly correlate with anxiety behaviours in fish (Egan et al. 2009; Cachat et al. 2010). The link between behavioural responses and stress hormones in fish under elevated CO_2 conditions is currently unknown and would be an interesting area for future work.

All fast start non-locomotor and locomotor variables in juvenile *A. polyacanthus* were unaffected by exposure to elevated CO_2, regardless of whether a stable or cycling regime was experienced. This contrasts with most previous studies on coral reef fish were significant effects of elevated CO_2 on fast starts have been observed (Allan et al. 2013, 2014; Munday et al. 2016). Negative effects of elevated CO_2 on fast start performance were also observed in the temperate species yellowtail kingfish, *S. lalandi* (Watson et al. 2018). My results are comparable to those reported for the coral reef damselfish *Pomacentrus wardi*, where elevated CO_2 was shown to have no effect on escape speed and distance (Allan et al. 2017).

The methods and CO_2 levels used in all studies, including this one, were very similar and so differences are most likely due to species-specific responses.

Temperature effects

Elevated temperature had a clear negative effect on routine activity and boldness of juvenile *A. polyacanthus*. These results contrast with previous experiments on coral reef damselfish were activity and boldness were generally shown to increase at higher temperatures (Biro et al. 2010; McCormick and Meekan 2010). However, the temperatures used in these studies were within the species preferred thermal range. When this is the case fish become more active to seek food so that increased metabolic costs associated with higher temperatures can be met (Biro et al. 2010). In contrast, when temperatures exceed a species optimum, as was the case in this study, reduced activity could represent an energy-saving strategy (Johansen and Jones 2011). Additionally, elevated temperatures have been shown to increase stress hormone levels in fish (Davis 2004) which is potentially another mechanism driving the observed reduction in activity and boldness, although additional testing is needed to confirm this.

In contrast to routine activity, elevated temperature had a small positive effect (4 to 8%) on fast start locomotor variables. Allan et al. (2017) also showed that elevated temperature had a stonger effect on fast start performance compared to elevated CO_2, although in this case the effects were negative. Watson et al. (2018) observed enhanced fast start performance in larval yellowtail kingfish at temperatures above their preferred range, although this was attributed to increased larval size under elevated temperature. While elevated temperature had a negative effect on size of juvenile *A. polyacanthus* in this study there was no difference

in the size of fish selected for fast start trials between treatments. The increased locomotory performance observed in this study may therefore have been linked to the combined effects of reduced viscosity and increased muscle efficiency in warmer water (Fuiman and Batty 1997; Wieser and Kaufmann 1998). The underlying reason for the opposing effects elevated temperature had on routine activity and fasts starts is unknown but may be related to the fact routine activity is under aerobic control whereas fast starts are driven by anaerobic metabolism (Domenici and Blake 1997).

Summary

Diel CO_2 cycles had a significant effect on three ecologically important traits in *A. polycanthus*. Firstly, diel CO_2 cycles were shown to mediate the negative effects of elevated CO_2 on absolute lateralization, confirming results of a previous study. Secondly, diel CO_2 cycles had a significant positive effect on growth under elevated CO_2. Finally, routine activity of fish from the larger diel-cycling CO_2 treatment was significantly lower compared to control fish. Importantly, these effects were observed at both current-day and future elevated temperatures, demonstrating that higher temperature did not significantly alter the effects of diel CO_2 cycles in the traits measured here. These findings highlight the importance of incorporating natural CO_2 variability in experiments to more accurately assess the impacts of future OA on shallow water coastal organisms (Cornwall et al. 2013; Frieder et al. 2014; Ou et al. 2015; Wahl et al. 2018; **Chapter 3**).

In contrast to elevated CO_2, elevated temperature had a significant effect on every trait except behavioural lateralization. Furthermore, in all cases when a trait was significantly affected by both climate change drivers' temperature had the strongest affect. While this is

unsurprising for survival and growth, it is perhaps unexpected for behavioural traits where elevated CO_2 has often been shown to have a significant impact. My results and those of **Chapter 3** suggest that behavioural responses that involve decision making (e.g. lateralization and response to cues) may indeed be more impacted by elevated CO_2, whereas responses which are governed by energetic constraints (e.g. activity, fast starts and competitive interactions) will be more impacted by elevated temperature. This is consistent with other recent studies where elevated temperature had a stronger effect on fast start performance and swimming activity compared to elevated CO_2 (Allan et al. 2017; Watson et al. 2018).

A limitation of this study was that I was unable to include a diel-cycling control treatment. It is possible that fish reared under a diel-cycling control may perform differently than to those reared under a stable control (Cornwall et al. 2013). For example, as was observed under elevated CO_2, it is possible that fish reared under a diel-cycling control may perform better compared to those reared under a stable control and thus the negative effects of elevated CO_2 may still be present. However, based on our current understanding of the impacts elevated CO_2 has on fishes I do not believe this would be case, although further work is needed to confirm this. In this study I only incorporated diel cycles with elevated CO_2 as this was a main stressor of interest. However, shallow water habitats also experience diel cycles in temperature (e.g. Kline et al. 2015), which are also known to affect fish performance (e.g. Hokanson et al. 1977). Importantly, the highest level of temperature and CO_2 cycles occurs at opposite times of the day, with CO_2 cycles having their high peak during the night and temperature cycles having their high peak during the day. Thus, the responses of fish to a cycling CO_2 and temperature environment could be different to a static one, or when one stressor is static as was the case in this study. Future experiments should incorporate both

diel-cycling CO_2 and temperature to further our understating on how marine fish will respond to climate change.

Chapter 5: Diel CO_2 cycles and parental effects have similar benefits to the growth of a coral reef fish under ocean acidification

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5.1 Summary

Parental effects have been shown to buffer the negative effects of within-generation exposure to ocean acidification conditions on the offspring of shallow water marine organisms. However, it remains unknown if parental effects will be impacted by the presence of diel CO_2 cycles that are prevalent in many shallow water marine habitats. Here, I examined the effects that parental exposure to stable-elevated (1000 µatm) and diel-cycling elevated (1000 ± 300 µatm) CO_2 had on the survival and growth of juvenile coral reef anemonefish, *Amphiprion melanopus*. Juvenile survival was unaffected by within-generation exposure to both elevated CO_2 treatments, but was significantly increased (8%) by parental exposure to diel-cycling elevated CO_2. Within-generation exposure to stable elevated CO_2 caused a significant reduction in juvenile growth (10.7-18.5 %), however, there was no effect of elevated CO_2 on growth when diel CO_2 cycles were present. Parental exposure to stable elevated CO_2 also ameliorated the negative effects of elevated CO_2 on juvenile growth, and parental exposure to diel CO_2 cycles did not alter the effects of diel CO_2 in juveniles. The results demonstrate that within-generation exposure to diel-cycling elevated CO_2 and parental exposure to stable elevated CO_2 had similar outcomes on juvenile condition. This study illustrates the importance of considering natural CO_2 cycles when predicting the long-term impacts of ocean acidification on marine ecosystems.

5.2 Introduction

Rapidly increasing atmospheric carbon dioxide (CO_2) levels are driving a reduction in surface ocean water pH, a process referred to as ocean acidification (OA) (Orr et al. 2005; Fabry et al. 2008; Doney et al. 2009). Near-future OA conditions are expected to negatively affect the performance of many marine species (Pörtner and Farrell 2008; Widdicombe and Spicer 2008; Kroeker et al. 2013b; Przeslawski et al. 2015), ultimately impacting community dynamics, ecosystem functioning and services (Hoegh-Guldberg and Bruno 2010; Doney et al. 2012; Kroeker et al. 2012, 2013a; Christen et al. 2013; Nagelkerken and Connell 2015; Nagelkerken and Munday 2016). However, most studies documenting the negative effects of OA on marine organisms have been short-term (i.e. days to weeks) and have focused on a single life-stage, or a single generation (Kroeker et al. 2013b; Calosi et al. 2016). Thus, they do not consider that the conditions experienced in one life-stage or generation can influence the performance of later life-stages and/or generations (Marshall and Morgan 2011; Burton and Metcalfe 2014; Donelson et al. 2018). Understanding such responses is imperative if we are to accurately forecast the long-term persistence of marine populations in a rapidly changing ocean (Munday et al. 2013; Calosi et al. 2016; Donelson et al. 2018).

Parental effects occur when the environmental conditions experienced by the mother and/or father alters the phenotype of their offspring *via* non-genetic inheritance mechanisms (Salinas et al. 2013; Burgess and Marshall 2014; Lane et al. 2015; Guillaume et al. 2016). Thus, parental effects represent a form of phenotypic plasticity that spans generations. Parental effects have the potential to increase offspring fitness, although they can also act to reduce offspring performance (Marshall and Uller 2007; Donelson et al. 2012; Griffith and Gobler 2017). Consequently, parental effects have gained interest as a potential mechanism that may

assist marine organisms to persist in the face of ongoing rapid OA (Munday 2014; Ross et al. 2016; Donelson et al. 2018). Many experimental studies have shown that within-generation exposure to OA conditions predicted to occur by the end of the century can negatively impact a range of traits (including survival, development and growth) in early life-stages of various marine taxa (Harvey et al. 2013; Kroeker et al. 2013b; Przeslawski et al. 2015). However, in some instances early life-stage tolerance to OA is increased when parents experience similar conditions to the offspring (Miller et al. 2012; Murray et al. 2014; Parker et al. 2015; Chakravarti et al. 2016; Rodríguez-Romero et al. 2016).

Many ecologically and economically important marine species live in shallow water coastal habitats. These habitats can experience substantial natural fluctuations in CO_2 on a variety of temporal scales (Hofmann et al. 2011; Duarte et al. 2013; Baumann et al. 2015; Pacella et al. 2018). Despite this, most OA experiments to date on shallow water species have used stable elevated CO_2 levels consistent with open ocean environments, and thus may have limited ecological relevance (McElhany and Busch 2013; Wahl et al. 2016). Perhaps the most well-known CO_2 fluctuations are those that occur over a 24h period, primarily driven by the net effects of photosynthesis and respiration over a day-night cycle (Falter et al. 2013). These diel CO_2 cycles occur in many shallow water coastal ecosystems, including coral reefs, and are expected to increase in magnitude with ongoing OA due to the change in seawater buffering capacity as the ocean absorbs more CO_2 (Shaw et al. 2013a; Pacella et al. 2018). Recent studies accounting for diel CO_2 cycles have demonstrated that they can either alleviate or intensify the within-generation responses of marine organisms to OA (Cornwall et al. 2013; Ou et al. 2015; Chan and Eggins 2017; Jarrold et al. 2017; Mangan et al. 2017; Onitsuka et al. 2018; Wahl et al. 2018). However, it is unknown if diel CO_2 cycles will alter the outcome of

parental effects on offspring traits, which limits our ability to accurately predict how marine organisms will respond to OA in the long-term.

The influence that parental effects have on offspring fitness is related to how predictable variations in environmental conditions are over time (Burgess and Marshall 2014). Consequently, the outcome of parental effects in the presence of diel CO_2 cycles may differ from those expressed in a stable CO_2 environment. To test this, I conditioned adult pairs of coral reef anemonefish, *Amphiprion melanopus*, to control (500 µatm), stable elevated (1000 µatm) and diel-cycling elevated (1000 ± 300 µatm) CO_2 (**Figure 5.1** and **Table 5.1**). Juveniles from control parents were reared in all CO_2 treatments, whereas juveniles from the two elevated CO_2 treatments were reared in the same conditions of their parents. Comparisons between treatments allowed me to determine the within-generation effects of stable-elevated and diel cycling-elevated CO_2 on juvenile growth and survival, and how these responses were modified by parental exposure to either stable-elevated or diel cycling-elevated CO_2.

5.3 Materials and methods

Parental conditioning

Adult breeding pairs of the cinnamon anemone fish, *Amphiprion melanopus*, were collected from the Bramble and Trunk Reef region of the Great Barrier Reef in September 2016. Diel CO_2 cycles on shallow coral reefs in the region have been shown to range between ±50-200 µatm (Albright et al. 2013; Kline et al. 2015), although a threefold amplification is expected to occur by the year 2100 (Shaw et al. 2013a). Breeding pairs were housed in 60 L aquaria and

maintained at temperature conditions matching the collection location (22.5°C winter - 28.5°C summer) for one year prior to the experiment. Pairs were assigned to the control (500 µatm), stable elevated (1000 µatm) and diel-cycling elevated (1000 ± 300 µatm) CO_2 treatments at the end of August 2017 (control CO_2 = 6 pairs, stable elevated CO_2 = 3 pairs and diel-cycling elevated CO_2 = 4 pairs). This allowed pairs to be conditioned in their CO_2 treatments for three months before the start of the breeding season in December 2017. Two pairs in the diel-cycling elevated CO_2 treatment failed to rear enough offspring through to hatching. Therefore, at the beginning of March three pairs from the control treatment (pairs 2,3 and 26), from which offspring had been obtained, where transferred to diel-cycling elevated CO_2. These pairs were allowed to produce two clutches before a clutch was taken for the experiment. Breeding pairs were provided with half of a terracotta pot as a shelter and a spawning site. Temperatures were increased from winter temperatures of 22.5°C at a rate of 0.5°C per week until the summer breeding temperature of 28.5°C was reached in the first week of December 2017 and maintained for the rest of the experiment. A total of 22 clutches were sourced from all pairs between January-May 2018.

Larval and juvenile rearing

Breeding pairs were checked daily for the presence of eggs. On the night of hatching, pots were removed from the 60 L breeding tanks and transferred to an aerated 100 L larval rearing aquarium. During the 12-day pelagic larval stage that occurs immediately after hatching, larvae from pairs reared under diel-cycling elevated CO_2 were reared under stable CO_2 conditions (1000 µatm) to represent the more stable pelagic ocean environments they occupy. Larvae from control and stable elevated CO_2 pairs were reared under the same CO_2 conditions as their parents. On the night of hatching, pots with egg clutches were removed

from the parental aquarium and transferred to an aerated 100 L larval rearing aquarium. Larvae were fed rotifers (*Brachionus* sp.) at 100 individuals' mL^{-1} each morning for the first 5 days. On these days 5 mL of non-viable *Nannochloropsis* algal paste was also added to the tanks to feed the rotifers. During these first 5 days aquariums had no water flow during the day (8am-4pm) and were then slowly flushed with filtered seawater each night. This daily cycle ensured that larvae could feed *ad libitum* throughout daylight hours and that any unconsumed food was removed each night. Half a teaspoon worth of freshly hatched *Artemia* naupli were added from days 3-12. A commercial weaning fish feed (INVE Aquaculture Nutrition Wean-S 200-400 µm) was added from day 10. A summer light cycle of 13 h of light/11 h of dark was simulated with fluorescent lights.

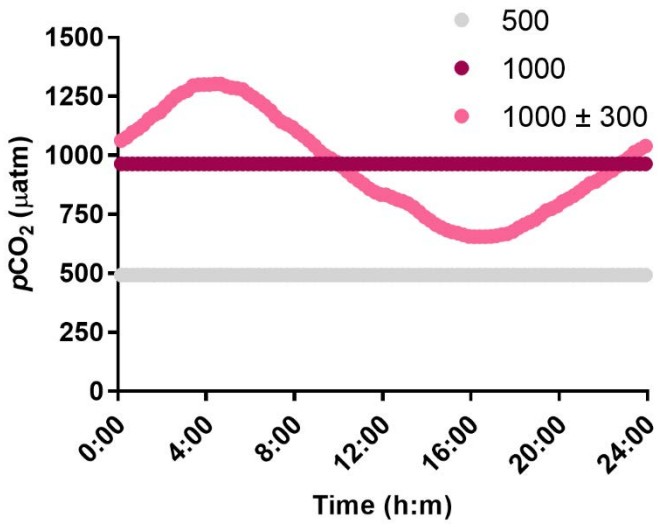

Figure 5.1 Mean daily pCO_2 profiles for the 500, 1000 and 1000 ± 300 µatm CO_2 treatments.

Table 5.1 Experimental Seawater parameters. Values are means ± 1 S.D. for mean, min and max pH (total scale) and pCO_2. Means ± 1 S.D. for total alkalinity (TA), temperature and salinity are also shown

	pCO_2 treatment (µatm)		
Parameter	500	1000	1000 ± 300
Mean pH_T	7.99 ± 0.03	7.75 ± 0.03	7.76 ± 0.09
Min. pH_T	–	–	7.63 ± 0.06
Max. pH_T	–	–	7.88 ± 0.03
Mean pCO_2	493 ± 24	964 ± 50	957 ± 233
Min. pCO_2	–	–	681 ± 63
Max. pCO_2	–	–	1270 ± 127
TA (µmol Kg^{-1})	2495 ± 155	2494 ± 150	2458 ± 129
Temperature (°C)	28.5 ± 0.07	28.5 ± 0.1	28.5 ± 0.09
Salinity (‰)	35.4 ± 0.7	35.4 ± 0.7	35.4 ± 0.8

At 12 days post hatch (dph), settlement stage fish (identified by transition to dark colouration) were randomly transferred into 40 L juvenile rearing tanks (5-12 fish per tank). Juveniles from control CO_2 pairs were split into control (control-control), stable elevated (control-stable) and diel-cycling elevated (control-cycling) CO_2 conditions. Juveniles from stable elevated and diel-cycling elevated CO_2 pairs were only reared in the same conditions as their parents (stable-stable and cycling-cycling). CO_2 treatments were duplicated and juveniles from each clutch were split between duplicate tanks (i.e. one tank per replicate CO_2 treatment). In some cases, there were insufficient juveniles to stock duplicate tanks and so another clutch from that pair was used, with juveniles being placed into a tank on the other replicate CO_2 treatment. Juveniles were reared for 28 days. They were fed a combination of freshly hatched *Artemia* nauplii (100 mL from a stock of 1.5L in which one teaspoon of cysts were hatched) and weaning fish feed (0.05g) daily for the first two dph. From 3-7 dph they were fed once daily (0.05g) on the weaning feed. From 8-28 dph juveniles were fed twice daily (0.05g each time) on the weaning feed. At the end of the rearing period juveniles were euthanised with clove oil anaesthetic. The number of fish remaining in each tank was recorded. Individuals were

then blotted dry, weighed (nearest mg) on an analytical balance (AX224, Sartorius, Bradford, USA) and photographed in a lateral position next to a ruler. Standard length (SL) to the nearest 0.1 mm was estimated for each fish from the digital photographs using ImageJ software (http://rsb.info.nih.gov/ij/).

Experimental system, CO_2 manipulation and seawater parameters

Larval rearing system

The larval rearing system comprised of two independent 8000 L recirculating seawater systems (one for control CO_2 (500 µatm) and one for stable-elevated CO_2 (1000 µatm)). A pH control system (AT Control, Aqua Medic, Germany) dosed CO_2 into 3000 L sumps to achieve the desired pH level for each CO_2 treatment. Each sump fed three 100 L larval rearing aquarium and another experimental room. pH_{NBS} was measured daily using a pH meter (InLab Expert Pro electrode and Seven2Go Pro meter, Mettler Toledo, Switzerland), which was calibrated weekly using standard NBS buffers (InLab Solutions, Mettler Toledo, Switzerland). Temperature was recorded daily with a digital thermometer (Comark C26, Norfolk, UK). Salinity readings were taken weekly using a conductivity sensor (HQ15d; Hach, USA). Total alkalinity was measured weekly using Gran Titration (Metrohm 888 Titrando Titrator Metrohm AG, Switzerland) and certified reference material from Dr. A. G. Dickson (Scripps Institution of Oceanography). pCO_2 values were calculated as a function of pH_{NBS}, temperature and salinity using CO_2SYS (Pierrot *et al*., 2006) employing constants from Mehrbach *et al* (1973) refit by Dickson & Millero (1987) and the $KHSO_4$ dissociation constant from Dickson (1990). Mean values for each of these seawater parameters are presented in **Table 5.2**.

Table 5.2 Experimental Seawater parameters for the larval rearing systems. Values are means ± 1 S.D. for pH (NBS scale) and pCO_2. Means ± 1 S.D. for total alkalinity (TA), temperature and salinity are also shown.

	pCO_2 treatment (µatm)	
Parameter	450	1000
pH_{NBS}	8.15 ± 0.01	7.75 ± 0.03
pCO_2	452 ± 23	1023 ± 72
TA (µmol Kg^{-1})	2369 ± 96	2377 ± 96
Temperature (°C)	28.5 ± 0.08	28.5 ± 0.1
Salinity (‰)	36.1 ± 0.5	35.8 ± 0.6

Juvenile rearing and adult system

Experiments were conducted in a 14,300 L recirculating seawater system. Briefly, the system consisted of an external 3,700 L sump tank connected to a bio-filter, protein skimmer, UV steriliser and a 1000 L algal bio-remediation tank. The external sump supplied water to six separate 1,600 L recirculating systems (two per CO_2 treatment) that comprised of one 1000 L sump tank, eleven 40 L juvenile rearing tanks and three 60 L breeding pair tanks, contained within a temperature-controlled room. Water was supplied to the internal sumps at a rate of approximately 1,600 L *per* day allowing for a complete exchange with the external sump. Holding tanks were supplied with water at a rate of 1 L min^{-1}. Both the internal sumps and holding tanks were aerated with ambient air.

Elevated pCO_2 treatments were achieved by dosing the 1000 L internal sumps with CO_2. This was controlled by solenoid valves (M-Ventil Standard, Aqua Medic, Germany) connected to a pH control system (Aqua Medic AT Control System, Aqua Medic, Germany) with laboratory grade pH electrodes (Neptune Systems, USA). The Aqua Medic AT Control System has a curve function which allowed us to create fluctuating pCO_2 profiles. pH profiles in the fluctuating pCO_2 treatments were recorded using a pH datalogger (Model 850060, Sper Scientific, USA)

set to take a reading every 15 min. For the stable pCO_2 treatments pH_{NBS} was measured daily using a pH meter (InLab Expert Pro electrode and Seven2Go Pro meter, Mettler Toledo, Switzerland). Seawater pH on the total hydrogen ion concentration scale (total scale, pH_T) was measured each week with a spectrophotometer following standard operating procedures (Dickson et al. 2007) using the indicator dye meta/*m*-cresol purple (mCP) (*m*-cresol purple sodium salt 99%, non-purified, Acros Organic). Daily and fluctuating pH_{NBS} measurements were converted to pH_T based on the offset between weekly pH_T and pH_{NBS} measurements. Temperature was recorded daily with a digital thermometer (Comark C26, Norfolk, UK). Salinity readings were taken weekly using a conductivity sensor (HQ15d; Hach, USA). Total alkalinity was measured weekly using Gran Titration (Metrohm 888 Titrando Titrator Metrohm AG, Switzerland) and certified reference material from Dr. A. G. Dickson (Scripps Institution of Oceanography). All seawater parameters were measured in randomly chosen tanks. pCO_2 values were calculated as a function of pH_T, temperature and salinity using CO_2SYS (Pierrot et al. 2006) employing constants from Mehrbach et al. (1973) refit by Dickson and Millero (1987) and the $KHSO_4$ dissociation constant from Dickson (1990). Mean values for each of these seawater parameters are presented in **Table 5.1**.

Statistical analyses

The effect of CO_2 treatment on juvenile survival was tested using a general linearized model with a binomial distribution, which was weighted to the number of fish initially stocked into a tank. The effects of CO_2 treatment on wet weight and standard length were tested using linear mixed effects models. Pair, clutch and tank were included as random effects with tank nested within clutch nested within pair. The number of fish remaining in a tank at the end of the experiment was included as a covariate to account for density-dependent effects.

Additive models were used (i.e. number + CO_2 treatment) based on Akaike information criterion. Pairwise comparisons were made between the control-control and other CO_2 treatments based on the linear model summary outputs. All analyses were conducted using R version 3.4.0. (R Core Team 2017) using the 'lme4' and 'nlme' packages.

5.4 Results

Juvenile survival ranged from 82.2% to 96.7% in the control-stable and cycling-cycling treatments, respectively, and was significantly affected by CO_2 treatment (**Figure 5.2a**, $\chi 2$ = 17.68, df = 4, P = 0.001). Survival in the control-control treatment (88.7 ± 3.6%, mean ± s.e.) was significantly lower than in the cycling-cycling treatment (96.7 ± 2.2%) (z = 2.09, P = 0.037). Juvenile wet weight and standard length were also significantly affected by CO_2 treatment (**Figure 5.2b, c**, min. $F_{4,31}$ = 3.48, P = 0.019). Juvenile exposure to stable elevated CO_2 (control-stable) caused a 18.5% and 10.7% reduction in weight wet and standard length, respectively, compared to the control-control treatment (max. t = -2.08, P = 0.045). However, there were no significant differences between the control-control and other treatment groups (min. t = -2.19, P = 0.051), indicating the both diel CO_2 cycles and parental exposure to elevated CO_2 restored growth to the same level as control. The number of fishes remaining in a tank at the end of the experiment had a significant effect on wet weight and standard length (min. $F_{4,31}$ = 5.66, P = 0.024). Positive relationships between the number of fish in each tank and wet weight/standard length were observed (min. $F_{1,454}$ = 17.59, R^2 = 0.037, P < 0.0001).

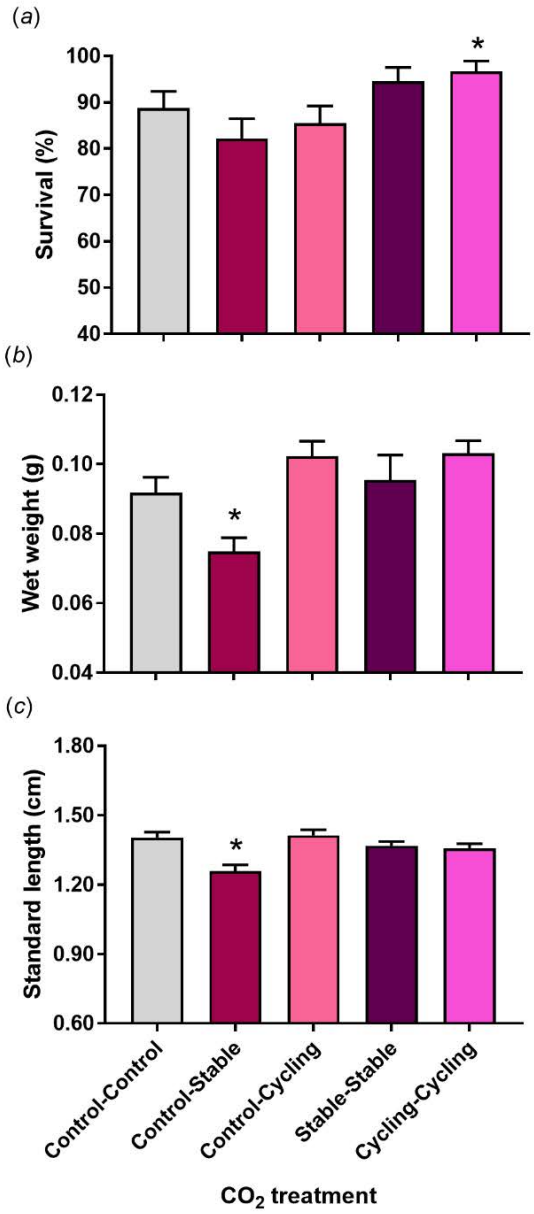

Figure 5.2 Effects of within-generation and parental exposure to stable elevated and diel-cycling elevated CO_2 on (*a*) survival, (*b*) wet weight and (*c*) standard length of juvenile cinnamon anemone fish, *Amphiprion melanopus*. Bars represent means ± S.E. Asterix (*) indicates a significant difference compared to the control-control treatment.

5.5 Discussion

My findings show that both parental effects and diel CO_2 cycles can significantly modify the growth of a coral reef fish under OA conditions. Parental exposure to stable elevated CO_2 (1000 µatm) alleviated the negative effect of high CO_2 on the growth of juvenile *A. melanopus*. This is consistent with past work on the same species (Miller et al. 2012) and the Atlantic silverside, *Menidia menidia*, (Murray et al. 2014). In contrast to juvenile fish reared at stable elevated CO_2, no negative effect on growth was observed in juveniles that experienced within-generation exposure to diel cycling-elevated CO_2 (1000 ± 300 µatm). Diel CO_2 cycles were also shown to alleviate the negative effect of elevated CO_2 on the growth of pink salmon, *Oncorhynchus gorbuscha*, larvae (Ou et al. 2015). Importantly, the beneficial effect of diel CO_2 cycles under elevated CO_2 on growth of juvenile fish in this study was unchanged by parental exposure to the same treatment, suggesting that a diel cycling regime is predictable enough to prevent negative parental effects from occurring.

This work demonstrates that parental exposure to stable elevated CO_2 and within-generation exposure to diel-cycling elevated CO_2 both alleviate the negative effect of elevated CO_2 on juvenile growth of a marine fish. One possible explanation is that energetic costs for juvenile fish living under elevated CO_2 are reduced when parents experience the same CO_2 conditions and when diel CO_2 cycles are present. Consistent with this hypothesis, a previous study on *A. melanopus* showed that parental exposure restored juvenile resting metabolic rates and size under elevated CO_2, demonstrating that the cost of living was lower if parents experienced the same conditions (Miller et al. 2012). Possible mechanisms for restoration of metabolic rates across generations is epigenetic modification of gene expression (Ryu et al. 2018) or the inheritance of acclimated mitochondria from mothers (Shama et al. 2014), as has been

observed in marine fish exposed to elevated temperature. Finally, it is important to mention that improved juvenile growth, caused by altered metabolic rates/energy re-allocation, could have negative consequences later in life as a result of trade-offs between traits (Pechenik 2006; Marshall and Morgan 2011).

The physiological mechanisms responsible for the beneficial effect of diel CO_2 cycles on the growth of juvenile fishes under elevated CO_2 are uncertain. However, there is evidence to suggest that acclimation of metabolic rates may be responsible. For example, in addition to alleviating the negative effects of elevated CO_2 on growth, a diel cycling CO_2 regime also restored maximal metabolic rates of *O. gorbuscha* larvae to levels seen in control fish (Ou et al. 2015). Alterations in metabolic rates under elevated CO_2 are thought be linked to the costs associated with defending acid-base status (Pörtner and Knust 2007). Under elevated CO_2 fishes actively increase intracellular and extracellular HCO_3^- concentrations to prevent plasma and tissue acidosis, a process which takes place over a time-scale of hours to days depending on the level of acidification experienced (Heuer and Grosell 2014). Consequently, the presence of diel CO_2 cycles could mean that elevated CO_2 levels are not experienced for long enough to allow complete acid-base regulation to occur. This could potentially result in more energy being available for other processes and warrants further investigation.

This study shows that parental effects are not required to improve juvenile fish growth under elevated CO_2 if diel CO_2 cycles are present. Thus, my research adds to a growing body of literature which highlights the importance of incorporating natural CO_2 variability in OA experiments to accurately predict the responses of shallow water coastal marine species to rising CO_2 levels (Cornwall et al. 2013; Ou et al. 2015; Jarrold et al. 2017; Mangan et al. 2017;

Wahl et al. 2018). Further work is needed to determine how parental exposure modifies the responses of other marine organisms to diel-cycling elevated CO_2, especially those that are more sensitive to OA than most fishes, as a more challenging parental environment might lead to negative outcomes for offspring fitness in some species (Marshall and Uller 2007).

Chapter 6: General Discussion

Accurately predicting the responses of shallow water coastal marine organisms to ocean acidification is a major challenge for marine scientists. To assess how an organism will respond to future ocean acidification consideration of the CO_2 conditions it currently experiences and how these will change is crucial. Many shallow water coastal marine habitats are characterised by natural diel CO_2 cycles that are expected to amplify in the future. To date, very few studies have considered these diel CO_2 cycles when investigating the potential impacts of ocean acidification on shallow water marine organisms, instead using stable CO_2 treatments consistent with atmospheric and open ocean projections. This thesis investigated how the presence of diel CO_2 cycles affected the early life-history development and behaviour of coral reef fishes under elevated CO_2. In general, I found that diel CO_2 cycles improved the performance of coral reef fishes under ocean acidification. Furthermore, I found that these beneficial effects were still present under elevated temperature and where not altered by parental effects.

Effects of ocean acidification and diel CO_2 cycles on early development

Juvenile survival in all three species tested was unaffected by both stable and diel-cycling elevated CO_2 (**Chapter 2,4 and 5)**. However, elevated temperature altered the effects of diel-cycling elevated CO_2 on juvenile survival in *Acanthochromis polyacanthus* (**Chapter 4**). While there was a trend of decreased survival in all the CO_2 treatments at 31°C, significant differences were only observed in the two diel-cycling elevated CO_2 treatments. Interestingly, I noticed that most mortality was observed in the mornings and within the first week of the

experiment. These observations indicate that deaths were occurring at night during the high CO_2 peaks. Thus, it appears that for newly hatched fish, which might not have fully developed acid-base regulation capacity, and for which the cost of homeostasis is greater (Brauner 2009), the metabolic demands to tolerate the higher CO_2 levels caused by diel cycles may become too great under elevated temperature. For reef fishes, this result suggest that the most vulnerable time could be when they transition from being pelagic larvae to benthic juveniles. Not only must they invest considerable energy in metamorphosis to a benthic life form, they also transition from a relatively stable open water CO_2 environment to the much more variable CO_2 environment on coral reefs, with diel CO_2 cycles. Hypoxia tolerance is known to increase dramatically at the settlement stage in reef fishes (Nilsson et al. 2007), and because O_2 and CO_2 levels are likely to be strongly correlated, they might also become more CO_2 tolerant at this stage. Nevertheless, fish might be most vulnerable to elevated CO_2 and temperature in the first few days after settlement, as was observed in this experiment. Finally, this result demonstrates for the first time that elevated temperature has the potential to modify the interaction between elevated CO_2 and diel CO_2 cycles, thus highlighting the importance of performing multi-stressor experiments to more accurately predict organism responses to climate change (Riebesell and Gattuso 2015; Gunderson et al. 2016).

There was a non-significant trend of decreased growth in juvenile *A. polyacanthus* and increased growth in juvenile *Amphiprion percula* reared under stable elevated CO_2 (**Chapter 2 and 4**). In contrast, exposure to stable elevated CO_2 had a significant negative effect on growth in juvenile *Am*. melanopus (**Chapter 5**). These results are generally in agreement with past studies on the same species (Munday et al. 2009a, 2011b; Miller et al. 2012). In **Chapter 2**, there was a non-significant trend for fish reared under diel-cycling elevated CO_2 being more

similar in size to control fish, compared to those reared under stable elevated CO_2. I argue that this lack of statistical significance may be due to low statistical power as only three clutches per species were used in that experiment. This is confirmed in **Chapter 4,** which used four clutches of *A. polyacanthus*, and where fish reared under diel-cycling CO_2 were significantly larger compared to fish reared under stable elevated CO_2. Similarly, diel CO_2 cycles alleviated the significant negative effects of elevated CO_2 on growth in juvenile *Am*. melanopus (**Chapter 5**). Importantly, the beneficial effects of diel CO_2 on growth were still present for *A. polyacanthus* under elevated temperature (**Chapter 4**) and where not altered by parental effects in *Am*. melanopus (**Chapter 5**). Positive effects of diel CO_2 cycles on growth under elevated CO_2 were also reported in the freshwater stage of larval pink salmon, *O. gorbuscha* (Ou et al. 2015).

The observation that diel CO_2 cycles can have a positive effect on growth under elevated CO_2 (**Chapter 4 and 5**) infers that it is energetically less expensive for fish to live in a diel-cycling elevated CO_2 environment compared to a stable elevated CO_2 environment. In line with this, a concurrent study to **Chapter 4** showed that exposure to stable elevated CO_2 increased resting metabolic rates of juvenile *A. polyacanthus* highlighting an increased cost of living; however diel CO_2 cycles (1000 ± 500 µatm) completely mitigated this cost, restoring metabolic rates to control levels (Laubenstein et al. submitted). Additionally, Ou et al. (2015) reported that maximal metabolic rates of larval pink salmon after transition to saltwater were negatively impacted by stable elevated CO_2, but not a by diel-cycling conditions. It has been hypothesized that elevated CO_2 induces a metabolic cost in fish because of increased acid-base regulation (Perry and Gilmour 2006; Pörtner and Knust 2007; Baker and Brauner 2012). If this is the case, my results would suggest that the cost of acid-base regulation under

elevated CO_2 is reduced by the presence of diel CO_2 cycles, ultimately resulting in more energy available for life-history processes such as growth. Indeed, complete acid-base regulation upon exposure to elevated CO_2 in fish occurs on time-scales of hours to days, depending on the species and level of CO_2 (Heuer and Grosell 2014). Thus, acid-base regulation may be dampened when diel CO_2 cycles are present, as environmental CO_2 will be declining before complete pH compensation has occurred.

Effects of ocean acidification and diel CO_2 cycles on behaviour

Consistent with past research (Dixson et al. 2010; Munday et al. 2010; Nilsson et al. 2012; Welch et al. 2014; Heuer et al. 2016), I observed negative effects of exposure to stable elevated CO_2 on behavioural responses in *A. polyacanthus* and *Am. percula* (**Chapter 3** and **Chapter 4**). Exposure to stable elevated CO_2 has been shown to negatively impact a range of ecologically important behaviours in numerous species of marine fish (reviewed in Clements and Hunt 2015; Nagelkerken and Munday 2016; Cattano et al. 2018). However, a number of studies have reported no impacts of elevated CO_2 on fish behaviour (e.g. Jutfelt and Hedgärde 2013; Heinrich et al. 2016; Cattano et al. 2017; Kwan et al. 2017; Schmidt et al. 2017; Sundin et al. 2017; Laubenstein et al. 2018). The reasons for discrepancies between studies are unclear but may be related to the behavioural traits chosen, as there is evidence of trait-specific responses to elevated CO_2. For example, in **Chapter 4** stable elevated CO_2 had a negative effect on behavioural lateralization in *A. polyacanthus* but had no effect on routine activity and fast start performance (see also Hamilton et al. 2014; Sundin and Jutfelt 2015; Schmidt et al. 2017). Another potential reason for differences between studies is the method in which behavioural traits were measured (Jutfelt et al. 2017). For example, Hamilton et al.

(2014), using two established methods, a light/dark preference test and a shelter test, tested the effect of stable elevated CO_2 on anxiety in juvenile Californian rockfish, *Sebastes diploproa*, but only observed a significant effect in the light/dark test. Future studies, especially those investigating untested species, should therefore test a diverse range of behavioural responses and/or use multiple methods to test the same behaviour to more accurately assess behavioural sensitivity to ocean acidification.

The presence of diel CO_2 cycles reduced the negative effects of elevated CO_2 on behavioural lateralization in *A. polyacanthus* (**Chapter 3 and 4**) and response to a predator cue in *Am. percula* (**Chapter 3**). The extent of reduction was dependent on both the magnitude of the cycles (*A. polyacanthus* only - **Chapter 3 and 4**) and the mean CO_2 level experienced (*A. polyacanthus* and *Am. percula* - **Chapter 3**). In **Chapter 3** and **Chapter 4**, exposure to 1000 ± 300 and 1000 ± 500 µatm CO_2 caused partial and full recovery, respectively, of behavioural lateralization in *A. polyacanthus*. In **Chapter 3**, behavioural impairments were not observed in either species at a mean CO_2 of 700 µatm when a diel CO_2 cycle was present but were still evident at a mean CO_2 of 1000 µatm. Overall, these results mean that earlier studies may have overestimated the effects of elevated CO_2 on the benthic phase of reef fishes, because they did not include the ameliorating effects of diel CO_2 cycles. Furthermore, they indicate that diel CO_2 cycles will delay the onset of behavioural abnormalities in natural populations of reef fishes. In the only other study to investigate the effects of diel CO_2 cycles on behavioural performance, Kwan et al. (2017) showed that they did not modify individual light/dark preferences and shoaling behaviour in juvenile Blacksmith, *Chromis punctipinnis*. However, in this case exposure to stable elevated CO_2 also had no effect on behaviour. Consequently, the research presented here is the first to demonstrate that diel CO_2 cycles can

significantly modify the behavioural performance of shallow water coastal marine organisms under ocean acidification. The observation that diel CO_2 cycles can reduce and/or delay the onset of behavioural abnormalities in juvenile coral reef fish under ocean acidification is a particularly important finding given that past research has shown a limited capacity for acclimation/adaptation of behavioural traits to stable elevated CO_2 (Munday et al. 2014; Welch et al. 2014; Welch and Munday 2017).

In contrast to the behavioural lateralization results, diel cycling CO_2 conditions (1000 ± 500 µatm) had an apparent negative effect on routine activity of *A. polyacanthus* compared to control fish (**Chapter 4**). Impaired metabolic performance can be ruled out a as a potential underlying mechanism for the observed decrease in routine activity because increased growth was observed under these conditions. I therefore propose that the decrease in activity was linked to altered anxiety levels. While not one of the designated anxiety traits, routine activity in a novel environment can be used as an indicator of anxiety, where reduced activity is usually an indicator of increased anxiety (Egan et al. 2009; Maximino et al. 2010).

Behavioural abnormalities in fish under ocean acidification conditions are thought to be linked to the effects of acid-base regulation on the functioning of type A γ-aminobutyric acid ($GABA_A$) neurotransmitter receptors, which have specific conductance for HCO_3^- and Cl^- (Nilsson et al. 2012; Hamilton et al. 2014; Heuer et al. 2016). Under elevated CO_2 fishes increase intracellular and extracellular HCO_3^- concentrations, with a corresponding decrease in Cl^-, to prevent plasma and tissue acidosis (Wood et al. 1990; Baker et al. 2009; Heuer and Grosell 2014). This altered ion gradient is thought to turn some $GABA_A$ receptors from inhibitory to excitatory, ultimately leading to behavioural impairments (Heuer et al. 2016).

The behavioural lateralization and response to a predator cue results (**Chapter 3** and **Chapter 4**) suggest that $GABA_A$ receptors were functioning more normally under elevated CO_2 when a diel cycling CO_2 regime was present. This could indicate that fish kept under diel CO_2 cycles were undergoing less acid-base compensation compared to those reared under stable elevated CO_2. Anxiety in fish is also linked to $GABA_A$ receptor functioning, with normal function acting to reduce anxiety (Stewart et al. 2011; Hamilton et al. 2014). Therefore, the observed decrease in activity of fish reared under the large diel-cycling elevated CO_2 regime (**Chapter 4**) may indicate that fish were less anxious, despite this being a non-typical response. Whether reduced activity in the large diel cycling CO_2 treatment was driven by increased or reduced anxiety remains unclear. Either way, another physiological mechanism, other than the $GABA_A$ pathway, is likely responsible. One possible explanation is that the higher CO_2 peaks may have altered stress hormone levels, such as cortisol and serotonin, which are known to directly correlate with activity and anxiety-associated behaviours in fish (Winberg and Nilsson 1993; Egan et al. 2009; Cachat et al. 2010; Maximino et al. 2013; Hamilton et al. 2016).

Ion and hormone regulation in fish are under circadian control (Peterson and Gilmore 1988; Pavlidis et al. 1999; Dmitriev and Mangel 2000; Balment et al. 2006; Nikaido et al. 2010; Falcón et al. 2011). An important feature of circadian clocks is that they are entrained or reset by daily changes in environmental conditions. In fish, daily cycles in light and temperature are the main cues used to entrain their circadian rhythms, due to their reliability as timing cues (Zachmann et al. 1991; Pavlidis et al. 1999; Falcón et al. 2011). However, it is possible that daily CO_2 cycles may also play a part in controlling circadian rhythms in fish from shallow water habitats. In a recent study on *A. polyacanthus*, variation in behavioural tolerance to stable

elevated CO_2 was linked to the differential expression of genes related to circadian rhythm control in brain tissue (Schunter et al. 2016). Specifically, offspring of CO_2 sensitive parents (i.e., those that exhibited behavioural abnormalities) upregulated the enzyme that catalyses the final reaction in the synthesis of melatonin. Melatonin is a key molecule involved in the entrainment of circadian rhythms in vertebrates and its daily variation in production plays an important role in controlling ion-regulation, behaviour (including locomotor activity) and other hormone levels (e.g. cortisol) (Azpeleta et al. 2010; Falcón et al. 2011). The results of Schunter et al. (2016) suggest that behaviourally sensitive fish may have been displaying more pronounced acid-base regulation due to stronger influence of circadian rhythm control. Indeed, disruptions of circadian rhythms are known to have behavioural ramifications in vertebrates (Barnard and Nolan 2008; Karatsoreos et al. 2011). The observations that diel CO_2 cycles can modify behavioural responses under elevated CO_2 (**Chapter 3 and 4**) suggests that fish were displaying a different level of circadian control over processes which can influence behavioural responses. Consequently, it appears that circadian rhythm control of some physiological processes in coral reef fish may be disrupted under stable elevated CO_2, which could affect the conclusions of studies that fail to incorporate natural diel CO_2 cycles in their design.

Future research priorities

Diel CO_2 cycles had a positive effect on growth and behaviour of coral reef fishes under ocean acidification (**Chapters 3-5**). These results indicate that fish reared under diel-cycling elevated CO_2 were undergoing less acid-base regulation compared to fish reared under stable elevated CO_2. Thus, it may be that for fish reared under diel-cycling CO_2, exposure to lower CO_2 levels

for several hours each day is enough to prevent the physiological changes that would normally occur at a stable high CO_2. Furthermore, it is possible that changes in acid-base regulation under diel-cycling elevated CO_2 may be linked to altered expression of circadian rhythm genes (Schunter et al. 2016). Further work is needed to determine if and how acid-base regulation under elevated CO_2 is altered when diel CO_2 cycles are present to develop a mechanistic understanding behind the responses observed here.

In addition to diel CO_2 cycles, coral reefs and other shallow water coastal habitats also experience diel cycles in temperature (McCabe et al. 2010; Baumann et al. 2015; Kline et al. 2015; Challener et al. 2016; Page et al. 2016, 2017; Takeshita et al. 2018). Diel temperature cycles on coral reefs are typically in the range of 0.5-2°C, but in extreme cases (e.g. shallow reef flats and tidal lagoons) can be as large as 6-8°C. Cycling temperature conditions can also modify organism performance (Hokanson et al. 1977; Dame and Vernberg 1978; Akesson and Costlow 1991; Podrabsky and Somero 2004; Biro et al. 2010; Paganini et al. 2014). Importantly, the highest level of temperature and CO_2 cycles occurs at opposite times of the day, with CO_2 cycles having their high peak during the night and temperature cycles having their high peak during the day. Thus, the responses of fish to a cycling CO_2 and temperature environment could be different to when temperature is kept static as was the case in **Chapter 4**. For example, I would expect cycling temperature conditions to mediate the negative effects elevated temperature had on juvenile survival of *A. polyacanthus* under diel-cycling elevated CO_2 conditions (**Chapter 4**). Future experiments should incorporate both diel-cycling CO_2 and temperature to further our understating on how shallow water coastal marine organisms will respond to future climate change.

Concluding remarks

This thesis presents the first works to investigate how diel CO_2 cycles affects the early development and behaviour of coral reef fishes under ocean acidification conditions. Furthermore, I test if elevated temperature and parental effects influence the interaction between elevated CO_2 and diel CO_2 cycles. I show that diel CO_2 cycles can improve growth and behavioural performance of coral reef fishes under ocean acidification conditions. Importantly, these beneficial effects were still present under elevated temperature and where not altered by parental effects. Similar beneficial effects of diel CO_2 cycles have also been observed in corals (Dufault et al. 2012; Comeau et al. 2014; Chan and Eggins 2017; Enochs et al. 2018). The results of this thesis add to a growing body of work that highlights the critical importance of incorporating natural CO_2 variability in ocean acidification experiments to more accurately assess the impacts of future ocean acidification on shallow water coastal marine organisms (e.g. Cornwall et al. 2013; Frieder et al. 2014; Ou et al. 2015; Mangan et al. 2017; Onitsuka et al. 2018; White et al. 2018).

While coral reefs have been relatively well characterised in terms of diel CO_2 cycles, other shallow water coastal habitats remain under- or un-sampled (Wahl et al. 2016). Consequently, there is an urgent need for more high resolution *in situ* studies that characterise natural CO_2 variability both spatially and temporally. Such data will be essential to establish ecologically relevant CO_2 treatments to be used in laboratory experiments and allow us to better interpret results from past ocean acidification studies that have employed stable CO_2 levels (McElhany and Busch 2013). This will be critical for accurately assessing the potential effects of ocean

acidification on shallow water marine organisms and which species and ecosystems may be at greatest risk.

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