Intergenerational effects of climate change on a coral reef fish, *Amphiprion melanopus*

Thesis submitted by

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Declaration of Ethics

The research presented in this thesis was conducted in accordance with the national Health and Medical Research Council (NHMRC) Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 7th Edition (2004) and the Queensland Animal Care and Protection Act (2001). The research received and was conducted under the animal ethics approval from the JCU Animal Ethic Committee Approval number #A1427.

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General Abstract

The marine environment is facing the dual threats of rising temperature and ocean acidification. Coral reef ecosystems are thought to be especially sensitive to these threats due to evolving in a relatively stable thermal environment and the susceptibility of reef building corals to ocean acidification. For populations of marine organisms to persist under climate change conditions, they must be able to reproduce and produce offspring that will survive in the environment. This study examined the effects of ocean acidification and increasing temperature on reproduction in a coral reef fish, the cinnamon anemonefish (*Amphiprion melanopus*) and tested how parental effects influence the susceptibility of the offspring to these dual stressors.

Ocean acidification is predicted to negatively affect reproduction of marine organisms, but few studies have tested this prediction in marine fishes. In **Chapter 2**, I determined the effect of ocean acidification on reproduction of *A. melanopus* by exposing adult breeding pairs to near-future CO_2 levels for a period of 9-months. A current-day control (~400µatm) and two CO_2 treatments (moderate (~600µatm) and high (~1000µatm)) were used based on CO_2 projections for the year 2100. Contrary to expectations, high CO_2 conditions stimulated reproduction. Pairs exposed to high CO_2 produced twice as many clutches, 60% more eggs and had 80% higher reproductive output compared with controls. Despite the increase in fecundity there were no changes in egg or hatchling size. This suggests, that for some species, increased CO_2 may not be as stressful as previously thought. Further, it suggests that a relatively small increase in CO_2 could potentially have stimulatory effects (a hormetic response) in reef fish. While no negative effects of increased reproductive effort were detected for either the adults or the offspring, it is possible that there could be effects in the longer-term that were not possible to investigate in this study.

Increasing CO_2 is the main driver of rising temperature and ocean acidification; consequently these stressors will affect the marine environment simultaneously. In **Chapter 3** I examined the interactive effects of increased temperature and ocean acidification on reproduction in fish using a current-day

control (~400µatm) and two CO_2 treatments (moderate (~600µatm) and high (~1100µatm)) fully crossed with a current-day temperature control (+0.0°C (+28.5°C)), and 2 elevated temperature treatments (+1.5°C (30.0°C) and +3.0°C (31.5°C)). Reproductive activity was recorded throughout the breeding season and adult body condition was determined at the end of the breeding season. Elevated CO_2, by itself, only affected some hatchling traits, significantly reducing hatchling length at high CO_2 and reducing yolk area in both CO_2 treatments. Increased temperature, in contrast, had a more detrimental effect on reproductive performance. Notably there was a decline in reproduction with increasing temperature across all CO_2 treatments, with no reproduction occurring at +3.0°C. There was no effect of increasing CO_2 or temperature on adult body condition (Fulton's K) or hepatosomatic index, suggesting that the decline in reproduction was not due to increased energy expenditure at higher temperatures. Instead the decline in reproduction may be due to changes in hormone concentrations or efficacy. Plasma 17β-estradiol concentrations were highest in the treatment groups with the highest reproduction and were significantly lower with +3.0°C increase. However, whether this was a treatment effect or due to a correlation between hormone production and the stage of oocyte development is unknown. These results suggest that increasing temperature is a greater threat than ocean acidification to reproduction in reef fishes.

The impact of ocean acidification on marine organisms may depend on the strength and direction of intergenerational effects. In **Chapter 4**, I examined the potential intergenerational effects of increased CO_2 by comparing the performance of offspring reared at the same CO_2 as their parents (control, moderate and high), with the performance of offspring from control parents that were reared at high CO_2 from hatching. This design allowed me to examine the acute (within generation) effects of elevated CO_2 on juvenile growth and survival and to determine if parental effects altered this response. Offspring in each CO_2 treatment were also reared at 3 different temperatures to examine the interactive effects of ocean acidification and global warming. Juvenile survival, weight, length and routine metabolic rate were measured at 31-days post hatching. Juveniles from control parents that were reared under high CO_2 had significantly lower performance compared with juveniles reared in control

conditions. However, offspring from elevated CO_2 parents performed as well, if not better under high CO_2 conditions than control offspring. A decline in performance was detected with increasing temperature irrespective of CO_2 treatment. These results show that there is scope for transgenerational acclimation to ocean acidification, with the parental effects appearing to prime the offspring for the environment they will experience, thereby improving performance in a high CO_2 environment. Studies that neglect parental or transgenerational effects may not provide a true representation of the effects of ocean acidification.

Recent studies have shown that increased CO_2 can increase otolith size in fish, however parental effects have not yet been investigated. In **Chapter 5** I examined size and shape morphometrics of otoliths from juvenile *A. melanopus* using the same experimental design as Chapter 4. This allowed me to determine both the acute and parental effects of increased CO_2 on this important sense organ. I detected no significant differences in otolith size shape or asymmetry associated with acute or parental exposure to elevated CO_2 or increased temperature. These findings are consistent with earlier studies on coral reef fish otoliths and, combined, suggest that not all fish will be sensitive to elevated CO_2. I suggested reasons for differences in sensitive and provide caveats for interpretation and extrapolation of the literature on otolith calcification under elevated CO_2, and provide suggestions for future research.

This research is the first to examine the interactive effects of high CO_2 and elevated temperature on fish reproduction and to test the potential for acclimation to ocean acidification in coral reef fish. My research shows that life history traits of reef fish may be relatively resilient to ocean acidification as a result of the capacity for transgenerational acclimation. However, reef fish reproduction is very sensitive to increasing temperature. Future studies should examine interactions between multiple climate change stressors, include the potential for transgenerational acclimation, and investigate how reproduction may change over multiple generations of exposure to rising temperature and ocean acidification.

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Chapter 1: General Introduction

Anthropogenic climate change is affecting ecosystems across the globe. Shifts in species distributions and the timing of key life history events have already been observed (Parmesan, 2006; Poloczanska *et al.,* 2013) and there are predictions of massive losses in biodiversity and fundamental changes in ecosystem function (Hoegh-Guldberg & Bruno, 2010) if climate change continues unabated. While temperature rise is a general phenomenon of global climate change, marine ecosystems will also face ocean acidification, caused by the increased uptake of CO_2 from the atmosphere (Royal Society, 2005; Doney *et al.,* 2012). Although many marine organisms already cope with diurnal and seasonal fluctuations in pH (Hofmann *et al.,* 2011; Albright *et al*., 2013) and temperature (Lough, 2012: Albright *et al.,* 2013), these variables are now changing at a rate that may outpace the ability for many marine organisms to adapt (Hoegh-Guldberg *et al.* 2013). Recent research has documented the potential for some animals, including fish, to acclimate to rising temperature within one or two generations (Donelson *et al.,* 2012, 2014; Salinas & Munch, 2012). Whether marine fishes will be able to acclimate in a similar way to ocean acidification is unknown. Furthermore, ocean acidification and increased temperature are predicted synergistically affect performance (Pörtner & Farrell, 2008). Despite this, research on climate change effects on marine organisms has predominantly focused on these stressors in isolation, with relatively few studies addressing the potential combined effects. With that in mind, this thesis aims to elucidate the combined effects of ocean acidification and elevated

temperature on performance in fishes and to determine whether they are able to acclimate to ocean acidification across generations.

The other CO_2 problem

Since industrialization, fossil fuel combustion, cement production and land use change have been emitting increasing amounts of carbon dioxide (CO_2) and other greenhouse gases into the atmosphere. Average global atmospheric CO_2 concentrations have risen from ~280ppm preindustrial to over 395ppm (Meehl *et al.*, 2007; Peters *et al.,* 2012). As recently as May 2013 atmospheric CO_2 concentrations reached 400ppm (Tans & Keeling, 2014). If CO_2 emissions continue unabated atmospheric CO_2 is projected to exceed 900ppm by 2100 (Meinshausen *et al.,* 2011; Collins *et al.,* 2013, Fig. 1.1).

The surface oceans are in approximate gas equilibrium with the atmosphere, consequently, as atmospheric CO_2 rises, oceanic CO_2 also increases. Since industrialization, the oceans have absorbed ~30% of the excess CO_2 that has been released by anthropogenic activities (Sabine *et al.,* 2004; The Royal Society, 2005). As CO_2 dissolves in the oceans it reacts with water molecules and carbonate ions according to the following net reaction:

$$H_2O + CO_2(\text{aqueous}) + CO_3^{2-} \rightarrow 2HCO_3^- + H^+$$

As a result of additional CO_2 being absorbed by the oceans, pCO_2, hydrogen and bicarbonate ions increase, carbonate ions and seawater pH decrease (Caldeira & Wickett, 2003; Sabine *et al.,* 2004). Average ocean pH has already decreased from pre-industrialization levels by 0.1 pH units, and is currently declining at a rate of 0.0013 pH units per year (Hoegh-Guldberg *et al.,* 2013). Under current emissions scenarios (RCP8.5), rising CO_2 levels would lead to a

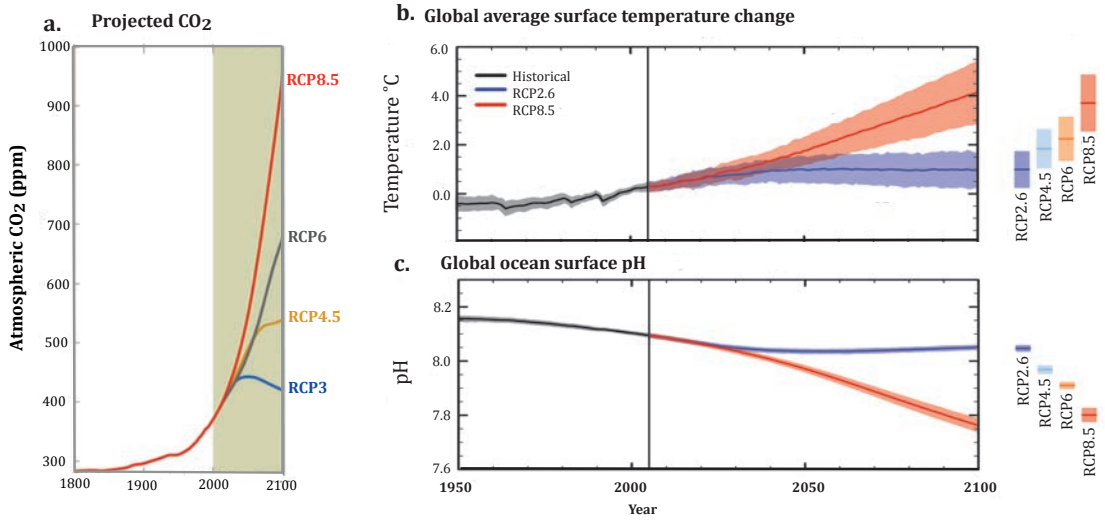

Figure 1.1 Atmospheric CO_2 levels projected to occur by 2100 (a) caused by anthropogenic activities under different representative concentration pathways (RCP) and the resulting increase in global surface temperatures relative to 1986-2005 (b) and surface ocean pH (c). Figures are modified from Meinshausen *et al.,* 2011 (a) and IPCC Summary for Policy Makers (2013) (b,c).

further 0.3-0.5 decline in pH and a 56% reduction in available carbonate ions by 2100 (Caldeira & Wickett, 2003; Collins *et al.,* 2013).

Carbonate declines result in a decrease in the saturation state of calcium carbonate, which is required by many calcifying organisms as part of the shell or skeleton formation. Due to this constraint calcification is likely to decrease in a number of species as the saturation state of calcium carbonate (and it's polymorphs) decreases. The saturation level of calcium carbonate has been steadily declining and is predicted to decrease to W_{arag} = 2.8 by the 2100 due to ocean acidification (Kleypas *et al.,* 1999). The increase in CO_2 and decline in CO_3^{2-} and W_{arag} associated with ocean acidification have been shown to reduce calcification in a number of different species including coccolithophores (Riebesell *et al.,* 2000), corals (Gattuso *et al.*, 1998; Langdon *et al.,* 2000) and sea urchins (Byrne *et al.,* 2013; Courtney *et al.,* 2013; see review Hofmann *et*

al., 2010). However the effect of ocean acidification on calcification rates differs among taxa (Ries *et al.,* 2009) with higher order species, such as echinoderms and crustaceans, generally increasing calcification under elevated CO_2 (Kroeker *et al.,* 2010). The variability in calcification responses is suggested to be due to variability in the form of $CaCO_3$ utilized by the organism (Ries *et al.,* 2009), the ability of the organism to control pH at the site of calcification (Cohen & McConnaughey, 2003; Taylor *et al.,* 2007) and the buffering capacity of symbiotic organisms (Kroeker *et al.,* 2010).

In addition to calcification, ocean acidification has been shown to affect other aspects of invertebrate life history. Reproduction (Fitzer *et al.,* 2012; Cohen-Rengifo *et al.,* 2013), larval settlement and growth (Albright & Langdon, 2011; Hettinger *et al.,* 2013), and survival (Watson *et al.,* 2012) have all been shown to decline with increasing CO_2 in various invertebrates. However, as with calcification, these effects vary between species and between life history stages. For example in echinoderms, reproduction (Kurihara & Shirayama, 2004; Havenhand *et al.,* 2008), larval and juvenile growth and survival (Dupont *et al.,* 2008; Brennand *et al.,* 2010) are reported to be negatively affected by elevated CO_2. But these effects have not been seen in all echinoderms, with just as many studies reporting no negative, and sometimes positive effects of ocean acidification (Dupont *et al.,* 2010; Havenhand & Schlegel, 2009; Pecorino *et al.,* 2014). The variability between life history stages and species suggests that some species will experience bottlenecks of survival with serious consequences for population survival during the coming century. However, ocean acidification will not exclusively affect invertebrates, with fishes predicted

to be sensitive to ocean acidification, though for different reasons than invertebrates.

Ocean acidification and fishes

For fishes the main concern of ocean acidification is not the decrease in carbonate ions, but the increase in ambient pCO_2 and decrease in pH. Elevated CO_2 in the surrounding water causes an increase in pCO_2 in the blood and tissues of fishes, which in turn causes acidosis (Ishimatsu *et al.,* 2004). Early studies on elevated CO_2 in fishes focused on CO_2 levels (>5000µatm) far in excess of projected levels associated with contemporary climate change. Nevertheless, these studies demonstrated that fishes have good acid-base regulatory mechanisms. In the event of acidosis, fishes are able to compensate or minimize the disturbance by a number of mechanisms, including but not limited to: a) physico-chemical buffering with bicarbonate (HCO_3^-) for non-bicarbonate ions (usually Cl^-) or through Na+/K+/ATPase activity and b) exchanging acid-base equivalent ions between the cells and the blood or the blood and the surrounding environment (Claiborne *et al.,* 2002; Evans *et al.,* 2005; Brauner & Baker, 2009). These processes occur at the gills, kidneys or intestines, with the gills being the major organ involved in acid-base regulation and ion exchange (Evans *et al.,* 2005). Fishes experiencing acidosis caused by elevated CO_2 actively compensate for the disturbance in acid-base balance by accumulating HCO_3^- in their blood (Brauner & Baker, 2009; Heuer *et al.,* 2012; Esbaugh *et al.,* 2012).

These compensatory mechanisms are predicted to be costly (Brauner & Baker, 2009; Melzner *et al.,* 2009a; Ishimatsu *et al.,* 2008) and could reduce

energy available for other processes, resulting in declines in growth, reproduction and survival. Yet, early research at levels higher than projected to occur by 2100 suggests that adult marine fishes are generally quite tolerant to elevated CO_2. No declines in growth were detected in adult wolfish (*Anarhichas minor*; Foss *et al*., 2003) or salmon (*Salmo salar*; Fivelstad *et al.,* 1998, 2003) at levels as high as 5900µatm, and the energetically costly process of maximum swimming speed was also not affected when Atlantic cod were exposed to similar levels of CO_2 (Melzner *et al.,* 2009a). Aspects of reproduction also appear to be tolerant to elevated CO_2, with no negative effects on reproductive propensity detected in pipefish (*Sygnathus typhle*; Sundin *et al.,* 2012). Nor has sperm motility been affected in 12 different species of fish across two studies (Inaba *et al.,* 2003; Frommel *et al.,* 2010). Sperm motility was only affected in 5 flatfish species, with motility arresting under elevated CO_2 (Inaba *et al.,* 2003).

Fish early life history stages are predicted to be more sensitive to ocean acidification as their homeostatic mechanisms are not fully developed (Ishimatsu *et al.,* 2008; Melzner *et al.,* 2009b; Brauner, 2009). Consequently, many studies of ocean acidification effects on fishes have focused on early life history and juvenile stages. Despite predictions, few studies have documented significant negative effects of ocean acidification on the growth and development of larval or juvenile fishes. Early and recent studies alike have generally reported a tolerance to elevated CO_2 with few studies noting significant declines in growth or survival from egg through to juvenile life stages (Munday *et al.,* 2011a; Bignami *et al.,* 2012; Frommel *et al.,* 2012a; Hurst *et al.,* 2012, 2013). Additionally, the energetic costs associated with acid-base

regulation were predicted to reduce performance in energetically costly activities, such as swimming. Yet, declines in swimming speed or kinematics have not been detected as a result of elevated CO_2 in juveniles (Munday *et al.,* 2009a; Maneja *et al.,* 2012) or in adult fish (Melzner *et al.,* 2009a).

Despite this apparent tolerance in fishes to elevated CO_2 some recent studies at CO_2 levels relevant to current ocean acidification have observed declines in survival of eggs (Forsgren *et al.,* 2013; Chambers *et al.,* 2014) and juvenile stages (Baumann *et al.,* 2012; Frommel *et al.,* 2012b). Additionally, some of the negative effects of ocean acidification that have been detected have been subtle. Declines have been detected in larval energy stores (e.g yolk sac), which could affect future growth and survival (Munday *et al.,* 2009a; Pope *et al.,* 2014). Damaged organ tissue has also been noted, with two species displaying damage to liver tissues when reared under high CO_2 (Atlantic cod, Frommel *et al.,* 2012a; Flounder, Chambers *et al*., 2014). While these effects are less severe than direct effects on growth and survival, they could still result in serious declines in performance, and possibly survival for fishes, under elevated CO_2 (CO_2 effects summarized Fig. 1.2).

The research conducted to date suggests that ocean acidification can affect fishes in unexpected ways and sometimes in ways that are contradictory to predictions. One of the most consistent effects of elevated CO_2 has been the increase in otolith size (ear stones). Otoliths are calcium carbonate structure.

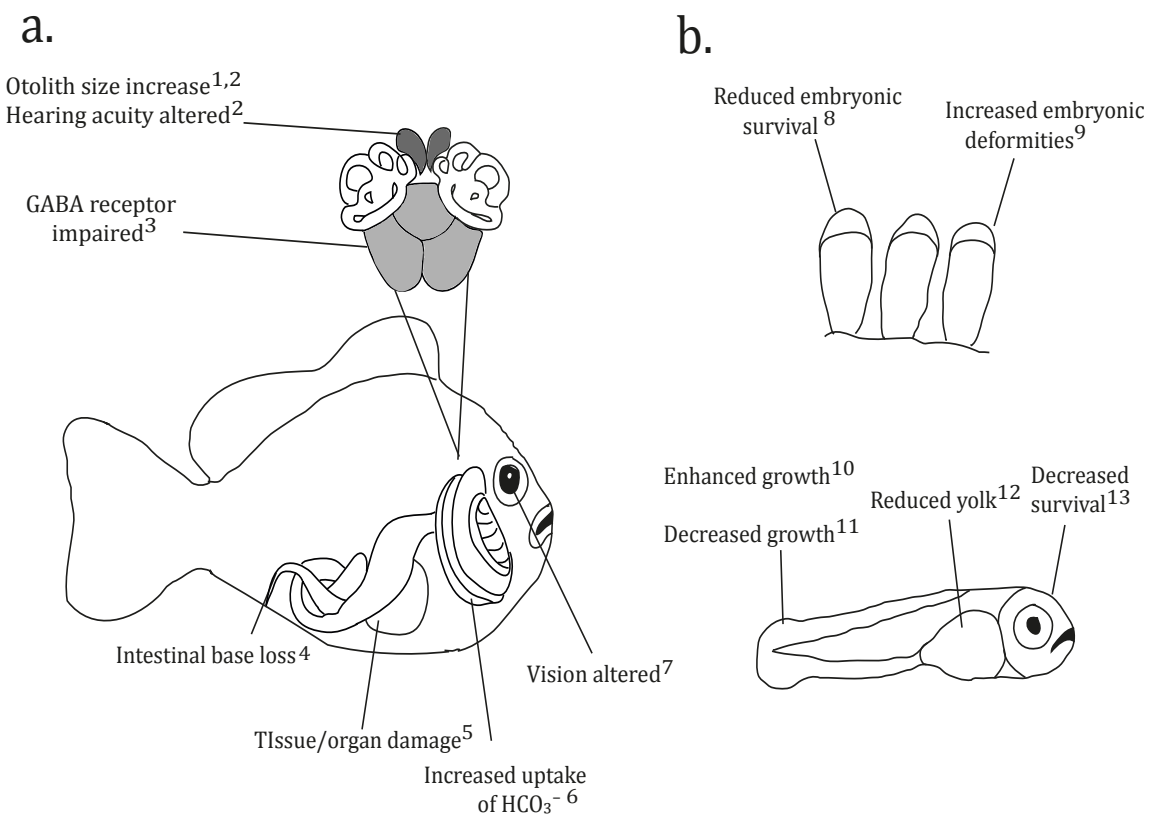

Fig. 1.2 The majority of research has noted no negative effects of CO_2 on fish life history traits, however, some studies do note subtle but important negative effects in both adult/juvenile (a) and early life history (b) stages of fishes. 1, Checkley *et al.,* 2009; 2. Bignami *et al.,* 2013; 3, Nilsson *et al.,* 2012; 4, Heuer *et al.,* 2012; 5, Frommel *et al.,* 2012; 6, Esbaugh *et al.,* 2012; 7, Chung *et al.,* 2014, 8, Chambers *et al.,* 2014; 9 Forsgren *et al.,* 2013 ;10, Chambers *et al.,* 2014; 11, Miller *et al.,* 2012 ;12, Munday *et al.,* 2009a ;13, Baumann *et al.,* 2012

located near the brain and are an important sensory organ used for sensing vibration, spatial orientation, speed and acceleration (Popper & Lu, 2000). Increased otolith size in response to elevated CO_2 has been documented in five species (Checkley *et al.,* 2009; Munday *et al.,* 2011b; Hurst *et al.,* 2012; Bignami *et al.,* 2012, 2013; Maneja *et al.,* 2013). These species are from varying habitats with differing life history characteristics, yet all display a similar increase in otolith size, albeit at different levels of CO_2. It is suggested that the increase in otolith size is a consequence of the change in inorganic carbon concentrations in the blood and endolymph surrounding the otolith, as a result

of acid-base regulation increasing HCO_2 / HCO_3^-. Similarly, behavioural disruptions (reviewed in Briffa *et al.,* 2012) documented in many species are also suggested to be due to changes in blood chemistry interfering with neurotransmitter function (Nilsson *et al.,* 2012; Chung *et al.,* 2014; Hamilton *et al.,* 2014).

Even with the increase in research focusing on the effects of ocean acidification on fishes, there are still areas that are not well understood. Two areas that require a better comprehension are: 1) how ocean acidification may interact with other climate change stressors, particularly increasing temperature, to affect fishes and, 2) given that climate change will occur over generational time scales for some species, how multigenerational exposure to ocean acidification may influence species responses.

Increasing temperatures

In addition to absorbing excess CO_2, the oceans are a heat sink, absorbing roughly 93% of excess heat energy created by the enhanced greenhouse effect from 1971-2000 (Hoegh-Guldberg *et al.,* 2013). The result is that the sea surface (0-700m) is warming. In regions with coral reefs the average temperature has already increased by between 0.07-0.13°C (Hoegh-Guldberg *et al.,* 2013) per decade till now, and is predicted to increase by up to 3°C by 2100 (Lough, 2007; Lough, 2012; Fig. 1.1). This increase in temperature will undoubtedly cause changes in species biology, distributions and phenology (Pörtner, 2002; Poloczanska *et al.,* 2013).

Temperature plays a critical role in physiological processes; from the shape of proteins, enzymes and hormones, to the speed of chemical and

enzymatic reactions through to whole organism metabolic demands. For ectothermic animals, that do not physiologically regulate their internal temperature, changes in environmental temperature can affect their physiological processes (Bret, 1971; Houde, 1989). As temperatures increase, the energetic cost of maintaining cellular function rises, increasing the basic cost of living and, unless available energy increases to match requirements, reduces the energy available for other processes (Bret 1971; Houde, 1989; Sokolova *et al.,* 2012) and can manifest in declines in growth, body condition, energy reserves, reproduction and, at temperature extremes, survival.

As maintaining plasticity to a range of temperatures is costly, populations have evolved specific thermal niches that they are best suited to living in (Kleypas *et al.,* 1999; Tewksbury *et al.,* 2008). Generally, as temperatures increase from the thermal minimum, performance is enhanced, up to the thermal optimum. Past the thermal optimum performance rapidly declines (Pörtner & Farrell, 2008; Fig. 1.3). For tropical species, a small increase in average temperature can have more serious consequences than for their temperature counterparts as tropical species often have narrower thermal niches due to evolving in a more thermally stable environment (Tewksbury *et al.,* 2008; Deutsch *et al.,* 2008; Fig. 1.3).

There is increasing evidence that many tropical species are living close to or at their thermal optimum (Neuheimer *et al.,* 2011; Rummer *et al.,* 2013a) and consequently even a small increase in temperature could exceed their thermal optimum. Within tropical fishes, attributes such as metabolic capacity (Johansen & Jones, 2011; Rummer *et al.,* 2013a), swimming performance and

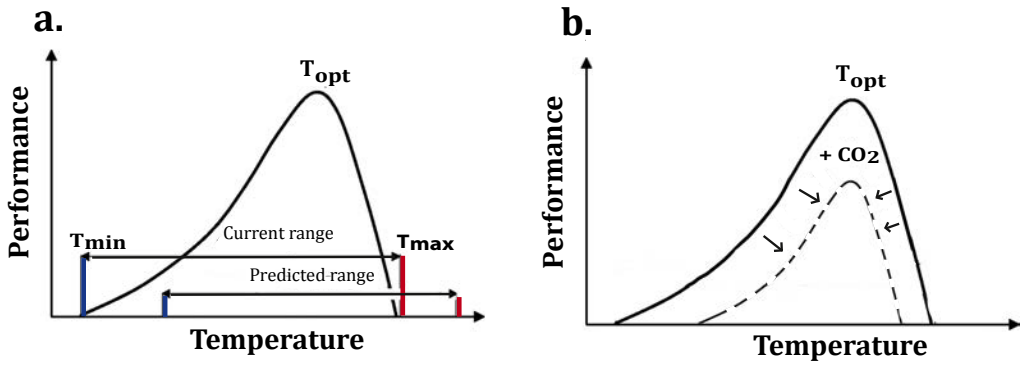

Figure 1.3. As temperature increases (a) past a species thermal optimum (Topt), a given performance trait will decline rapidly to the thermal maximum (Tmax). Currently most species experience temperatures that are well within their thermal limits, however temperature increases will result in species experiencing temperatures above their optimum more regularly. When elevated CO_2 is included as a stressor (b), species performance for a given temperature is predicted to be reduced. Modified from Tewksbury *et al.,* 2008 and Pörtner & Farrell, 2008.

activity patterns (Johansen & Jones, 2011; Johansen *et al.,* 2013), growth (McCormick & Molony, 1995; Green & Fisher, 2004; Sponaugle *et al.,* 2006; Donelson *et al.,* 2012) and survival (Gagliano *et al.,* 2007; Donelson *et al.,* 2012) have been shown to decline rapidly when fishes are exposed to temperatures in excess of those usually experience by the population.

Elevated temperature also impacts on reproduction in fishes. Reproduction only occurs within the confines of a specific thermal window, which is often narrower than the organism's thermal window (Pankhurst & King, 2010; Pankhurst & Munday, 2011). Rising temperature affects reproduction at a number of different levels. As mentioned above, the cost of maintaining cellular processes increases when temperatures exceed the thermal optimum for a given species or population, reducing the amount of energy available for reproduction and altering how parents partition that energy (Bernardo, 1996;

Pörtner & Farrell, 2008). This reduction in energy can manifest as declines in the number of offspring produced, both in terms of clutches produced across a season and the number of eggs produced per clutch (Stearns, 1992; Hilder & Pankhurst, 2003; Brown & Sibly, 2006). Increasing temperature can also reduce the amount of energy provided to the offspring (e.g. yolk sac), potentially reducing the offspring performance and survival (Donelson *et al.,* 2010).

Elevated temperatures can also directly influence reproductive physiology. Reproductive hormones are thermally sensitive and elevated temperatures can reduce their efficacy, shape and solubility (Pankhurst & Munday, 2011). This can result in the appropriate hormones not reaching or being recognized by the correct receptor, or being excreted (Van Der Kraak & Pankhurst, 1997; Pankhurst & Munday, 2011). Whatever the particular alteration to the hormone, these changes disrupt the hormonal cascade and can lead to reproductive failure.

Synergistic effects of CO_2 and temperature

Rising temperatures will occur simultaneously to ocean acidification, and these stressors combined are expected to exert a larger response than either alone (Pörtner & Farrell, 2008). A number of studies have examined the interactive effects of ocean acidification and increasing temperature on performance traits in invertebrates, often showing that temperature exerts a strong influence on the given traits (Byrne *et al.,* 2009; Pansch *et al.,* 2012; Byrne & Przeslawski, 2013). There is comparatively little research on the potential interactive effects of ocean warming and acidification on performance in fishes, with a few

exceptions (Munday *et al.,* 2009b; Frommel *et al.,* 2010; Nowicki *et al.,* 2012; Pope *et al.,* 2014). Given the predicted declines in growth and reproduction under acidified conditions, the known declines in growth and reproduction in response to elevated temperatures and the predicted combined effects of ocean acidification and elevated temperatures, understanding how these two stressors will affect reproduction and the resulting offspring is crucial.

Transgenerational effects

Conspicuously absent, given the interest in how ocean acidification will affect invertebrate reproduction, is research on how ocean acidification will affect fish reproduction. While some attempts have been made to investigative fish reproduction under acidified conditions (Inaba *et al.,* 2003; Frommel *et al.,* 2010; Sundin *et al.,* 2012; Forsgren *et al.,* 2013), these studies were all short-term exposure, from minutes (Inaba *et al.,* 2003; Frommel *et al*., 2010) to days (Sundin *et al.,* 2012; Forsgren *et al.,* 2013). Such a short-term exposure may not be long enough to allow for the more subtle or chronic effects of ocean acidification to occur. Furthermore, the effects of parental exposure to high CO_2 on the performance of offspring has not been investigated. For example, while Forsgren *et al.,* (2013) detected declines in hatchling yolk size, it is not known whether effects on the eggs and larvae will be amplified or potentially compensated by parental exposure (parental effects).

Parents have the ability to influence the phenotype of their offspring – termed parental effects. One way this can occur is through genetic inheritance, for example larger individuals produce larger offspring because of the genes they pass on to their offspring. In addition, parents can influence the phenotype

of their offspring through non-genetic mechanisms (Uller, 2008). Non-genetic parental effects can encompass a wide range of mechanisms including nutritional provisioning (e.g. yolk reserves), transfer of somatic elements (e.g. proteins and hormones), and behavioural learning (Bonduriansky *et al*., 2012). Epigenetic mechanisms, including DNA methylation and changes in chromatic structure are another way in which parents can influence the quality and performance of their offspring (Bonduriansky *et al*., 2012). Critically, epigenetic effects can prime physiological processes for optimum performance in the environment that offspring will likely experience. Either way, parents take cues from the environment they experience (e.g. temperature shifts, increasing CO_2) and may match their offspring's phenotype to improve the offspring's performance in it's natal environment (Burgess & Marshall, 2014).

Parental effects include energy provisioning, such as changes in quality or quantity of yolk or milk and hormonally induced behavioural and morphological changes, that can influence early life history characteristics, such as size at hatching and larval growth rates (Mousseau & Fox, 1998; Uller, 2008). Generally, parental effects are thought to be adaptive, with parents (usually mothers) providing their offspring with sufficient energy for a good start. However, reproduction often causes a trade-off between the current and future reproductive events, with mothers altering energy expenditure in one reproductive event for the benefit, or at the expense of, future reproductive events (Smith & Fretwell, 1974; Bernardo, 1996).

Recently, interest has been increasing in another form of parental effects, epigenetic mechanisms (Bonduriansky *et al.,* 2012). Epigenetics refers to stable alterations in gene expression that occur through processes of DNA

methylation, chromatin structure, and RNA modifications (Jaenisch & Bird, 2003). Through such mechanisms the environment can induce changes in gene expression, altering the phenotype of the individual, often adaptively. These changes are then held stable within the organism through the process of mitosis and can be passed on from parents to offspring, resulting in transgenerational acclimation (Jablonka & Raz, 2009; Bonduriansky *et al.*, 2012).

Transgenerational acclimation occurs when the environment that the parents experience directly shapes the offspring reaction norm for that environment (Salinas & Munch, 2012), thus resulting in offspring that are better suited to that environment. Transgenerational acclimation effectively allows for faster transmission of adaptive phenotypes, even if the genetic variation is absent (Bonduriansky *et al.,* 2012; Salinas *et al*., 2013). The speed of transgenerational acclimation is particular important, as it potentially allows populations to respond to rapidly changing environmental conditions, such as climate change. Recent studies have examined the potential for transgenerational acclimation to environmental changes in a variety of taxa (Whittle *et al.,* 2009; Steigenga & Fischer, 2007; Donelson *et al*., 2012; Salinas & Munch, 2012; Parker *et al*., 2012). Through these studies, it is clear that transgenerational acclimation could play a strong role in the survival of populations in the coming century. Furthermore, parental effects and transgenerational acclimation have the potential to influence adaptive evolution in response to climate change (Benton *et al.,* 2005; Burgess & Marshall, 2011). Consequently, it is important that studies investigating species' responses to climate change consider transgenerational acclimation in their design.

Figure 1.4: Adult *Amphiprion melanopus* on the Great Barrier Reef with its host the bubble tip anemone, *Entacmaea quadricolor*.

Study species

This thesis examined the potential effects that elevated CO_2 and temperatures projected to occur in the ocean by the end of this century (Collins *et al.,* 2013; Kirtman *et al*., 2013) have on the reproduction and early life history traits of the cinnamon anemonefish, *Amphiprion melanopus* (Fig. 1.3). The species chosen is commonly found on coral reefs of the Great Barrier Reef and the broader Indo-Pacific region and is amenable to being reared in captivity. In its natural habitat *A. melanopus* live in social groups, sometimes containing multiple breeding pairs (Myers, 1991; Drew *et al*., 2008). Throughout the summer breeding season (November – April) *A. melanopus* lay multiple benthic clutches containing oblong shaped eggs, which usually hatch within 7-9 days. During this time, the male of the breeding pair provides the majority of parental care, tending to the eggs, providing oxygenation by fanning, and removing any eggs

that are diseased or not developing correctly (Breder & Rosen, 1966; Michael, 2008). At the end of the egg stage, the larvae hatch around an hour after dark (Green & McCormick, 2001). Larvae are pelagic for approximately 11 days, before they are competent to settle back to the reef (Bay *et al.,* 2006).

For the research conducted in this thesis, adult *A. melanopus* were collected from colonies on reef in the central Great Barrier Reef: Orpheus Island (18.62°S, 146.49°E), Bramble Reef (18.42°S, 147.64°E) and Slasher's Reef (18.47°S, 147.08°E). The anemonefish on these reefs are expected to genetically panmictic due to their close proximity (Bay *et al.,* 2006; Jones *et al*., 2010). Further, these reefs experience approximately the same water temperature range, including a summer breeding temperature of 28.5°C.

Aims

This thesis investigates the effects of near-future ocean conditions on the reproduction and early life history traits of a reef fish. While it is predicted that increasing pCO_2 will negatively affect reproduction, there is very little direct evidence to support this assumption. Only a small number of studies have examined reproduction under elevated CO_2 in fish and all previous studies focused on short-term exposure rather than an entire breeding season. The effect of elevated CO_2 on reproductive attributes and offspring condition of a reef fish across an entire breeding season is examined in Chapter 2. This chapter examined a current-day control and two elevated CO_2 levels that could occur in the surface waters by 2100 (~600µatm and ~1000µatm). Increasing water temperatures are also predicted to effect reproduction, and will occur concurrently with elevated CO_2. In combination, these stressors are predicted

to have a stronger effect that either stressor alone, but few studies on fishes have examined these stressors in combination. Chapter 3 utilizes a fully orthogonal 3 x 3 design of a current-day control and two near-future CO_2 levels (moderate and high) and a current-day control (+0.0°C) and 2 temperature (mid century (+1.5°C), and end century (+3.0°C)) treatments on the reproductive attributes, adult physiological and hormonal condition, and offspring condition. As with Chapter 2, Chapter 3 examined these reproductive attributes across an entire breeding season, providing a comprehensive assessment of the effects of elevated CO_2 and temperature on reproduction in this reef fish.

Once the effects of ocean acidification on reproduction are established, the focus of this thesis shifts to the potential for the environment experienced by the parents to modify the phenotype of the resulting offspring, through non-genetic inheritance. To do so, the offspring from Chapter 2 were reared in either their parental CO_2 conditions, or clutches produced by control parents were acutely exposed to high CO_2 from hatching. In addition temperature effects were overlaid on the CO_2 effects, in a fully orthogonal design of four CO_2 groups and three temperature treatments, to determine the combined effects of these stressors. Chapter 4 specifically examined the growth, survival and oxygen consumption of the juveniles produced from the parents in elevated CO_2. Chapter 5 then examined otolith growth in these juveniles, as changes in otolith growth are consistently documented in response to elevated CO_2. Evidence presented in this thesis greatly expands our knowledge of how reef fish populations may be affected by future ocean conditions.

Chapter 2: Increased CO_2 stimulates reproduction in a coral reef fish

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Abstract

Ocean acidification is predicted to negatively impact the reproduction of many marine species, either by reducing fertilization success or diverting energy from reproductive effort. While recent studies have demonstrated how ocean acidification will affect larval and juvenile fishes, little is known about how increasing partial pressure of carbon dioxide (pCO_2) and decreasing pH might affect reproduction in adult fishes. We investigated the effects of near-future levels of CO_2 on the reproductive performance of the cinnamon anemonefish, *Amphiprion melanopus*, from the Great Barrier Reef, Australia. Breeding pairs were held under three CO_2 levels (current-day Control (430μatm), Moderate (584μatm) and High (1032μatm)) for a 9-month period that included the summer breeding season. Unexpectedly, increased CO_2 dramatically stimulated reproductive activity in this species of fish. Over twice as many pairs bred in the Moderate (67% of pairs) and High (55%) compared to the Control (27%) CO_2 treatment. Pairs in the High CO_2 group produced double the number of clutches per pair and 67% more eggs per clutch compared to the Moderate and Control groups. As a result, reproductive output in the High group was 82% higher than the Control group and 50% higher than the Moderate group.

Despite the increase in reproductive activity, there was no difference in adult body condition between the three treatment groups. There was no significant difference in hatchling length between the treatment groups, but larvae from the High CO_2 group had smaller yolks than Controls. This study provides the first evidence of the potential effects of ocean acidification on key reproductive attributes of marine fishes and, contrary to expectations, demonstrates an initially stimulatory (hormetic) effect in response to increased CO_2. However, any long-term consequences of increased reproductive effort on individuals or populations remains to be determined.

Introduction

Successful reproduction is critical for ensuring an individual's genes are carried forward to the next generation and replenishing the population with new individuals. However, reproduction can be expensive due to the energy spent provisioning gametes and offspring, or time spent on courting or nest defense behaviours (Stearns, 1992; Cox *et al.,* 2010). In females, egg or offspring provisioning requires the allocation of significant amounts of energy, which often comes from energy stored during non-reproductive periods (Watson *et al*., 1998; Visser & Lessells, 2001; Grazer & Martin, 2011). For males, energy expenditure usually comes in the form of mate acquisition, nest construction and nest defense (Gillooly & Bayliss, 1999; Husak & Swallow, 2011). Due to the costs involved, reproduction often occurs within a narrow range of environmental conditions that favour offspring survival (Cushing, 1969; Visser *et al*., 2009). Reproduction is also sensitive to environmental cues (Dawson, 2008), with many species requiring specific environmental conditions to trigger

breeding (Pankhurst & Porter, 2003). As reproduction is energetically costly and reliant on specific environmental conditions, if those environmental conditions change or place extra energetic demands on the individual, reproduction might decline or cease altogether with consequences for population sustainability.

Semelparous species reproduce once during their lifetime, putting all available energy into a single reproductive event with the trade-off being the mortality of the adults (Roff, 1992). In contrast, iteroparous species reproduce multiple times throughout their lifetime and are able to adjust their reproductive effort to suit current environmental conditions (Roff, 1992). Iteroparous species can respond in one of two different ways to environmental stressors. Individuals can reduce their investment in current reproduction, thereby saving energy for future reproduction (Clutton-Brock, 1984; Hamel *et al.,* 2011). Alternatively, individuals can invest more energy into current reproduction, in an attempt to offset any negative impacts of environmental stressors on reproductive performance and to potentially increase offspring survival (Paul *et al.,* 1993). In this circumstance there would be additional demand placed on energy acquisition or energy stores to meet the energetic requirements of increased reproductive activity. If environmental conditions are so poor that the adult is unlikely to survive to reproduce again when conditions improve, it may invest all available energy in a final reproductive event, but at the expense of its own survival (terminal investment; Clutton-Brock, 1984).

Reproduction in fishes is highly regulated and reliant on specific environmental conditions (Munro *et al.,* 1990; Conover, 1992; Van Der Kraak & Pankhurst, 1997). Salinity, temperature, photoperiod, water flow and food availability are all known to influence breeding and to determine reproductive

success in fishes (Munro *et al.,* 1990; Hilder & Pankhurst, 2003). In many marine fishes, gamete maturation and spawning are dependent on increasing photoperiod and temperatures in spring (Pankhurst & Porter, 2003), which coincide with optimal conditions for larval growth and survival. Many fish species only reproduce within a narrow range of the temperatures they normally experience (Van Der Kraak & Pankhurst, 1997). Furthermore, if temperatures exceed their normal breeding conditions, or a temperature increase occurs during a particularly sensitive phase (such as gamete maturation), reproduction may cease (Donelson *et al.,* 2010; Pankhurst & Munday, 2011). Consequently, reproduction in fish may be particularly sensitive to the impacts of global climate change (Van Der Kraak & Pankhurst, 1997; Pankhurst & Munday, 2011).

For marine fishes the impacts of climate change will not be limited to increasing temperature. Marine fish must also cope with the increase of partial pressure of carbon dioxide (pCO_2) in their environment, due to increased uptake of atmospheric CO_2 at the ocean surface (Caldeira & Wickett, 2005; Doney, 2010). For fish, exposure to high pCO_2 in seawater causes an increase in plasma pCO_2, which acts to acidify blood and tissue (Brauner & Baker, 2009). Fish have well-developed acid-base regulatory systems and are able to restore their pH, despite the acidifying effects of higher pCO_2, by the exchange of acid-base relevant ions with the external environment (Brauner & Baker, 2009; Esbaugh *et al.,* 2012). However, increased regulation of pH through ion transport is predicted to be energetically expensive (Pörtner *et al.,* 2004; Ishimatsu *et al*., 2008). An increase in energy use to maintain acid-base balance could reduce the amount of energy available for other activities, including reproduction (Ishimatsu *et al.,* 2008; Sokolova *et al.,* 2012). Increased

energy requirements for pH homeostasis could affect female reproductive output, or reduce the provisioning of eggs, with potential consequences for offspring quality. However, one recent study found that aerobic scope, which is an indicator of individual performance, increased in marine fish exposed to near future CO_2 levels (Rummer *et al.,* 2013b). While the mechanisms responsible for increased aerobic scope at near-future CO_2 remains uncertain, this result suggests that the energy available for reproduction could potentially increase under acidified conditions in some species, and therefore, reproduction could potentially increase as well.

To date only a handful of studies have examined the effects of ocean acidification on reproduction in fish. Inaba *et al.* (2003) tested the effect of direct CO_2 gas application on the motility of activated sperm from 16 species of fish from a range of different families. CO_2 affected the sperm motility of 5 species, all of which were flatfishes, with activity arresting within 30ms of CO_2 being applied; however, there was no effect of CO_2 on sperm motility in the 11 other species tested. Similarly, Frommel *et al.* (2010) found no effect of increased CO_2 on sperm motility of Baltic cod (*Gadhus morhua*) when sperm was activated using acidified seawater. Sundin *et al.* (2012) found no effect of decreased pH on the reproductive propensity of a pipefish (*Sygnathus typhle*), however animals were exposed to altered pH for a maximum of 5 hours. No studies have yet been conducted for extended periods of time to allow for any impacts of increased pCO_2 on gametogenesis or energy provisioning of the gametes to occur, or examined reproductive performance across a breeding season. Given the predicted increased cost of acid-base balance, it might be expected that adults exposed to continuous acidified conditions will exhibit a

decline in reproductive activity through reductions in the number of clutches or eggs produced, or declines in egg provisioning, with subsequent consequences for offspring quality.

We tested the hypothesis that long-term exposure to increased pCO_2 would decrease reproductive activity, either through reductions in gamete production, provisioning or offspring quality. To do this we placed breeding pairs of the cinnamon anemonefish *Amphiprion melanopus* into either a current-day control or one of two increased pCO_2 treatments prior to the start of the breeding season and allowed them to reproduce naturally over a 9-month period. These pCO_2 treatments were representative of current day conditions and predicted mid century (541ppm) and end of century (936ppm) pCO_2 levels in keeping with the RCP8.5 scenario (Meinshausen *et al.,* 2011). We monitored reproductive activity on a daily basis and compared the number of clutches produced, the average number of eggs produced, reproductive output, offspring quality and adult body condition.

Material and methods

Study species and collection

The anemonefish, *Amphiprion melanopus*, is common on coral reefs throughout the Indo-Pacific where it occurs in colonies containing multiple breeding pairs (Drew *et al.,* 2008). *A. melanopus* is a serial benthic spawner, laying multiple clutches of oblong shaped eggs during the summer breeding season. Embryonic duration is usually between 7 and 9 days, during which time the male tends the eggs (Michael, 2008). The larvae have a pelagic phase of approximately 11 days, after which they are competent to settle to the reef.

Breeding pairs of *A. melanopus* were collected in the austral winter (late May - early July) from 4 adjacent reefs within the Orpheus Island region of the Central Great Barrier Reef: Orpheus Island (18.6183°S, 146.4936°E), Bramble Reef (18.417°S, 146.700°E), Davies Reef (18.83°S, 147.63°E) and Slasher's Reef (18.467°S, 147.083°E). Anemonefish populations on these reefs are expected to be genetically panmictic because of their close proximity (Bay *et al*., 2006; Jones *et al*., 2010). These reefs experience approximately the same mean summer water temperature of 28.5°C. Pairs were collected using hand nets and dilute clove oil (Munday & Wilson, 1997). Breeding pairs were transported to the Marine and Aquaculture Research Facility at James Cook University where each pair was housed in an individual 45l aquarium.

Experimental systems and CO_2 manipulation

The experiment used three 8000l recirculating aquarium systems, each set to a different CO_2 and corresponding pH level. The CO_2 treatments were a current day Control (430µatm), a mid century Moderate CO_2 (584µatm) and an end of century High CO_2 (1032µatm). The Moderate and High treatment levels are consistent with the RCP8.5 (Meinshausen *et al.,* 2011) scenario for atmospheric CO_2 levels projected for the middle and end of this century. An Aqua Medic AT Control system was used to maintain the desired pH level of each system, by dosing CO_2 into a 3000l sump. The equilibrated seawater was then delivered to the individual aquaria at a rate of ~1.5lmin^{-1}. pH_{NBS} and temperature (°C) in the aquariums were recorded daily using a Hach pH (HQ40d) probe and a Comark C26 temperature probe. Total alkalinity was estimated weekly by Gran Titration (Metrohm 888 Titrando Titrator) and using

certified reference material from Dr. A.G. Dickson (Scripps Institute of Oceanography). Salinity was measured weekly using a Hach multimeter (HQ15d). Aqua Medic dosing set points were adjusted as needed to maintain the desired pCO_2 concentrations. Average pCO_2 for the experimental period was determined in CO2SYS v2.1 (http://cdiac.ornl.gov/oceans/co2rprt.html) using the daily temperature (°C) and pH_{NBS} readings and weekly total alkalinity and salinity measurements (Table 2.1).

Experimental design

Eighteen pairs of *A. melanopus* were placed into each of the three CO_2 treatment groups (Control, Moderate and High). All individuals were weighed (wet weight; g) and standard length (mm) was measured immediately before being placed into treatment in August 2010 and again at the end of the breeding season (ensuring that there was an even distribution of weights among treatment groups). Fulton's K condition factor (body condition) was calculated at the end of the breeding season using the formula $K=100*(W/L^3)$ where W is the wet weight in grams and L is the standard length in centimeters. At the start of the experiment, August 2010, pairs were placed into individual 45l tubs with continuous water flow at winter non-breeding temperatures (22.5°C) and at ambient pCO_2. pCO_2 was slowly adjusted over a 2-week period to the desired levels and maintained at those levels from late August 2010 to the end of the breeding season in May 2011. This allowed for an acclimation to CO_2 treatment for two months prior to the start of the breeding season. Temperature was increased by 0.5°C weekly until the average summer breeding temperature (28.5°C) was reached the first week of November 2010.

Each pair was provided with a half terracotta pot as a shelter and breeding substrate. Pairs were fed 0.1 grams of commercial fish feed pellet (INVE NRD 12/24) 3 times a day (1.21% of average body weight; Donelson *et al.,* 2010) during the breeding season (November 2010-May 2011).

Data collection

From October 2010 through to the end of May 2011, representative of a single breeding season, the terracotta nesting pots were checked daily between 9am and 10am for the presence of a new egg clutch. As *A. melanopus* lay clutches after dark, this ensured that digital photographs of the clutch were taken within 12 hours of being laid. The total number of eggs in each clutch was counted using image analysis software (ImageJ). A random sample of 10 - 20 eggs (1-2% of the total number of eggs) was taken from each clutch and preserved in 4% phosphate buffered formaldehyde. Digital photographs of the sampled eggs were taken within 3 days of preserving using a Leica camera attached to a stereomicroscope. The eggs were placed in a horizontal position, with the longest axis visible on a 5mm grid. The individual area of 5 eggs from the sample was then determined using ImageJ by tracing around the egg and estimating area to the nearest 0.01mm^2. Reproductive output, per clutch, was calculated by multiplying the total number of eggs in the clutch by the average individual egg area for that clutch, to give an overall area (mm^2) of eggs, and therefore an estimate of maternal investment per clutch. On the night of hatching egg clutches were transferred into 70l hatching tanks containing the same treatment water as their parents. A sample of 10 - 20 hatchlings (between 2-5% of the hatchlings) was collected within an hour of hatching (approximately

9.30pm, or 1 hour after dark). Hatchlings were euthanized using an overdose of clove oil before being preserved in 4% phosphate buffered formaldehyde. The hatchlings were photographed on a 5mm grid using a stereomicroscope. Hatchling standard length was determined to the nearest 0.01mm and yolk area was determined by tracing the yolk sac and estimating the area to the nearest 0.1mm^2 in ImageJ.

Data analysis

Female weight at the start and end of the experiment and Fulton's K body condition factor at the end of the breeding season were compared among treatment groups using ANOVA. The proportion of pairs that reproduced was compared among treatments using a Chi^2 test of homogeneity. The average number of clutches produced per pairs was analysed using an ANCOVA with the number of clutches as the dependent variable, the treatment group as the predictor variable and females weight as the covariate. All other reproductive characteristics were analyzed using Linear Mixed Effects (LME) models (Pinheiro & Bates, 2000) with the reproductive characteristic (number of eggs, egg area, reproductive output, hatchling size and yolk area) being the response variable, CO_2 treatment being a fixed explanatory variable and female weight included as a random explanatory variable. Data were grouped by breeding pair (number of clutches, eggs per clutch and reproductive output per clutch) or egg clutch (egg area, hatchling length and yolk area) according to the level at which replication occurred for the measure of interest and then heterogeneous variance was allowed to occur at this level. Linear mixed effect models control for inherent variation between female reproductive levels by estimating error

terms for variation among individuals as well as estimating residual error terms for variation within individuals. Akaike information criteria (AIC) were used to determine which model best fit the data, whereby the model with the smallest AIC value was the best fit. All statistical analyses were conducted using S-Plus v.8.0.4.

Results

Parental condition and timing of breeding

Female weight at the start of the experiment (mean 24.82±1.1g SE) did not differ between the three CO_2 treatment groups (ANOVA; $F_{2,50}$= 0.042, p=0.387). Increased CO_2 significantly stimulated reproduction, with approximately twice the number of reproductive pairs breeding in the Moderate and High CO_2 treatment groups (21 of 36 pairs in the two groups) compared to the Control (5 of 18 pairs; χ^2_1 =3.741, p<0.05). There was no difference in the proportion of pairs that reproduced in the Moderate and High groups. In the Moderate group 61% (11 of 18) of pairs reproduced and in the High group 55% (10 of 18) of pairs reproduced, compared with only 27% (5 of 18) pairs that reproduced in the Control group. Despite the increase in reproductive activity in the High CO_2 group, the onset of breeding was the same for all treatments, with the first clutch of the season from each treatment group being laid during the last week of October 2010 (Fig. 2.1). The length of the breeding season was also unaffected by CO_2, with all groups breeding until the end of April 2011. All treatments displayed two peaks in reproductive activity: one in December - January, the other in April.

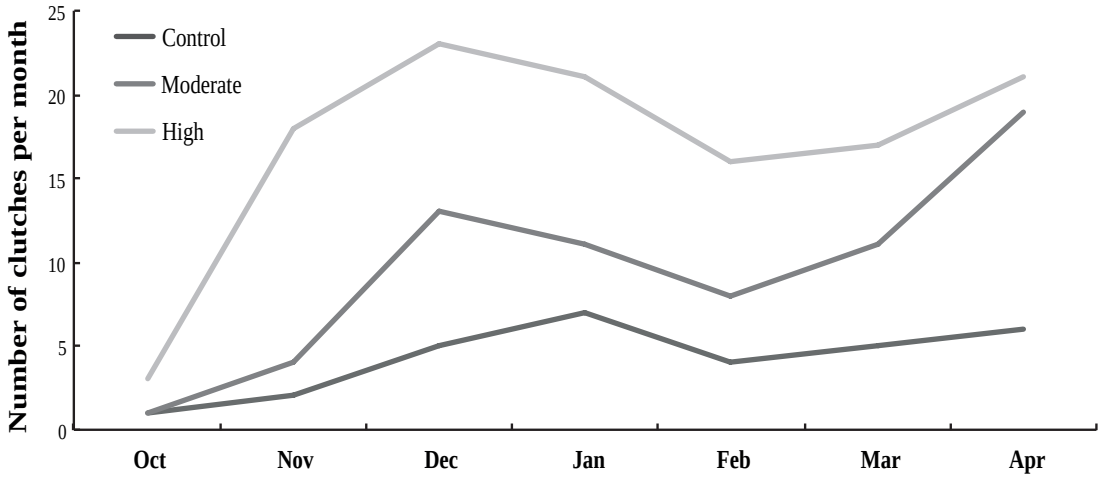

Figure 2.1: The number of clutches laid for each month from breeding pairs of *Amphiprion melanopus* held under Control, Moderate or High CO_2. Total numbers of clutches produced for each treatment group were Control N=30, Moderate N=67 and High N=119.

Reproductive frequency and egg production

Elevated CO_2 affected the frequency of reproduction and the number of clutches produced. Overall, the Control group produced a total of 30 clutches, the Moderate group produced 67 and the High group produced 119 clutches (Fig. 2.1). On average, pairs in the Control and Moderate groups produced 6.6±2.1 and 6.45±1.2 (mean±SE) clutches respectively during the breeding season. In contrast pairs in the High group produced on average 12.6±0.9 clutches throughout the breeding season, approximately double the number from the Control and Moderate groups (ANCOVA: $F_{2,22}$=7.33, p=0.003; Fig. 2.2a). Embryonic duration was not affected by CO_2, with all successful clutches hatching in 7 - 8 days.

The number of eggs per clutch increased with increasing CO_2 (Fig. 2.2b). The Control group produced 504±92 eggs per clutch, the Moderate produced 559±123 and the High group 847±121 eggs per clutch (LME model: Intercept

p<0.001; Table 2.2). The High group produced 67.16% more eggs compared to Control (LME model: High p<0.01; Table 2.2). There was no significant difference between the Moderate and Control group (LME model: Moderate p=0.654; Table 2.2). There was no evidence of a decline in male reproductive capacity, with few unfertilized eggs observed in any of the clutches.

Reproductive characteristics

CO_2 treatment group had a significant effect on egg size (LME model: Intercept p<0.001; Table 2.2) with the Moderate treatment producing significantly smaller eggs (1.96±0.08mm^2) compared to the Control (2.15±0.06mm^2; Moderate p=0.02, Table 2.2, Fig. 2.2c). However, eggs from fish in the High CO_2 treatment were not significantly different in area (2.12±0.08mm^2; High p=0.708) from the Control CO_2 treatment (2.15±0.06mm^2). CO_2 treatment also had a significant effect on reproductive output (LME model: Intercept p<0.0001). Reproductive output was significantly higher in the High CO_2 group compared to the Control (High p=0.003, Table 2.2), but there was no difference between the Moderate and Control CO_2 groups (Moderate p=0.437, Table 2.2, Fig. 2.2d). There was no significant difference in hatchling length between the Control and either of the elevated CO_2 groups (Moderate p=0.664; High p=0.9162; Table 2.2, Fig. 2.3a). However, CO_2 did have a significant influence on yolk provisioning (LME model: Intercept p<0.0001, Fig. 2.3b). The Moderate parents produced larvae with the largest yolks (0.559±0.02 mm^2; Moderate p=0.338; Table 2.2), though not significantly different from Control (0.054±0.17 mm^2). Larvae from the High CO_2 parents had significantly smaller yolks compared to the Control group (0.5±0.01 mm^2; High p=0.02; Table 2.2).

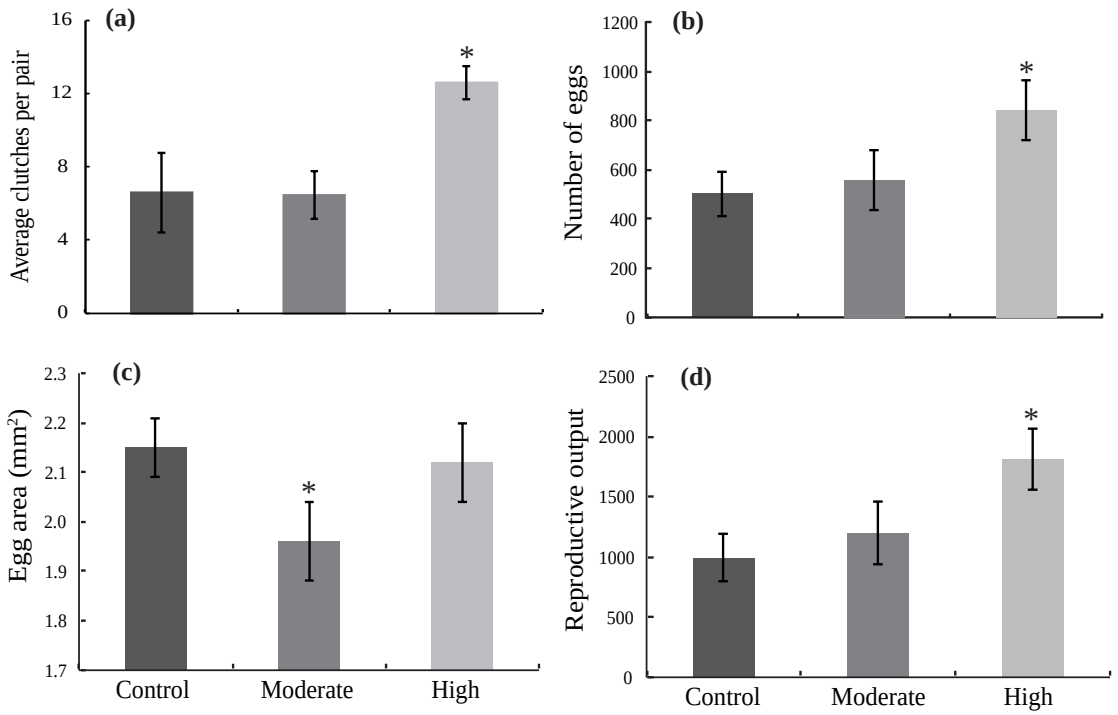

Figure 2.2: Effect of increased CO_2 on reproductive traits of a coral reef fish. The average number of clutches produced per pair (a), the average number of eggs produced per clutch (b), the average area of the eggs produced (c) and the reproductive output (number of eggs per clutch X average egg size to give a standard metric to compare clutches by) per clutch (d) of adult breeding pairs of *Amphiprion melanopus* held under three different CO_2 conditions. * indicates a significant difference from the Control group. Data are means± standard error.

The increase in reproduction at elevated CO_2 did not appear to come at a cost to adult body condition, with no significant difference in Fulton's K body condition factor among the treatment groups (All females: mean±SE Control=4.89±0.08, Moderate=5.02±0.08, High=5.00±0.09, ANOVA: $F_{2,47}$=0.67, p=0.517; Reproductive females only: Control=4.86±0.15, Moderate=4.95±0.11, High=4.86±0.11, ANOVA: $F_{2,23}$=0.234, p=0.79). During the breeding season, treatment groups on average gained mass (Control=2.06±0.72g; Moderate=1.05±0.69g; High=1.49±0.65g, ANOVA: $F_{2,47}$=0.499, p=0.609). Similarly, when only females that had reproduced were considered all treatment groups on average gained weight (weight gain: Control=0.61±0.76g,

Moderate=1.79±0.51g, High=0.88±0.54g, ANOVA: $F_{2,23}$=1.125, p=0.341). There were no deaths in any group during the experiment.

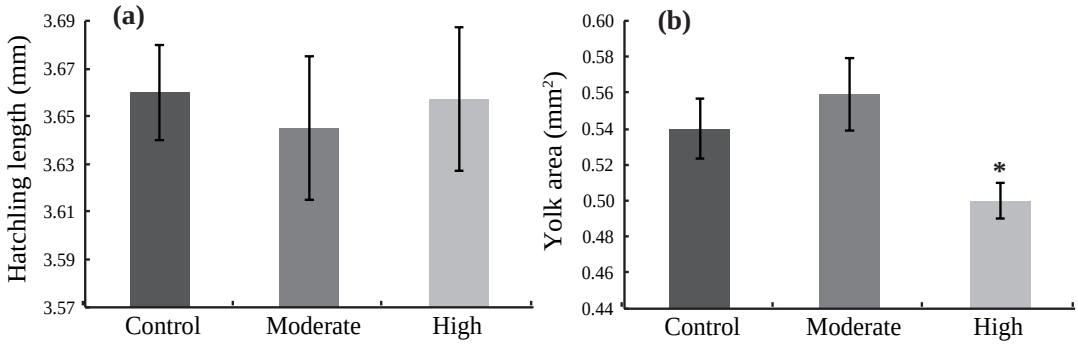

Figure 2.3: The average length of hatchlings (a) and the average area of the yolk (b) of newly hatched larvae of *Amphiprion melanopus* from reproductive adults, held under different pCO_2 treatments. * indicates where the treatment value is significantly different from the Control CO_2 group. Data are means ± standard error.

Discussion

Previous studies in a range of invertebrates (copepods: Fitzer *et al.,* 2012; sea urchins: Havenhand *et al*., 2008; oysters: Parker *et al.,* 2009) have found that reproduction is negatively impacted by elevated pCO_2 and predictions were that fishes would be similarly affected. Contrary to expectations, our study showed a distinct stimulation of reproductive activity with increasing CO_2. To our knowledge this is the first study to show an increase in reproduction in response to ocean acidification in any marine organism. The increase in reproduction was most marked in the High treatment group with a doubling in the number of clutches produced, 67% more eggs per clutch and an increase of 82% in reproductive output per clutch compared to Control pairs. The number of pairs breeding also doubled in the Moderate group, however these pairs did not increase reproductive effort to the same extent of the High CO_2 group,

producing 11% more eggs per clutch and with a 21% increase in reproductive output compared to Control pairs. This increase did not appear to come at a cost to adult body condition in either elevated CO_2 group. These results contradict the hypothesis that increased CO_2 will negatively impact reproduction due to the increased costs associated with acid-base regulation.

Increases in reproduction have been documented in birds (Velando *et al.,* 2006; Hall *et al*., 2009; Bowers *et al.,* 2012), insects (Nielsen & Holman, 2012; Copeland & Fedorka, 2012) and mammals (Hoffman *et al.,* 2010) that are senescing and/or facing a lethal stressor. This response is known as terminal investment. Individuals that display terminal investment use all available energy for reproduction rather than homeostasis or growth. As such, terminal investment has two clear outcomes: adults do not survive and there is often a decline in the quality of the offspring as the adults are already in poor condition (Bonneaud *et al.,* 2003). In this experiment we saw a dramatic increase in reproduction, however, this increase was not associated with adult mortality or a decline in adult body condition. While there was a decline in yolk sac area of the offspring in the highest CO_2 group, there was no significant difference in hatchling length, which is a key fitness-associated trait (Miller *et al.,* 1998). Consequently, the increase in reproduction seen in response to elevated CO_2 is not consistent with the occurrence of terminal investment.

The increase in reproduction observed here could be a hormetic response to an increase in pCO_2. A hormetic response occurs where an organism's response to an environmental stressor varies with the dose of the toxicant, specifically a mild dose of a stressor results in an increase in a given performance measure (Constantini, 2008). This initial increase in performance

could occur either through speeding up physiological reactions or by stimulating the organism to perform activities at a higher rate (Schreck, 2010; Costantini, 2008). Our results show that a mild increase in pCO_2 appears to stimulate reproduction with no readily apparent cost to the organism. We detected no negative impacts of increased reproduction or pCO_2 on adult body condition over a period of 9-months, suggesting that the elevated CO_2 treatments were not harmful in the short-term. This is consistent with previous findings where growth and survival of adult fish are not affected until CO_2 levels reach at least an order of magnitude higher than those used here (Ishimatsu *et al.,* 2008; Melzner *et al.,* 2009a,b). The exact mechanisms that allow this dramatic increase in reproduction to occur with no apparent costs are not known, however it is plausible that there could be alterations to the endocrine pathways leading to a stimulation of reproductive activity (Pankhurst & Munday, 2011) or this species may have increased energetic efficiency under these levels of CO_2, and could therefore have more energy available for reproduction.

While we did not detect any effects of increased reproduction on adult body condition or offspring standard length, it is possible that there will be trade-offs with other life history traits, or over longer time scales (Creighton *et al.,* 2009). For example, longer-term exposure to increased pCO_2 could result in declines in longevity and lifetime fecundity (Pörtner & Peck, 2010) that could not be measured in our experiment. It is also possible that the adults used in this experiment may show reduced reproduction in subsequent breeding seasons. Research on reproduction in invertebrates and birds has suggested that increased reproduction can lead to reduced reproduction or survival of the parents, potentially due to increased oxidative stress (Costantini, 2008).

Furthermore, there could be effects on offspring condition. While the size at hatching of offspring did not differ among treatments, the average yolk area of larvae in the highest CO_2 treatment was smaller than larvae from current-day controls. This suggests that high CO_2 may have had some energetic cost to the larvae. Reduced yolk size could potentially affect juvenile growth or survival in the wild (Hoey & McCormick, 2004; Grorud-Colvert & Sponaugle, 2006). However, while there was a decline in yolk area at the highest CO_2, there was no significant difference in hatchling length, which may be equally important to larval survival (Meekan & Fortier, 1996; Miller *et al.,* 1998). Furthermore, Miller *et al.* (2012) showed that, rather than displaying negative impacts, juveniles from parents held under high pCO_2 were better acclimated to increased pCO_2. When reared at high CO_2 these offspring displayed similar growth and survival compared to offspring reared under control conditions. Consequently, any differences in yolk area to not appear to have negative effects on growth and survival, presumably because the offspring are better acclimated to the high CO_2 conditions.

Interestingly, different levels of increased CO_2 appeared to lead to differing levels of provisioning for the offspring. The Moderate group had significantly smaller eggs compared to the High group and also appeared to gain more weight across the breeding season. This suggests that different levels of increased CO_2 may lead to different investment strategies, with fish exposed to moderate levels of CO_2 choosing to invest more energy into adult body condition at the expense of high rates of reproduction.

The dramatic increase in reproductive effort, with no apparent cost to adult body condition, suggests that there may be more energy available for

reproduction in fish exposed to the near future CO_2 levels used in our study. Fish were fed the same amount in each treatment, so there was no difference in nutritional availability among treatments that could explain the similar increase in mass of breeding females across treatments despite the vastly differing levels of energy allocated to reproduction. Although activity levels were not quantified, there was no apparent difference in activity among treatments that could explain the different levels of energy allocation. Previous studies have shown that juvenile *A. melanopus* exposed to high CO_2 and control temperature do not increase their foraging rate (Nowicki *et al*., 2012). Our results suggest that near future levels of CO_2 may not be as costly for adult reef fish as expected. Reef fish experience large variations in pCO_2 and pH levels in their environment on a diurnal basis (Gagliano *et al.,* 2010; Shaw *et al.,* 2013) and may be pre-conditioned or better adapted to increased CO_2 than would otherwise be expected. Indeed, Rummer *et al.* (2013b) found that the routine metabolic rate decreased and maximum oxygen uptake increased, in a common reef fish under levels of CO_2 similar to levels used in our experiment. The changes in metabolic rates suggests that some reef fish require less energy for routine activities, such as homeostasis, when CO_2 levels are slightly elevated, and could therefore put more energy into reproduction.

In direct contrast to the prediction that the energetically costly phase of reproduction would be particularly sensitive to increased CO_2, here we show that increased pCO_2 stimulates reproduction in a marine fish. Further we found limited evidence of negative impacts of increased reproductive effort on either adult body condition or offspring standard length. Although yolk area of hatching was reduced, previous experiments suggest this might not have

significant effects on offspring performance in a high CO_2 environment. These results suggest that some species may have a much greater capacity to tolerate increased pCO_2 than has been predicted. This is the first study to examine the impacts of ocean acidification by exposing reproductive adults to increased CO_2 prior to and during a breeding season, allowing for any impacts on gametogenesis to occur. We also investigated the effects of ocean acidification on multiple stages of the reproductive cycle, from potential effects on the adult condition through to the condition of the resulting offspring. Given the unexpected results found here, future experiments on the impacts of ocean acidification will need to examine the potential impacts on all stages of reproduction, including on gametogenesis and adult condition, to provide a more realistic indication of the effects of future levels of ocean acidification on marine populations.

Tables

Table 2.1: Experimental system seawater parameters for the reproductive adults held under Control, Moderate and High CO_2 concentrations.

Treatment	Salinity (ppt)	Temp (°C)	Total alkalinity (µmol kg^{-1} SW)	pH_{NBS}	pCO_2 (µatm)
Control	36.4	28.5 ±0.3	2049 ±79	8.11 ±0.04	430 ±54
Moderate	36.4	28.5 ±0.3	2089 ±143	8.01 ±0.05	584 ±77
High	36.2	28.5 ±0.3	1945 ±23	7.77 ±0.05	1032 ±137

Table 2.2: Linear mixed effects tables for reproductive characteristics. * represents significant effects (p<0.05) are shown in bold. Intercept is the Control group; Moderate and High are compared to the Control.

Reproductive variable	Treatment	Variable value	SE	DF	t-value	p-value
Number of Eggs	(Intercept)	503.74	91.65	196	5.49	<0.001*
	Moderate	55.67	122.71	23	0.45	0.654
	High	338.35	121.45	23	2.78	0.01*
Egg Area	(Intercept)	2.15	0.06	1084	30.86	<0.0001*
	Moderate	-0.19	0.08	23	-2.33	0.02*
	High	-0.03	0.08	23	-0.37	0.708
Reproductive Output	(Intercept)	991.31	196.54	189	5.04	<0.0001*
	Moderate	207.97	263.19	23	0.79	0.437
	High	816.51	253.12	23	3.22	0.003*
Hatchling Length	(Intercept)	3.66	0.02	718	122.52	<0.0001*
	Moderate	-0.015	0.03	82	-0.43	0.664
	High	-0.003	0.03	82	-0.11	0.9162
Yolk Area	(Intercept)	0.54	0.01	707	31.13	<0.0001*
	Moderate	0.019	0.02	81	0.96	0.338
	High	-0.04	0.01	81	-2.23	0.02*

Chapter 3: Temperature is the evil twin: Effects of increased temperature and ocean acidification on reproduction in a reef fish.

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Abstract

Reproduction in many organisms can be disrupted by changes to the physical environment, such as those predicted to occur during climate change. Marine organisms face the dual climate change threats of increasing temperature and ocean acidification, yet no studies have examined the potential interactive effects of these stressors on reproduction in marine fishes. We used a long-term experiment to test the interactive effects of increased temperature and CO_2 on the reproductive performance of the anemonefish, *Amphiprion melanopus*. Adult breeding pairs were kept for 10 months at three temperatures, 28.5°C (+0.0°C), 30.0°C (+1.5°C) and 31.5°C (+3.0°C), cross-factored with 3 CO_2 levels, a current day control (417µatm) and moderate (644µatm) and high (1134µatm) treatments consistent with the range of CO_2 projections for the year 2100 under RCP8.5. We recorded each egg clutch produced during the breeding season, the number of eggs laid per clutch, average egg size, fertilization success, survival to hatching, hatchling length and yolk provisioning. Adult body condition, hepatosomatic index,

gonadosomatic index, and plasma 17β-estradiol concentrations were measured at the end of the breeding season to determine the effect of prolonged exposure to increased temperature and elevated CO_2 on adults, and to examine potential physiological mechanisms for changes in reproduction. Temperature had by far the stronger influence on reproduction, with clear declines in reproduction occurring in the +1.5°C treatment and ceasing altogether in the +3.0°C treatment. In contrast, CO_2 had a minimal effect on the majority of reproductive traits measured, but caused a decline in offspring quality in combination with elevated temperature. We detected no significant effect of temperature or CO_2 on adult body condition or hepatosomatic index. Elevated temperature had a significant negative effect on plasma 17β-estradiol concentrations, suggesting that declines in reproduction with increasing temperature were due to the thermal sensitivity of reproductive hormones rather than a reduction in energy available for reproduction. Our results show that elevated temperature exerts a stronger influence than high CO_2 on reproduction in *A. melanopus*. Understanding how these two environmental variables interact to affect the reproductive performance of marine organisms will be important for predicting the future impacts of climate change.

Introduction

Reproduction is critical to individual fitness and the persistence of populations. Reproduction in most organisms is also sensitive to changes in the physical environment. For example, the timing of reproduction can be influenced by variation in temperature (Kjesbu, 1994; Visser *et al.,* 2009), photoperiod (Duston & Bromage, 1986; Dawson *et al.*, 2001), rainfall (Donnelly & Guyer,

1994; Hau *et al.,* 2004) and flow regimes (Schlosser, 1982; Bunn & Arthington, 2002). Similarly, reproductive output is affected by temperature (King *et al*., 2003; Saino *et al*., 2004) and food availability (Brown & Shine, 2007; Donelson *et al*., 2010). Consequently, anthropogenic climate change is predicted to affect reproductive success of many species (Parmesan, 2006; Poloczanska *et al*., 2013) and could be the primary driver of population declines due to climate change (Van Der Kraak & Pankhurst, 1997; Zeh *et al.*, 2012).

For marine organisms, increasing temperature and ocean acidification are the most serious climate change threats (Hoegh-Guldberg *et al.,* 2007; Doney *et al.,* 2009) and they are predicted to be additive or synergistic in their effect on performance, potentially leading to greater effects in combination than in isolation (Pörtner & Farrel, 2008). Many studies have examined the effects of one or other of these two stressors on reproduction in marine organisms (including Miller *et al.*, 2013; Donelson *et al.,* 2010), but few have examined the potential interactive effects on reproduction. While an increasing number of studies are testing the interacting effects of ocean warming and ocean acidification on invertebrates (Byrne *et al.,* 2009, 2010; Parker *et al.,* 2009; Albright & Mason 2013, Cohen-Rengifo *et al*., 2013) relatively few studies have tested these two stressors in combination for fish (but see Munday *et al.,* 2009a; Nowicki *et al*., 2012; Grans *et al.,* 2014) and none have tested the interacting effects on reproduction in fishes. In fishes, increased temperature has been shown to negatively affect reproduction in many species (see reviews, Pankhurst & Porter, 2003; Pankhurst & King, 2010). In contrast, ocean acidification, while predicted to have negative impacts (Pörtner & Farrel, 2008; Ishimatsu *et al*., 2008) has been found to have little impact, or even positive

effects, on reproduction in multiple species of fish (Frommel *et al*., 2010; Sundin *et al.,* 2012, Miller *et al*., 2013; Forsgren *et al*., 2013). Yet, whether elevated temperature and ocean acidification will interact to affect reproduction in fishes is not known.

For many species of fish, temperature is one of the main cues for reproduction, signaling the beginning and the end of the breeding season (Van Der Kraak & Pankhurst, 1997; Pankhurst & Munday, 2011). For spring-summer spawner's, the increase in water temperatures following the winter minimum, elicits physiological changes, production of sex steroids, maturation of gonads, and spawning (Kjesbu, 1994; Pankhurst *et al*., 1996). Nevertheless, reproduction only occurs within a narrow range of temperatures that the population normally experiences (Van der Kraak & Pankhurst, 1997). If temperatures exceed this thermal window, reproduction can quickly decline and may cease altogether (Donelson *et al*., 2010; Dorts *et al.*, 2011). Tropical fishes may be especially sensitive to changes in temperature, as they inhabit a more thermally stable environment than higher latitude species (Tewksbury *et al.,* 2008; Rummer *et al.,* 2014). This means that even a relatively small increase in average temperature, such as predicted by climate change, could have serious effects on reproductive performance in tropical species (Donelson *et al.,* 2010; Zeh *et al*., 2012).

Reproduction may decline at elevated temperatures as a result of energetic constraints or through the effects of temperature on hormonal pathways. As temperatures increase past the thermal optimum, individuals need to expend more energy maintaining cellular function (Pörtner & Farrell, 2008). Organisms have a finite amount of energy available and as more of the

energy is used for homeostasis less is available for other activities, such as reproduction (Somero, 2002; Sokolova *et al.,* 2012). Under energy constraints, adults could opt to produce the same number of offspring as under normal conditions, but at a cost to offspring provisioning. Alternatively, individuals may produce fewer offspring that have adequate levels of provisioning in an attempt to ensure offspring survival (Stearns, 1992).

Reproduction may also decline with increasing temperature due to the thermal sensitivity of reproductive hormones. Reproduction in fish is tightly controlled through the interplay of multiple hormones and steroids created by the hypothalamus, the pituitary and the gonads (Hypothalamic-Pituitary-Gonadal axis (HPG axis)) (Yaron & Levavi-Sivan, 2011). Elevated temperatures have the ability to inhibit the HPG axis at multiple sites, through changes in hormone synthesis, action and structures (Pankhurst & Munday, 2011). The inhibitory effects of temperature can occur through changes in protein and hormone structures, resulting in a reduced uptake or insolubility of the hormones. These changes can then lead to the hormones failing to reach the correct receptor, or passing straight through the kidneys and being excreted, thereby impairing the particular reproductive process (Van Der Kraak & Pankhurst, 1997; Pankhurst & Munday, 2011). Ultimately, wherever the disruption to the hormonal cascade occurs, elevated temperatures result in declines in reproductive activity, egg size and offspring survival.

In addition to increasing temperatures, marine fishes will have to cope with increasing partial pressure of carbon dioxide (pCO_2) in the ocean. Increasing pCO_2 has been documented to negatively impact reproduction in a number of invertebrates (see Ross *et al.,* 2011). Fishes, however, have well-

developed mechanisms for acid-base regulation and are able to maintain their internal pH against an elevated CO_2 gradient, through active transport of ions across the gills and in their blood and tissues (Brauner & Baker, 2009; Esbaugh *et al.,* 2012). This process is not cost free and it has been predicted that the increase in energy required to maintain acid-base balance should result in a decline in energy available for reproduction and other activities (Pörtner *et al.,* 2004; Ishimatsu *et al*., 2008). However, only one study (Inaba *et al.,* 2003) has documented a negative impact of increasing pCO_2 on a reproductive trait, with sperm motility being reduced in some flatfishes, but not in a range of other species. Other studies have reported little to no effects of increased CO_2 on reproduction. For example, Frommel *et al.* (2010) found no effect on sperm motility in Baltic cod (*Gadhus morhua*), Sundin *et al.* (2012) found no difference in reproductive propensity in pipefish (*Syngnathus typhle*) and Forsgren *et al.* (2013) found no differences in clutch size but did see a significant decline in egg survival with increasing CO_2 in a temperate goby (*Gobiusculus flavescens*). Interestingly, several studies have documented increases in reproduction or reproductive related traits in response to elevated CO_2 (Miller *et al*., 2013; Schade *et al.,* 2014) Furthermore, Preus-Olsen *et al.* (2014) documented increased levels of sex steroid hormones in Atlantic cod at high CO_2 which is consistent with greater rates of reproduction in the other studies. Neither Miller *et al.* (2013) or Schade *et al.* (2014) found negative consequences of the increased reproductive activity in high CO_2 on the condition of the adults or the resulting offspring. Instead these studies show transgenerational acclimation of the offspring to elevated CO_2 due to parental exposure to high CO_2 (Miller *et al.,* 2012: Schade *et al.,* 2014).

Increases in temperature and pCO_2 will not occur in isolation from each other, and for that reason, it is important to understand how they may interact to affect reproduction. As these two variables have been documented to have contrasting effects on reproduction in fish, it is especially important to understand how they might interact to affect this critical process. The aim of this study was to document the effect of elevated CO_2 and increased temperature on reproductive activity, offspring quality, and any effect on adult condition (physical and reproductive) in a tropical reef fish. Adult pairs of *Amphiprion melanopus* were kept in current-day control CO_2 or elevated CO_2 treatments (moderate and high). CO_2 treatments were fully cross-factored with 3 temperature treatments, current-day summer average water temperature, 28.5°C (+0.0°C), or two elevated temperatures, 30.0°C (+1.5°C) and 31.5°C (+3.0°C). Adult pairs were placed in CO_2 treatment during winter, slowly brought up to the required temperature treatments through spring and then allowed to reproduce naturally during the summer breeding season. Throughout the breeding season we assessed the effect of elevated temperature and increased CO_2 on key reproductive traits related to breeding and spawning, egg production and survival, and offspring provisioning (Fig. 3.1). At the end of the reproductive season we assessed adult physiological (Fulton's K body condition index, hepatosomatic index) and reproductive condition (gonadosomatic index, plasma hormone concentration) (Fig. 3.1) to determine if difference in reproductive performance were potentially associated with the energetic cost of reproduction or effects on reproductive hormones. 17β-estradiol (E_2) was chosen as the focal sex steroid due to its well-defined role in vitellogenesis and

oocyte maturation in female fish (Lubzen *et al.,* 2010; Yaron & Levavi-Sivan, 2011).

Methods

Study species and husbandry

The cinnamon anemonefish, *Amphiprion melanopus* (Pomacentridae) inhabits coral reefs throughout the Indo-Pacific region, including the Great Barrier Reef, Australia (Drew *et al.,* 2008). *Amphiprion melanopus* occur in large social groups containing multiple breeding pairs that reproduce repeatedly during the summer. Eggs are laid in clutches attached to hard substratum near their host anemone (Michael, 2008). Embryonic duration is 7-9 days, during which time the males tend the eggs. Larvae hatch after dark and are pelagic for approximately 11 days, at which point they metamorphose and become competent to settle to reef habitat (Bay *et al*., 2006). Adults reach a maximum length of 12cm (Lieske & Myers, 1994) and have a reported maximum age of 5 years (Allen, 1975).

Adult breeding pairs of *A. melanopus* were collected between June 2009 and June 2011 from 4 reefs in the central Great Barrier Reef: Orpheus Island (18.6183°S, 146.4936°E), Bramble Reef (18.417°S, 146.700°E), Davies Reef (18.83°S, 147.63°E) and Slasher's Reef (18.467°S, 147.083°E) and transferred to James Cook University, Townsville. Pairs were housed individually in 45L aquaria and provided with a half terracotta post as a nest site and shelter. Aquaria were provided with continuous flow of seawater at 1.5Lmin^{-1}. Pairs were fed 0.1g of commercial fish feed (INVE NRD 12/24) three times a day, equivalent to 1.21% of the average body weight (Donelson *et al.,* 2010).

Experimental design

Between seven and eight adult pairs of *A. melanopus* were assigned to each of the nine treatment groups and held individually, i.e. one pair per aquaria. Three CO_2 groups were cross-factored with three temperature groups reflective of pCO_2 and temperatures projected to occur in the ocean by 2100 under RCP6 and RCP8.5 (Collins *et al.,* 2013). The three CO_2 levels used were a current-day control (~417µatm), a moderate (~644 µatm) and a high (~1134 µatm) CO_2 treatment. The temperatures were the current-day summer average of the collection region, 28.5°C (+0.0°C), a moderate 30.0°C (+1.5°C) and a high, 31.5°C (+3.0°C) temperature treatments (See Table 3.1 for full experimental parameters). These temperatures reflect the current summer average water temperature for the Orpheus Island region where the adults were collected, and the 1.5 to 3.0°C warming predicted to occur in the tropical oceans over the coming century due to climate change (Poloczanska *et al.,* 2007; Ganachaud *et al.,* 2011).

Three 8000L liter aquarium systems were utilized, each dosed to the desired pCO_2. Due to the scale of the study it was not possible to have separate CO_2 and temperature treatments applied to each aquarium. Pairs were in their respective CO_2 treatments by June 2011 at current day winter water temperatures (22.5°C). Temperatures were subsequently increased over a two month period to achieve the required temperature separation and then increased by 0.5°C weekly to reach experimental breeding temperatures in the second week of November 2011.

Data collection of key reproductive traits

Throughout the breeding season, key reproductive traits related to reproductive activity, egg survival, offspring provisioning and adult physiological and reproductive condition were collected (Fig. 3.1). Terracotta pots were checked daily between 0900 and 1100 for the presence of egg clutches. A digital photograph (Canon G12) was taken of each new clutch. A sample of 10-20 eggs was then taken from the clutch and preserved in 6% formalin. Daily photographs of each clutch were taken until the eggs hatched or there were no more eggs due to mortality. Parents often eat the eggs if development is abnormal or if eggs are diseased. Clutches were considered successful if any eggs survived to 6 – 8 days post-spawning. Surviving egg clutches were hatched into 70L aquaria at the same pCO_2 and temperature treatment as their parents. A sample of between 10-20 larvae was taken the morning after hatching between 0730 and 0830, within 12 hours of hatching. Larvae were euthanized with an overdose of clove oil before being preserved in 4% phosphate buffered formaldehyde. A digital photograph (Canon G12) of each larva was taken on a 5mm grid in a horizontal position within 3 days of sampling. The number of eggs laid in each clutch and the number of eggs remaining at hatching was counted from the digital image with the aid of ImageJ. The percentage of surviving eggs was then determined from these two counts. Fertilization success was determined by counting the number of unfertilized eggs in the initial clutch photograph. Unfertilized eggs were identified by their white colouration, whereas fertilized eggs had an orange colouration.

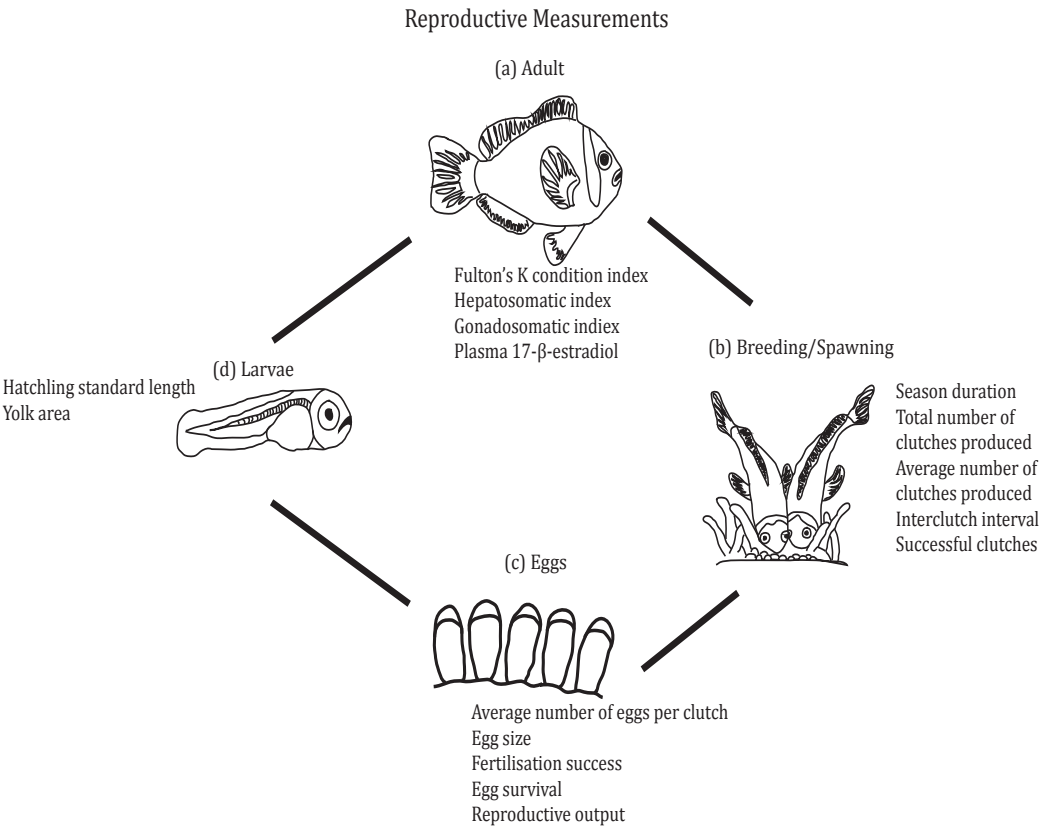

Figure 3.1: The data collected at each stage of reproduction in *A. melanopus*, (a) reproductive adults (b) clutches produced, (c) eggs collected form reproductive adults and (d) the resulting hatchlings. Breeding pairs were kept at control, moderate or high CO_2 cross-factored with either +0.0°C (28.5°C), +1.5°C (30.0°C) or +3.0°C (31.5°C) temperature treatment.

To determine egg size the eggs sampled from each clutch were photographed (Canon G12) while placed horizontally on a 5mm grid so that the longest axis was visible. The image was viewed on a computer screen and ImageJ was used to trace the outside of 5 eggs from each sample and the average egg area (mm^2) was determined. Reproductive output for each clutch was estimated by multiplying the total number of eggs by the average egg area for that clutch providing a relative estimate of investment for each clutch (mm^2). Hatchling standard length (SL) was measured to the nearest 0.1mm from a photograph using ImageJ, by drawing a line from the tip of the mouth to the beginning of the tail. Yolk area was determined to the nearest 0.1mm^2 by tracing the yolk sac in

ImageJ from photographs viewed on a computer screen. *Data collection of adult physiological and reproductive traits*

Adults were euthanized at the end of the breeding season to examine the effects of increased temperature and pCO_2 on body condition, liver condition, oocyte production and gonadal steroidogenesis (17β-estradiol, E_2). Each fish was weighed (wet weight W, nearest 0.01g) and measured (standard length SL, to the nearest 0.01mm). Fulton's K body condition factor (body condition) was then calculated using the formula $K=100*(W/SL^3)$, where W is wet weight in grams and SL is standard length in centimeters. To maintain genetic material and allow for potential genetic analysis, liver and gonads were dissected and snap frozen in liquid nitrogen. After freezing they were weighed to the nearest 0.0001g and then fixed in 4% phosphate buffered formaldehyde for several days before storage in 100% ethanol. Hepatosomatic index and gonadosomatic index were determined by the formula HSI/GSI= (liver weight or gonad weight (g)/fish weight (g))*100. *Plasma 17β-estradiol quantification*

Blood samples were taken from females prior to euthanasia to estimate 17β-estradiol (E_2) concentrations. 17β-estradiol was chosen due to its role in vitellogenesis and oocyte maturation in female fish. Thus, changes in E_2 concentration could result in changes yolk provisioning, egg size, the number of eggs per clutch, the number of clutches, hatchling survival and hatchling length (Lubzens *et al.,* 2010; Yaron & Levavi-Sivan, 2011). Changes in E_2 concentration can therefore provide a direct endocrine pathway to any changes in reproductive output.

Liver and gonad samples

Fixed ovaries were embedded in histoparaffin and 5-µm sections were taken at 3 points along the longest axis. Sections were mounted on a glass slide and stained with Mayer's alum haematoxylin and Young's eosin-erythrosine. To determine the reproductive status of individuals, a transect was run along each representative section and the type of sex cell under 100-graticules marked on an eyepiece micrometer was recorded at a 10 times magnification. Female cells were categorized into: oogonia (Stage 1), perinucleolus (Stage 2), cortical alveolus (Stage 3), early vitellogenic oocytes (Stage 4) and late vitellogenic oocytes (Stage 5), following Genten *et al*. (2009). The relative abundance of each cell type in each section was calculated.

Aquarium systems and seawater analysis

The pCO_2 in each system was controlled by an AquaMedic AT-controller that dosed a 3000L sump with CO_2 to maintain the pH at the appropriate level for the desired pCO_2. The control temperature (+0.0°C) was maintained by circulating seawater through a SolarWise heater/chiller on each system. The +0.0°C temperature seawater was either delivered directly to the aquaria, or was sent through Toyesi inline 2.5kW heaters to raise the temperature +1.5°C or +3.0°C, prior to delivery to the aquaria.

pH_{NBS} (Hach HQ40d) and temperature (Comark C26 thermometer) were recorded daily from replicate aquariums for each treatment. Total alkalinity was estimated weekly by Gran Titration (Metrohm 888 Titrando titrator) and validated against certified reference material (A.G. Dickson Scipss Institute of Oceanography). Salinity (Hach HQ15d) was measured weekly. The Aqua

Medic pH set points were adjusted as needed to maintain the desired pCO_2 on each system. In addition, regular NDIR measurements were recorded in each treatment group to ensure that the CO_2 that was required was being achieved and that pH_{NBS} was reflecting the given pCO_2.

pCO_2 was calculated in CO2SYS (http://cdiac.ornl.gov/oceans/co2rprt.html) using the daily pH_{NBS} and temperature (°C) readings and the weekly total alkalinity and salinity measurements. As temperature affects seawater pCO_2, CO_2 levels were not exactly the same among temperature treatments within each CO_2 treatment group. Nevertheless, CO_2 treatments remained well separated and for simplicity, the average CO_2 levels are reported (417µatm, 644µatm and 1134µatm) and are referred to as control, moderate or high CO_2 treatments.

Data analysis

ANCOVA was used to compare the number of clutches produced per pair in each treatment group. Prior to analysis the assumptions of the ANCOVA were tested and the data was found not to violate either non-normality or display heterogeneity of variance. The number of clutches produced was the dependent variable, CO_2 and temperature treatments were the fixed variables, and female weight the covariate.

Linear mixed effects models (LME) (Pinheiro & Bates, 2000) were used to analyze the reproductive characteristics: interclutch interval, average number of eggs laid per clutch, average fertilization success, average egg area and reproductive output per clutch. As the experiment aimed to determine the interactive effect of increased temperature and CO_2 on reproduction, only

clutches that were produced after experimental temperatures were attained were included in the analysis. The simplest LME constructed included the reproductive characteristic of interest as the dependent variable, CO_2 and temperature treatments as the fixed variables, and female weight was included as a random variable because reproductive traits can be strongly weight-dependent in fishes (Model A). Model B was constructed as above, but also grouped the data according to the breeding pair. Model C added the step of allowing for heterogeneity of variance within each pair, as pairs will have naturally fluctuating reproductive effort. The model that best represented the data set was determined by comparing the Akaike Information Criterion. Hatchling length and yolk area were also analyzed using linear mixed effects models. These analyses were constructed in the same order as described above (Model A, Model B and Model C). For hatchling length and yolk area, data was grouped according to clutch ID and heterogeneity of variance was allowed to occur within each clutch, as this was the level of replication.

The proportion of clutches that survived to hatching, and the proportion of eggs within each clutch that survived to hatching, were analysed with a penalized quasi-likelihood general linear mixed model (Splus Mass library). Temperature and CO_2 treatment were fixed variables, and breeding pair was a random variable in each model. For clutch survival, the proportion of the number of clutches that survived was weighted against the number of clutches that were produced by each pair. All mixed effects models were constructed and compared in Splus.

Fixed factor ANOVA (Type III) was used to compare Fulton's K, hepatosomatic index, gonadosomatic index and plasma E_2 concentration of

females. The physiological trait was the dependent variable and CO_2 and temperature treatment were the fixed variables. Where a significant difference was detected a Fisher's LSD test was used to determine which treatment groups were significantly different.

Factor analysis was used to identify which reduce explanatory variable and identify gonad stages that had the largest influence on the data set (Manly 1994, Kroon *et al*., 2003). The data was transformed using a varimax raw rotation to differentiate the original variables by extracted factor. Initial analysis identified two factors, and these two factors were used as the independent variables in a multiple regression analysis, where the plasma concentration of E_2 was the dependent variable.

Results

Reproductive characteristics

Reproduction in all treatment groups began in early October 2011, a month prior to summer breeding temperatures being achieved. Reproduction continued throughout the breeding season at +0.0°C for all CO_2 treatments (Fig. 2a). In contrast, reproduction in the +3.0°C treatment groups, irrespective of CO_2 level, and in moderate CO_2 +1.5°C, effectively ceased within a month of experimental temperatures being attained (Fig. 2b,c). Reproduction in all other groups continued from late September 2011 through to mid April 2012 with no obvious peaks in reproductive activity (Fig. 3.2a,b). An unequal number of pairs reproduced in the treatment groups, with 5 pairs reproducing in the moderate +0.0°C but only 2 reproducing in the moderate +3.0°C (Fig. 3.2). The total number of egg clutches produced was greatest at +0.0°C (N=152) and declined

markedly with increasing temperature (N=53 at +1.5°C and N=8 at +3.0°C) (Fig. 3.2). At +0.0°C the moderate and high CO_2 groups produced more clutches in total compared to the control CO_2 group (n= 55, 58 and 39 respectively), but this trend was not apparent at higher temperatures. Temperature appeared to have a stronger effect on the moderate CO_2 breeding pairs, as the moderate +1.5°C did not reproduce successfully once temperatures were attained, whereas, the control and high CO_2 at the same temperature continued to reproduce successfully (Fig. 3.2b).

The average number of clutches produced per pair declined with increasing temperature (Table 3.2, Fig. 3.3a). At +3.0°C there was a decline of between 78% to 87% in the average number of clutches produced per pair in all CO_2 treatments compared to the respective +0.0°C clutches produced per pair (Fig. 3.3a). In contrast, elevated CO_2 had no significant effect on the number of clutches produced per pair (Table 3.2, Fig. 3.3a). There was no effect of female weight on the average number of clutches produced per pair (Table 3.2)

Unsurprisingly, given the differences in the number of clutches produced among treatments, both temperature and elevated CO_2 significantly increased the interclutch interval (Table 3.2). This was most marked in the moderate CO_2 group, where interclutch interval increased from 15±0.8 (SE) days at +0.0°C to 96.5±115 (SE) days at +3.0°C. A similar, though less marked effect, was seen in the control CO_2 group where interclutch interval increased from 18±0.8 at +0.0°C to 35±4 at +3.0°C. In contrast, interclutch interval decreased from 17±2 days at +0.0°C to 14±1 days at +1.5°C in the high CO_2 group before increasing to 19±6 days at +3.0°C.

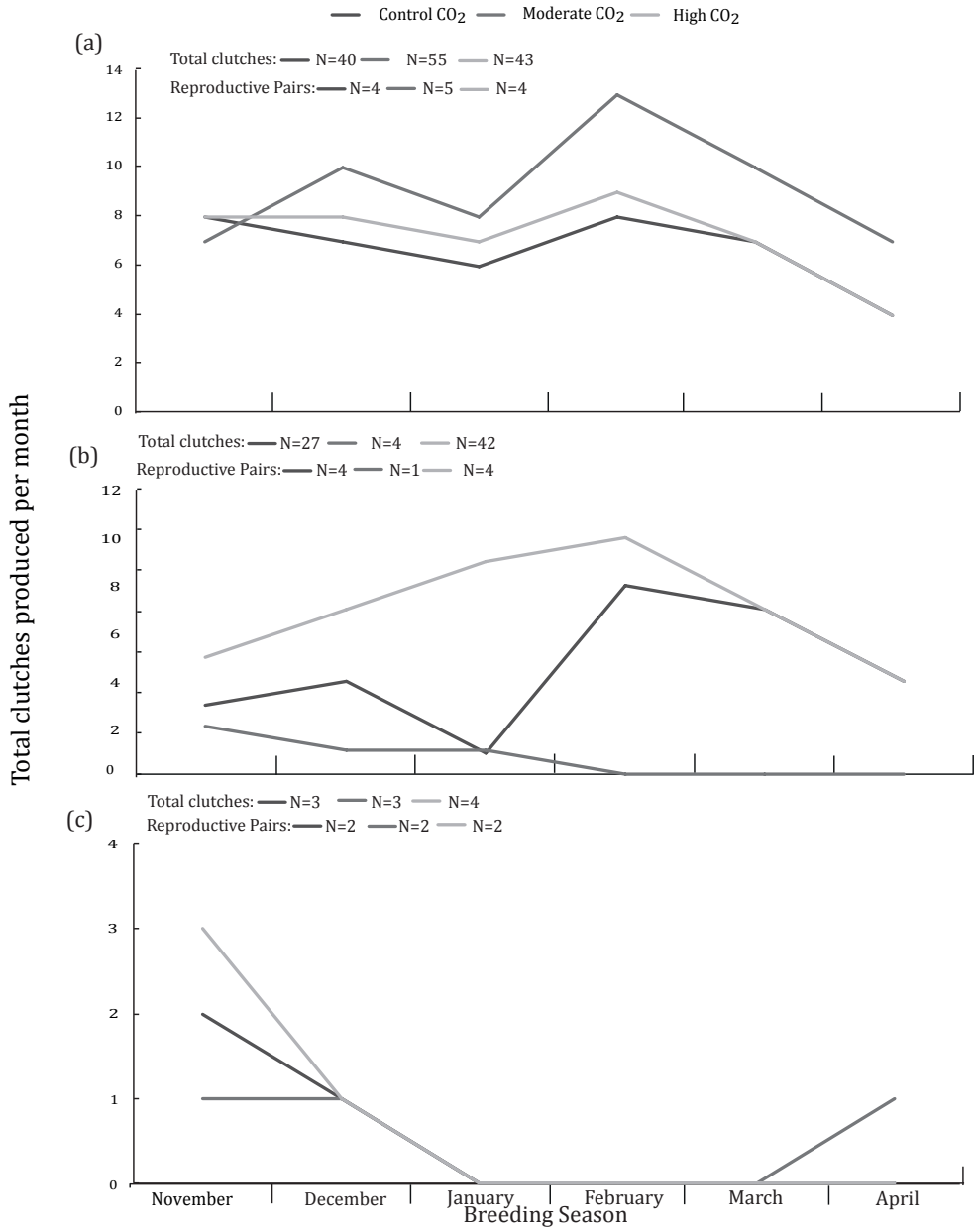

Figure 3.2: The total egg clutches produced per month across the breeding season for each CO_2 treatment at (a) +0.0°C (28.5°C), (b) +1.5°C (30.0°C) and (c) +3.0°C (31.5°C). Shown is the total number of clutches produced in each treatment for that month. The total number of clutches produced in each temperature by CO_2 treatment group and the number of pairs that reproduced in each treatment group are shown on the figure.

On average pairs in the control temperature (+0.0°C) produced 1017±26 eggs per clutch (Fig. 3.3b). There was a trend for the number of eggs produced per clutch to decrease with increasing temperature (Fig. 3.3b). The decline was most obvious in the high CO_2 group, which decreased from 872 eggs per clutch

at +0.0°C to just 12 eggs in the only clutch that was produced at 3.0°C. Despite the large decrease in the number of eggs produced, no treatment groups were significantly different from control +0.0°C (Table 3.3). Across all treatments, female weight had a significant positive effect on the number of eggs produced per clutch (Table 3.3).

Fertilization success was generally high, with fertilization being above 95% for 8 out of the 9 treatment groups. Moderate +3.0°C was the only group to exhibit a significant difference from control +0.0°C (Table 3.3) with fertilization being 51.42±48%.

Egg area was affected by a significant interaction between temperature and CO_2 (Table 3.3, Fig. 3.3c). At +0.0°C egg area increased with CO_2, though not linearly, with moderate +0.0°C producing the largest eggs overall and high +0.0°C producing an egg area intermediate to the moderate and control CO_2 treatments. Egg area declined with increasing temperature in all CO_2 treatments (Table 3.3, Fig. 3.3c). The moderate group exhibited the greatest reduction in egg area with increasing temperature with egg area of the +1.5°C and +3.0°C groups 83% and 75% of the moderate +0.0°C eggs. The control and high CO_2 displayed a similar trend with egg area of the control +1.5°C and +3.0°C groups being 92 and 74% and high groups being 88% and 72% of their +0.0°C groups respectively (Fig. 3.3c). Reproductive output showed a similar pattern to the number of eggs produced, with temperature having a negative effect on output, however only the control +3.0°C was different from the control +0.0°C (Table 3.3, Fig. 3.3d). There was no effect of CO_2 on reproductive output (Table 3.3).

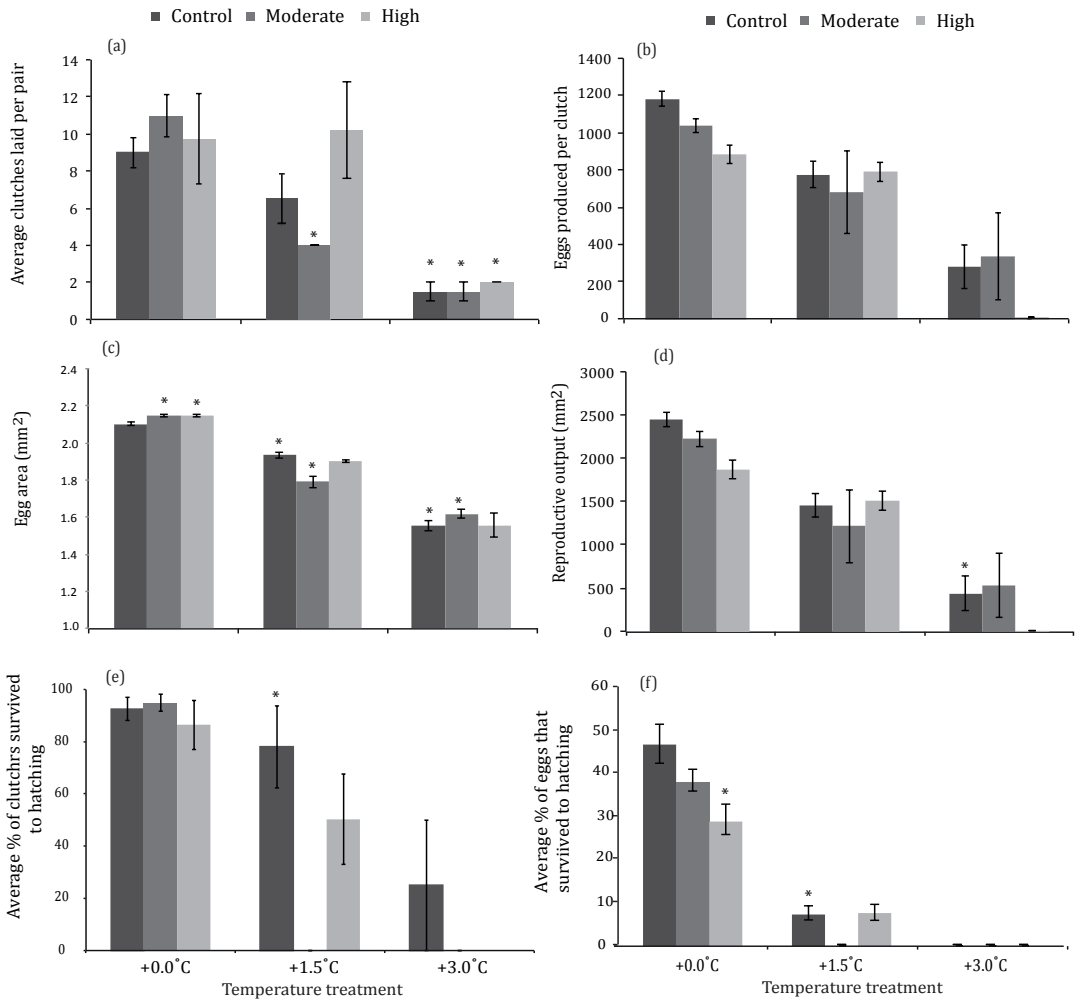

Figure 3.3: The reproductive characteristics of breeding pairs kept at control, moderate or high CO_2 cross-factored with either +0.0°C (28.5°C), +1.5°C (30.0°C) or +3.0°C (31.5°C) temperature treatments. The reproductive characteristics are: (a) the average number of clutches produced per pair for the breeding season, (b) the average number of eggs produced per clutch, (c) the average egg area (mm^2), (d) the average reproductive output, being the average egg area per clutch multiplied by the number of eggs in the clutch to give an estimate of energy (mm^2), (e) the average survival rate of the clutches produced and (f) the average egg survival. All measures are the raw data means ± standard error. * denotes a treatment group that is significantly different from Control CO_2 +0.0°C.

Egg area was affected by a significant interaction between temperature and CO_2 (Table 3.3, Fig. 3.3c). At +0.0°C egg area increased with CO_2, though not linearly, with moderate +0.0°C produced the significantly largest eggs and high +0.0°C producing an egg area intermediate to the moderate and control CO_2 treatments, though still significantly different to control CO_2. Egg area

declined with increasing temperature in all CO_2 treatments (Table 3.3, Fig. 3.3c). The moderate group exhibited the greatest reduction in egg area with increasing temperature with egg area of the +1.5°C and +3.0°C groups 83% and 75% of the moderate +0.0°C eggs. The control and high CO_2 displayed a similar trend with egg area of the control +1.5°C and +3.0°C groups being 92 and 74% and high groups being 88% and 72% of their +0.0°C groups respectively (Fig. 3.3c). Reproductive output showed a similar pattern to the number of eggs produced, with temperature having a negative effect on output, however only the control +3.0°C was different from the control +0.0°C (Table 3.3, Fig. 3.3d). There was no effect of CO_2 on reproductive output (Table 3.3).

More than 85% of the clutches produced in +0.0°C survived to hatching regardless of the CO_2 treatment. However, the number of clutches that survived to hatching markedly declined with increasing temperature (Table 3.4, Fig. 3.3e). No clutches survived to hatching at moderate or high +3.0°C and only one clutch survived to hatching in control +3.0°C (Fig. 3.3e). Egg survival to hatching was quite low, the highest average survival being 47% in the control CO_2 +0.0°C. Egg survival decreased with increasing CO_2 down to 29% survival in high +0.0°C (Table 3.4, Fig. 3.3f). In addition, temperature increase also decreased egg survival to 7% in the control and high CO_2 +1.5°C and no survival in the moderate CO_2 +1.5°C or the +3.0°C groups (Table 3.4, Fig. 3.3f). There was insufficient reproduction in the moderate 1.5°C and the +3.0°C treatment groups for significant differences to be detected.

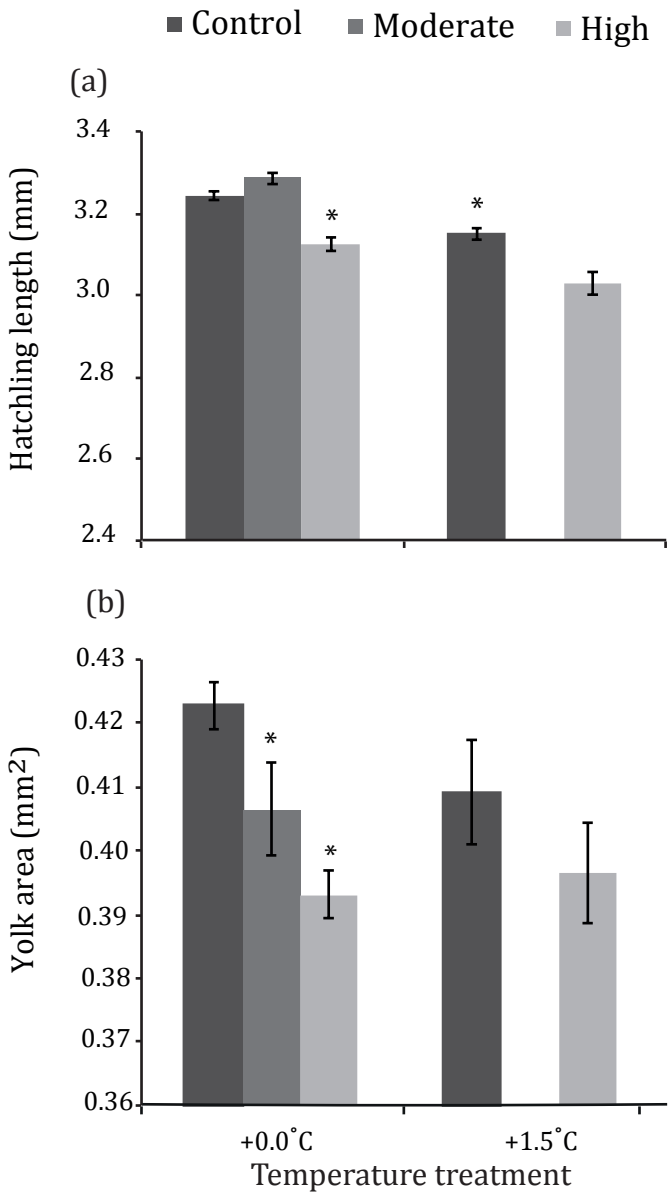

Figure 3.4: Offspring characteristics from parents kept at control, moderate or high CO_2 cross-factored with either +0.0°C (28.5°C) or +1.5°C (30.0°C). No eggs survived to hatching in the moderate +1.5°C or the +3.0°C treatment groups, consequently they are not shown. The offspring characteristics were (a) hatchling standard length (mm) and (b) yolk area (mm^2). The raw means ± standard error are presented in the figure. * denotes a treatment group that is significantly different from control CO_2 +0.0°C.

Offspring characteristics

No clutches survived to hatchling in moderate CO_2 +1.5°C or the +3.0°C groups, consequently only the +0.0°C and control and high +1.5°C were analyzed. Both temperature and CO_2 had a significant effect on hatchling length

(Table 3.5, Fig. 3.4a). Newly hatched larvae in the high +0.0°C and in the control +1.5°C groups were significantly shorter than the control +0.0°C (Fig. 3.4a). Elevated CO_2 but not temperature, had a significant negative impact on yolk area, with both the moderate and high +0.0°C treatment group larvae having smaller yolk reserves compared to control +0.0°C (Table 3.5, Fig. 3.4b).

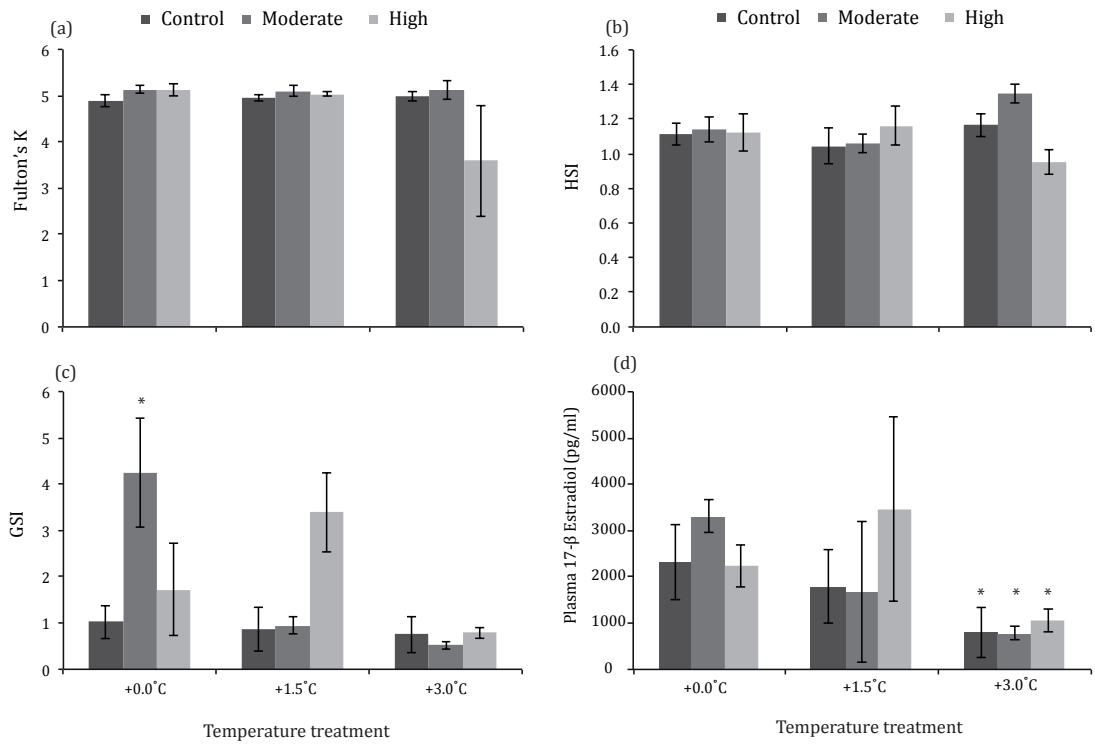

Figure 3.5: Adult physiological condition and hormone concentrations at the end of the breeding season, (a) Fulton's K body condition index, (b) hepatosomatic index, (c) gonadosomatic index and (d) plasma 17β-E_2 concentrations of females. The raw means± standard error are presented in the figure. * represent groups that are significantly different.

Adult body and reproductive condition

Neither Fulton's K body condition factor or hepatosomatic index (HSI) were significantly affected by either temperature or CO_2, for reproductive females (Table 3.6, Fig. 3.5a,b). Fulton's K and HSI levels were generally high and only reduced at the most extreme treatment, high CO_2 +3.0°C. Temperature significantly effected gonadosomatic index (GSI) and there was an interaction

between temperature and CO_2 treatment (Table 3.6). At +0.0°C, moderate CO_2 was significantly different from all other treatment groups (Fig. 3.5c). GSI was unaffected by temperature in control CO_2. At moderate CO_2, GSI was highest at +0.0°C and declined at the higher temperature. In contrast, GSI was highest at +1.5°C before decreasing at +3.0°C in the high CO_2 treatment (Fig. 3.5c).

Plasma E_2 concentrations

Plasma E_2 concentrations were significantly negatively affected by increasing temperature (Table 3.6). Concentrations in the +3.0°C were significantly lower than the +0.0°C E_2 concentrations (Fig. 3.5d). Factor analysis identified two main factors, First, the presence of stage 2 oocytes and the presence of stage 4 and 5 oocytes (Table 3.7) accounting for nearly 50% of the variation in the data set. The second factor, accounting for 25% of the variation, was influenced by the presence of stage 3 oocytes (Table 3.7). Multiple regression analysis showed significant overall relationship for plasma E_2 concentration ($F_{2,47}$=25.63, $p<0.0001$) which included a significant relationship to factor 1 ($F_{1,47}$=7.11, $p<0.000001$) but not factor 2 ($F_{1,47}$=-0.88, $p=0.38$).

Discussion

Higher temperatures and elevated CO_2 can act additively or synergistically to reduce individual performance (Pörtner & Farrell, 2008). Previous studies have found that reproduction declines at elevated temperatures in a range of marine fishes (Donelson *et al.,* 2010; Hilder & Pankhurst 2003; Van Der Kraak & Pankhurst 1997). Similarly, increased CO_2 is predicted to increase the energy required for maintaining homeostasis, and therefore reduce the amount of energy available for reproduction (Ishimatsu *et al*., 2008, Melzner *et al*., 2009;

Pörtner, 2012). Despite this prediction, recent studies have found that exposure to increased CO_2 does not, on its own, cause reproduction to decline in fish (Sundin *et al.,* 2012; Frommel *et al.,* 2010; Miller *et al.*, 2013; Schade *et al.,* 2014). This study is the first to examine the interaction between temperature and CO_2 on reproduction in reef fish for an entire reproductive season. We found that the interaction between CO_2 and temperature was complex, but that overall, elevated temperature had a much greater effect on reproduction than did projected future CO_2 levels. At control temperatures there was an apparent decline in reproductive output and offspring quality with increasing CO_2. At +1.5°C above current-day temperatures, breeding pairs in the moderate CO_2 treatment didn't produce successful clutches; however the control and high CO_2 pairs at this temperature continued to reproduce, though at a reduced rate compared to the same CO_2 treatments at control temperatures. By far the most obvious result from this study, was the complete cessation of reproduction at +3.0°C above current-day summer average temperature, irrespective of CO_2 level.

Reproductive and offspring characteristics: CO_2

In a previous experiment, Miller *et al.* (2013) observed an increase in reproduction at elevated CO_2 levels similar to those used in this experiment. A similar increase in reproduction has since been documented in the Three-spine stickleback (Schade *et al.,* 2014) and elevated CO_2 resulted in increased levels of reproductive hormones in Atlantic cod (Preus-Olsen *et al.,* 2014). Together, these results suggest that stimulation of reproduction by elevated CO_2 could occur in a variety of fish species from a range of families.

In this study, however, reproduction in the high CO_2 group was not significantly increased compared to the control or moderate groups (at control temperatures matching Miller *et al*. 2013). In this instance, the control and moderate groups doubled their reproductive activity compared to Miller *et al.* (2013), while the high group maintained reproductive levels in comparison to the previous study. The high CO_2 group did produce a similar number of eggs per clutch, and more clutches over the season, compared to Miller *et al.* (2013). The reason for the increase in reproductive performance of the control and moderate CO_2 groups compared to reproductive performance of control and moderate pairs in Miller *et al*. (2013) is unclear, but may be related to difference in the time required to acclimate to laboratory conditions. Breeding of wild-caught fish can improve with time in captivity, which could explain why the control and moderate group performed better in their second year of captivity (this study) compared with the earlier study (Miller *et al*., 2013). Behavioural studies show that reef fish exposed to CO_2 >700µatm tend to be bolder and more active (reviewed Munday *et al*., 2012) which may compensate for the stress response to captivity (Pankhurst & Van Der Kraak, 1997), leading to greater breeding in the first year in this group. Whatever the mechanism, our results suggest that examining just one reproductive season may not provide a full picture of the effect of elevated CO_2 on reproduction.

Unlike our previous study (Miller *et al.,* 2013) we also detected significant negative impacts on reproduction, with clear declines in egg survival and yolk provisioning. A similar decline in embryonic survival has recently been seen in a temperate goby (Forsgren *et al.*, 2013), but was not detected in a closely related species, *Amphiprion percula*, (Munday *et al.*, 2009b). The decline

detected here equates to the high CO_2 +0.0°C group having less than half the number of surviving eggs per clutch (~250 eggs) compared to the control group (~540 eggs). A decline in reproductive output of this magnitude, if it occurs in wild populations, could potentially have a significant effect on population replenishment.

Further to the decline in embryonic survival, the larvae that were produced under high CO_2 were both shorter and had less yolk compared to the control and moderate CO_2 larvae. A reduction in yolk area was also detected in *A. percula* larvae reared at similar CO_2 levels (Munday *et al.,* 2009b). Yolk reserves provide the energy for growth until the larvae are able to feed, therefore a reduction in yolk provisioning could lead to reduced somatic growth at least in the early larval stage. Yolk reserve is also a good indicator for future growth and performance (Hoey & McCormick, 2004; Grorud-Colvert & Sponaugle, 2006). In addition, the high CO_2 offspring were significantly shorter at hatching compared to control offspring. Hatchling length is a key fitness-related trait (Miller *et al.*, 1998). Reductions in both yolk reserve and hatchling length could reduce juvenile performance, potentially increasing mortality.

Reproductive characteristics: Temperature and interaction

The most obvious result from our data was the negative impact of increasing temperature on every reproductive characteristic investigated, except fertilization success, regardless of CO_2 level. This was particularly obvious in the decline in number of eggs produced per clutch, with an increase of +3.0°C reducing the egg output of the control group by 75%. Even more startling was the decline in the number of eggs that survived to hatching. An increase of

+1.5°C reduced survival to hatching from ~49% to ~7% in the control group. The same temperature increase resulted in no surviving eggs in the moderate CO_2 group, and at +3.0°C there were no surviving eggs regardless of CO_2 treatment. This trend of declining reproduction with increasing temperature has been shown in a number of tropical and temperate fishes (Donelson *et al*., 2010; Lansteiner & Kletzl, 2012; Warren *et al.,* 2012) and in other ectothermic animals (Snell 1986; Lee *et al.,* 2003). Given this trend, there could be serious declines in fish populations by 2100, through reduced reproduction, unless there is sufficient scope for thermal acclimation or adaptation of reproduction over the next few decades.

No studies have yet examined the potential for genetic adaptation of reproduction in fishes to ocean warming. However, one study has tested the potential for acclimation of reproduction to projected future warming in a reef fish. Donelson *et al.* (2014) found that reproductive traits in *Acanthochromis polyacanthus* were restored to control levels when fish complete development and are reaed their entire life at +1.5°C, but there was no reproductive acclimation when fish were reared at +3.0°C for their entire lives. Consequently, there appear to be constraints on the potential for acclimation, at least for some reef fishes, particularly at the higher temperatures (+3.0°C) that caused the greatest declines in reproduction in our study. Whether there is potential for transgenerational acclimation of reproduction is currently unknown.

In addition to transgenerational acclimation, it is possible that species may extend their ranges, either shifting to higher latitude or deeper waters to avoid or escape the effects of elevated temperatures (Poloczanska *et al*, 2013). However, range shifting may only be relevant to populations that live at the

edge of the species range. For populations in the middle of the range expanding polewards may not alleviate the impacts of climate change. A concerted effort needs to be made to understand how effective range shifts will be in allowing for species survival in the face of climate change.

As with elevated CO_2, we detected a significant negative effect of increased temperature on hatchling length in the control CO_2 group, and a similar, though non-significant, trend in the high CO_2 group. There may be a minimum size for hatchling length, similar to the minimum or optimal length required for metamorphosis of juveniles in fish and other species (Chambers & Leggett, 1987; Altwegg & Reyer, 2007). If so, the effect of elevated CO_2 treatment may have already reduced hatchling length close to the minimum viable length, to a point that increased temperature did not have a further significant impact. This hypothesis is supported by the declines in yolk provisioning that occurred in +1.5°C control offspring, not being present in the +1.5°C high CO_2 offspring. A minimum energy requirement may be needed for embryo's to survive to hatching.

Potentially the most surprising result in this study was the cessation of reproduction in the moderate CO_2 +1.5°C group. This was not due a delay in reproduction, as there was reproduction in this group prior to experimental temperatures being attained. The fact that the moderate CO_2 +0.0°C and the control +1.5°C groups both reproduced suggests that, on their own, neither stressor is enough to restrict reproduction. However, when the two occur in combination they cause sufficient stress on the organism, causing reproduction to cease. Interestingly, despite the clear decline in reproduction in the moderate CO_2 +1.5°C, there was not a significant decline in plasma E_2 concentrations in

this group. This suggests that changes in concentrations of this particular sex steroid are not responsible for the decline in reproduction at moderate CO_2 +1.5°C. One possible explanation for the cessation of reproduction in this group, but not the high CO_2 +1.5°C, is that the moderate CO_2 level lies below the threshold at which physiological acclimation occurs. In Miller *et al.* (2013) we suggested that the increased reproduction in the high CO_2 group could be a hormetic response. It is possible that at the high CO_2 level, a change, caused by the increased CO_2 occurs that "switches on" reproduction. This switch could explain why the high CO_2, but not the moderate CO_2 group reproduced at +1.5°C.

The behavioural impacts of elevated CO_2 begin to occur, somewhere between 600 and 700µatm in most reef fishes studied to date (Munday *et al.,* 2010). It is possible that whatever causes the change in behaviour (hypothesized to be disrupted neural activity, Nilsson *et al.,* 2012; Hamilton *et al.,* 2014) could also cause the stimulation of reproduction. At a more practical level, the cessation of reproduction at projected mid-century CO_2 and temperature levels is quite disturbing. The synergistic effects of these stressors could result in reproductive failures for tropical fishes within the next 40 years potentially impacting on commercial and non-commercial species alike. While there is evidence for transgenerational acclimation of life history-traits to ocean acidification (Miller *et al.*, 2012), and the at least potential for reproductive acclimation to moderate warming (+1.5°C) (Donelson *et al*., 2014), as yet, there is no evidence for acclimation to both these stressors in combination.

Hormonal and physiological impacts

The results suggest that reproductive, but not physiological condition, of females caused the changes in reproductive and offspring characteristics. First, elevated temperature resulted in a decrease in E_2 concentrations. Moreover, there was a strong correlation between E_2 concentration and the average number of clutches produced per pair. In contrast, there was little effect of elevated CO_2 or temperature on the physiological condition of either reproductive or non-reproductive females. Both Fulton's K body condition index and hepatosomatic index were high for all treatment groups, indicating no decline in body condition or energy stores. This suggests that the dramatic declines in reproductive output observed at higher temperature were not due to energetic constraints in the females. Previous studies have shown that under elevated temperatures the enzyme CYP19 aromatase, which catalyses the irreversible conversion of testosterone into E_2 in inhibited (Watts *et al.,* 2004). Inhibition of CYP19 aromatase will result in a decline in E_2 synthesis, and a subsequent reduction in vitellogenesis and oocyte maturation (Piferrer & Blázquez, 2005; Yaron & Levavi-Sivan, 2011). Hence the results strongly suggest that a reduction of plasma E_2 concetrations, likely through inhibition of CYP19 aromatase may be the cause for decrease reproduction at higher temperature in *A. melanopus*.

Conclusions

This is the first time that the potential interactive effects of projected future CO_2 and temperature conditions have been tested in regards to fish reproduction. As with similar studies conducted on invertebrate reproduction (Chua *et al.*, 2013; Byrne *et al*., 2009), we found that temperature had a much stronger impact that

CO_2. Our data, similar to other studies on tropical fish, showed that there was complete reproductive failure at +3.0°C above the current-day average temperature. Given that sea surface temperatures within the tropics are projected to rise up to +3.0°C (Ganachaud *et al.,* 2011) by 2100, there could be significant consequences for reproduction in tropical fish populations. Nevertheless, we did detect interactions between temperature and CO_2 at the combined moderate levels. Previous studies have shown that when temperature has increased +1.5°C that reproduction is reduced, as we saw in the control CO_2 group. Yet when the extra stress of increased CO_2 was added, and without any compensatory mechanisms, there appears to be a major reproductive failure. Our data suggest that, without reproductive acclimation or adaptation, there could be reproductive failure for this species as early as the middle of the century. These results reinforce the importance of examining how multiple stressors will interact, so that accurate climate change predictions can be made.

Our results also show that, for this species, reproduction may not occur when an individual is in otherwise good physiological condition and that one will not necessarily predict the other in future warmer and more acid conditions. Reproduction involves a complex series of interactions between environmental conditions and hormonal pathways. Further research will be required to determine the mechanisms responsible for declining reproduction at higher temperature, but it will likely involve thermal sensitivity of hormonal pathways. As yet there is no easy way to predict how reproduction will respond to climate change scenarios other than to experimentally test the population.

Table 3.1: Seawater parameters for adult *Amphiprion melanopus* held under control, moderate or high CO_2 cross-factored with +0.0°C (28.5°C), +1.5°C (30.0°C) or +3.0°C (31.5°C) water temperature treatments. Temperature, salinity, total alkalinity and pH_{NBS} were measured in situ, while pCO_2 was calculated using CO2SYS.

Treatment	Temp (°C)	Salinity (ppt)	Total alkalinity (µmol kg^{-1} SW)	pH_{NBS}	pCO_2 (µatm)
Control +0.0°C	28.4±0.01	33.32±0.12	2058±16	8.15±0.005	400±6
Control +1.5°C	29.8±0.02	33.32±0.12	2064±16	8.14±0.005	411±6
Control +3.0°C	31.4±0.02	33.32±0.12	2077±16	8.12±0.005	441±7
Moderate +0.0°C	28.5±0.01	32.7±0.12	2152±10	8.001±0.007	634±13
Moderate +1.5°C	30.1±0.01	32.7±0.12	2117±7	8.00±0.006	642±12
Moderate +3.0°C	31.5±0.01	32.7±0.12	2130±8	8.00±0.007	658±13
High +0.0°C	28.5±0.01	33.62±0.09	2168±7	7.81±0.008	1087±25
High +1.5°C	29.8±0.02	33.62±0.09	2167±7	7.79±0.008	1126±24
High +3.0°C	31.5±0.01	33.62±0.09	2169±7	7.78±0.008	1191±27

Table 3.2: ANCOVA (type III) results for the average number of clutches produced by each adult pair kept at control, moderate or high CO_2 cross-factored with +0.0°C (28.5°C), +1.5°C (30.0°C) or +3.0°C (31.5°C) water temperature treatments.

Treatment	DF	Sums of Squares	Mean Squares	F-value	p-value
Weight	1	1.252	1.252	0.095	0.7619
Temperature	2	207.932	103.9663	7.856	<0.01*
CO_2:Temperature	4	16.702	5.176	0.316	0.8629
Residuals	18	238.1976	13.233		

Table 3.3: Linear mixed effects model tables for the reproductive characteristics from adults kept at control, moderate or high CO_2 cross-factored with +0.0°C (28.5°C), +1.5°C (30.0°C) or +3.0°C (31.5°C) water temperature treatments. Treatment groups that are significantly different to Control +0.0°C are marked by *.

Characteristic	Variable	Value	SE	DF	t-value	p-value
Interclutch Interval	(Intercept)	17.811	0.745	161	23.921	<0.0001*
	Control +1.5°C	1.174	1.669	19	0.703	0.4905
	Control +3.0°C	12.722	0.980	19	12.982	<0.0001*
	Moderate +0.0°C	-3.997	0.897	19	-4.458	<0.001*
	Moderate +1.5°C	4.678	3.832	19	1.221	0.2371
	Moderate +3.0°C	69.963	80.941	19	0.864	0.3982
	High +0.0°C	-6.249	0.816	19	-7.662	<0.0001*
	High +1.5°C	-0.319	1.763	19	-0.181	0.8582
	High +3.0°C	-5.784	5.416	19	-1.068	0.2989
Number of Eggs	(Intercept)	369.260	353.156	157	1.046	0.2974
	Female Weight	24.495	9.979	17	2.455	<0.05*
	Control +1.5°C	-230.128	196.044	17	-1.174	0.2566
	Control +3.0°C	-486.441	313.976	17	-1.549	0.1397
	Moderate +0.0°C	74.833	193.248	17	0.387	0.7034
	Moderate +1.5°C	95.137	388.244	17	0.245	0.8094
	Moderate +3.0°C	-322.907	409.861	17	-0.788	0.4416
	High +0.0°C	-139.385	214.176	17	-0.651	0.5239
	High +1.5°C	172.361	258.544	17	0.667	0.5139
	High +3.0°C	-209.533	450.492	17	-0.465	0.6477
Fertilisation success	(Intercept)	90.160	3.791	157	23.783	<0.0001*
	Control +1.5°C	9.409	6.405	18	1.469	0.1591
	Control +3.0°C	7.322	8.223	18	0.890	0.3850
	Moderate +0.0°C	9.362	6.161	18	1.520	0.1460

	Moderate +1.5°C	-8.931	13.081	18	-0.682	0.5035
	Moderate +3.0°C	-55.415	12.019	18	-4.611	<0.001*
	High +0.0°C	4.527	3.791	18	1.194	0.2479
	High +1.5°C	-3.439	8.528	18	-0.403	0.6915
	High +3.0°C	-2.009	13.196	18	-0.152	0.8807
Egg Area	(Intercept)	2.563	0.061	1627	41.684	<0.0001*
	Female Weight	-0.014	0.002	174	-7.943	<0.0001*
	Control +1.5°C	-0.277	0.034	174	-8.194	<0.0001*
	Control +3.0°C	-0.794	0.076	174	-10.428	<0.0001*
	Moderate +0.0°C	-0.095	0.032	174	-3.011	<0.01*
	Moderate +1.5°C	-0.197	0.075	174	-2.621	<0.01*
	Moderate +3.0°C	0.338	0.117	174	2.897	<0.01*
	High +0.0°C	-0.128	0.036	174	-3.571	<0.001*
	High +1.5°C	0.018	0.043	174	0.413	0.6804
	High +3.0°C	0.184	0.157	174	1.252	0.2122
Reproductive output	(Intercept)	1161.624	741.883	157	1.566	0.1194
	Female Weight	39.682	20.922	17	1.897	0.0750
	Control +1.5°C	-701.157	413.854	17	-1.694	0.1085
	Control +3.0°C	-1462.128	589.445	17	-2.481	<0.05*
	Moderate +0.0°C	104.453	411.792	17	0.254	0.8028
	Moderate +1.5°C	64.732	802.208	17	0.081	0.9366
	Moderate +3.0°C	-398.727	793.082	17	-0.503	0.6216
	High +0.0°C	-405.597	452.430	17	-0.896	0.3825
	High +1.5°C	412.802	547.022	17	0.755	0.4608
	High +3.0°C	-49.727	887.772	17	-0.056	0.9560

Table 3.4: Quasi-likelihood general linear model results for the average proportion of clutches that survived to hatching and average proportion of eggs that survived to hatching for adults kept at control, moderate or high CO_2 cross-factored with +0.0°C (28.5°C), +1.5°C (30.0°C) or +3.0°C (31.5°C) water temperature treatments. Treatment averages that are significantly different from Control +0.0°C are marked with an asterix (*).

Treatment	Value	SE	DF	t-value	p value
Successful clutches (%)					
Control +0.0°C	-0.211	0.2	64	-1.125	0.2646
Control +1.5°C	-2.220	0.4	19	-5.217	<0.0001*
Control +3.0°C	-1.706	0.8	19	-2.069	0.0525
Moderate +0.0°C	0.020	0.3	19	0.079	0.9378
Moderate +1.5°C	-24.661	116809.1	19	0.000	0.9998
Moderate +3.0°C	-24.550	110522.5	19	0.000	0.9998
High +0.0°C	-0.444	0.3	19	-1.481	0.1549
High +1.5°C	0.348	0.6	19	0.555	0.585
High +3.0°C	-23.584	54075.0	19	0.000	0.999
Egg survival					
Control +0.0°C	-0.156	0.15	179	-1.050	0.2953
Control +1.5°C	-2.250	0.36	19	-6.230	<0.0001*
Control +3.0°C	-1.762	0.87	19	-2.018	0.0580
Moderate +0.0°C	-0.292	0.2	19	-1.451	0.1630
Moderate +1.5°C	-23.271	65787.90	19	0.000	0.9997
Moderate +3.0°C	-23.237	71691.82	19	0.000	0.9997
High +0.0°C	-0.659	0.23	19	-2.828	<0.05*
High +1.5°C	0.661	0.49	19	1.258	0.1904
High +3.0°C	-22.369	35076.46	19	0.000	0.995

Table 3.5: Linear mixed effects ANOVA results for physical characteristics (hatchling length and yolk area) of offspring resulting from adults kept at control, moderate or high CO_2 cross-factored with +0.0°C (28.5°C) or +1.5°C (30.0°C) water temperature treatments. No clutches survived to hatching in the Moderate +1.5°C or +3.0°C treatment groups. Significant effects ($p<0.05$) are denoted by *.

Characteristic	Variable	Value	Standard Error	DF	t-value	p-value
Hatchling length	(Intercept)	3.245	0.128	557	25.358	<0.0001*
	Female Weight	-0.000	0.004	64	-0.012	0.9901
	Control +1.5°C	-0.083	0.036	64	-2.322	<0.05*
	Moderate +0.0°C	0.047	0.057	64	0.820	0.4152
	High +0.0°C	-0.111	0.055	64	-2.004	<0.05*
Yolk area	(Intercept)	0.486	0.036	557	13.685	<0.0001*
	Female Weight	-0.002	0.001	64	-1.903	0.0615
	Control +1.5°C	-0.006	0.010	64	-0.623	0.5356
	Moderate +0.0°C	-0.032	0.016	64	-2.001	<0.05*
	High +0.0°C	-0.046	0.015	64	-3.078	<0.01*

Table 3.6: Table 6. Fixed factor type III ANOVA table for adult physiological parameters. Adults were kept at Control, Moderate or High CO_2 cross-factored with +0.0°C (28.5°C), +1.5°C (30.0°C) or +3.0°C (31.5°C) water temperatures. Significant effects ($p<0.05$) are denoted by *.

Characteristic	Variable	DF	MS	F-value	p-value
Fulton's K	CO_2	2	0.079	1.184	0.313
	Temperature	2	0.211	3.157	0.051
	CO_2:Temperature	4	0.024	0.356	0.839
	Residuals	54	0.067		
H.S.I.	CO_2	2	0.016	0.374	0.689
	Temperature	2	0.041	0.971	0.385
	CO_2:Temperature	4	0.038	0.887	0.478
	Residuals	54	0.042		
G.S.I	CO_2	2	3.365	2.266	0.114
	Temperature	2	11.655	7.848	<0.01*
	CO_2:Temperature	4	4.157	2.800	<0.05*
	Residuals	54	1.486		
17β-estradiol	CO_2	2	1765838	1.044	0.361
	Temperature	2	10229356	6.050	<0.01*
	CO_2:Temperature	4	1584219	0.937	0.452
	Residuals	43	1690826		

Table 3.7: Eigenvector values from factor analysis examining the trends in variance of gonadal development in relation to plasma 17β-estradiol concentration in female *A. melanopus*. The percentage of variation in plasma 17β-estradiol concentration explained by the first two factors in given. Gamete stages that contributed >70% to the factors are bolded.

Gonadal cell stage	Factor 1 48%	Factor 2 25%
Stage 1 Oogonia	-0.691	0.499
Stage 2 Perinucleolus	**-0.793**	0.170
Stage 3 Cortical alveolous	0.007	**0.975**
Stage 4 Early vitellogenic	**0.891**	0.011
Stage 5 Late vitellogenic	0.719	0.204

Chapter 4: Parental environment mediates impacts of elevated CO_2 on a coral reef fish

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Introduction

Carbon dioxide (CO_2) concentrations in the surface ocean are increasing due to rising CO_2 concentrations in the atmosphere (Doney, 2010). Higher CO_2 levels are predicted to affect essential physiological processes of many aquatic organisms (Pörtner *et al*., 2004; Melzner *et al*., 2009b), leading to widespread impacts on marine diversity and ecosystem function, especially when combined with the effects of global warming (Hoegh-Guldberg *et al.,* 2007; Pörtner & Farrell, 2008; Fabry *et al*., 2008). Yet the ability for marine species to adjust to rising CO_2 levels over multiple generations is an unresolved issue. Here we show that ocean conditions projected for the end of the century (approximately 1000µatm CO_2 and a temperature rise of 1.5°C - 3.0°C) cause an increase in metabolic rate and decreases in length, weight, condition and survival of juvenile fish. However, these effects are absent or reversed when parents also experience high CO_2 concentrations. Our results show that non-genetic parental effects can dramatically alter the response of marine organisms to

increasing CO_2 and demonstrate that some species have more capacity to acclimate to ocean acidification than previously thought.

Elevated CO_2 can affect acid-base regulation, oxygen transport and metabolic rate (Pörtner *et al.,* 2004; Melzner *et al.,* 2009b: Rosa & Seibel, 2008), with consequences for individual growth, survival and reproduction. Increased temperature also affects growth and survival of marine organisms, primarily through limitations to oxygen transport (Pörtner & Farrell, 2008). In fish, a declining capacity for oxygen delivery to the tissues with increasing temperature sets the limits for individual performance, which ultimately determines the viability of local populations (Pörtner & Knust, 2007; Eliason *et al*., 2011; Nilsson *et al*., 2009). Although both elevated CO_2 and higher temperature can constrain individual performance, a major limitation to predicting the effects of rising CO_2 concentrations on marine species and ecosystems is the lack of information on acclimation or adaptation to elevated CO_2 over time scales relevant to climate-change predictions (Pandolfi *et al*., 2011; Pörtner *et al*., 2011). There is increasing evidence that the capacity for acclimation to environmental stress may depend on the history of previous life stages (Marshall & Morgan, 2011; Parker *et al*., 2012). For example, recent studies show that the aerobic capacity of some thermally sensitive fish can fully acclimate to warmer water, but only if their parents have experienced the same increase in temperature (Donelson *et al*., 2012; Salinas & Munch, 2012). Whether parental effects can similarly mediate the effects of elevated CO_2, or the interacting effects of elevated CO_2 and temperature, is unknown.

We conditioned adult anemonefish, *Amphiprion melanopus*, to current-day (control, 430µatm) and elevated CO_2 treatments (moderate, 581µatm; high,

1032µatm) consistent with projections for CO_2 concentrations in the atmosphere and ocean over the next 50-100 years (Meehl *et al*., 2007). Breeding pairs were allowed to spawn naturally in their CO_2 treatments, and their offspring were reared in a cross-factored CO_2 x temperature design. Juveniles from control parents were reared at either control CO_2 (control-control CO_2) or transferred to high CO_2 (control-high CO_2) at each of three temperatures (28.5°C, 30.0°C and 31.5°C). Rearing temperatures represent the current-day average summer temperature for the study population (28.5°C) and the 1.5°C - 3.0°C increase in tropical sea surface temperature predicted for the next 50-100 years (Poloczanska *et al*., 2007). Juveniles from parents in the moderate and high CO_2 treatments were reared in similar CO_2 conditions as their parents (moderate-moderate CO_2 and high-high CO_2) at each of the three temperatures (See Table 4.1 for all seawater parameters). Comparisons between treatments allowed us to determine the acute (within-generation) effects of elevated CO_2 and temperature on juvenile performance and to test if such effects were mediated by exposure of parents to elevated CO_2.

Methods

Experimental system and seawater chemistry

Adult and juvenile anemonefish were reared in an environmentally controlled aquarium facility at James Cook University, Townsville, Australia. The facility consisted of 3 x 8000l recirculating seawater systems that were maintained at different CO_2 levels (control, moderate, high) and supplied seawater to individual aquariums. Moderate and high CO_2 treatments were achieved by CO_2 dosing to a set pH following standard techniques (Gattuso *et al*., 2010). A

pH computer (Aquamedic AT-Control, Germany) regulated CO_2 dosing in a 3000l temperature-controlled sump within each system. Seawater was heated to three temperatures (28.5°C, 30.0°C and 31.5°C) with electronic in-line heaters before delivery to individual aquariums. Temperature (Comark C22) and pH_{NBS} (Hach HQ40d) were recorded daily in the adult and juvenile aquaria. Total alkalinity was estimated weekly by Gran titration from water samples of replicate tanks in each system (Metrohm 888 Titrando) and salinity was measured weekly (Hach HQ15d). Accuracy of titrations was within 1% of certified reference material (Prof. A. Dickson, Scripps Oceanographic Institute). Average seawater pCO_2 was calculated in the program CO2SYS (Pierrot *et al*., 2006) using the constants of Mehrbach *et al*. (1973) refit by Dickson & Millero (1987). Seawater parameters are shown in Table 4.1.

Study Species and Brood Stock Maintenance

To examine the potential for transgenerational acclimation to ocean acidification, adult breeding pairs of the cinnamon anemonefish, *Amphiprion melanopus*, were collected from 4 reefs in the Palms Island region of the central Great Barrier Reef, Australia (18° 37’ S, 146° 30’ E). Breeding pairs were housed in individual 60l aquaria and maintained at control temperatures (22.5°C winter and 28.5°C summer). Eighteen breeding pairs were randomly assigned to each of the 3 CO_2 treatments (control (~430µatm), moderate (~581µatm) and high (~1032µatm)) at the end of August 2010 and pH was slowly adjusted to desired levels over a 2-week period. This allowed pairs to be condition to their CO_2 treatments for two months before the start of the breeding season in November. Temperatures were increased from winter temperatures

of 22.5°C at 0.5°C per week until the summer breeding temperature, 28.5°C, was reached in the first week of November 2010. Breeding pairs were maintained in their CO_2 treatments until May 2011. Breeding pairs were provided with a half terracotta pot as a hide and a spawning site. Pairs were allowed to spawn naturally during the breeding season (November 2010-May 2011) and spawning sites were checked daily for the presence of a new egg clutch.

Juvenile Rearing

Juveniles were reared in the designated CO_2 from hatching. On the night of hatching, terracotta pots with clutches were moved to 60L larval rearing tanks. Tanks were filled with treated system water and aerated with pre-mixed air to the desired level (Munday *et al*., 2009a). To examine the difference between acute exposure to CO_2 and parental effects of CO_2 on juvenile reef fish, the juveniles were either hatched into the parental CO_2 treatment, or some clutches from control parents were hatched into high CO_2 treatment. The four juvenile CO_2 treatment groups are therefore named by both their parental CO_2 and by the CO_2 that they have spent their post-hatching life in, i.e. control-control, control-high, moderate-moderate and high-high. Between 4 and 8 different clutches per CO_2 treatment were used in the experiment, depending on the number of parents that reproduced successfully at each CO_2 level (Control-control=4, control-high=5, moderate-moderate=4, high-high=8). A subsample of newly hatched larvae was taken from each clutch to estimate size at hatching. The remaining juveniles were reared using standard protocols (Munday *et al*., 2009a) in clutch groups until the end of the pelagic larval stage at 11-days post-

hatching. At the end of their pelagic larval phase a total of 45 juveniles from each clutch were randomly selected and assigned to the three temperature treatments (28.5°C 30.0°C and 31.5°C) within their CO_2 treatment group (N=15 per temperature). Juveniles were transferred to 1.5l aquaria where they were reared individually at the required CO_2 level x temperature treatment for a further 21 days. Juveniles were fed equal rations once per day of *Artemia spp* and INVE pellets. After 21 days, 5 juveniles from each CO_2 x temperature treatment, from each clutch, were selected to have their routine metabolic rate determined. All surviving juveniles were then euthanized and preserved in 4% phosphate buffered formaldehyde before being weighed and photographed to determine their standard length at a later date.

For each clutch, routine metabolic rate (RMR) was estimated for 5 randomly selected juveniles from each temperature treatment at the end of the rearing period. RMR in mg O_2 kg^{-1} hr^{-1} was individually measured using closed system respirometry. Fish were starved for 24hr before testing. Juveniles were acclimated to individual 25ml glass respirometers blacked out with tape for at least 1hr with constant water flow prior to testing. At the end of the acclimation period the chambers were sealed and the oxygen concentration measured every 30 sec with an oxygen electrode (WTW CellOx325, Germany) for 30 min. Oxygen concentration remained above 70% of air saturation during all trials. Juveniles were tested in their rearing treatments.

Statistical Analysis

Partially nested factorial ANOVAs were used to compare SL, weight and RMR among CO_2 treatments and temperature, with egg clutch nested within CO_2

treatment. Fisher's LSD post-hoc tests were used to compare treatment means. Specifically, the mean of each CO_2 treatment (control-high, moderate-moderate, high-high) was compared to the mean of the control-control. The frequency of survival was compared among CO_2 and temperature treatment using logistic regression. As the sample size for each CO_2 x temperature treatment for each clutch was small (N=15), it was not possible to include clutch in the survival analysis and the data were pooled for this analysis. All data analysis was conducted using StatisticaX (Statsoft, Tulsa).

Results

Standard length (SL) and mass of fish at the end of the experiment were significantly less in the control-high CO_2 group compared with the control-control group at all three temperatures (SL: $F_{16,470}$=18.4, p<0.0001; Weigh: $F_{16,470}$=18.08, p<0.0001) (Fig. 4.1a,b), demonstrating a clear effect of high CO_2 on juvenile growth. However, these effects were absent, or reversed, when both parents and juveniles experienced elevated CO_2 (Fig. 4.1a,b). SL of juveniles in the moderate-moderate CO_2 and high-high CO_2 treatments was not significantly different from control-controls at 28.5°C and 30.0°C, or at 31.5°C for the high-high CO_2 group (Fig. 4.1a). At 31.5°C, SL of juveniles in the moderate-moderate CO_2 group was less than in the control-control group, but they were still significantly larger than the control-high CO_2 group (Fig. 4.1a).

Weight in the moderate-moderate CO_2 and high-high CO_2 treatments were greater than in the control-control group, although the magnitude of the effect declined with increasing temperature (Fig. 4.1b). Weight of both moderate-moderate CO_2 and high-high CO_2 treatments was greater than in the

control-control group at 28.5°C, but only the high-high CO_2 group exhibited significantly greater mass than controls at 30.0°C and 31.5°C. Increased weight in the elevated CO_2 groups may be due to the higher feeding rate that has been observed in juvenile anemonefish exposed to elevated CO_2 and increased temperatures (Nowicki *et al.,* 2012). The comparisons of length and weight among treatments demonstrate that within-generation effects of high CO_2 on juvenile growth are highly dependent on the CO_2 environment experienced by their parents.

Juvenile survival was also affected by CO_2 and parental treatments. Survival in the control-high CO_2 group was lower than the control-control group at all temperatures (Wald statistic$_3$=43.22, p<0.001) (Fig. 4.1c). In contrast, there was no significant difference in survival between the control-control group and either the moderate-moderate or high-high CO_2 group (Fig. 4.1c), demonstrating that parental effects mediate the effect of high CO_2 on juvenile survival.

As expected, temperature had an independent effect on SL (CO_2:Temperature; $F_{6,470}$=0.7, p=0.62 Temperature $F_{2,470}$=8.9, p<0.001) and weight (CO_2:Temperature; $F_{6,470}$=0.51, p=80; Temperature $F_{2,470}$=3.7, p<0.05), with both SL and weight declining with increasing temperature (Fig. 4.1a,b). The declines in SL and weight were most apparent between 30.0°C and 31.5°C for all CO_2 treatments. The additional decline in SL and weight associated with temperature in the control- high CO_2 group indicates that the acute (within-generation) effects of higher temperature and elevated CO_2 are additive. There was no effect of temperature on survival (Wald statistic$_2$=4.15, p=0.13). Length

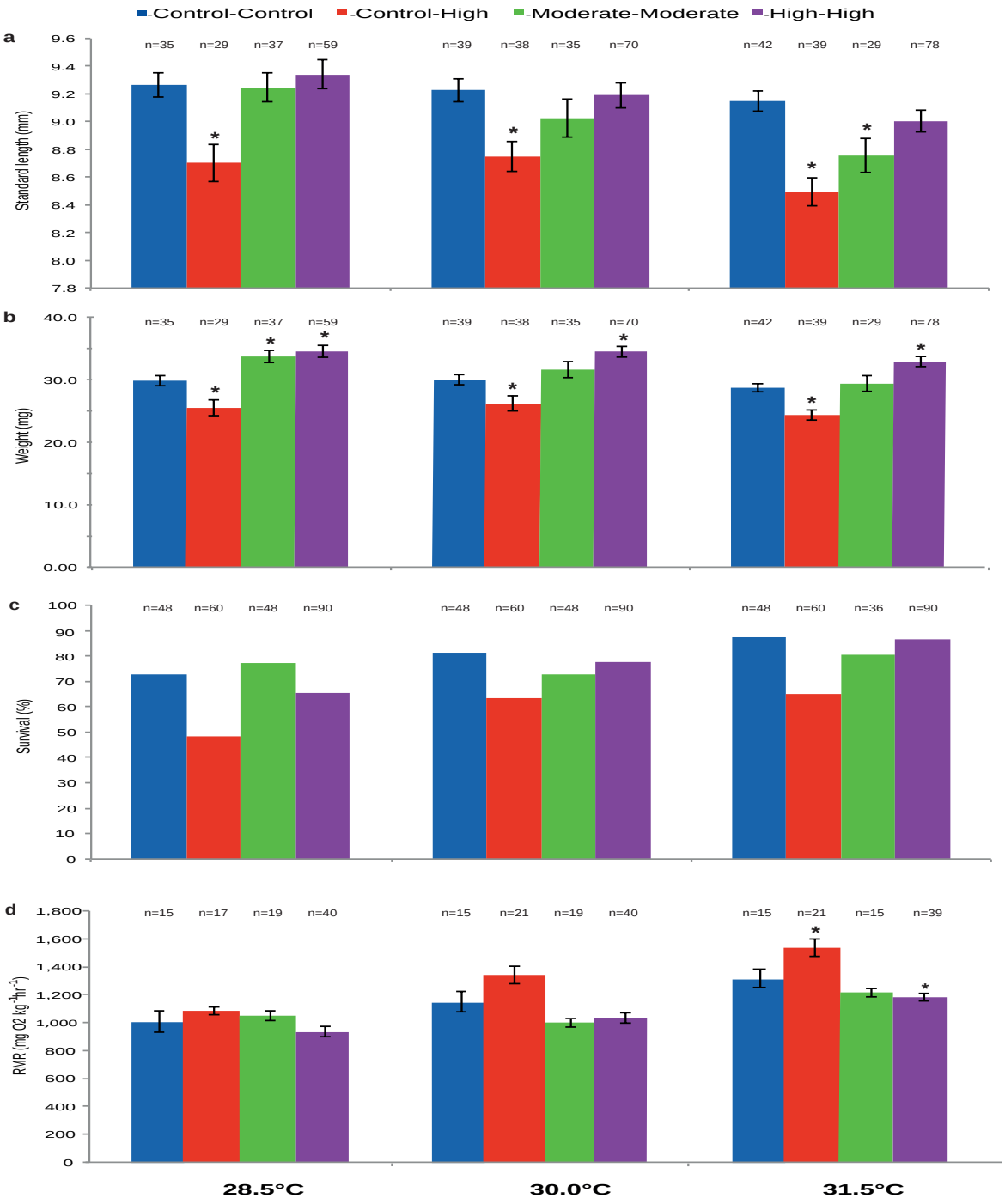

Figure 4.1: Effect of parental environment on life history and metabolic traits of juvenile anemonefish exposed to high CO_2. Standard length (**a**), weight (**b**) survival (**c**) and routine metabolic rate (**d**) of 31d-old juveniles in three different parent-offspring CO_2 treatments (control-high, moderate-moderate, high-high) were compared with a control-control treatment, where both parents and offspring experienced control conditions. A significant difference between a treatment group and the control-control group is indicated with an asterisk.

and weight of juveniles also varied among clutch (nested within CO_2 treatment) (Length: $F_{16,470}$=18.4, p<0.0001; Weight: $F_{16,470}$=18.08, p<0.0001).

The within and between generation effects of elevated CO_2 on growth and survival of juvenile fish was associated with changes in metabolic rate. As expected, routine metabolic rate (RMR) of 31d-old juveniles increased with rearing temperature (Fig. 4.1d) ($F_{2,219}$=26.495, p<0.0001). RMR also increased under elevated CO_2 ($F_{3,219}$=7.952, p<0.0001), but not consistently across temperature treatments. There was no difference in RMR between control-control and control-high CO_2 groups at 28.5°C, but acute CO_2 significantly increased RMR at 31.5°C (Fig. 4.1d). Consequently, juveniles in the control-high CO_2 group exhibited a greater increase in oxygen consumption between 28.5°C and 31.5°C (Q_{10} = 3.2) than juveniles in the control-control group (Q_{10} = 2.6). In contrast, juveniles in the moderate-moderate and high-high CO_2 groups exhibited a marked reduction in RMR compared to juveniles in the control-high CO_2 group (Fig. 4.1d). RMR of these two groups was similar to the control-control group at 28.5°C and 30.0°C, and for the high-high CO_2 group was marginally lower than the control-control group at 31.5°C (Fig. 4.1d) (Q_{10} = 1.7 moderate-moderate CO_2 and 2.2 high-high CO_2). This demonstrates that parental effects can completely compensate for the effects of high CO_2 on juvenile metabolic rate.

Discussion

Our results show that under CO_2 concentrations that could occur in the ocean by the end of this century the energy required for basic maintenance and activity (RMR) in a coral reef fish is increased. Furthermore, elevated CO_2 had

a greater influence on RMR at temperatures beyond those normally experienced by the population, thereby exacerbating the effects of rising temperature on oxygen demand. Changes in the energy budget caused by increased RMR in the control-high CO_2 group are reflected in patterns of somatic growth, with fish in this treatment both shorter and lighter than control-control fish after 31 days. Juvenile survival was also significantly reduced in juvenile fish exposed to high CO_2. Consequently, there appears to be a link between changes in basal energy turnover and individual performance. Although such associations between the physiological effects of elevated CO_2 and whole organism life-history traits have been predicted (Pörtner *et al*., 2004; Pörtner *et al*., 2011), they have not previously been demonstrated for marine fish.

Importantly, the adverse effects of elevated CO_2 on RMR, growth and survival did not occur when parents were exposed to the same CO_2 conditions as the juveniles. The conditions experienced by adults can have significant carry-over effects on the performance of their offspring (Marshall & Morgan 2011; Donelson *et al*., 2012; Bonduriansky & Day 2009), often leading to improved capacity to cope with environmental stress (Donelson *et al*., 2009; Bernardo 1996). However, with the exception of one recent study on oysters (Parker *et al*., 2012), this important mechanism has not been investigated in ocean acidification research. Many studies have reported negative effects of near-future CO_2 and pH levels on the early life history stages of marine species (Fabry *et al*., 2008; Kurihara, 2008; Hendriks *et al*., 2010; Kroeker *et al*., 2010), including larval fish (Baumann *et al*., 2012; Frommel *et al*., 2012b). Our results clearly show that non-genetic parental effects have a highly significant influence

on the performance of juvenile fish exposed to high CO_2, with the potential to fully compensate for metabolic and life history effects caused by acute (within generation) exposure to elevated CO_2.

Parental effects were equally effective in moderating the acute effects of high CO_2 on juvenile performance at moderate (581μatm) and high (1032μatm) CO_2 concentrations. In both these future CO_2 scenarios, where adults experienced the same CO_2 conditions as their offspring, the patterns of growth, survival and RMR of juveniles were similar to the control-control group. This suggests that parental effects prepare juveniles for similar conditions to those experienced in the parental generation. Although CO_2 levels in the atmosphere and ocean are rising rapidly (Doney, 2010), most species will experience a gradual increase in CO_2 over several generations. Consequently, parental effects could be highly effective in moderating the impacts of ocean acidification to rising CO_2 concentrations over coming decades.

Non-genetic parental effects may have a molecular (e.g. epigenetic inheritance) or nutritional (e.g. maternal provisioning) basis (Bonduriansky & Day, 2009). There was no difference in size at hatching between any of the treatments ($F_{82,718,}$= 0.886, p=0.75) that would be indicative of differences in maternal provisioning. Furthermore, maternal provisioning could not account for the underlying improvement in RMR of juveniles from parents reared in high CO_2. More efficient acid-base regulatory processes and/or mitochondrial function could explain the dramatic improvement in juvenile RMR in the high-high CO_2 group, and the corresponding improvement in growth and survival of juveniles from high CO_2 parents. Transgenerational epigenetic inheritance (Jablonka & Raz, 2009) is a likely mechanism by which changes in gene

expression for key enzymes involved with acid-base regulation or mitochondrial metabolism could be passed between generations, thereby enabling developing juveniles to improve their performance in a high CO_2 environment. Deigweiher and colleagues (Deigweiher *et al.,* 2008) found that gene expression for the major ion transporters (Na^+/HCO_3^- and Cl^-/HCO_3^-) in fish gills involved with acid-base regulation is initially downregulated following exposure to high CO_2, but then returns to pre-disturbance levels, or is up-regulated, over a period of 4-6 weeks. Na^+-K^+-ATPase capacity, which is expected to be the main enzyme driving the ion transport process, is also up-regulated on similar time scales. Such changes in the epigenetic state of parents exposed to high CO_2 may prime their offspring to develop more efficient physiological pathways for a high CO_2 environment. Regardless of the precise mechanism involved, our results show that the parental environment has a highly significant influence on the performance of offspring in high CO_2 conditions.

Although no life-history costs of parental effects were identified in our study, it is possible that they exist, potentially affecting traits such as maximum size or longevity (Donelson *et al.,* 2012). Furthermore, while conditioning parents to high CO_2 had a positive effect on the performance of juvenile *Amphiprion melanopus*, parental effects could be negative in other circumstances. For example, there would likely be consequences for juvenile growth and survival if mothers exposed to high CO_2 experience energetic constrains that lead to a reduction in egg provisioning (Marshall & Morgan, 2011; Green, 2008). We predict that parental effects are most likely to be positive for fish and other organisms with well-developed mechanisms for acid-base regulation, which allow them to cope with elevated CO_2, and where there

is little evidence that exposure of adults to near-future CO_2 levels has significant energetic costs (Melzner *et al.,* 2009b; Ishimatsu *et al.,* 2008).

Our observation of decreased SL and weight in 31-day-old juvenile *A. melanopus* contrasts with the lack of negative effects at similar CO_2 levels in 11-day-old larval *A. percula* (Munday *et al.,* 2009a; Munday *et al.,* 2011). Previous experiments with *A. percula* have focused on the larval stage, during which time the fish had a continuous supply of food and were able to feed *ad libitum*. The continuous supply of food may provide the larvae with sufficient energy to overcome any detrimental effects of increased CO_2 on growth. In contrast, juveniles in this experiment were fed a fixed ration once per day after they passed the larval phase. The reduced access to food by juveniles, compared with earlier studies with larvae, may have allowed the effects of elevated CO_2 on growth to become apparent. The fixed ration may also have contributed to the decline in SL and weight at the highest temperature (31.5°C) because RMR increased most markedly at this temperature, indicating an increase in the energetic cost of maintenance.

Publications on the impacts of ocean acidification on marine organisms are increasing exponentially; however most studies are short term and almost none consider more than one generation. Research into the effects of ocean acidification is set to increase even further in coming years due to targeted research funding by national governments and international consortiums. Our results show that parental effects can have a highly significant influence on the performance of marine organisms under conditions simulating future ocean acidification. Such effects will need to be considered in order to make robust predictions about future impacts on marine diversity and ecosystem function.

Table 4.1: Seawater parameters for parents and juveniles reared at control, moderate and high CO_2. Values are means ± SD

Life stage	Treatment	Salinity (ppt)	Temp (°C)	Total alkalinity (µmol kg^{-1} SW)	pH_{NBS}	pCO_2 (µatm)
Parents	Control 28.5°C	36.39	28.49 ±0.32	2049 ±79	8.11 ±0.04	430 ±54
	Moderate 28.5°C	36.38	28.51 ±0.32	2089 ±143	8.01 ±0.05	584 ±77
	High 28.5°C	36.19	28.51 ±0.33	1945 ±23	7.77 ±0.05	1032 ±137
Juveniles	Control 28.5°C	36.39	28.57 ±0.54	2054 ±56	8.12 ±0.04	438 ±46
	Control 30.0°C	36.39	29.96 ±0.26	2054 ±56	8.13 ±0.04	443 ±52
	Control 31.5°C	36.39	31.69 ±0.50	2054 ±56	8.11 ±0.04	462 ±52
	Moderate 28.5°C	36.38	28.53 ±0.19	2069 ±67	8.05 ±0.05	588 ±76
	Moderate 30.0°C	36.38	29.94 ±0.28	2069 ±67	8.02 ±0.05	610 ±82
	Moderate 31.5°C	36.38	31.47 ±0.29	2069 ±67	8.01 ±0.05	632 ±74
	High 28.5°C	36.19	28.59 ±0.18	1937 ±27	7.81 ±0.04	926 ±109
	High 30.0°C	36.19	30.01 ±0.36	1937 ±27	7.79 ±0.04	974 ±114
	High 31.5°C	36.19	31.44 ±0.59	1937 ±27	7.78 ±0.04	1012 ±123

Chapter 5: Otolith growth in *Amphiprion melanopus* juveniles is unaffected by acute or parental exposure to elevated CO_2

In preparation "Miller GM, Jones RE, Villacorta-Rath C, McCormick MI & Munday PL, Otolith growth in *Amphiprion melanopus* juveniles is unaffected by acute or parental exposure to elevated CO_2."

Abstract

The dual threats of ocean acidification and ocean warming will occur simultaneously over multiple generations for marine organisms. Yet many studies examine these environmental attributes as independent, single generation stressors. Otoliths are one of the main sensory organs in fish and are sensitive to ocean acidification. However, it is currently unknown whether otolith growth will acclimate to near-future CO_2 and temperature levels, or their interactive effects. We used a multi-generational experiment to examine the effects of ocean acidification, elevated temperature and parental effects on the growth of otoliths in juvenile reef fish. We detected no significant effects of acute or parental exposure to elevated CO_2 on the otolith shape and size morphometrics or in the asymmetry of the otolith pairs. We provide suggestions for why otolith calcification in some species is not sensitive to near future CO_2 and some caveats for interpretation and extrapolation of the literature.

Introduction

Success of larval and juvenile stages is crucial for ensuring population persistence of marine organisms. Yet these early life stages are highly sensitive to variation in the physical environmental and even relatively small changes in environmental parameters can dramatically affect growth, development and survival (Sogard 1992; Abrams *et al.* 1996; Takasuka & Aoki 2006;). Furthermore, the impact of environmental changes on early life stages may depend on the environment experienced by parents (Marshall, 2008). Consequently, complex interactions between parental and juvenile environments are likely to influence the success of early life history stages of many marine organisms. Rising carbon dioxide levels, due to anthropogenic activities will result in two major environmental changes, ocean warming and ocean acidification (Harley *et al.* 2006; Hoegh-Guldberg & Bruno 2010; Doney *et al.* 2012; Poloczanska *et al.* 2013) that will affect the future performance of marine organisms. The interacting effects of these two stressors on the early life history stages of marine organisms and how they may interact with the parental environment remain poorly understood (Dupont & Thorndyke 2009; Dupont *et al.* 2013; Sunday *et al.* 2014).

Marine fishes are expected to be relatively tolerant to ocean acidification due to their well-developed acid-base regulatory systems that can maintain blood and tissue pH balance during hypercapnia (Ishimatsu *et al.* 2008; Brauner & Baker 2009; Melzner *et al.* 2009). While the effects on growth and survival appear to vary between species, a consistent effect of increased CO_2 in fishes is an increase in otolith (ear bone) size (Checkley *et al.* 2009, Munday *et al.* 2011a,b; Hurst *et al.* 2012; Bignami *et al.* 2013; Maneja *et al.* 2013).

Otoliths are paired carbonate structures that are used in hearing, orientation and gauging movement in fish (Popper & Lu 2000; Lychakov & Rebane 2005). White sea bass (Checkley *et al.* 2009), cobia (Bignami *et al.* 2013), orange clownfish (Munday *et al.* 2011b), walleye pollock (Hurst *et al.* 2012) and Atlantic cod (Maneja *et al.* 2013) all exhibited increased otolith growth or increment width when reared in elevated CO_2 conditions. However, the level of CO_2 at which enhanced otolith growth occurred was higher in the clownfish and Atlantic cod (~1800µatm) compared to the other species (~800µatm). Furthermore, although otolith size was affected in these species, shape and symmetry was not (Munday *et al.* 2011a,b; Bignami *et al.* 2013; Hurst *et al.* 2012; Maneja *et al.* 2013). The increase in otolith growth is thought to be a consequence of altered dissolved inorganic carbon concentrations in the otolith endolymph (fluid surrounding the otoliths) of fish exposed to high CO_2 (Checkley *et al.* 2009; Munday *et al.* 2011b). While the exact process in not known, as increase in HCO_2 and/or HCO_3^- ions in the endolymph as a result of acid-base regulation could promote otolith accretion (Payan *et al.* 1999; Payan *et al.* 2004; Checkley *et al.* 2009; Munday *et al.* 2011b).

In addition to ocean acidification, otolith growth can be influenced by water temperature. In general, otolith growth tends to increase with increasing temperature (Campana & Neilson 1985; Gauldie & Radtke 1990). Otolith growth has been connected to metabolic rates, with higher metabolic rates leading to larger otolith sizes relative to fish standard length (Mosegaard *et al,* 1988; Metcalfe *et al,* 1995; Maillet & Checkley 1990); however, unlike somatic growth, otolith growth does not cease when temperatures exceed the optimum for somatic growth (Mosegaard *et al.* 1988; Metcalfe *et al.* 1995). Consequently,

otoliths will keep accreting even in temperatures that are not favorable for the individual. This is due to otolith formation being an extracellular process, and reliant on chemical processes (Gauldie & Nelson 1990), unlike somatic growth, which is reliant on sufficient energy being available for growth to continue. Consequently, the expectation is that as temperatures increase, so too will size-independent otolith growth.

Parental effects are typically non-genetic or epigenetic effects that influence the offspring's phenotype, often enhancing the offspring's performance in their environment (Marshall 2008). Recent studies have documented parental effects enhancing offspring performance under climate change scenarios (Salinas & Munch 2011; Donelson *et al,* 2011, Burgess & Marshall 2011), including ocean acidification (Parker *et al.* 2011, Miller *et al.* 2012; Murray *et al.* 2014). Critically, in juvenile anemonefish, parental exposure to elevated CO_2 resulted in juvenile growth, metabolism and survival, being equal to that of control juveniles, and much better than juveniles that were acutely exposed to elevated CO_2 (Miller *et al.* 2012). While such studies have shown that parental exposure to ocean acidification can improve the performance of the offspring reared under ocean acidification (Parker *et al.* 2012; Miller *et al.* 2012; Murray *et al*. 2014), it is not known whether similar effects will extend to otolith growth and development. If parental effects are indeed altering acid-base regulatory mechanisms, change in physiology may temper the influence of high CO_2 on otolith development.

Any changes to the size, shape and symmetry of fish otolith due to ocean acidification or warming could alter fish hearing, the ability to navigate and survival during the larval phase (Gagliano *et al.* 2008; Lemberget &

McCormick 2009; Bignami *et al.* 2013). In the present study we used a multigenerational experiment to determine whether parental exposure to elevated CO_2 alters otolith calcification rates, either through the additive effects of multigenerational exposure increasing otolith calcification further, or epigenetic effects of multigenerational exposure correcting the increased calcification. Adult *A. melanopus* were maintained in either current day control (430µatm), or high (1032µatm) CO_2 conditions for 9 months including the summer breeding season. Offspring were then reared from hatching at their parental pCO_2 treatment, except for some offspring from control parents that were reared at high CO_2. Each clutch of juveniles was split into three temperature treatments that reflect predicted ocean warming (+0.0°C, +1.5°C and +3.0°C) that will occur concurrently with increased pCO_2 conditions (Collins *et al.* 2013; Kirtman *et al.* 2013). Offspring were individually reared for 31 days post hatching, at which time sagittal otoliths were extracted and the size, shape and asymmetry (difference between left and right otoliths) were determined. This design allowed us to test the acute and parental effects of CO_2 exposure as well as the potential interacting effects between pCO_2 and ocean warming.

Methods

Study species and brood stock maintenance

The cinnamon anemonefish, *Amphiprion melanopus*, is widely distributed throughout the Indo-Pacific, where it occurs in large social groups in association with a host anemone (Drew *et al.* 2008). *A. melanopus* is a serial benthic spawner, laying multiple egg clutches during a spring-summer breeding season. Embryonic duration is between 7-9 days (Michael 2008) and after

hatching the larvae have a pelagic phase lasting 8 to 14 days (mean = 11 days) (Bay *et al.* 2006). Once developmentally competent to settle, the larvae use a variety of auditory, visual and olfactory cues to find suitable reef habitats (Leis *et al.* 2011).

Adult breeding pairs of *A. melanopus* were collected from mid-shelf reefs off the coast of Townsville, Australia in the central Great Barrier Reef: Orpheus Island (18.6183°S, 146.4936°E), Bramble Reef (18.417°S, 146.700°E), Davies Reef (18.83°S, 147.63°E) and Slasher's Reef (18.467°S, 147.083°E). Breeding pairs were randomly assigned to either control or an elevated CO_2 treatment in August 2010 (Austral winter) and placed into the treatment systems at ambient CO_2 (430µatm). In the elevated CO_2 treatment [high (1032µatm)], CO_2 was slowly increased over a two-week period until the desired level was reached. Pairs were initially held at winter non-breeding temperatures (22.5°C). Water temperature was slowly increased at 0.5°C per week until the summer breeding temperature of 28.5°C (the average summer water temperature for the collection sites) was reached in the first week of November. This slow increase to breeding temperatures allowed breeding pairs a long acclimation period to the increased CO_2 (two months from September to November) before reproducing. Breeding pairs were held under CO_2 treatment and control temperature (28.5°C) from November 2010 through to May 2011 and allowed to spawn naturally during that period. Pairs were provided with a half terracotta pot as a spawning site and fed 0.1g of commercial pellet (INVE NRD 12/24) three times per day. Terracotta pots were checked daily for new egg clutches.

Experimental treatments

The experiment utilized two 8000L recirculating aquarium systems, maintained at the desired pCO_2 and corresponding pH levels. The pCO_2 levels used were a current–day control CO_2 (430µatm), and a high CO_2 (1032 µatm) consistent with CO_2 levels projected to occur under RCP8.5 by the year 2100 (Collins *et al*. 2013). An AquaMedic AT Controller was used to maintain the desired CO_2 and pH levels in the high treatment by dosing CO_2 into a 3000L sump. The pH_{NBS} of the adult and juvenile rearing aquaria were recorded daily using a Hach Multimeter (HQ40D). Total alkalinity (TA) was estimated weekly by Gran titration (Metrohm 888 Titrando Titrator) and validated with reference material supplied by Dr. A.G. Dickson (Scripps Institution of Oceanography). Salinity was recorded weekly using a Hach Multimeter (HQ15D). pCO_2 was determined in CO2SYS (http://cdiac.ornl.gov/oceans/co2rprt.html) using salinity, TA, pH_{NBS}, and temperature (°C) and seawater pCO_2 was validated using a portable infrared sensor (GMP343, Vaisala, Helsiniki, Finland) (Munday *et* al, 2014: Watson *et al*, 2014). The pH dosing set points of each system was adjusted as needed during the experiment to maintain the desired pCO_2 levels. Average experimental pCO_2 levels were calculated using the daily pH_{NBS} and temperature and weekly salinity and TA measurements (Table 5.1).

Three temperature treatments were used to represent the current-day summer average water temperature for the study populations, 28.5°C (+0.0°C), and the projected 1.5°C to 3°C temperature increase, (30.0°C (+1.5°C) and 31.5°C (+3.0°C)), projected to occur in the tropical ocean over the coming century (Ganachaud *et al.* 2011; Kirtman *et al.* 2013). The control temperature was maintained using a SolarWise 341 heater-chiller unit. Elevated

temperatures were achieved by sending the seawater through a series of Toyesi 2.5kW inline heaters. Heating the seawater after CO_2 dosing resulted in slightly different pCO_2 levels among the 3 temperatures within a treatment (Table 5.1), but there was no overlap among CO_2 treatments. There were minor differences in total alkalinity between the 3 systems (Table 5.1). Otolith calcification occurs in a semi-permeable saccular epithelium, and surrounded by endolymph. The ionic concentration of the endolymph is highly regulated against environmental variation, consequently otolith calcification is less sensitive to changes in environmental carbonate (Campana, 1999). Adult aquaria were provided with a continuous flow of control or treatment seawater at ~1.5L min^{-1} and juveniles' aquaria were provided with continuous flow of control or treatment seawater at ~0.1L min^{-1}.

Juvenile rearing

On the night of hatchling, egg clutches were removed from their parents and hatched into 60L larval rearing aquaria at the desired pCO_2 level. Larvae were either hatched into the same CO_2 treatment as their parents (parental effects), or some clutches from control parents were hatched into the high CO_2 treatment (acute CO_2 effect). The CO_2 treatment groups are referred to as either control, acute CO_2 or parental CO_2 to reflect the relevant CO_2 exposure period Larvae were reared in clutch groups until the end of the pelagic larval stage at 11 day post-hatching. During the pelagic-stage larvae were fed according to standard protocol (Munday *et al.* 2009). Briefly, larvae were fed a diet of live rotifers (*Branchionus* sp.) and newly hatched *Artemia* nauplii each morning. Larval rearing tanks had no water exchange during daylight hours,

which allowed the larvae to feed *ad libitum* during the day. Tanks were then flushed continuously with treatment water during the night to ensure complete exchange of treatment water and to remove any waste and uneaten food. All tanks were continuously bubbled with air (control) or CO_2 enriched air set to the desired CO_2 concentration to maintain treatment levels. Temperature was maintained at 28.5°C in the larval rearing tanks by 500W aquarium heaters.

At 11 days post-hatching, when larvae were competent to settle, 45 individuals from each clutch were randomly selected and assigned to the three juvenile temperature treatments (+0.0°C, +1.5°C or +3.0°C; n=15 per temperature treatment) but kept in their CO_2 treatment. Juveniles were individually reared in 1.5L containers, one fish per container, for 20 days (31 days post hatch). Individual tanks were supplied with a continuous flow of seawater at the appropriate pCO_2 and temperature (Table 5.1). Juveniles were fed once per day and slowly weaned off *Artemia* nauplii and onto a commercial fish feed pellet (INVE NRD 2/4 pellet) at 5mg of pellets once per day.

At 31 days post-hatching juveniles were euthanized and fixed in 4% phosphate buffered formaldehyde. Within three days of preservation each fish was removed from the fixative, blotted dry and the wet weight determined to the nearest mg. Individuals were then photographed in a lateral position using a Olympus DP26 camera mounted on an Olympus S761 stereo microscope. Standard length (SL) was measured to the nearest 0.01mm from the photo using the program ImageJ. After the image had been taken, fish were transferred to 70% ethanol and stored individually until the otoliths were removed.

Pairs of sagittal otoliths were extracted with the aid of a dissecting microscope and cleaned of adhering tissue. A calibrated grey-scale image of left and right sagittal otoliths was taken with a digital camera mounted on a compound microscope, using QCapture. Images of the right otolith were flipped horizontally so that left and right otoliths would mirror each other if symmetrical. Otoliths were photographed on a white background so that the edge was clearly visible. Each image was then imported into the visual analysis software Optimas (v6.5). An automatic trace was conducted using the distal edge of the rostrum as a common landmark. Four size (otolith area, maximum breadth, maximum length and perimeter) and two shape characteristics (circularity and rectangularity) were determined for each otolith. The degree of otolith size symmetricality within individuals and within a population were determined. Absolute asymmetry (asymmetry within an individual) examines the size difference between otolith pairs irrespective of which side is larger. Directional asymmetry (whether one side is consistently larger than another in a population) was obtained by subtracting the value of the left otolith from the value of the right otolith (Lemberget & McCormick 2008).

Statistical Analysis

Otolith size and shape characteristics were expected to show high levels of co-linearity. To overcome this issue, principle components analysis was used to reduce the number of dependent variables for the left side, right side and absolute asymmetry. The first (size) and second (shape) components of the analysis accounted for >90% of the variance in otolith data set and were therefore used in further statistical tests. .

Linear mixed effects models were used to determine if there were statistically significant differences between the treatment groups. The models were created using either the first (size) or second (shape) component from the principle components analysis as the dependent variable, CO_2 and temperature treatment as the independent variables, fish standard length as a covariate and clutch was a random factor and, if necessary, variation was allowed within the clutch. Including standard length in the linear mixed effects model results in coefficients that are for a fish of a 0mm length, effectively removing any differences in size due to differences in fish length between treatments.

Logistic regression was used to test for differences in directional asymmetry of all otolith traits. Individuals were scored for whether the left or right otolith was larger for each otolith trait measured. The frequency of left versus right dominance was the dependent variable in the model and CO_2 treatment and temperature treatment were the categorical variables. The model tested included the interaction between CO_2 and temperature treatments. Juvenile length was the continuous variable.

Results

Principle Component Analysis

Principle component analysis confirmed that the otolith size and shape characteristics displayed high levels of co-linearity. Two principle components (PC) were identified as significant for the three data sets and accounted for 92.5%, 91.2% and 79.5% of the variation in the left, right and absolute asymmetry. PC1 account for 70.2, 72.8 and 60.7% of the variance and PC2 accounted for a further 22.3, 18.4 and 18.8% in the left and right otoliths and

the absolute asymmetry respectively (Fig. 5.1). Examination of the loadings for the components showed that PC1 was dominated by the size characteristics, and PC2 by the shape characteristics. Consequently, PC1 referred is “size” and PC2 as “shape”. The size components all tracked in a negative direction, such

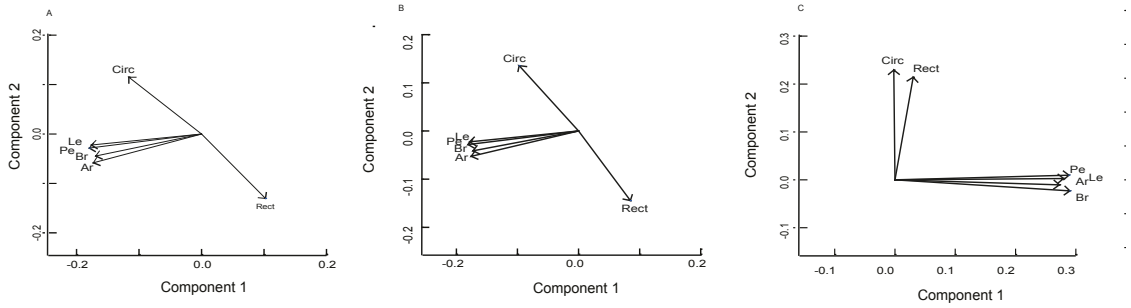

Figure 5.1 Biplots from the principle components analysis of the a) left, b) right and c) absolute asymmetry measures of juveniles reared under control, acute high CO_2 and parental high CO_2 cross-factored with temperature (+0.0°C, +1.5°C, +3.0°C). Circ= circularity, Rect= rectangularity, Pe=perimeter, Le= length, Ar= area, Br= breadth.

that otoliths that were larger had a more negative size component score. Otoliths that were more circular had positive shape component scores, while rectangular otoliths had a negative shape component score.

Left and Right Otoliths

As expected there was a significant positive relationship between absolute size and fish standard length. Conversely, larger otoliths had a negative size component score and as such a significant negative relationship between fish standard length and the size component scores was evident (Fig. 5.2, Table 5.2). There were clear differences in fish standard length between the treatment groups however, the LME compared coefficients for a fish of 0mm length, removing any effect of treatment group driven size differences. Neither CO_2

exposure nor temperature treatment significantly affected left or right otolith size.

There was no effect of either CO_2 exposure or temperature treatment on otolith shape, for either the left or right otoliths (Fig. 5.3; Table 5.3). There was a significant negative relationship with fish standard length, such that larger fish tended to have more rectangular otoliths (Table 5.3).

Absolute asymmetry

Absolute asymmetry was not correlated with fish standard length for either size or shape components (Fig. 5.2,5.3; Table 5.2,5.3), except for a significant interaction between fish standard length and temperature for shape asymmetry. There was a significant interaction between CO_2 and temperature treatment that affected the absolute asymmetry of the size characteristics (Fig. 5.3). CO_2 exposure, both parental and acute, resulted in lower size asymmetry component scores compared to control at +0.0°C. Levels of asymmetry in the CO_2 treatment groups increased to control levels at +1.5°C, but then reduced at +3.0°C.

Directional asymmetry

There was no effect of CO_2 exposure or temperature treatment on the directional asymmetry in otolith area, perimeter, length or circularity (Table 5.4).

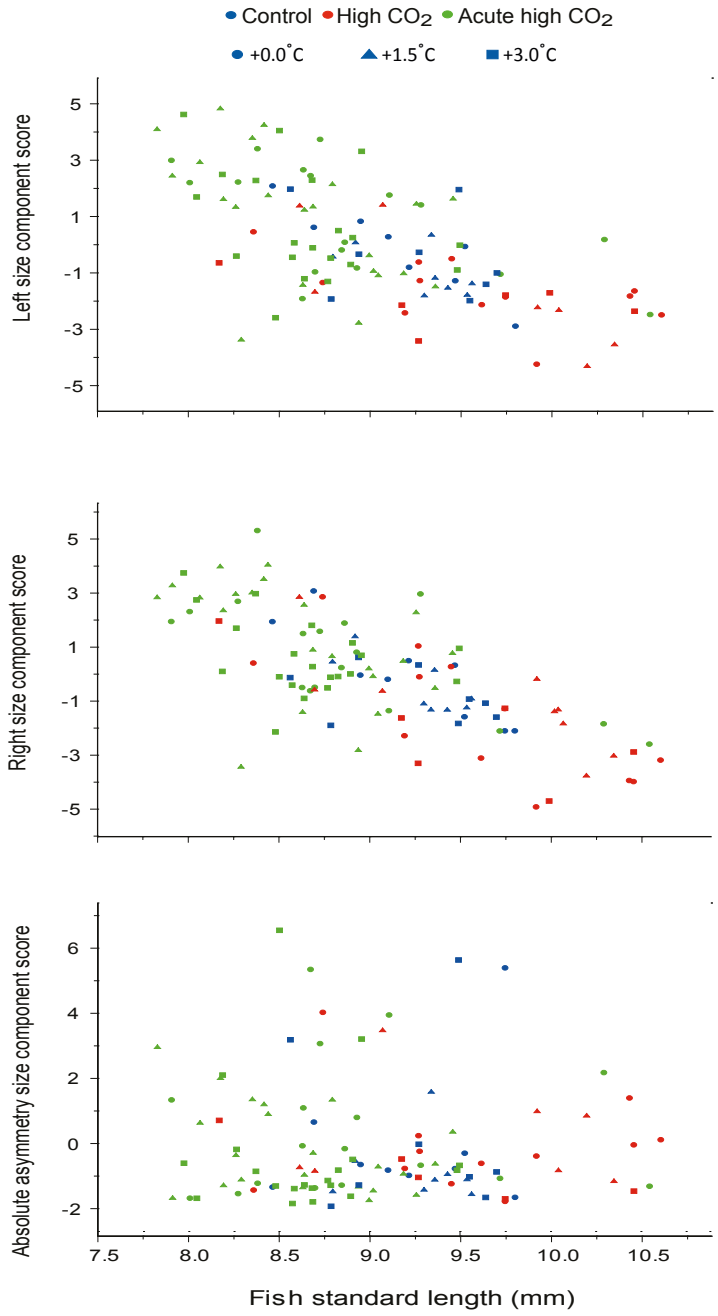

Figure 5.2 Raw component 1 (size) scores on fish standard length for the left, right and absolute asymmetry for juveniles reared under control, acute high CO_2 and parental high CO_2 cross-factored with temperature (+0.0°C, +1.5°C, +3.0°C). CO_2 treatment groups are represented by different colours; control= blue, acute high CO_2 = green, parental High CO_2= red. Temperature treatments are represented by different shapes.

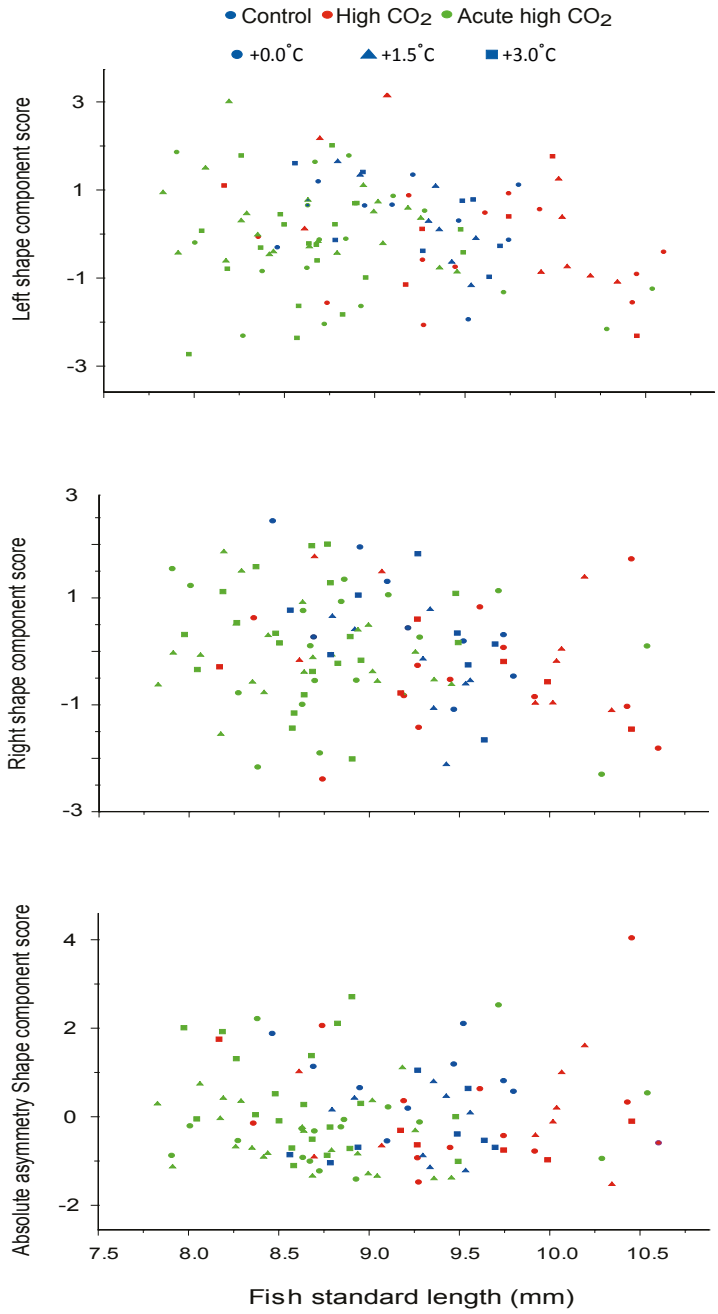

Figure 5.3 Raw component 2 (shape) scores on fish standard length for the left, right and absolute asymmetry for juveniles reared under control, acute high CO_2 and parental high CO_2 cross-factored with temperature (+0.0°C, +1.5°C, +3.0°C). CO_2 treatment groups are represented by different colours: ; control= blue, acute high CO_2 = green, parental High CO_2= red. Temperature treatments are represented by different shapes.

Discussion

In this study we did not detect significant changes in otolith size, shape or asymmetry in response to acute or parental exposure to elevated CO_2 or to juvenile exposure to increased temperatures. These findings are in direct contrast to other studies (8 of 11 studies to date) that have found that otolith are larger in larval and juvenile fishes reared at elevated CO_2. Yet, our results are consistent with studies on the effects of elevated CO_2 on otolith growth in reef fish (Munday *et al.,* 2011a, Munday *et al.,* 2011b, Wilcox Freeburg, 2014) where otolith size is no greater at near-future CO_2 levels ≥1000µatm. Together, these earlier studies and the current study suggest that reef fish otolith calcification is less sensitive to elevated CO_2 than in other fishes tested to date. Understanding the how and why of fish responses to elevated CO_2 vary so markedly is an issue that should now be examined in more detail.

Within the ocean acidification literature there is an overwhelming suggestion that climate change relevant elevated CO_2 will cause an increase in otolith size. However, when the existing literature is examined it appears that the predicted increase in otolith size in response to near-future CO_2 levels is not be as ubiquitous as initially suggested. To date 12 studies have examined the effects of elevated CO_2 on otolith morphometrics. Of these, 7 found an increase in otolith size parameters (Checkley *et al.,* 2009; Munday *et al.,* 2011a, Bignami *et al.,* 2013a,b; Hurst *et al*., 2012; Maneja *et al.,* 2013; Schade *et al.,* 2014). Four of these used <1000µatm with the remaining 3 not observing an effect until CO_2 levels ≥1700µatm (Munday *et al.* 2011a; Bignami *et al.,* 2013b; Maneja *et al.,* 2013). Based on a close examination of the literature, a universal increase in otolith size in response to near-future CO_2 is unlikely.

The increase in otolith size observed in larval and juvenile fishes in previous studies involving exposure to elevated CO_2 may have been caused by an acid-base regulatory response preventing acidosis in the blood and tissues of fish (Checkley *et al.* 2009; Munday *et al.* 2011a; Maneja *et al.,* 2013). If bicarbonate is the primary substrate for otolith accretion the increase in otolith size at high pCO_2 could be caused by higher concentrations of bicarbonate ions as a result of extracellular acid-base regulation to prevent acidosis (Munday *et al.,* 2011a;; Heuer *et al.,* 2012; Heuer & Grosell, 2014). At what level of environmental CO_2 effect occurs will likely vary between species based on their sensitivity to elevated CO_2. Species that live in areas of high variation in CO_2, such as some reef environments, may not acid-base regulate until levels are in excess of near-future CO_2 (≥1000µatm). This hypothesis is supported by Munday *et al.,* (2011a) who reported effects on otolith size at 1700µatm but not at 1050µatm CO_2. Consequently, understanding a species ecology and the CO2 variation that occurs in the environments they inhabit may increase our understanding of sensitivity to elevated CO_2.

In this study we did not detect any effects of parental exposure to elevated CO_2 on otolith growth. However, we also found no effect of acute exposure to elevated CO_2, suggestion that reef fish otolith formation may not be as sensitive to elevated CO_2 as other species. Consequently any parental effects may be masked by this insensitivity. Only one other study (Schade *et al.,* 2014) has examined the effects of parental exposure to elevated CO_2 on otolith growth. In that study, acute exposure to elevated CO_2 caused an increase in otolith size, suggesting that this species is sensitive to elevated CO_2, A further increase in otolith size was detected when both parents were

exposed to elevated CO_2. This suggests that parental effects can alter otolith traits in offspring, although not necessarily in a fitness improving manner. Future studies should test for parental effects in other species that appear to be sensitive to climate change stressors, such as among the 4-species that have shown increased otolith growth in response to acute near-future pCO_2.

Otoliths are a crucial sensory organ for hearing and orientation in fishes. However, it is not clear how changes in otolith size due to elevated CO_2 will impact on the overall fitness of these individuals. To date, Bignami *et al.* (2013a) is the only study to try to link changes in otolith size caused by elevated CO_2 to changes in organ function. Micro-CT images of the otolith were used to model the effects of increased otolith accretion due to ocean acidification on hearing acuity. Using technologies like the above, to determine how changes in otolith size and shape parameters may affect fitness characteristics will aid our understanding of the full impact of these effects.

Rates of otolith accretion change across ontogeny, with early life history stages accreting at a faster rate compared to adult life stages. The current literature has focused on the early life history stages, with only two (excluding the present study) rearing individuals past metamorphosis and none following through to adulthood. Larval stages are thought to be especially susceptible to elevated CO_2 because they have a reduced capcacity for ion regulation and other homestatic activities compared with more developed life stages (Ishimatsu *et al.,* 2008). Extrapolating the effects seen in larvae and juvenile into adulthood could lead to an overestimation of the impacts of elevated CO_2. Further, by focusing on the susceptible larval stages, there may be creating a bias for detecting negative effects of elevated CO_2.

Otolith growth and calcification is a complex process influenced by genetic, metabolic and environmental factors (Campana, 1999). As a crucial sense organ, changes to otolith growth could have consequences for the fitness for an individual and therefore understanding how climate change may affect these organs is necessary. In this study we did not detect any significant effects of either increased temperature or elevated CO_2, nor did we detect any effects of parental exposure to elevated CO_2 on otolith size or shape characteristics. An examination of the literature suggests that reef fish otolith calcification may not be as sensitive to elevated CO_2 as some species. Understanding why these species are less sensitive, and conversely why some species more sensitive, will be central to predicting which species are more likely to be affected by climate change.

Table 5.1 Seawater chemistry and temperature parameters for adult brood stock and juvenile *A. melanopus*. Values are mean ± standard deviation.

Stage	Treatment	Salinity	Temperate (°C)	TA (µmol/kg seawater)	pH_{NBS}	pCO_2 (µatm)	DIC (µmol/kg seawater)	HCO_3 (µmol/kg seawater)	CO_3^- (µmol/kg seawater)	CO_2 (µmol/kg seawater)
Adult	Control +0.0°C	36.39±0.59	28.53±0.30	2049±79	8.11±0.04	430±54	1775±75	1577±74	187±18	11±1
	Moderate +0.0°C	36.38±0.82	28.53±0.30	2089±143	8.01±0.05	584±77	1865±124	1691±110	159±23	15±2
	High +0.0°C	36.18±0.85	28.53±0.30	1945±23	7.77±0.05	1032±132	1830±27	1712±30	92±9	27±4
Juvenile	Control +0.0°C	36.39±0.59	28.57±0.50	2054±56	8.12±0.04	438±46	1782±61	1584±65	186±15	8±1
	Control +1.5°C	36.39±0.59	29.96±0.26	2054±56	8.13±0.04	443±52	1775±60	1572±62	191±14	11±1
	Control +3.0°C	36.39±0.59	31.69±0.50	2054±56	8.11±0.04	462±52	1770±58	1564±61	194±15	11±1
	Moderate +0.0°C	36.38±0.82	28.53±0.19	2069±67	8.05±0.05	588±75	1851±65	1680±64	155±14	15±2
	Moderate +1.5°C	36.38±0.82	29.94±0.28	2069±67	8.02±0.05	610±82	1847±67	1673±66	157±14	15±2
	Moderate +3.0°C	36.38±0.82	31.47±0.29	2069±67	8.00±0.05	644±86	1846±66	1672±65	158±14	15±2
	High +0.0°C	36.18±0.85	29.59±0.18	1937±27	7.81±0.04	926±109	1814±30	1685±29	99±10	25±3
	High +1.5°C	36.18±0.85	30.01±0.36	1937±27	7.79±0.04	974±114	1815±30	1684±29	99±9	25±3
	High +3.0°C	36.18±0.85	31.44±0.59	1937±27	7.78±0.04	1012±122	1812±3	1680±30	101±9	25±30

Table 5.2 Linear mixed effects of the size component of left and right otolith and absolute asymmetry for the size component from juveniles reared under control, acute high CO_2 and parental high CO_2 cross-factored with temperature (+0.0°C, +1.5°C, +3.0°C).

Left	Numerator DF	Denominator DF	F-value	p-value
Intercept	1	94	0.02	0.885
Standard length	1	94	29.25	<0.0001
CO_2	2	94	2.02	0.138
Temperature	2	94	1.17	0.314
CO_2: Temperature	4	94	1.17	0.33
Right				
Intercept	1	94	0.13	0.721
Standard length	1	94	69.05	<0.0001
CO_2	2	94	1.02	0.366
Temperature	2	94	2.42	0.095
CO_2: Temperature	4	94	0.38	0.825
Absolute Asymmetry				
Intercept	1	94	0.07	0.798
Standard length	1	94	<0.001	0.998
CO_2	2	94	0.53	0.593
Temperature	2	94	0.03	0.970
CO_2: Temperature	4	94	2.68	0.036

Table 5.3 Linear mixed effects model of the shape component for the left and right otoliths and absolute asymmetry of shape of otolith from juveniles reared under control, acute high CO_2 and parental high CO_2 cross-factored with temperature (+0.0°C, +1.5°C, +3.0°C).

Left	Numerator DF	Denominator DF	F-value	p-value
Intercept	1	94	0.07	0.789
Standard length	1	94	14.95	<0.001
CO_2	2	94	2.21	0.115
Temperature	2	94	2.20	0.117
CO_2: Temperature	4	94	0.55	0.699
Right				
Intercept	1	94	0.030	0.863
Standard length	1	94	33.127	<0.0001
CO_2	2	94	0.163	0.850
Temperature	2	94	0.496	0.610
CO_2: Temperature	4	94	2.328	0.062
Absolute Asymmetry				
Intercept	1	86	0.095	0.758
Standard length	1	86	0.567	0.454
CO_2	2	86	2.361	0.100
Temperature	2	86	2.604	0.080
Standard length: CO_2	2	86	0.551	0.578
Standard length: Temperature	2	86	4.880	<0.01
CO_2: Temperature	4	86	2.146	0.082
Standard Length: CO_2: Temperature	4	86	1.521	0.203

Table 5.4 Logistic regression on the effects of CO_2 and temperature on directional asymmetry of otolith area, perimeter, length and circularity of juveniles reared under control, acute high CO_2 and parental high CO_2 cross-factored with temperature (+0.0°C, +1.5°C, +3.0°C).

	DF	Wald statistic	p-value
Area			
Intercept	1	0.26	0.61
CO_2	2	0.19	0.91
Temperature	2	0.39	0.82
CO_2:Temperature	4	3.98	0.41
Perimeter			
Intercept	1	0.30	0.58
CO_2	2	1.30	0.52
Temperature	2	0.83	0.66
CO_2:Temperature	4	2.00	0.73
Length			
Intercept	1	0.03	0.86
CO_2	2	0.21	0.90
Temperature	2	0.50	0.78
CO_2:Temperature	4	4.16	0.38
Circularity			
Intercept	1	0.44	0.51
CO_2	2	1.83	0.40
Temperature	2	1.18	0.55
CO_2:Temperature	4	2.07	0.72

Chapter 6: General Discussion

For populations to persist, individuals must be able to successfully reproduce and produce offspring that can survive within that environment. Populations can become adapted to predictable or regular environmental changes over multiple generations through an increase in favoured phenotypes or plasticity within phenotypes. However, with the advent of anthropogenic climate change, environmental conditions are changing rapidly, potentially faster than it is possible for individuals to acclimate or adapt to the new conditions (Sunday *et al.,* 2014). As a direct result of increasing atmospheric carbon dioxide, marine organisms will be subjected to increasing sea surface temperatures and ocean acidification concurrently. These stressors could potentially have serious consequences for reproduction and for the ability of the offspring to survive. Presented within this thesis is one of the first studies to examine the effect of ocean acidification and warming on reproduction in fish, the first studies to examine the combined effects of ocean acidification on reproduction in fish, and experimental tests of multigenerational exposure to elevated CO_2 on key life history traits of the resulting offspring. Together, these chapters clearly demonstrate that future research should consider not only multi-stressor effects, but also multigenerational effects of ocean acidification and warming to gain a greater understand of how marine organisms will respond to rapid climate change.

Reproduction

Due to the energetic costs associated with maintaining acid-base balance, ocean acidification is predicted to cause declines in fish reproduction (Pörtner *et al*., 2004; Ishimatsu *et al.,* 2008; Melzner *et al*., 2009; Brauner & Baker, 2009). Contrary to prediction, **Chapter 2** demonstrated that exposure to elevated CO_2 increased reproductive output in the cinnamon anemonefish with no apparent effects on the condition of the adults or on the offspring. This study was the first to detect a positive influence of elevated CO_2 on reproduction, with other studies finding no effect of elevated CO_2 (Sundin *et al.,* 2012; Frommel *et al*., 2010). In this chapter, I made the case that the increase in reproduction was a hormetic response. Hormetic responses are biphasic in nature and a continued increase in the environmental agent causing the response will inevitably result in a decline for the given trait (Costantini *et al*., 2010). As such, it is equally important to know where this tipping point lays within the concentration of the environmental agent, in this case CO_2. Additionally, as hormesis is effectively a physiological coping mechanisms, there are likely to be trade-offs between various life history traits; for example the trade-off between longevity and reproduction in heat stress fruit flies (Le Bourg, 2005; Sørensen *et al*., 2007, 2008). While I did not detect a trade-off between reproduction and adult or juvenile condition, it is possible that there may be trade-offs later in life for the adults, or indeed that may show up in the resulting offspring. Examining if there are trade-off between increased reproduction and other life history characteristics is an important area for consideration.

There is well-documented evidence that elevated CO_2 disrupts normal behavioural traits in a range of species (Briffa *et al*., 2012; Hamilton *et al*.,

2014). These disruptions are caused by the accumulation of HCO_3^- and the resulting decrease on Cl^- in the blood as fish compensate for the acidosis triggered by elevated CO_2 (Ishimatsu *et al.,* 2008; Brauner & Baker, 2009). The decline in Cl^- ions results in activation of the neurotransmitter receptor for $GABA_A$ and a depolarization and excitation of the neurons (Nilsson *et al.,* 2012). This then leads to the major behavioural disturbance noted above.

Interestingly, $GABA_A$ also plays a role within the reproductive hormonal cascade (Khan & Thomas, 1999; Chyb *et al.,* 2001). In goldfish and Atlantic Croaker, $GABA_A$ has been found to stimulate the release of gonadotropin releasing hormone (GnRH) (Trudeau *et al.,* 1993; Khan & Thomas, 1999), a neuropeptide that regulates gonadotropin, and as such is a key regulator of reproduction (Yaron & Levavi-Sivan, 2011). While the role of GABA in fish reproduction is still unclear, it is possible that the stimulation of reproduction that was seen in Chapter 2 could be caused by interference of GABA functionality. Additionally, as GABA seemingly exerts a strong influence on the secretion of reproductive hormones, other aspects of life history that are controlled by reproductive hormones could be altered, including sex allocation and sex change.

Courtship and mate choice were not covered in this study. Many reef species exhibit strong mate selection and complex courtship behaviours (see Petersen *et al.,* 1992). Ocean acidification has been shown to affect a wide range of behaviours in fishes (Briffa *et al.,* 2012) and the performance of sensory systems, including visual and olfactory systems (Munday *et al.,* 2009c; Chung *et al*., 2014), which are often used in courtship and mate choice. A change in any of these in relation to reproduction could cause significant

alterations in the reproductive ability of reef fish species, before spawning even occurs. How CO_2 affects reproductive behaviours could be an important topic for future study and would help provide a more complete picture of the potential effects on reproduction.

In **Chapter 3**, I examined the effects of elevated CO_2 in combination with increased temperature on reproduction. Unlike in Chapter 2, there was no significant increase in reproduction with elevated CO_2 at control temperatures. Elevated CO_2, by itself, only affected some hatchling characteristics, significantly reducing hatchling length and yolk area. The most important result from Chapter 3 was the sharp decline in reproduction with increasing temperature. There was no successful reproduction at +3.0°C regardless of CO_2 treatment. This is consistent with other studies that have shown that fish reproduction in tropical marine fishes occurs within a limited temperature range and can be severely impacted by even a relatively small increase in average temperature (Pankhurst & Porter, 2003; Donelson *et al.,* 2010). Because elevated temperature and CO_2 did not diminish energy stores (hepatosomatic index) or body condition (Fulton's K), it is unlikely that the decline in reproduction was due to energetic constraints. Instead, reduced reproductive performance could be due to the thermal sensitivity of reproductive hormones (King *et al*., 2007; Donelson *et al.,* 2010; Pankhurst & Munday, 2011).

Startlingly, there was no successful reproduction at moderate CO_2 +1.5°C, suggesting a strong interaction between elevated CO_2 and increased temperature as has been predicted (Pörtner & Farrell, 2008). This treatment group represents temperatures and CO_2 levels that could occur as soon as 2050, 35 years from now. Without reproductive acclimation, it is possible that

there could be complete reproductive failure of some fishes within 40 years, with serious consequences not only for fish populations, but also for fisheries. The effect of elevated CO_2 on commercial important species is gaining interest (Bromhead *et al*., 2014; Hurst *et al*., 2013; Frommel *et al.,* 2012a). All three studies examined reproductive processes from fertilization onwards. Consequently, any effects on oogenesis or of parental effects on the resulting eggs and larvae would not be detected. Whether elevated CO_2 will effect reproduction, from oogenesis through to provisioning and parental effects in these commercial species is currently unknown.

Parental effects

Chapter 4 and **Chapter 5** focused on the effects of multigenerational exposure to elevated CO_2 on key life history traits on juvenile reef fish. By exposing parents to elevated CO_2 I was able to show that transgenerational acclimation to ocean acidification can occur in fishes. Further, I was able to rule out traditional parental effects of provisioning as the mechanism driving this acclimation, as I detected no significant difference in hatchling length or yolk area between the CO_2 treatment groups. This suggests that the documented acclimation occurred through another form of non-generational inheritance, including but not limited to, hormonal effects, DNA methylation or RNA inheritance (Bonduriansky *et al*., 2012). For ease, the term transgenerational acclimation is used to encompass these different mechanisms.

Trade-offs between life history characteristics are known to occur both within and between generations. Trade-offs have already been documented in response to transgenerational acclimation to elevated temperature, whereby

individuals acclimated to elevated temperature but at a cost to overall growth (Donelson *et al*., 2012, 2014). A natural follow on from Chapter 4 would be to grow out the offspring for longer to determine if there are trade-offs later in life. Further, whether the changes in reproduction seen in Chapter 2 occur in the second generation needs to be determined. Continuing experimentation through to a 3rd of 4th generation would also allow a fuller understanding of whether transgenerational acclimation will encompass multiple generations.

While transgenerational acclimation was able to compensate for decline in growth, RMR and survival, it is unclear whether the same mechanisms will be able to compensate or correct other traits that are being altered by elevated CO_2. In **Chapter 5** I examined the potential for transgenerational acclimation to affect otolith size. In contrast to Chapter 4, I found no effect of acute or parental exposure to elevated CO_2 on otolith growth. While this result is in contrast to other studies on the effects of elevated CO_2 on otolith growth, it is consistent with studies that have examined these effects in reef fish. In combination with the previous studies, Chapter 5 shows that not all fishes will be sensitive to elevated CO_2.

Despite the prevalence of increased otolith size at elevated CO_2, there is very little understanding of how changes in otolith size may affect the individuals. A recent study (Bignami *et al*., 2013) utilized micro computed tomography (micro-CT) to collect images of the otolith in situ and to provide a 3D image. These images were then used to model the effect of increased otolith accretion due to ocean acidification on hearing acuity. These models suggest that fish with larger otoliths may be able to detect sounds from a greater distance, potentially leading to impaired orientation and navigational

abilities. These are fascinating results and highlight the need for experimental approaches to determine if the changes in otolith size observed in ocean acidification have an effect on individual performance.

A clear message from Chapter 4 is that parental effects are not only important for our understanding of climate change, but they can manifest differently depending on the trait examined and in interaction with other stressors. Transgenerational acclimation research is still in its infancy with regards to fishes and the potential for acclimation to climate change. Yet, there appears to be considerable capacity for physiological acclimation (growth, metabolic rate) to temperature and CO_2 (Donelson *et al.,* 2012; Miller *et al.,* 2012; Salinas & Munch *et al.,* 2012; Murray *et al.,* 2014; Schade *et al.,* 2014). Few studies have examined the potential for other traits to acclimate, but those that have, have found less capacity for acclimation (Allan *et al.,* 2014; Donelson *et al.,* 2014). It seems that traits that are comparatively less plastic compared to growth, may not possess the essential phenotypic flexibility to allow for acclimation. Traits, such as reproduction, that have evolved to occur under very specific environmental conditions may not be able to readily acclimate. The same may be true of behavioural traits that are caused by hard-wired neuronal activity. The current research into transgenerational acclimation is a start to fill the knowledge gap concerning the capacity of marine fishes to acclimate and adapt to the new environmental conditions that are associated with climate change. However there are questions that still need to be answered about the breadth of traits that will be improved through transgenerational acclimation and how many generations the acclimation may take to occur.

This thesis demonstrates the potential effects of climate change and the remarkable ability of reef fish to rapidly acclimate to these conditions. Understanding how climate change will affect multiple life history stages and the transitions between these stages is critical to develop a more complete picture of how species will respond to a rapidly changing climate over the coming century. Selection operates on all stages of an organism's life cycle, and as such, while an organism may be robust to alterations in their environment as an adult, vulnerability in another life stage may regulate population survival; therefore examining all life history stages is essential for gauging the full effects of climate change. Exposing reproductive pairs for an entire breeding season to near-future CO_2 and temperature conditions demonstrated that without either climate change mitigation or acclimation by fishes, there could be complete reproductive failure by mid-century, and almost certainly by the end of the century. It also became evident that rapid physiological acclimation to ocean acidification was possible. However, this acclimation did not occur in all traits examined and was differentially expressed depending on the temperature experienced by the offspring. Ocean acidification and elevated temperature will likely have complex, interacting influences on all stages of the life cycle, that will be difficult to predict based on theory alone. As such it is vital for future research to consider multiple life stages, multiple generations and multiple stressors so that better informed predictions as to the biological impacts of climate change can be made.

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