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Transgenerational acclimation and adaptation of reef fishes to ocean acidification

Thesis submitted by

Megan Joan WELCH BSc (Hons)

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James Cook University

Statement on the Contribution of Others

This thesis includes collaborative work with my supervisors Prof. Philip Munday and Prof. Mark McCormick, as well as Dr. Sue-Ann Watson and Dr. Justin Welsh. While undertaking these collaborations I was responsible for project design, data collection, analysis and interpretation of my results. My co-authors provided intellectual guidance, editorial assistance, financial support and technical assistance.

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General Abstract

Ocean acidification, caused by the uptake of additional CO_2 from the atmosphere, will have far-reaching impacts on marine ecosystems. Many experiments have demonstrated negative effects of projected future CO_2 levels and reduced seawater pH on a diverse range of marine species. However, most of these studies have been of short duration and may not accurately predict the longer-term effects of ocean acidification on marine populations. In particular, few studies have considered the potential for marine organisms to acclimate or adapt to ocean acidification. Recent experiments show that short-term exposure to elevated CO_2 can affect a range of life history and behavioural traits in marine fishes, yet few studies have tested if these effects are maintained across multiple generations, or if fish populations might adapt to projected future CO_2 levels. A multigenerational perspective will enable us to better predict how ocean acidification will affect marine populations. This study therefore examines the potential effects of transgenerational exposure to ocean acidification in coral reef fishes.

Reproduction is critical for individual and population success, and is where transgenerational effects will originate. However, reproduction is energetically expensive and could be adversely affected by rising CO_2 levels in the ocean. Therefore, Chapter 2 investigates the effects of projected future CO_2 levels on reproductive output of two species of coral reef damselfish, *Amphiprion percula* and *Acanthochromis polyacanthus*. Adult breeding pairs were maintained at current-day control (446 μatm), moderate (652 μatm) or high CO_2 (912 μatm) levels for a 9-month period that included the summer breeding season. Reproductive output increased in *A. percula*, with 45-75 % more egg clutches produced and a 47-56 % increase in the number of eggs per clutch in the two elevated CO_2 treatments. In contrast, reproductive output decreased at high CO_2 in *Ac. polyacanthus*, with approximately one-third as many clutches produced compared with controls. Egg survival was not affected by CO_2 for *A. percula*, but was greater in elevated CO_2 for *Ac. polyacanthus.* Hatching success was also greater for *Ac. polyacanthus* at elevated CO_2, but there was no effect of CO_2 treatments on offspring size. Despite the variation in reproductive output, body condition of adults did not differ between control and CO_2 treatments in either species. These results demonstrate different effects of high CO_2 on fish reproduction, even among species within the same

family.

Previous studies have shown that the behaviour and sensory performance of juvenile coral reef fishes are impaired at CO_2 levels projected to occur in the ocean in the next 50–100 years. However, it is unknown whether parental exposure to elevated CO_2 can allow for behavioural acclimation. Chapter 3 tests the potential for transgenerational acclimation of reef fish olfactory preferences and behavioural lateralization at moderate (656 μatm) and high (912 μatm) end-of-century CO_2 projections. Juvenile spiny damselfish, *Ac. polyacanthus*, from control parents (446 μatm) exhibited an innate avoidance to chemical alarm cue (CAC); however, juveniles lost this innate response and even became attracted to CAC when reared at elevated CO_2 levels. Juveniles from parents maintained at mid-CO_2 and high-CO_2 levels also lost their innate avoidance of CAC when reared in elevated CO_2, demonstrating no capacity for transgenerational acclimation of olfactory responses. Behavioural lateralization was also disrupted for juveniles reared under elevated CO_2, regardless of parental conditioning. These results demonstrate minimal potential for transgenerational acclimation in this fish, suggesting that genetic adaptation will be necessary to overcome the effects of ocean acidification on behaviour.

Changes to the morphology of fish otoliths (aragonitic ear bones) have been observed under higher levels of CO_2, with potential implications for hearing, balance and orientation in a future high CO_2 environment. However, no studies have tested for possible transgenerational effects of high CO_2 on otolith morphology. Chapter 4 assesses transgenerational effects of high CO_2 on otolith area, perimeter, maximum length and circularity in juvenile *Ac. polyacanthus*. Offspring from parents maintained at control (446 μatm), moderate CO_2 (652 μatm) and high CO_2 (912 μatm) were reared for 6 weeks in the three treatments, in a fully crossed design. Otolith development in juveniles was affected by moderate CO_2 exposure, but these effects were absent when parents also experienced moderate CO_2. However, transgenerational exposure to high CO_2 resulted in increased otolith area and perimeter. These results show that transgenerational acclimation can mitigate the effects of moderate CO_2 on otolith development in *Ac. polyacanthus*, but higher CO_2 levels lead to marked effects on otolith development across generations.

The lack of transgenerational acclimation in behaviours to high CO_2 (Chapter 3) indicates that genetic adaptation will be required to maintain behavioural performance in the future. Adaptation depends on the presence of heritable phenotypic variation in the trait, which may differ between populations and environments. Previous studies have shown that some individuals exhibit greater behavioural tolerance to high CO_2 than others, but whether this behavioural tolerance is heritable is unknown. In Chapter 5, I used father-midoffspring regressions to estimate the heritability of behavioural tolerance to high CO_2 (754 µatm) in both field and laboratory-reared populations of *Ac. polyacanthus*. The field population of *Ac. polyacanthus* exhibited high heritability of olfactory behaviour phenotype ($h^2 = 0.56$) when offspring were acutely exposed to high CO_2 for 4-5 days. The laboratory population exhibited similarly high heritability of olfactory behaviour phenotype ($h^2 = 0.65$) when offspring were acutely exposed to high CO_2. However, this heritability was completely lost when juveniles where chronically exposed to high CO_2 for 6 weeks. Parental exposure to high CO_2 did not alter this relationship between acute and chronic CO_2 treatments: heritability of behavioural phenotype was high when offspring were acutely exposed to high CO_2, but lost when offspring when chronically exposed to high CO_2, regardless of parent treatment. The loss of heritability occurred because juveniles that were relatively tolerant to high CO_2 in the acute treatment lost this tolerance in the chronic CO_2 treatment. This indicates that genetic variation in behavioural tolerance to high CO_2 is obscured by non-adaptive plasticity when offspring are chronically exposed to high CO_2. These results demonstrate that behavioural tolerance to high CO_2 is heritable, but adaptive potential may be severely limited by non-adaptive plasticity when a high CO_2 environment is experienced across several generations, as will occur due to rising CO_2 levels in the ocean.

This research is among the first to use a multigenerational approach to test the effects of ocean acidification on reef fishes and estimate their adaptive potential. The results demonstrate how CO_2 levels predicted for the end of the century affect the behaviour and life history traits of reef fish in various ways. Critically, I found no evidence for transgenerational acclimation of impaired behaviours to CO_2, and that heritable variation in behavioural tolerance to high CO_2 was obscured by non-adaptive plasticity when offspring were reared from hatching in a high CO_2 environment. These results are concerning because they indicate little scope for acclimation or adaptation of impaired behavioural responses to high CO_2, at least in *Ac. polyacanthus*. Further research should examine potential trade-offs of behavioural and physiological processes

in elevated CO_2 environments to better predict overall survival and success of marine populations. This research demonstrates the important of incorporating a long-term, multigenerational perspective when assessing the likely impacts of ocean acidification on marine ecosystems.

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Chapter 1: General Introduction

The Industrial Revolution sparked the rapid rise of anthropogenic carbon dioxide (CO_2) emissions, leading to an increase in atmospheric levels from 280 ppm (preindustrial) to over 400 ppm in 2016 (www.esrl.noaa.gov/gmd/ccgg/trends/), the highest level in at least 800,000 years (Lüthi et al. 2008). The oceans have absorbed approximately one-third of all anthropogenic CO_2 emitted into the atmosphere, leading to a decline in pH through a process called ocean acidification (Caldeira and Wickett 2003). Ocean pH has decreased by 0.1 units and become approximately 30 % more acidic than before the Industrial Revolution. If the current rate of emissions continues, atmospheric and oceanic CO_2 concentrations are predicted to exceed 900 ppm by the end of this century, with a further drop of 0.3-0.4 units in ocean pH (Collins et al. 2013).

The ongoing acidification of the oceans threatens the well-being of marine ecosystems. Decreased seawater pH is accompanied by a reduction in the saturation state of carbonate ions (Sabine et al. 2004), which reduces the ability of marine organisms to produce calcium carbonate shells and skeletons (Sabine et al. 2004; Kleypas et al. 2006). The chemical changes associated with ocean acidification are predicted to severely affect many marine species, especially calcifiers such as corals and some molluscs that will experience increased difficultly building their aragonitic skeletons (Feely et al. 2004; Orr et al. 2005; Kleypas et al. 2006). Fundamental life history processes, such as nitrogen fixation, metabolism and reproduction, can also be affected by elevated CO_2 levels and reduced pH in seawater (Doney et al. 2009). Ecological ramifications will resonate throughout marine ecosystems if ocean acidification continues unabated (Gattuso and Hansson 2011).

Most of the biological effects of ocean acidification have been reported from experiments where animals are exposed to projected future CO_2 and pH levels for periods ranging from days to weeks or months (Kroeker et al. 2013; Riebesell and Gattuso 2015). Many of these short-term experiments have demonstrated negative effects of future ocean acidification on a wide-range of marine species and ecological processes (Doney et al. 2009; Kroeker et al. 2013), but some studies have also reported potentially positive effects, such as enhanced photosynthesis and growth rates in some species (Munday et al. 2009b; Vogel et al. 2015). Vastly different effects of ocean acidification have also been observed among species. For example, elevated CO_2 reduced calcification rates for 10 out of 18 benthic marine species, whereas seven other species exhibited increased net calcification and one species was not affected by elevated CO_2 (Ries et al. 2009). Additionally, fertilization success in the Sydney rock oyster was reduced by elevated CO_2 (Parker et al. 2009), yet no effects of high CO_2 on egg production were seen in multiple copepod species (Weydmann et al. 2012; McConville et al. 2013). Differences in sensitivity to changes in seawater pH and CO_2 levels among taxonomic groups (Ries et al. 2009; Fabricius et al. 2011; Chan et al. 2015) and even among closely related species (Langer et al. 2006; Ferrari et al. 2011b; Pandolfi et al. 2011; Strahl et al. 2015) suggest that some species are already adapted to the projected changes in ocean chemistry, and thus other species could potentially adapt too, given sufficient time.

Ocean acidification will progress over a much longer period of time than is currently being investigated in short-term experimental studies. For most species, the changes in ocean chemistry will occur over a number of generations. Consequently there is potential for individuals and populations to acclimate or adapt to these changes through time. To date, however, relatively few studies have attempted to assess the adaptive potential of marine organisms to ocean acidification. Nevertheless, the few studies that have been conducted illustrate how important it is to assess the capacity for acclimation and adaptation. Some studies demonstrate individual acclimation through improved performances after long-term exposure to high CO_2; for example, reduced fecundity seen in green sea urchins after 4 months exposure to high pCO_2 (1200 μatm) was eliminated after 16 months exposure (Dupont et al. 2013). Other studies demonstrate the presence of genetic variation within populations that may promote adaptive responses (Sunday et al. 2011; Kelly et al. 2013). However, there may also be constraints to the pace of adaptive responses such as negative genetic correlations with other environmental traits (e.g. temperature) (Blows and Hoffmann 2005). Without consideration of long-term exposure and adaptive potential, the effects of ocean acidification on marine ecosystems may be over- or underestimated (Sunday et al. 2014; Gaylord et al. 2015; Foo and Byrne 2016).

Implications of ocean acidification for fishes

The majority of ocean acidification studies have examined calcifying species, because of the likely effects of reduced carbonate saturation state on the ability of these species to produce their calcium carbonate shells and skeletons (Kleypas et al. 2006). However, rising CO_2 levels in the ocean can also have a direct effect on other marine organisms (Pörtner et al. 2004). In water breathing organisms, the gradient in pCO_2 (partial pressure of CO_2) between the plasma and the external environment is much lower than in air-breathers. For example, plasma pCO_2 in fish can be less than 3000 μatm, whereas it is above 50,000 μatm in humans (Heuer and Grosell 2014). Elevated CO_2 levels in seawater causes plasma pCO_2 levels to rise in fish, which could lead to acidosis (Claiborne et al. 2002; Heuer and Grosell 2014). Fish have a well-developed acid–base regulatory system that allows them to defend against intra- and extracellular acidosis in a high-CO_2 environment (Brauner and Baker 2009; Heuer and Grosell 2014), primarily by the accumulation of HCO_3^- ions in exchange for Cl^- across the gills (Brauner and Baker 2009). However, the increased ion transport required to maintain pH in a constant high-CO_2 environment could incur additional energetic costs (Pörtner et al. 2004; Ishimatsu et al. 2008; Strobel et al. 2012), which may either require enhanced foraging, or be supplemented from other important activities (Ishimatsu et al. 2004; Barry et al. 2011). For example, declines in growth rate observed in some fishes at high CO_2 levels (Baumann et al. 2011) could potentially be a result of energy reallocation for acid-base maintenance.

Reproduction, while essential for population replenishment and critical to individual fitness, is energetically expensive and may be constrained by a range of environmental factors. Reproductive females allocate significant amounts of energy to egg or offspring provisioning (Watson et al. 1998; Visser and Lessells 2001; Grazer and Martin 2011), while males may expend large amounts of energy in courtship or nest development (Cox et al. 2010). These energetic costs restrict reproduction to specific environmental conditions and make it highly sensitive to environmental fluctuations (Cushing 1969; Dawson 2008; Visser et al. 2009). An increased energetic demand for acid-base regulation in a high CO_2 environment might therefore affect the energy available for reproduction (Aze et al. 2014). Reduced energy for reproduction could potentially result in reduced clutch size, smaller eggs, smaller hatchlings or fewer clutches produced in a breeding season, which could have important ramifications for population dynamics. Despite these predictions, little is known of the effects of elevated CO_2 on fish reproduction. The paucity of studies into the effects of high CO_2 on reproduction might stem from the logistical difficulty of maintaining large breeding populations of most marine fishes in captivity and simultaneously manipulating their CO_2 environment. However, this is a critical life process that requires more attention for predictions of population dynamics in a high CO_2 world. In one of the only studies conducted to date, Miller et al. (2013) surprisingly found positive effects of elevated CO_2 on reproduction in the cinnamon anemonefish, *Amphiprion melanopus*. Contrary to expectations, reproductive output was substantially higher in the elevated CO_2 treatment compared with present-day controls. It is not yet known if other reef fishes experience similar stimulation in reproductive output, or why such effects should occur.

One of the most unexpected consequences of high CO_2 on marine fishes is altered behaviours and impairment of sensory systems. Recent studies show that CO_2 levels predicted for the end of the century have adverse effects on a wide range of behaviours in marine fishes (Briffa et al. 2012; Clements and Hunt 2014; Heuer and Grosell 2014) with consequences for key ecological processes such as larval dispersal, habitat selection, competition and predator-prey interactions (Nagelkerken and Munday 2016). Behavioural changes include altered activity levels, loss of behavioural lateralization, inability to learn, slowed visual acuity, as well as altered auditory and olfactory preferences (Briffa et al. 2012; Branch et al. 2013; Leduc et al. 2013; Chung et al. 2014). These alterations have significant consequences for ecological processes, such as predator-prey (Munday et al. 2010; Ferrari et al. 2011b) and competitive interactions (McCormick et al. 2013), that may have far-reaching implications for fish populations and communities (Ferrari et al. 2011a,b; Chivers et al. 2013). Behavioural changes that increase predation risk in juvenile fishes, such as reduced response to predator odour and chemical alarm cue (CAC) (Dixson et al. 2010; Ferrari et al. 2011b), are especially significant as they raise concerns for the success of populations in a high CO_2 environment (Munday et al. 2010).

Interference of $GABA_A$ neurotransmitter function in the fish brain is believed to be the underlying mechanism of these behavioural changes (Nilsson et al. 2012; Chivers et al. 2014; Hamilton et al. 2014). The $GABA_A$ receptor is a gated ion channel that conducts HCO_3^- and Cl^-. The altered concentrations of HCO_3^- and Cl^- that occur in the plasma during acid-base regulation in a high CO_2 environment could change the flow of these ions through the $GABA_A$ receptor (Heuer and Grosell 2014). This would result in a reversal in receptor polarization, leading to changes in behaviour of fish that are permanently exposed to high CO_2. The role for $GABA_A$ in behavioural changes is supported by experiments showing that gabazine, a $GABA_A$ antagonist, reverses the behavioural changes that occur in elevated CO_2 (Nilsson et al. 2012; Chung et al. 2014; Ou et al. 2015). Behavioural changes have been observed in juvenile fish after exposure to elevated CO_2 for from a few days to weeks, and longer-term experiments and studies at natural CO_2 seeps indicate behavioural impairment persists when fish are permanently exposed to elevated CO_2 for many weeks to months (Munday et al. 2013, 2014a). This suggests that there is limited potential for within-generation acclimation of impaired behavioural responses. One of the key questions remaining is whether fish behaviour can acclimate or adapt to high CO_2 over longer time frames.

Fish skeletons are primarily composed of calcium phosphate (Toppe et al. 2007) and are therefore not sensitive to the decline in carbonate saturation state that accompanies ocean acidification. Yet, fish do still possess some calcium carbonate structures, most notably aragonitic ear bones (otoliths). Otoliths are calcified structures that sit within a saccular epithelium filled with endolymph (Tohse et al. 2004). Otoliths are much denser than water, and therefore move at different amplitudes to the body (Popper and Lu 2000). The differences in movement result in a bending of the sensory hair cells, enabling the direct detection of sound and perception of movement (Popper and Lu 2000). The otoliths are critical for sound detection, orientation and acceleration (Popper et al. 2005; Simpson et al. 2011). Otoliths continue to grow throughout the life of the fish, and periodic increments that are deposited within the otolith are often used to estimate the age of fish (Fowler 1990; Checkley et al. 2009). However, otolith growth is sensitive to changes in environmental conditions (Gagliano and McCormick 2004), and it might be expected that the reduced saturation state of aragonite in more acidic water would affect the precipitation rate of otoliths. Contrary to this prediction, otoliths have commonly been found to increase in size when fish are exposed to the CO_2 levels predicted to occur in the ocean by the end of this century (sea bass at 993 μatm, Checkley et al. 2009; cobia at 800 μatm, Bignami et al. 2013a). Otolith growth is proportional to HCO_3^- concentrations of the fluid surrounding the saccular epithelium (Tohse and Mugiya 2001; Checkley et al. 2009). Fish use acid-base regulation to avoid acidosis under high CO_2 environments, which results in elevated HCO_3^- levels in the plasma that surrounds the endolymph and thus likely contributes to increased otolith precipitation (Heuer and Grosell 2014). Changes to otolith shape and size may affect auditory capacity and swimming performance, ultimately resulting in ecological consequences for fish (Bignami et al. 2013a,b). However, just as with other traits, there is variation among species in how high CO_2 affects otolith development, with some fishes showing no change in otolith growth at near future CO_2 levels (spiny damsels at 900 μatm, Munday et al. 2011a; clownfish at 1050 μatm, Munday et al. 2011b; clownfish at 900 μatm, Simpson et al. 2011) whereas other species exhibit changes in otolith size and shape (Checkley et al. 2009; Bignami et al. 2013a). These varying effects of high CO_2 on otolith development have again mainly been studied under short-term CO_2 exposure, and it is not known if exposure of multiple generations will sustain or reverse these changes.

Acclimation to a changing environment

The majority of ocean acidification studies have exposed organisms to high CO_2 levels for days or weeks at a time. However, these studies may be over- or underestimating the consequences of climate change, as CO_2 levels are predicted to rise over a much longer time frame and organisms will be exposed to these conditions throughout life. A major limitation of most short-term experimental studies is that they do not consider the potential for acclimation and adaptation to rising CO_2 levels over multiple generations (Munday et al. 2013b; Sunday et al. 2014; Foo and Byrne 2016).

Acclimation is a form of phenotypic plasticity, and as such it involves the ability of a given genotype to express different phenotypes in different environments (Whitman and Agrawal 2009). Acclimation involves a phenotypic response to environmental change that improves the performance of the individual in a new environment (Munday 2014). For example, there was less change in metabolic rate of *Acanthochromis polyacanthus* when individuals were conditioned in high temperatures compared to individuals that were acutely exposed to warmer environments (Donelson et al. 2012). When full acclimation occurs, phenotypic change may not be observed in the trait of interest, because that trait would maintain its phenotype across levels of environmental change (i.e. rising CO_2 levels) due to underlying plasticity in gene expression or energy allocation (Sunday et al. 2014). This effect has been called phenotypic buffering (Reusch 2014).

Importantly, acclimation can occur over different timeframes: within a generation (reversible or developmental acclimation) or across generations (transgenerational acclimation) (Munday 2014). Reversible acclimation is commonly observed in species that live in variable environments and thus must regulate their physiological responses to daily or seasonal environmental change. Developmental acclimation involves permanent phenotypic changes that occur as a result of exposure to a particular environment during early life (Angilletta 2009). For example, swimming performance of guppies in warmer water was improved when the fish had experienced these temperatures as embryos (Scott and Johnson 2012). Transgenerational acclimation occurs when the environment experienced by the parents influences offspring response to the new environment, without genetic alterations (Fox and Mousseau 1998; Salinas and Munch 2012). For example, developmental impediments due to elevated CO_2 in juvenile Sydney rock oysters were reduced when adults were exposed to high CO_2 during reproductive conditioning, producing larger and faster developed larvae (Parker et al. 2012).

Transgenerational acclimation in fishes

Transgenerational acclimation, also referred to as transgenerational plasticity (TGP), has been observed to occur in a wide range of traits in a variety of taxa (Salinas et al. 2013). Importantly, transgenerational acclimation is a form of plasticity and thus does not involve the selection of different genotypes across generations. Instead transgenerational acclimation occurs through a variety of non-genetic mechanisms (Bonduriansky 2012). Parents can alter the phenotypes of their offspring through changes in nutritional provisioning and transfer of hormones or proteins, often established during egg production for fishes. Mothers and fathers might also transmit epigenetic marks to their offspring that alter gene regulation in the new environment (Jablonka and Raz 2009; Figure 1.1). Advantageous TGP (acclimation) occurs if offspring performance is benefitted by these parental influences in the new environment.

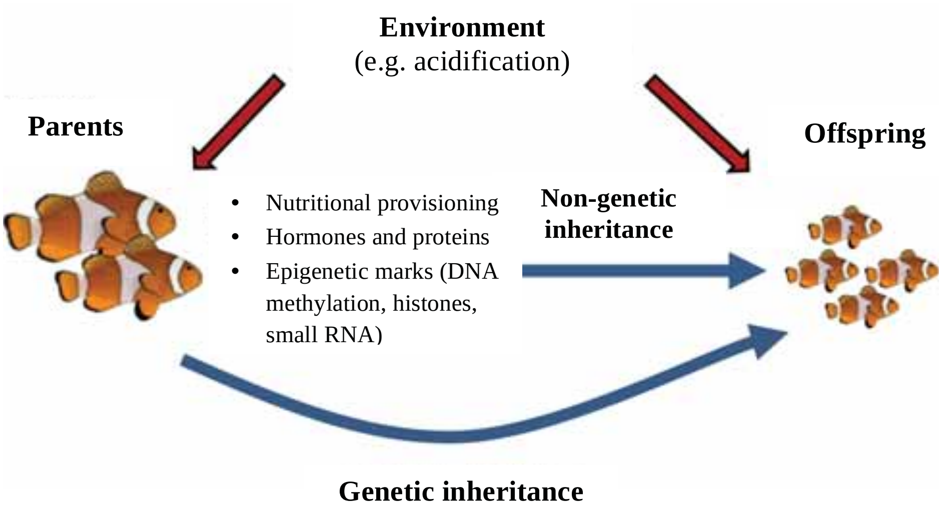

Figure 1.1. Modified from Munday (2014): Parents influence the phenotype of their offspring through both genetic and non-genetic pathways.

In the context of climate change, TGP has been observed in warmer environments with improvements in offspring aerobic scope (Donelson et al. 2012) and growth (Salinas and Munch 2012) when parents were reared under the same warmer environment of their offspring. There is also evidence of TGP to ocean acidification for some traits, where exposure of parents to higher CO_2 levels confers benefits to their offspring in a high-CO_2 environment (Miller et al. 2012; Murray et al. 2014). For example, parent Atlantic silverside *Menidia menidia* that spawned in low pH conditions late in the breeding season, produced offspring that were more tolerant to high CO_2 compared to offspring hatched earlier in the breeding season when the parents experienced much higher seawater pH (Murray et al. 2014). Similarly, the effects of elevated CO_2 on the metabolic rate, growth and survival of juvenile *A. melanopus* were alleviated when parents were also reared under high-CO_2 (Miller et al. 2012). Additionally, *A. melanopus* parents were able to lessen the effects of elevated CO_2 on the kinematics of predator-escape responses in their offspring when both generations experienced the same high-CO_2 environment (Allan et al. 2014). These studies indicate that short-term exposure to high CO_2 may overestimate the consequences of ocean acidification on marine species, and that transgenerational acclimation can allow for the amelioration of negative effects of high CO_2 in some organisms. Short-term exposure to high CO_2 affects anti-predator behaviour in juvenile fishes (Briffa et al. 2012; Clements and Hunt 2014; Heuer and Grosell 2014), which raises concern for individual survival, especially as coral reef fish experience the greatest risk of predation during the first few days of settlement to the reef (Almany and Webster 2006). There is no evidence for within generation acclimation for these behaviours under elevated CO_2 (Munday et al. 2010, 2014b), however the potential for transgenerational acclimation to overcome the impacts of rising CO_2 on olfactory and cognitive impairment in reef fishes has not been tested.

As with other aspects of ocean acidification research, studies on otolith development in a high CO_2 environment have involved short-term exposure to elevated CO_2 and have not considered parental effects on the observed developmental changes. Otoliths are key structures in fish for hearing, balance and orientation (Popper et al. 2005; Simpson et al. 2011), thus the changes detected in high CO_2 conditions could have serious consequences for individual performance. However, the possibility that parental effects could have either beneficial or detrimental effects on otolith development has rarely been considered. In the only study to date to examine transgenerational effects of high CO_2 on otolith development, juvenile three-spined sticklebacks in high CO_2 (1167 μatm) still had larger otoliths when their parents were also reared in high CO_2 (1013 μatm) (Schade et al. 2014). While some species, such as the spiny damsel, do not show effects of short-term exposure to elevated CO_2 on otolith development (Munday et al. 2011a), effects could be delayed over generations. More transgenerational studies are needed to better understand potential effects of changed environmental conditions across generations on otolith development.

Adaptation to a changing environment

Species adaptation to environmental changes involves selection on genetic variation within the population. For rapid adaptation to environmental change to occur there must be sufficient standing genetic variation present in the population (Hoffmann and Sgrò 2011). Furthermore, this genetic variation must be expressed as heritable phenotypic variation (Hoffmann and Parsons 1991). When selection acts upon this heritable phenotypic variation, there will be a shift in gene frequency and thus a shift in the distribution of the phenotype in the population. Heritability (h^2), a key metric for determining adaptive potential, is the amount of phenotypic variation observed in a particular trait that is due to inherited genetic factors (Wray and Visscher 2008; Foo and Byrne 2016). Heritability is estimated by comparing the phenotypes of individuals of known relatedness (Lynch and Walsh 1998), and is often measured with diallel breeding designs, where multiple males are crossbred with multiple females (Kelly et al. 2013). This design is most easily achieved with broadcast spawning species because it is possible to cross fertilise the gametes of many males and females (Munday et al. 2013b), and then decompose the maternal, sire and environmental components affecting the trait variation in the offspring. For example, a diallel breeding design was used to examine the tolerance of sea urchin embryos, *Pseudoboletia indiana*, in a multi-stressor (ocean acidification and temperature), finding heritable variation in tolerance through significant sire × environment interactions (Foo et al. 2014). Conversely, cross-breeding in the mussel, *Mytilus trossulus*, detected little additive genetic variance in larval performance at high $p\mathrm{CO}_2$ (Sunday et al. 2011), which could indicate limited scope for evolutionary responses to ocean acidification. However, a diallel breeding design is not always possible, for example where it is not possible to cross-breed a large number of males and females in the laboratory. In such instances, parent-offspring correlations are popular alternate methods for estimating heritability. In this approach the similarity in phenotypic traits is measured in parents and their offspring, and h^2 is the slope of the regression of the offspring phenotype on the parent phenotype (Lynch and Walsh 1998). A limitation of parent-offspring correlations is that they may not be able to decompose maternal, paternal and environmental effects. Consequently, these correlations are often considered to estimate broad-sense heritability because they might include multiple sources of genetic and non-genetic variation that are unable to be fully teased apart (Lynch and Walsh 1998). Father-offspring correlations provide the least biased estimate of narrow-sense heritability (additive genetic variation) as they can at least reduce the influence of maternal effects.

Evolutionary studies are complex and have commonly highlighted that heritability is not constant and can vary with environmental conditions (Hoffmann and Merilä 1999). Adaptation could therefore be less likely under some environmental conditions than others, which complicates the assessment of adaptive potential to ocean acidification. The environmental stress of a rapidly changing environment may increase or decrease phenotypic variation, resulting in different heritability among environments (Hoffmann and Hercus 2000). Unfavorable stress has resulted in decreased broad- and narrow-sense heritability in a variety of animals (Charmantier and Garant 2005; Wilson et al. 2006). On the other hand, stressful environments may also increase heritability if the stressful environment leads to greater expression of phenotypic variation compared with less stressful environments (Hoffmann and Parsons 1991; Hoffmann and Hercus 2000). Estimates of heritability may therefore differ in populations exposed to an acute environmental change compared with chronic environmental stress. The effects of acute versus chronic environmental stress are especially relevant to experiments testing adaptive potential to ocean acidification because previous experiments have exposed species to high CO_2 for just a few days or weeks (i.e. acutely), whereas ocean acidification will result in chronic exposure to high CO_2 throughout life. Thus short-term studies may incorrectly predict long-term effects of ocean acidification on marine species.

The limited number of acclimation and adaptation studies conducted to date illustrates heritable phenotypic variation in response to ocean acidification for some species (Kelly et al. 2013; Malvezzi et al. 2015). For example, variation in body size of the purple sea urchin was heritable, showing evolutionary potential for this trait (Kelly et al. 2013). Selection of CO_2 tolerant genotypes has also been seen concurrent with the maintenance of developmental and morphological traits (Pespeni et al. 2013). In other species, however, there appears to be limited heritable variation of phenotypic traits at high CO_2 (Sunday et al. 2011). Underlying genotypes and other environmental stressors may further influence the strength or direction of heritability in a given trait, which could limit adaptive potential (Foo et al. 2014). Adaptation assessments for a broader range of marine taxa and phenotypic traits are therefore a priority in predicting the performance of marine species in a high CO_2 world.

Adaptation to ocean acidification in fishes

Studies have not yet addressed the adaptive potential of fish to ocean acidification. The one exception is a novel study by Malvezzi et al. (2015) who found that genetically related juvenile Atlantic silverside, *M. menidia*, had a similar probability of survivorship, indicating a significant additive genetic component that may promote adaptation to ocean acidification. Following exposure to elevated CO_2 for 15 days post-hatching, offspring and parents were genotyped to construct a parent-offspring pedigree and estimate variance components (Malvezzi et al. 2015). This study provided the first heritability estimate for marine fish survival under elevated CO_2. The lack of quantitative genetic studies in other marine fishes may be due to difficulties in laboratory breeding. For instance, it is not possible to complete the life cycle of most tropical fish species in captivity (Rummer and Munday 2016), and those species that can be reared are mainly demersal spawners that exhibit paternal care. This makes the use of diallel breeding designs near impossible for most tropical marine fishes. Studies would therefore need to consider alternative methods to assess additive genetic variation such as cross-fostering studies (e.g. Johnson et al. 2010) or parent-offspring regressions (Lynch and Walsh 1998; Åkesson et al. 2008).

Weeks of elevated CO_2 exposure reveal persistently altered olfactory responses (Munday et al. 2013a, 2014a). If transgenerational plasticity is not apparent for fish behaviour, genetic adaptation will be necessary to overcome these behavioural impairments. Importantly, individual variation in olfactory tolerance to CO_2 has been observed, especially at near-future CO_2 levels around 700 µatm, where some individuals become strongly attracted to the cue while others maintain CO_2 tolerance and avoid the cue (Munday et al. 2010; Ferrari et al. 2011a). Furthermore, field-based studies have demonstrated selection for CO_2-tolerant behavioural phenotypes (Munday et al. 2012). This individual phenotypic variation and natural selection could be key for adaptation in reef fish; however, it is unknown if behavioural tolerance to high CO_2 is heritable. Determining the heritability of variation in behavioural tolerance to high CO_2 will be necessary to predict the potential for adaptation to ocean acidification depending on its strength and direction relevant to other traits or environmental factors.

Aims and thesis outline

This thesis examines the potential for reef fishes to acclimate and adapt to the high CO_2 environment predicted to occur by the end of this century. While there is some understanding of short-term exposure to elevated CO_2, there has been little consideration of how multiple generations will fare under these changed environmental conditions, as will occur in nature. In **Chapter 2**, I examine the impacts of elevated CO_2 on reproduction, the initial stage of cross-generational effects, for two coral reef fishes: the orange clownfish *Amphiprion percula* and the spiny chromis *Acanthochromis* *polyacanthus*. Both species are readily reared in captivity with high success. Additionally, *A. percula* is a close relative of *A. melanopus*, which had increased reproductive output due to high CO_2 (Miller et al. 2013), and *Ac. polyacanthus* is from the same family as both clownfish species (Pomacentridae). The use of these study species allowed me to explore if ocean acidification had similar effects among closely related marine fishes.

After assessing reproductive potential under future ocean acidification, I focus on the potential for acclimation and adaptation in one species, *Ac. polyacanthus*. In **Chapter 3** I test if parental exposure to high CO_2 can mitigate the negative effects of high CO_2 on the behaviour of juvenile fish (i.e. transgenerational acclimation). A full factorial design was used, where newly hatched *Ac. polyacanthus* from control, mid-CO_2 and high CO_2 parents were reared in control, mid-CO_2 and high-CO_2 treatments. This enabled me to test: 1) the short-term effects of elevated CO_2 on the behaviour of juvenile fish, 2) if CO_2 conditioning across generations had either positive or additional negative effects on fish behaviour and 3) any potential carry-over effects when offspring from mid- and high-CO_2 parents were reared in control conditions.

In **Chapter 4** I examine potential transgenerational effects of elevated CO_2 on otolith development. Previous experiments have reported no effects of otolith development in *Ac. polyacanthus* during short-term exposure to high CO_2, thus they were an ideal species to observe potential long-term effects of ocean acidification across generations. In this chapter I used the same experimental design as in Chapter 3. Therefore, I was able to test: 1) the short-term effects of elevated CO_2 on otolith development, 2) if CO_2 tolerance in otolith development was maintained with transgenerational exposure and 3) if parental CO_2 exposure had carry-over effects when offspring were reared in control conditions.

While transgenerational acclimation may provide one means for coping with environmental change, adaptive potential must also be simultaneously considered. In **Chapter 5** I test for heritability of variation in behavioural responses to chemical alarm cues (CAC) under control and elevated CO_2 conditions in both wild and laboratory populations of *Ac. polyacanthus*. The spiny chromis is unique among coral reef fishes in that it lacks a pelagic larval stage and offspring remain with their parents for several months after hatching (Kavanagh 2000). Consequently, this species is ideally suited to estimate heritability with parent-offspring correlations, which is the approach used in this chapter. I was able to identify parents and their offspring in the wild, as well as breed these fish in the laboratory, which enabled me to compare heritability in CO_2 tolerance for olfactory responses in both wild and laboratory settings. I also test for potential differences in heritability associated with acute and chronic exposure to high CO_2 in offspring, and due to high CO_2 exposure in the parental generation. Field estimates allow for comparison of heritability estimated in laboratory environments. Furthermore, the laboratory component enabled me to test for heritability of behavioural tolerance to high CO_2 following chronic exposure to high CO_2 from hatching, and to compare this with heritability estimated following acute exposure to high CO_2. The factorial breeding design used in the laboratory further allowed for examination of any effects of parental conditioning to elevated CO_2 on the heritability of behavioural responses to CAC in offspring exposed both acutely and chronically to high CO_2 conditions.

Chapter 2: Contrasting effects of ocean acidification on reproduction in reef fishes

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Authors: Megan J. Welch and Philip L. Munday

2.1 Summary

Differences in the sensitivity of marine species to ocean acidification will influence the structure of marine communities in the future. Reproduction is critical for individual and population success, yet is energetically expensive and could be adversely affected by rising CO_2 levels in the ocean. I investigated the effects of projected future CO_2 levels on reproductive output of two species of coral reef damselfish, *Amphiprion percula* and *Acanthochromis polyacanthus*. Adult breeding pairs were maintained at current-day control (446 μatm), moderate (652 μatm) or high CO_2 (912 μatm) for a 9-month period that included the summer breeding season. The elevated CO_2 treatments were consistent with CO_2 levels projected by 2100 under moderate (RCP6) and high (RCP8.5) emission scenarios. Reproductive output increased in *A. percula*, with 45–75 % more egg clutches produced and a 47–56 % increase in the number of eggs per clutch in the two elevated CO_2 treatments. In contrast, reproductive output decreased at high CO_2 in *Ac. polyacanthus*, with approximately one-third as many clutches produced compared with controls. Egg survival was not affected by CO_2 for *A. percula,* but was greater in elevated CO_2 for *Ac. polyacanthus*. Hatching success was also greater for *Ac. polyacanthus* at elevated CO_2, but there was no effect of CO_2 treatments on offspring size. Despite the variation in reproductive output, body condition of adults did not differ between control and CO_2 treatments in either species. These results demonstrate different effects of high CO_2 on fish reproduction, even among species within the same family. A greater understanding of the variation in effects of ocean acidification on reproductive performance is required to predict the consequences for future populations of marine organisms.

2.2 Introduction

Atmospheric carbon dioxide (CO_2) concentrations have increased from 280 ppm before the Industrial Revolution to 400 ppm in 2015 (www.esrl.noaa.gov/gmd/ccgg/trends/), the highest level in at least 800,000 yr (Lüthi et al. 2008). The oceans have absorbed approximately one-third of the additional CO_2 released by humans during this time, causing ocean pH to decline by 0.1 units, through a process known as ocean acidification (Caldeira and Wickett 2003). If the current anthropogenic rate of emissions continues, climate models project that CO_2 concentrations in the atmosphere and surface ocean will exceed 900 ppm by the end of this century and ocean pH will decline a further 0.3–0.4 units compared with current-day values (Collins et al. 2013). Higher CO_2 levels and reduced pH can have negative effects on a wide range of biological processes, including calcification, metabolism and nitrogen fixation (Doney et al. 2009). However, some studies have also reported positive effects such as enhanced photosynthesis and growth due to increased pCO_2 (Munday et al. 2009b; Vogel et al. 2015). Furthermore, the effects of ocean acidification can differ among taxonomic groups (Ries et al. 2009; Fabricius et al. 2011; Chan et al. 2015) and even among closely related species, with some species apparently more tolerant than others (Langer et al. 2006; Ferrari et al. 2011b; Pandolfi et al. 2011; Strahl et al. 2015). Differences in sensitivity to ocean acidification will influence which species could thrive in a future high-CO_2 world, and which will not, thus determining the future structure of marine communities.

Reproduction is essential to population replenishment and is also critical to individual fitness. Nevertheless, reproduction is energetically expensive and may be constrained by a range of environmental factors. For example, females allocate significant amounts of energy to egg or offspring provisioning (Watson et al. 1998; Visser and Lessells 2001; Grazer and Martin 2011), while males may expend large amounts of energy in courtship or nest development (Cox et al. 2010). These energetic costs restrict reproduction to specific environmental conditions and make it highly sensitive to environmental fluctuations (Cushing 1969; Dawson 2008; Visser et al. 2009). Ocean acidification has been shown to have varying effects on reproduction in marine organisms, depending on species and exposure time to elevated CO_2. For example, Dupont et al. (2013) found that four months' exposure to high pCO_2 (1200 μatm) reduced the fecundity of green sea urchins, but there was no effect on fecundity after 16 months' exposure. Elevated CO_2 reduced fertilization success in Sydney rock oyster (Parker et al. 2009), yet no effects of CO_2 on egg production were found in multiple copepod species (Weydmann et al. 2012; McConville et al. 2013). Despite the fundamental importance of reproduction to individual fitness and population success, surprisingly few studies have examined the potential effects of elevated CO_2 on reproductive performance.

Fish are generally considered to be relatively tolerant to ocean acidification because they have a well-developed acid–base regulatory system that can defend against intra and extra-cellular acidosis in a high-CO_2 environment (Brauner and Baker 2009; Heuer and Grosell 2014). However, the increased ion transport required to maintain pH in a high-CO_2 environment is expected to incur additional energetic costs (Pörtner et al. 2004; Ishimatsu et al. 2008; Strobel et al. 2012). The additional energy put into acid–base regulation may be supplemented from other important activities (Ishimatsu et al. 2004; Barry et al. 2011), including reproduction (Aze et al. 2014). Reduced energy availability could potentially result in reduced clutch size, smaller eggs, smaller hatchlings or fewer clutches produced in a breeding season. Despite these predictions, little is known about the effects of elevated CO_2 on fish reproduction, possibly due to the logistical difficulty of maintaining large breeding populations of most marine fishes in captivity and simultaneously manipulating their CO_2 environment. In one of the only studies conducted to date, Miller et al. (2013) found positive effects of elevated CO_2 on reproduction in the cinnamon anemonefish, *Amphiprion melanopus*. Contrary to expectations, reproductive output was substantially higher in the elevated CO_2 treatment compared with present-day controls. Whether reproduction is similarly stimulated in other reef fishes has not been investigated.

I tested the effects of elevated CO_2 on reproductive performance, offspring quality and adult body condition in two coral reef damselfishes (Pomacentridae): *A. percula and Acanthochromis polyacanthus*. These two damselfishes have overlapping geographic distributions and are found in similar reef habitat (Randall et al. 1990). Both species are benthic spawners that form monogamous breeding pairs. They are ideal for examining the effects of elevated CO_2 on fish reproduction because they are easily maintained in captivity, and replicate breeding pairs can be independently maintained under different environmental conditions. Furthermore, the anemonefish *A. percula* is in the same phylogenetic clade as *A. melanopus* studied by Miller et al. (2013), and therefore provides the opportunity to test whether reproduction of other anemonefishes is also stimulated by elevated CO_2. Breeding pairs of both species were kept under control (446 μatm), moderate (652 μatm) and high CO_2 (912 μatm) for a 9-month period that included the entire reproductive season and two months' CO_2 conditioning before the start of breeding. The moderate and high CO_2 treatments matched CO_2 levels projected for the end of the century under moderate (RPC6) and high (RCP8.5) emission scenarios, respectively (Meinshausen et al. 2011). Reproductive measurements included the number of pairs that reproduced in each treatment, the total and average number of egg clutches produced, number of eggs per clutch and egg survival. The body condition of parents was compared among treatments to determine whether there were substantial energetic costs to maintain reproduction in a high CO_2 environment. Finally, for one species, *Ac. polyacanthus*, hatching success, standard length and weight were measured. Standard length and weight measurements were used to estimate offspring quality under different CO_2 scenarios.

2.3 Methods

Study species and collection

The anemonefish, *A. percula*, occurs throughout the Indo-Pacific in monogamous breeding pairs, laying numerous clutches throughout the summer breeding season (Drew et al. 2008). Eggs are laid on a protected, hard substrate. The male tends to the eggs throughout embryonic duration, which lasts 7–9 d (Michael 2008). Once hatched, larvae have a pelagic phase of approximately 11 d before settling on the reef (Bay et al. 2006). The spiny chromis, *Ac. polyacanthus*, also occurs on coral reefs throughout the Indo-Australian region. They are demersal spawners and lay between one and three clutches during the summer breeding season. *Ac. polyacanthus* lays eggs on a protected substrate, often within small caves and crevices in the reef matrix. Eggs hatch into small juveniles, with both parents providing care to the eggs and offspring for up to 45 d post-hatching (Kavanagh 2000).

Adult breeding pairs of *A. percula* were collected from the Cairns Region of the Great Barrier Reef, Australia and housed at James Cook University. All pairs were supplied by commercial collectors. Adult breeding pairs of *Ac. polyacanthus* were collected from the Orpheus Island region (18.62 °S, 146.49 °E) of the Great Barrier Reef during September 2012 and transferred to James Cook University, Townsville. Breeding pairs of *A. percula* and *Ac. polyacanthus* were housed in 40 L aquaria supplied with a continuous flow of seawater at 22.5 °C. Each aquarium contained half of a terracotta pot for shelter and breeding site. Pairs were fed 0.1 g of commercial fish feed pellet (INVE Aquaculture Nutrition NRD 12/20) once per day. Feeding was increased to three times per day (Donelson et al. 2010) during the breeding season (November–May).

Experimental design and data collection

Nine breeding pairs of each species were randomly assigned to each of the three treatment groups (*N* = 27 pairs for each species). CO_2 levels were set at control, moderate and high CO_2 concentrations when the fish entered the systems. Temperatures were raised by 0.5 °C weekly from the winter temperature (22.5 °C) until they reached the average summer temperature (28.5 °C) on 1 November 2012. Lights were also adjusted weekly to reach the average summer photoperiod of 13L:11D by 1 November. This allowed for a 2-month acclimation period to CO_2 treatments before summer breeding temperatures and photoperiod were attained. Temperatures and photoperiod were maintained at summer conditions for the entire breeding season (Table 2.1). Breeding began at the start of November, when the water reached summer temperature. The presence of egg clutches in both species was checked daily between 0700 and 0800 hrs. Clutches were photographed with a waterproof camera on the day they were laid. A random sample of 10 eggs was then taken from the clutch and preserved in 6 % formalin. Sampled eggs were photographed 48 h after preservation and egg area calculated from the image using ImageJ software (National Institute of Health, Bethesda, MD, USA). Photographs of each clutch were taken again in the middle of development and on the day of hatching.

Clutch photographs were analysed with ImageJ to assess the number of eggs laid and the numbers remaining throughout development. Unfertilized eggs, noted by their opaque colouration, were not included in these counts. Percentage egg survival was determined from the counts.

Hatching success and hatchling condition were only measured for *Ac. polyacanthus.* These parameters were not estimated for *A. percula* because of logistical constraints in monitoring nocturnal hatching and collecting newly hatched larvae of this species. *Ac. polyacanthus* offspring were hatched into their parental aquaria and the number of hatchlings counted within 5 h of hatching. Hatching success was determined as the number of hatchlings as a proportion of the number of eggs laid. A sample of 20–25 hatchlings was taken and fixed in 4 % phosphate-buffered formaldehyde. The preserved hatchlings were photographed and weighed 48 h later. Hatchling weight was measured (to 0.01 mg) as wet weight, where the hatchlings were first blotted dry before being placed in the weigh boat. Photographs were analysed for standard length measurements using ImageJ. Hatchlings that were not sampled immediately were removed from their parental aquaria within 12 h of hatching.

Experimental systems and CO_2 manipulation

Three 8000 L recirculating aquarium systems were used, each set to a different pH and corresponding CO_2 level: a current-day control (446 μatm), mid-range CO_2 (656 μatm) and high CO_2 (912 μatm), mirroring projections for the end of this century. In the elevated CO_2 treatments, CO_2 was dosed into a 3000 L sump in each system using an Aqua Medic AT Control System (Aqua Medic, Germany), which maintained the desired pH level in each system. The equilibrated seawater was then delivered to the holding aquaria at a rate of 1.5 L min^{-1}. Water chemistry was measured directly in randomly selected aquaria on the relevant timescales. Daily pHNBS (pH calibrated to National Bureau of Standards) and temperature measurements were taken using a pH electrode (SevenGo Pro, Mettler Toledo, Switzerland) and temperature probe (Comark C26, Norfolk, UK). Weekly salinity readings were measured using a conductivity sensor (HQ15d; Hach, Loveland, CO, USA). Total alkalinity was also estimated weekly using Gran Titration (Metrohm 888 Titrando Titrator Metrohm AG, Switzerland) and using certified reference material from Dr. A. G. Dickson (Scripps Institution of Oceanography). CO2SYS was used to calculate carbonate chemistry parameters (Pierrot et al. 2006) with the constants K1 from Mehrbach et al. (1973) refit by Dickson and Millero (1987) and Dickson for KHSO4 (Table 2.1).

Table 2.1. Mean (± SD) seawater parameters in the experimental system during the breeding season. Temperature, pH, salinity and total alkalinity (TA) were measured directly. $p\mathrm{CO}_2$ was estimated from these parameters using CO2SYS.

Treatment	**$\mathrm{pH}_{\mathrm{NBS}}$**	**Temperature ºC**	**Salinity**	**TA (μmol.kg^{-1}SW)**	**$p\mathrm{CO}_2$ μatm**
Control	8.13 (±0.03)	28.5 (±0.1)	35.6 (±0.9)	2199 (±93)	446 (±40)
Mid-CO_2	7.98 (±0.06)	28.5 (±0.1)	35.6 (±0.9)	2125 (±76)	652 (±77)
High-CO_2	7.85 (±0.03)	28.5 (±0.1)	35.9 (±0.7)	2139 (±113)	912 (±74)

Data analysis

A Chi-square test for independence was used to compare the total number of clutches produced in control and elevated CO_2 treatments by each species. A two-way ANOVA was then used to compare the average number of clutches per reproductive pair among CO_2 treatments for the two species. CO_2 treatment and species were fixed factors in the analysis.

The mean number of eggs per clutch and average egg area per clutch were analysed with nested ANOVAs. The two species were analysed separately because they produce very different-sized clutches and eggs; *A. percula* egg clutches contain approximately twice as many eggs than *Ac. polyacanthus* clutches, but egg area of *A. percula* is approximately half that of *Ac. polyacanthus*. CO_2 treatment and parental pair were the main effects in each analysis, with parental pair nested within CO_2 treatment. For egg area, the average of the 10 measurements per clutch was used as the unit of replication in the analysis.

Two-way nested ANOVA was also used to compare egg survival to hatching among CO_2 treatment for each species. Percent egg survival data were logit transformed for analysis (Warton and Hui 2011). Breeding pairs of *A. percula* sometimes eat their egg clutches, even when the eggs appear viable. A Chi-square test showed that there was no difference in the frequency of eaten clutches versus non-eaten clutches among CO_2 treatments ($\chi^2 = 0.291$, df = 2, $p > 0.05$); thus, only non-eaten clutches were included in the egg survival analysis. Tukey's HSD post hoc tests were used to compare treatment means and identify homogeneous grouping among treatments in all ANOVA analyses.

There was greater variation in survivorship of embryonic and larval *A. percula* compared with *Ac. polyacanthus*, therefore only the latter species was used to assess hatching success and hatchling size. Two-way ANOVAs, with parents nested within CO_2 treatment, were used to test for effects of CO_2 treatment on these traits. Percent hatching success data were logit transformed for analysis. For length and weight measurements, the average of the 20 measurements of each trait per clutch was used as the unit of replication.

ANCOVA was used to compare body condition of adults among CO_2 treatments, with weight as the dependent variable and length as the covariant. Only females were included in the analysis for *A. percula* because of the substantial dimorphism in body size between males and females. Both males and females were included for *Ac. polyacanthus* because there is no size dimorphism. To limit any stress involved with taking length and weight measurements on live fish, only adults from control and high CO_2 treatments were measured, as any effect of CO_2 on body condition should be most apparent in this comparison. Weight data were square-root transformed for analysis.

2.4 Results

Elevated CO_2 had no effect on the number of *A. percula* pairs that reproduced, with 6–7 of the 9 breeding pairs reproducing in each treatment. For *Ac. polyacanthus*, seven of nine pairs reproduced in both control and moderate CO_2 treatments, but only four pairs reproduced in the high CO_2 treatment.

Elevated CO_2 had contrasting effects on reproductive output of the two species ($\chi^2 = 15.06$, df = 2, $p < 0.001$). Over the breeding season, *A. percula* produced a greater total number of clutches under elevated CO_2 than in controls. A total of 64 clutches were produced in moderate CO_2 and 77 clutches at high CO_2, compared with 44 clutches in the control treatment. In contrast, reproductive output of *Ac. polyacanthus* decreased under high CO_2. Control pairs produced a total of 24 clutches and moderate CO_2 fish laid 28 clutches, whereas high CO_2 fish produced only nine clutches over the breeding season.

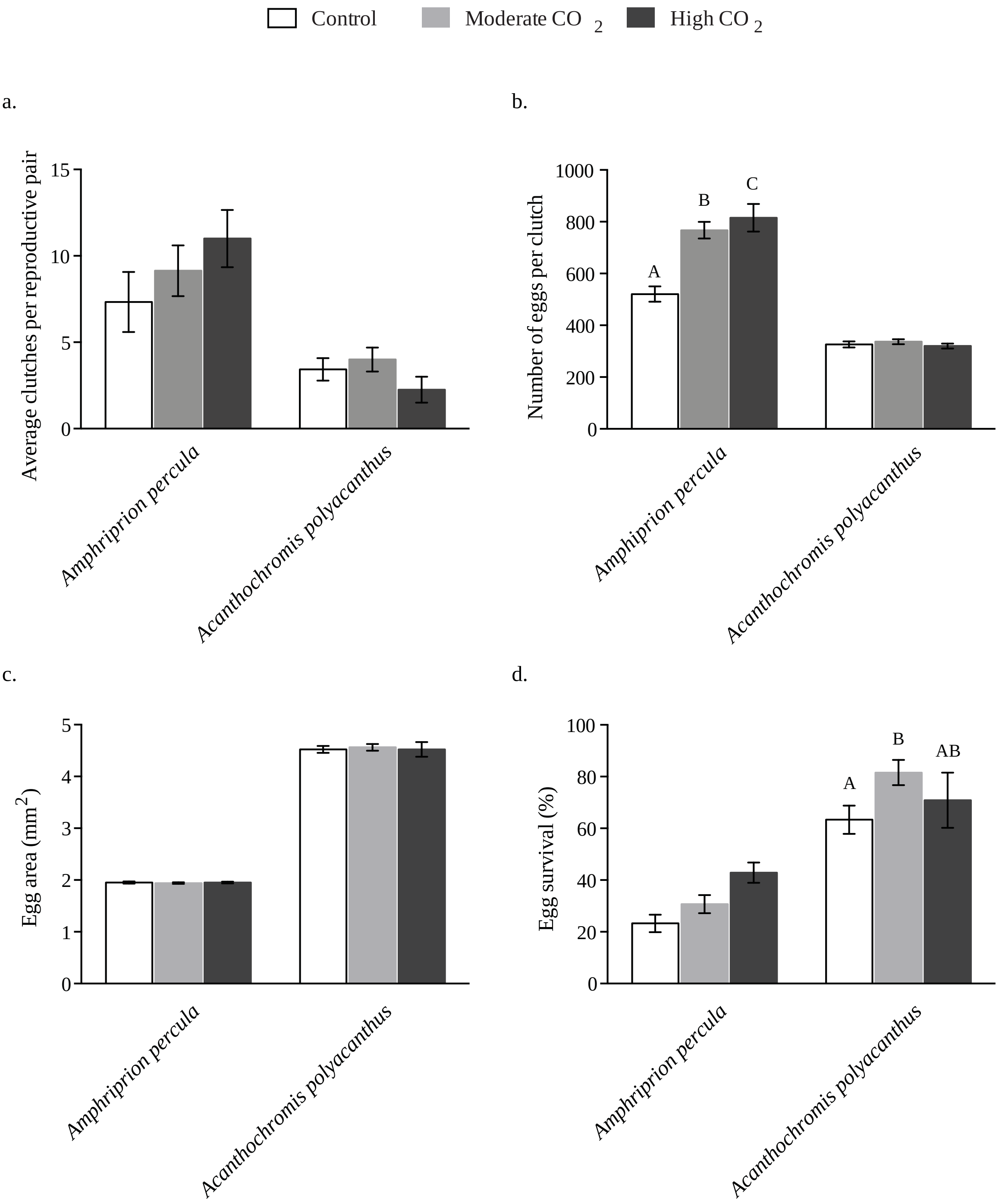

Figure 2.1. Reproductive characteristics of breeding pairs of *Amphiprion percula* and *Acanthochromis polyacanthus* in control (white bars), moderate CO_2 (light grey bars) and high CO_2 (dark grey bars) treatments. (a) Average number of clutches produced per breeding pair, (b) number of eggs laid per clutch, (c) egg area and (d) egg survival. Values are mean ± SE. *N* = 39 control, 56 moderate CO_2 and 63 high CO_2 clutches for *A. percula*. *N* = 17 control, 19 moderate CO_2 and 8 high CO_2 clutches for *Ac. polyacanthus.*

The average number of clutches produced per pair was not significantly affected by elevated CO_2 (Figure 2.1a). Breeding pairs of *Ac. polyacanthus* produced a lower mean number of clutches than *A. percula*, but there was no difference in the average number of clutches produced among CO_2 treatments for either species. There was a trend towards more clutches per pair in the elevated CO_2 treatments in *A. percula* (Figure 2.1a), but this was not statistically significant.

The average number of eggs per clutch increased in elevated CO_2 for *A. percula* (Figure 1b; $F_{2,181} = 45.497$, $p < 0.005$), but not for *Ac. polyacanthus*. For *A. percula*, there was a 47–56 % increase in the number of eggs produced per clutch between present-day control and the two elevated CO_2 treatments. The average number of eggs per clutch also differed among parental pairs for *A. percula* ($F_{17,181} = 52.632$, $p < 0.005$). In contrast, elevated CO_2 had no effect on the number of eggs produced per clutch for *Ac. polyacanthus* (Figure 2.1b). There was no effect of elevated CO_2 on egg area for either *A. percula* or *Ac. polyacanthus* (Figure 2.1c).

Egg survival appeared to increase with elevated CO_2 for both species (Figure 2.1d); however, it was not significantly different among CO_2 treatments for *A. percula*. Although the mean egg survival was approximately 20 % higher for *A. percula* eggs in the high CO_2 treatment compared with present-day control conditions (Figure 2.1d), this was due to some parents in the high CO_2 treatment having very high egg survivorship (Parental pair: $F_{16,118} = 4.097$, $p < 0.005$) rather than a direct effect of high CO_2. For *Ac. polyacanthus*, egg survival was significantly higher in the moderate CO_2 treatment ($F_{2,28} = 3.687$, $p = 0.044$). There was a 20 % increase in survival of eggs in the moderate CO_2 treatment compared to control eggs. Survival of high CO_2 eggs was intermediate to the moderate CO_2 treatment and control (Figure 2.1d).

Hatching success and hatchling size were only measured for *Ac. polyacanthus*. Hatching success increased at higher CO_2 levels (Figure 2.2; $F_{2,28} = 6.124$, $p = 0.008$). Hatching success was 21 % higher at high CO_2 compared with control conditions. A similar hatching success occurred in the moderate CO_2 treatment, but was not significantly different from the control. Conversely, elevated CO_2 had no effect on hatchling size. Neither hatchling length (Figure 2.3a) nor weight (Figure 2.3b) differed among CO_2 treatments.

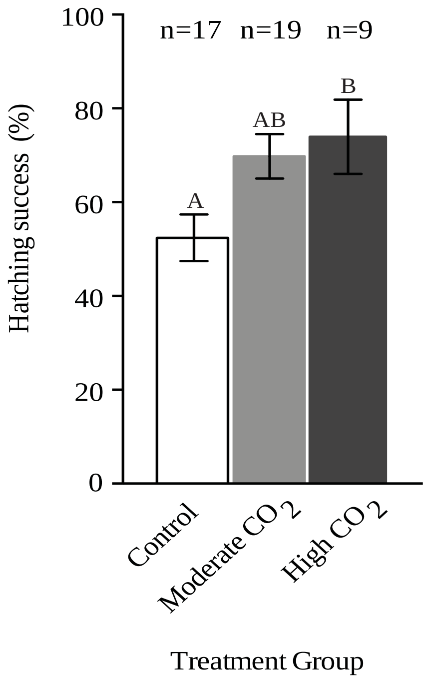

Figure 2.2. Hatching success of *Acanthochromis polyacanthus* egg clutches reared in control (white), moderate CO_2 (light grey) and high CO_2 (dark grey) treatments. Values are means for each clutch (± SE); letters indicate significant differences between treatments (Tukey's HSD test).

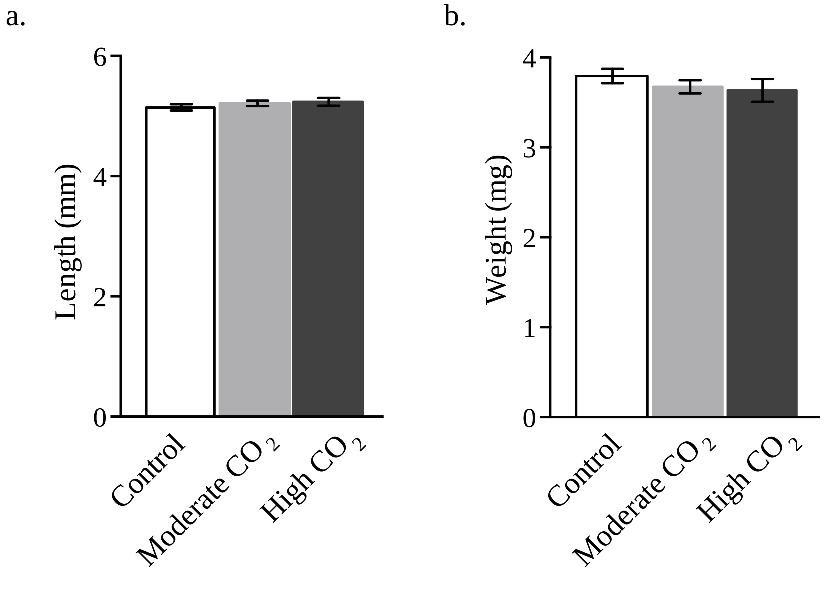

Figure 2.3. Size of *Acanthochromis polyacanthus* hatchlings in control (white), moderate CO_2 (light grey) and high CO_2 (dark grey) treatments. (a) Standard length (mm); (b) weight (mg). Values are means of clutch averages (± SE).

At the end of the breeding season, there was no apparent effect of CO_2 treatment, or differential reproductive effort among CO_2 treatments, on the body condition of either species (*A. percula*: $F_{2,22} = 0.584$, $p = 0.57$; *Ac. polyacanthus*: $F_{1,14} = 0.934$, $p = 0.35$). The length–weight relationship did not differ among CO_2 treatments for *A. percula* breeding females (Figure 2.4a), nor for breeding *Ac. polyacanthus* males and females (Figure 2.4b).

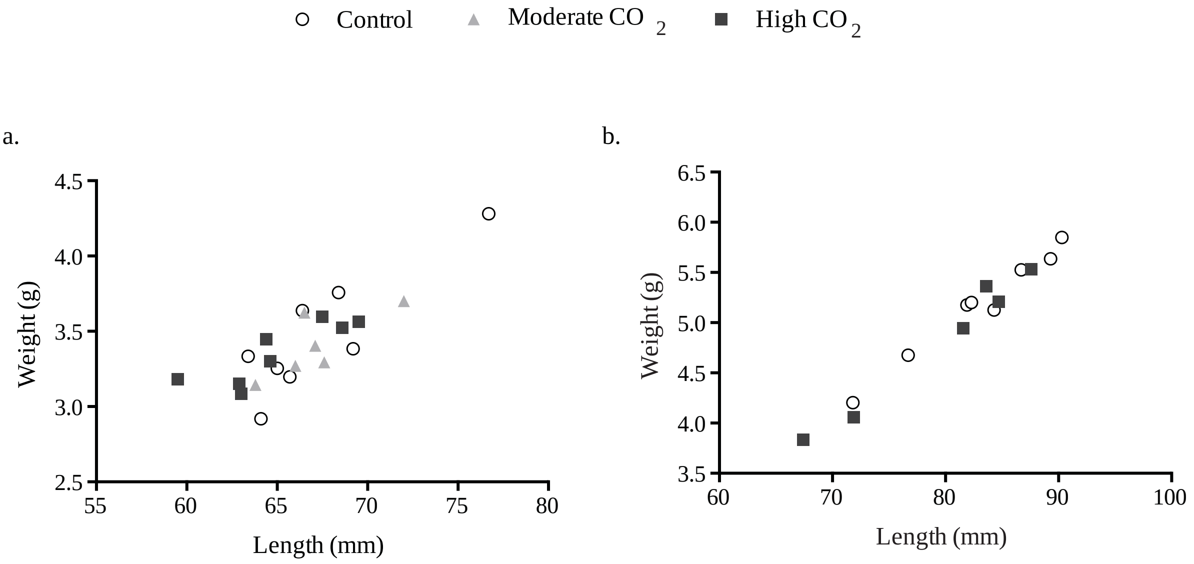

Figure 2.4. Adult body condition as indicated by length–weight relationships. (a) *Amphiprion percula* (females only) and (b) *Acanthochromis polyacanthus* (both males and females) in control (open circles), moderate CO_2 (grey triangles) and high CO_2 (black squares) treatments.

2.5 Discussion

I observed different effects of near-future CO_2 levels on the reproductive performance of two coral reef fishes. The anemonefish, *A. percula*, exhibited an overall increase in reproductive output, with more and larger egg clutches at higher CO_2 levels. In contrast, the damselfish, *Ac. polyacanthus*, produced a smaller total number of clutches at high CO_2, but with no effect on clutch size. While the total number of egg clutches was lower at high CO_2 for *Ac. polyacanthus*, egg survival and hatching success increased at elevated CO_2 in this species. These results indicate the effects of high CO_2 on fish reproduction can differ substantially, even among species within the same family. Furthermore, there may be interactions between reproductive output and embryo survivorship that need to be considered when assessing the overall impact of elevated CO_2 on reproductive performance.

The increase in reproduction was marked in both elevated CO_2 treatment groups for *A. percula*, with a near 50 % increase in the total number of clutches produced at moderate CO_2 and a 75 % increase at high CO_2 compared with controls. At the same time, the average number of eggs per clutch increased by approximately 250 and 300 eggs under moderate and high CO_2, respectively. Importantly, increased reproductive output was not associated with smaller eggs or reduced egg survivorship in *A. percula*. If anything, there was a trend for increased egg survival at high CO_2 in *A. percula*, as observed in *Ac. polyacanthus*. Consequently, there was a clear stimulation of reproduction at higher CO_2 levels for *A. percula* with no apparent trade-off in embryo quality. These results are consistent with the stimulation of reproduction by elevated CO_2 in another anemonefish, *A. melanopus* (Miller et al. 2013). In fact, the percentage increases in the total number of clutches produced and number of eggs per clutch in *A. melanopus* were similar to those I observed here for *A. percula*, indicating very similar effects of elevated CO_2 on reproduction in these two species of anemonefish.

Why reproduction was stimulated by elevated CO_2 in *A. percula* and *A. melanopus* is unknown, but could be related to the function of $GABA_A$ receptors. The main inhibitory neurotransmitter receptor in the vertebrate brain, the $GABA_A$ receptor, is a gated ion channel with high conductivity for Cl^- and HCO_3^-. Plasma concentrations of Cl^- and HCO_3^- change substantially when fishes are exposed to high CO_2, which could interfere with the function of the $GABA_A$ receptor (Heuer and Grosell 2014). This is believed to be the cause of altered behaviours and impaired sensory performances in fishes at high CO_2 (Nilsson et al. 2012; Hamilton et al. 2014; Ou et al. 2015). $GABA_A$ receptors also play an important role in reproduction by mediating hormone secretions necessary for normal reproductive output (Trudeau et al. 2000; Henderson 2007; Van Der Kraak 2009; Zohar et al. 2010). GABA influences the release of pituitary hormones, such as luteinizing hormone (LH), in goldfish (*Carassius auratus*) (Trudeau et al. 1993) and in rainbow trout (*Oncorhynchus mykiss*) (Mañanos et al. 1999). GABA further impacts the secretion of follicle-stimulating hormone (FSH) in rainbow trout

(Mañanos et al. 1999). However, the effects of GABA on reproductive hormones seem to vary between inhibition and stimulation depending on reproductive stage of the fish [goldfish: Trudeau et al. 1993; Atlantic croaker (*Micropogonias undulatus*): Khan and Thomas 1999]. It is possible that the altered function of the $GABA_A$ receptor in a high CO_2 environment affects the release of reproductive hormones in anemonefishes, thus stimulating reproduction in these species. Detailed examination of hormonal profiles in fish exposed to high CO_2 is needed to test this hypothesis.

In contrast to *A. percula*, there was no stimulation of reproduction in *Ac. polyacanthus*. The total number of clutches produced was similar to controls at moderate CO_2, but declined substantially at high CO_2. The lower total number of clutches at high CO_2 appeared to be due to the combined effects of both a decline in the number of pairs that reproduced and a decline in the number of clutches in those fish that did breed, although neither of these changes was statistically significant by themselves. Despite the reduction in the total number of clutches produced, there was no effect of elevated CO_2 on the average number of eggs per clutch or egg size in *Ac. polyacanthus*. The influence of GABA on reproductive hormones also appears to vary among fish species, and it may act in either a stimulatory or inhibitory fashion (Trudeau et al. 2000). Therefore, it is not surprising that high CO_2 may have a stimulatory effect on reproduction in some species and an inhibitory action in others. The exact reasoning for this variation, however, remains uncertain, but it does indicate that effects of high CO_2 can vary widely among species. Damselfishes further vary in their sensitivity to high CO_2, regarding metabolic rates (Couturier et al. 2013) and behavioural impairment (Ferrari et al. 2011b), indicating the complexity of biological responses that can occur in response to rising CO_2 levels and hypercapnia, even among closely related species.

While there was a reduction in the total number of clutches produced at high CO_2 in *Ac. polyacanthus*, there was a generally positive effect on egg survival and hatching success. Egg survival and hatching success could be increased under high CO_2 either because the embryos are of better quality, or because the parents provided better care of the eggs at high CO_2. There were no effects of elevated CO_2 on egg size, which suggest that parents did not provide additional provisioning to the embryos. Parents could have altered the quality of nutritional provisioning to the eggs at high CO_2, but this was not tested here and would require further investigation. Unquantified observations of parental behaviour during the experiment suggest that parents were more territorial at moderate and high CO_2 than parents in the control treatment, consistent with the idea that parents were more protective and careful of their developing offspring in the high CO_2 environment.

Hatchling size of *Ac. polyacanthus* was not affected by parental and embryonic exposure to elevated pCO_2. This is consistent with the findings of Munday et al. (2011a) that elevated CO_2 had no effect on growth, development or survival of juvenile *Ac. polyacanthus*. Together, these results suggest that early development of *Ac. polyacanthus* is highly tolerant to elevated CO_2 levels. Growth and development of *A. percula* juveniles also appear to be tolerant to elevated CO_2 (Munday et al. 2009b); however, other species may be more sensitive to projected future CO_2 levels (Baumann et al. 2012; Frommel et al. 2012; Pimentel et al. 2014). Consequently, just as I detected contrasting effects of near-future CO_2 levels of reproduction, there appear to be contrasting effects of high CO_2 on the early development of marine fishes.

Changes in reproductive output were not associated with changes in adult body condition in either of the two damselfishes studied here. In particular, there was no effect of increased reproduction on adult body condition for *A. percula*, suggesting that changes in reproduction may not be energetically demanding in a laboratory setting. Feeding was standardized and plentiful throughout the breeding season in the laboratory, which might enable adult fish to maintain their body condition despite a significant increase in reproductive output. In the wild, however, food could be more limited, and this may alter the relationship between body condition and increased reproductive output. Furthermore, energy required to forage, avoid predators and other daily activities in the wild may involve different energetic trade-offs that were not present in the controlled laboratory setting.

Ocean acidification and elevated CO_2 levels are usually considered to be stressors that will have a negative effect on marine organisms. Surprisingly, I found that projected future CO_2 levels increased the reproductive output of *A. percula* with no apparent effect on the parents or their offspring. However, it is possible that there could be energetic consequences over longer timescales or at later life stages in the offspring. For example, higher reproductive effort during one breeding season could be offset by reduced effort in the future, or enhanced performance during early life stages could be offset by reduced performance later in life. Furthermore, fish populations will experience both warming and acidification in the future, and warming might have opposing effects to high CO_2 levels. Elevated temperatures reduce reproductive output in *Ac. polyacanthus* (Donelson et al. 2010) and in the anemonefish *A. melanopus* (Miller et al. 2015). Consequently, future work should consider the interacting effects of these climate drivers on reproduction in marine species. Nevertheless, this study demonstrates that elevated CO_2 can have unexpected effects on reproduction in marine fishes and that these effects also vary substantially between species. Understanding the basis of this variation will be critical for predicting the fate of reef fish populations and communities in future high CO_2 world.

Chapter 3: Effects of elevated CO_2 on fish behaviour undiminished by transgenerational acclimation

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Authors: Megan J. Welch, Sue-Ann Watson, Justin Q. Welsh, Mark I. McCormick and Philip L. Munday

3.1 Summary

Behaviour and sensory performance of marine fishes are impaired at CO_2 levels projected to occur in the ocean in the next 50–100 years (Munday et al. 2009a, 2010; Briffa et al. 2012; Jutfelt et al. 2013; Hamilton et al. 2014), and there is limited potential for within-generation acclimation to elevated CO_2 (Munday et al. 2013a, 2014a). However, whether fish behaviour can acclimate or adapt to elevated CO_2 over multiple generations remains unanswered. I tested for transgenerational acclimation of reef fish olfactory preferences and behavioural lateralization at moderate (656 μatm) and high (912 μatm) end-of-century CO_2 projections. Juvenile spiny damselfish, *Acanthochromis polyacanthus*, from control parents (446 μatm) exhibited an innate avoidance to chemical alarm cue (CAC) when reared in control conditions. In contrast, juveniles lost their innate avoidance of CAC and even became strongly attracted to CAC when reared at elevated CO_2 levels. Juveniles from parents maintained at mid-CO_2 and high-CO_2 levels also lost their innate avoidance of CAC when reared in elevated CO_2, demonstrating no capacity for transgenerational acclimation of olfactory responses. Behavioural lateralization was also disrupted for juveniles reared under elevated CO_2, regardless of parental conditioning. These results show minimal potential for transgenerational acclimation in this fish, suggesting that genetic adaptation will be necessary to overcome the effects of ocean acidification on behaviour.

3.2 Introduction

Experiments and field studies have shown that the sensory performance and behaviours of marine fishes are impaired when they are exposed to CO_2 levels projected to occur in the ocean by the end of this century (Munday et al. 2009a, 2010, 2013a; Jutfelt et al. 2013; Hamilton et al. 2014). Changes include altered activity levels, loss of behavioural lateralization, inability to learn, slowed visual acuity, and altered auditory and olfactory preferences (Briffa et al. 2012; Branch et al. 2013; Leduc et al. 2013; Chung et al. 2014). These behavioural alterations have significant effects on ecological processes, such as predator-prey (Munday et al. 2010; Ferrari et al. 2011b) and competitive interactions (McCormick et al. 2013), that may have far-reaching implications for population replenishment, community structure and ecosystem function. Of particular concern are behavioural changes that can increase the risk of predation in juvenile fishes, such as reduced response to predator odour and chemical alarm cue (CAC) (Dixson et al. 2010; Ferrari et al. 2011b), and impaired cognitive function (Domenici et al. 2012; Chivers et al. 2014). The underlying mechanisms of these behavioural changes appear to be the interference with neurotransmitter function in the fish brain caused by elevated CO_2 levels (Nilsson et al. 2012; Chivers et al. 2014; Hamilton et al. 2014). A major unanswered question, however, is whether fish behaviour can acclimate or adapt (Sunday et al. 2014) to higher CO_2 over coming decades.

Long-term experiments and studies at natural CO_2 seeps indicate that behavioural impairment persists even when fish are permanently exposed to elevated CO_2 for weeks to months (Munday et al. 2013a, 2014a). This suggests that there is limited potential for within-generation acclimation of impaired behavioural responses. Furthermore, the greatest risk of predation for coral reef fishes occurs during the first few days of settlement to the reef (Almany and Webster 2006), and there is no evidence that acclimation of behaviour to elevated CO_2 occurs during this crucial life phase (Munday et al. 2010, 2014a). More promising, however, is new evidence of transgenerational acclimation to ocean acidification, where exposure of parents to higher CO_2 levels confers benefits to their offspring in a high-CO_2 environment (Miller et al. 2012; Parker et al. 2012; Murray et al. 2014). For example, effects of elevated CO_2 on the metabolic rate, growth and survival of juvenile anemonefish *Amphiprion melanopus* were absent when their parents were also reared under high-CO_2 (Miller et al. 2012). Furthermore, the effects of elevated CO_2 on the kinematics of predator-escape responses in juvenile anemonefish were less pronounced when parents experienced the same high-CO_2 environment as the offspring (Allan et al. 2014). These studies indicate that transgenerational acclimation can be a powerful mechanism by which some organisms may be able to adjust to projected future environmental changes, such as ocean acidification. Yet, the potential for transgenerational acclimation to overcome the negative consequences of rising CO_2 on olfactory and cognitive impairment in reef fishes has not been tested.

I tested for transgenerational acclimation of coral reef fish behaviour at CO_2 levels projected to occur in the ocean during this century under mid-CO_2 (656 μatm) and high-CO_2 (912 μatm) CO_2 emissions scenarios (Collins et al. 2013). I used a fully factorial design, where breeding pairs of the spiny damselfish, *Acanthochromis polyacanthus*, were reared in present-day control conditions (446 μatm) or one of the two elevated CO_2 treatments (mid and high) (Table 3.1). Clutches of newly hatched offspring from these breeding pairs were then divided equally into control, mid-CO_2 and high-CO_2 treatments (Figure 3.1), and reared for 40-45 days before testing (Table 3.1). This design enabled me to test for acute effects of elevated CO_2 on the behaviour of juvenile fish and the potential for mitigation of these effects when parents were maintained in elevated CO_2 conditions. This design also allowed me to distinguish if cross-generation CO_2 conditioning had additional negative influences on fish behaviour, or if there were carry-over effects when offspring from mid- and high-CO_2 parents were hatched into control conditions. Multiple clutches from each breeding pair were tested to examine the consistency in behavioural responses (Table 3.2). I tested olfactory preferences and behavioural lateralization in juveniles, as these are two of the most commonly affected behaviours in marine fishes exposed to elevated CO_2 (Dixson et al. 2010; Domenici et al. 2012; Jutfelt et al. 2013; Chivers et al. 2014; Munday et al. 2014a), and are key to predator avoidance and survival of juvenile fishes.

3.3 Methods

Study species and collection

The spiny chromis, *A. polyacanthus*, occurs on coral reefs throughout the Indo-Australian region. They are demersal spawners and lack a pelagic larval stage, with eggs hatching into small juveniles (Kavanagh 2000), making them ideal for laboratory experiments.

Adult breeding pairs of *A. polyacanthus* were collected from the Orpheus Island region (18.62 °S, 146.49 °E) of the Great Barrier Reef during September 2012. Pairs were transferred to James Cook University where they were housed in 40 L aquaria. Pairs were divided equally among control and elevated CO_2 treatments and were kept at winter temperatures (22.5 °C) until the start of the experiment. Each pair was given half of a terracotta pot for shelter and as a suitable artificial breeding site.

Experimental Design

Temperatures were raised by 0.5 °C weekly until they reached the average summer temperature (28.5 °C) in November 2012. Photoperiod was also adjusted weekly until the average summer hours were reached concurrently with summer temperatures (13L:11D). This allowed for a two-month acclimation period to CO_2 treatments before summer photoperiod and breeding temperatures were reached. Adult pairs were fed 0.1 g of commercial fish feed pellet (INVE Aquaculture Nutrition NRD 12/20) three times a day (Donelson et al. 2010) during the breeding season (November-May). The presence of egg clutches was checked daily between 07:00 and 08:00 h. Newly hatched juveniles were removed from the parental tanks within 12 hours of hatching, divided randomly and equally and immediately placed into control and CO_2 treatments (Figure 3.1). Newly hatched juveniles were fed live *Artemia* nauplii at 2 nauplii mL^{-1} of the rearing tank. One week after hatching, juveniles were fed INVE Aquaculture Nutrition NRD 2/4 larval pellets daily. Juveniles were reared for 40-45 days before testing of behavioural responses. Survival rate of juveniles from hatching to testing was not different between the three treatment groups, and was above 90 % regardless of CO_2 treatment.

Offspring were tested for alterations in their response to CAC and lateralization. The first three clutches for each breeding pair were used for behavioural testing to determine if there was consistent variation among parents in their effects on offspring performance. The only exception was one breeding pair in the high CO_2 group that only produced one clutch during the experiment (Table 3.2). For testing, fish were gently transferred into the choice flume or T-maze via a small beaker. Individuals were gently scooped into the beaker and allowed to swim out of the beaker directly into the chamber or maze.

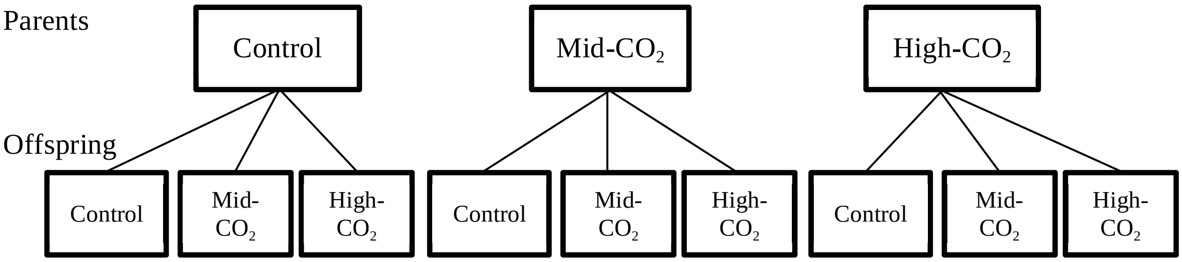

Figure 3.1. Factorial breeding and rearing design. The top row is the treatment groups for the adult breeding pairs of *A. polyacanthus*. Breeding pairs were maintained in CO_2 treatments for the entire breeding season (Table 3.1). Offspring from each clutch were divided randomly and equally amongst the control (446 μatm), mid-CO_2 (656 μatm), and high-CO_2 (912 μatm) treatments. Offspring from control parents demonstrated the acute effects of elevated CO_2, while comparisons with offspring from parents under the elevated CO_2 treatments allow for examination of potential transgenerational mitigation of CO_2 impacts. Comparisons of all control-reared offspring enabled assessment of carry-over effects of parental CO_2 on offspring.

Experimental systems and CO_2 manipulation

This experiment used three 8000 L recirculating aquarium systems, each set to a different pH and corresponding CO_2 level: a current-day control (446 μatm) and two elevated CO_2 treatments (656 and 912 μatm), consistent with mid-range and high-end projections for CO_2 levels at the end of this century (Murray et al. 2014). An Aqua Medic AT Control System (Aqua Medic, Germany) dosed CO_2 into a 3000 L sump to maintain the desired pH level for each system. The equilibrated seawater was then delivered to the holding aquaria at a rate of 1.5 L min^{-1}. pH_{NBS} and temperature were recorded daily using a pH electrode (SevenGo Pro, Mettler Toledo, Switzerland) and temperature probe (Cormark C26, Norfolk, UK). Total alkalinity was estimated weekly by Gran Titration (Metrohm 888 Titrando Titrator Metrohm AG, Switzerland) and using certified reference material from Dr. A.G. Dickson (Scripps Institution of Oceanography). Salinity was measured weekly using a conductivity sensor (HQ15d; Hach, Loveland, CO, USA). Carbonate chemistry parameters were calculated in

CO2SYS (Pierrot et al. 2006) using the constants K1 from Mehrbach et al. (1973) refit by Dickson and Millero (1987), and Dickson for KHSO4 (Table 3.1). Temperatures for each system reached 28.5 ± 0.1 °C on 1 November 2012 and remained at this summer level for the entire breeding season (Table 3.1), reflecting summer conditions in the Orpheus Island region.

Table 3.1. Mean (± SD) seawater parameters in the experimental system for adults and juveniles during the breeding season. Temperature, pH, salinity and total alkalinity (TA) were measured directly. $p\mathrm{CO_2}$ was estimated from these parameters using CO2SYS. Seawater parameters were consistent for breeding and experimental components of the study.

Treatment	**$\mathrm{pH_{NBS}}$**	**Temperature ºC**	**Salinity**	**TA (µmol.kg^{-1}SW)**	**$p\mathrm{CO_2}$ μatm**
Control	8.13 (±0.03)	28.5 (±0.1)	35.6 (±0.9)	2199 (±93)	446 (±40)
Mid-$\mathrm{CO_2}$	7.98 (±0.06)	28.5 (±0.1)	35.6 (±0.9)	2125 (±76)	656 (±116)
High-$\mathrm{CO_2}$	7.85 (±0.03)	28.5 (±0.1)	35.9 (±0.7)	2139 (±113)	912 (±74)

Olfaction

Response to chemical alarm cue (CAC) was tested in a two-channel choice flume (13 cm × 4 cm) as used in previous studies (Munday et al. 2009a; Dixson et al. 2010). The flume combination was conspecific chemical alarm cue versus untreated water. Water from the two different sources was gravity fed into the choice flume, which is divided down half of its length. A constant flow rate of 100 mL min^{-1} was maintained and monitored using a flow meter and dye test after every water change. Water chemistry in the flume matched the rearing condition for each juvenile and was consistent with seawater parameters during the breeding season (Table 3.1).

To produce CAC, control donor fish were euthanized with a quick blow to the head. Superficial cuts were made along the sides of the donor fish and then rinsed with 15 mL of treatment water (Ferrari et al. 2011a). This water was collected and immediately mixed with 10 L of treatment water in the tank used to supply CAC to the flume. This ensured a consistent concentration of fresh CAC for the duration of each trial. A ratio of one donor fish to one test fish was used.

Table 3.2. The number of breeding pairs and offspring tested in relation to the breeding design in Figure 3.1. Breeding pairs and clutches of offspring were reared in three different treatments: control (446 μatm), mid-CO_2 (656 μatm), and high-CO_2 (912 μatm). The first three clutches from each breeding pair were used for experiments, except for one breeding pair in high-CO_2 that produced only one clutch. Clutches were divided randomly and equally among the three treatments.

Water system	**Number of parent pairs**	**Number of pairs producing offspring**	**Number of clutches produced**	**Number of clutches tested**	**Number of juveniles tested for olfaction**			**Number of juveniles tested for lateralization**		
					Total	Treatment	Individual	Total	Treatment	Individual
Control	9	7	24	21	425	Control	148	659	Control	225
						Mid-CO_2	132		Mid-CO_2	212
						High-CO_2	145		High-CO_2	222
Mid-CO_2	9	7	28	21	476	Control	160	744	Control	245
						Mid-CO_2	155		Mid-CO_2	250
						High-CO_2	161		High-CO_2	249
High-CO_2	9	3	7	7	184	Control	62	315	Control	105
						Mid-CO_2	60		Mid-CO_2	105
						High-CO_2	62		High-CO_2	105

For each trial, a single test fish was placed in the centre of a downstream end of the choice flume and given a 2 min acclimation period. The position of the fish was then recorded every five seconds for a total of 2 min. A rest period of 3 min followed, during which time the water sources were switched to eliminate potential side preferences. The entire acclimation and trial process was then repeated. Fish from the first three clutches from each breeding pair were tested, except for one breeding pair in high-CO_2 that produced only one clutch (Table 3.2). Ten fish from each clutch were tested per CO_2 treatment, for a total of thirty fish per clutch. Each fish was only used once.

Lateralization

A T-maze was used to test lateralization. Dimensions were based on those used by Domenici et al. (2012) (60 cm × 30 cm × 20 cm), with a runway in the middle (25 cm × 3 cm, length × width), and at both ends of the runway (3 cm ahead of the runway) an opaque barrier (12 cm × 12 cm × 1 cm) was positioned perpendicular to the runway. Water depth in the maze was 4 cm and matched the CO_2 chemistry in which the fish were reared (Table 3.1).

A single fish was placed at one end of the T-maze and given a 5 min habituation period, during which time it could explore the apparatus. At the end of the habituation period the fish was gently guided to the beginning of the runway and left to swim along the runway until reaching the perpendicular barrier. Direction choice was recorded, with the criterion for choice being the first turning direction when the fish exited the runway. Twenty runs were recorded per fish. The first three clutches from each breeding pair were tested, except for one breeding pair in high-CO_2 that produced only one clutch (Table 3.2). Fifteen fish from each clutch were tested per CO_2 treatment, for a total of 45 fish per clutch. Each fish was only used once. To account for any possible asymmetry in the maze, turns were recorded alternately on the two ends of the runway (Domenici et al. 2012).

Data analysis

A total of 1,085 individuals were tested for changes in olfactory behaviour. A linear mixed effects model (LME) was used to test for transgenerational acclimation in offspring's responses to elevated CO_2. Percentage time in the CAC-water was arcsine-transformed before analysis to improve the distribution of the proportional data. Parental pair was included as a fixed factor in the model, while clutch number was a random factor nested within parental pair. A two-factor univariate LME was used to test for an effect of parental treatment, offspring treatment, and an interaction effect. Parental and offspring treatments were fixed factors in the models. A Tukey's HSD post hoc test was used to compare treatment means and identify homogeneous grouping among treatments.

A total of 1,728 individuals were tested in the lateralization experiments. Both absolute and relative lateralization were calculated and used to analyse potential for transgenerational acclimation (Domenici et al. 2012). The absolute lateralization index (L_A) of each fish was calculated to determine the strength of lateralization amongst individuals, regardless of a turning bias. An L_A of zero represents an equal proportion of turns to the right and to the left, and an L_A of 100 represents all turns in the same direction.

The absolute lateralization (L_A) of individuals was compared among treatments using LME models. Parental pair was included as a fixed factor in the model, with clutch number as a random factor nested within parental pair. A two-factor univariate LME was used to test for an effect of parental treatment, offspring treatment, and an interaction effect. Parental and offspring treatments were fixed factors in the models. A Tukey's HSD post hoc test was used to compare treatment means and identify homogeneous grouping among treatments. Transformations were not able to rectify the heterogeneity of variances in the lateralization data. Therefore, a more conservative alpha value of 0.01 was adopted when interpreting the results of the statistical tests on this dataset (Quinn and Keough 2002).

Relative lateralization (L_R) was calculated using the following formula (Bisazza et al. 1998):

$$L_R = [(\text{Turn to the right - Turn to the left}) / (\text{Turn to the right + Turn to the left})]*100$$

Individuals’ L_R means were grouped between -100 (fish that turned left for all 20 trials) and 100 (fish that turned right for all 20 trials). A L_R of zero reflects individuals’ lack of turning preference (10 turns to the left and 10 turns to the right). The relative lateralization index (L_R) was used to compare turning bias (that is, left versus right) among the nine groups. A Pearson's Chi-square test for independence was conducted on each CO_2 grouping (three parental groups x three offspring groups) using L_R data.

3.4 Results and Discussion

As expected, juveniles from control parents that were reared in present-day control conditions strongly avoided chemical alarm cue (CAC) when presented in a two-channel choice flume, spending less than 10 % of their time in the water stream containing CAC (Figure 3.2). In contrast, juveniles from control parents that were reared in elevated CO_2 treatments exhibited strikingly different responses ($F_{2,1077}$ = 291.17, $p < 0.01$). Control-bred juveniles reared under mid-CO_2 spent 50 % of their time on average in CAC water, while control bred juveniles reared at high-CO_2 became strongly attracted to CAC, spending nearly 80 % of their time in the cue (Figure 3.2). These results are consistent with previous studies testing the effect of elevated CO_2 on olfactory preferences in juvenile fishes (Munday et al. 2009a; Dixson et al. 2010; Ferrari et al. 2011a).

Maintaining parents in elevated CO_2 did not affect the response of their offspring to elevated CO_2, regardless of parent treatment ($F_{2,1077}$ = 2.18, $p > 0.05$, Figure 3.2). Consequently, there was no transgenerational acclimation of olfactory behavioural responses. On average, juveniles reared at mid-CO_2 spent approximately 50 % of their time in CAC water, and juveniles reared at high-CO_2 spent 75-80 % of their time in CAC water, regardless of the parental CO_2 treatment. The attraction to CAC exhibited by juveniles from mid-CO_2-treated parents that were reared at high-CO_2 was slightly less than the juveniles from control parents reared at high-CO_2 (Figure 3.2); however, the magnitude of the difference was minor in comparison with the marked change in response to CAC exhibited by all juveniles reared in the elevated CO_2 treatments versus juveniles reared in control conditions (Figure 3.2). Offspring from mid- and high-CO_2 parents that were reared in control conditions had a significantly greater attraction to CAC than offspring from control parents reared in control conditions (Figure 3.2), demonstrating a carry-over effect of parental mid- and high-CO_2 on offspring behaviour. There was no significant effect of parent pair ($F_{14,1044}$ = 1.41, $p > 0.05$) or clutch number ($F_{3,1044}$ = 1.65, $p > 0.05$) on the olfactory responses.

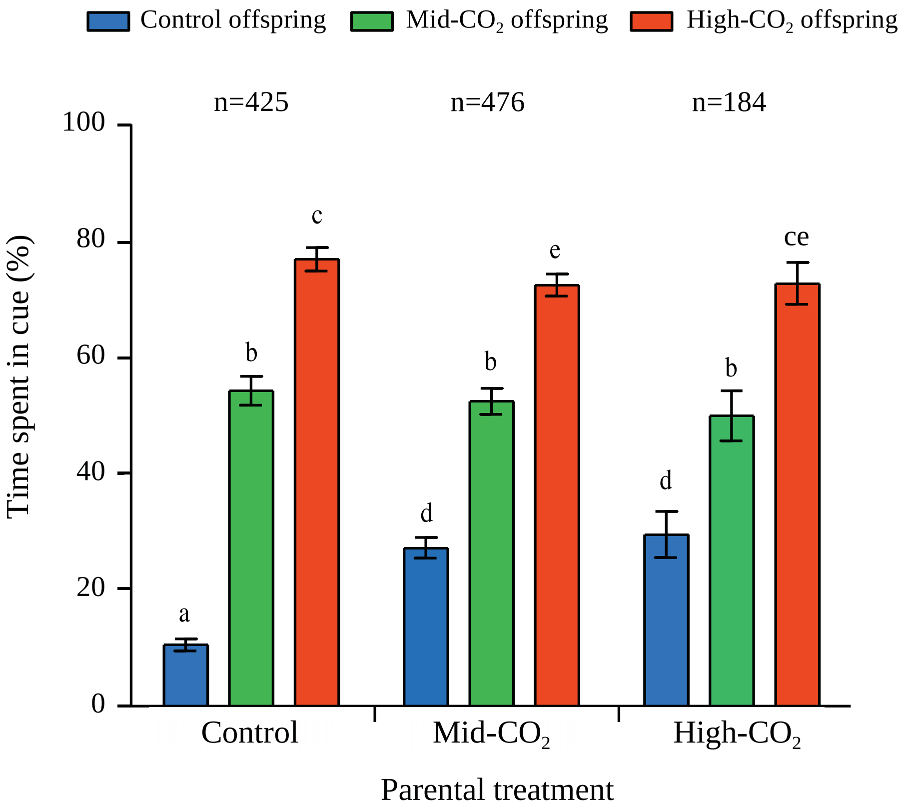

Figure 3.2. Preference of juvenile fish for water streams containing chemical alarm cues presented in a two-channel flume chamber. Juvenile fish from control (446 μatm), mid-CO_2 (656 μatm) and high-CO_2 (912 μatm) parental treatments, that had been reared in the same three CO_2 treatments (Figure 3.1), were given the choice of untreated water or water with conspecific chemical alarm cues (CAC). The graph shows mean percentage time (± SE) spent in the CAC water stream. Letters above bars represent Tukey's HSD groups. Less breeding occurred at high-CO_2, resulting in a smaller sample size of juveniles for this group (Table 3.2).

Mean L_A among individuals decreased in elevated CO_2 treatments compared with controls (Figure 3.3). Juveniles from control parents that were reared in control conditions were over 60 % lateralized. In contrast, lateralization for juveniles from control parents dropped below 40 % when reared at mid-CO_2 and below 35 % when reared at high-CO_2 (Figure 3.3). Offspring from mid-CO_2 parents showed a similar trend to those from control parents, with offspring less lateralized at higher CO_2 levels (Pearson Chi-square test, $p < 0.001$). Offspring from high-CO_2 parents exhibited a slight increase in L_A compared with juveniles from control parents reared at high-CO_2, but their overall lateralization was still significantly lower than present-day controls (Figure 3.3). These results indicate that transgenerational acclimation does not ameliorate the effects of elevated CO_2 on turning preferences in juvenile fishes, although juveniles from parents exposed to the highest CO_2 level (912 µatm) were slightly less affected by elevated CO_2 than juveniles from control parents.

As observed for the olfactory preferences, there was a carry-over effect of parental elevated CO_2 for offspring reared in control conditions (Figure 3.3), where L_A was reduced compared with control-bred offspring reared in control conditions. There was no effect of breeding pair on L_A, indicating that all pairs produced similar behaving offspring amongst CO_2 treatment groups ($F_{14,26} = 2.076$, $p > 0.5$). Clutch order nested within breeding pair was significant ($F_{26,1677} = 2.97$, $p < 0.001$), with individuals reared under high-CO_2 conditions showing a slight increase in lateralization in later clutches. Juveniles reared under control and mid-CO_2 conditions showed no consistent trend in relation to clutch order.

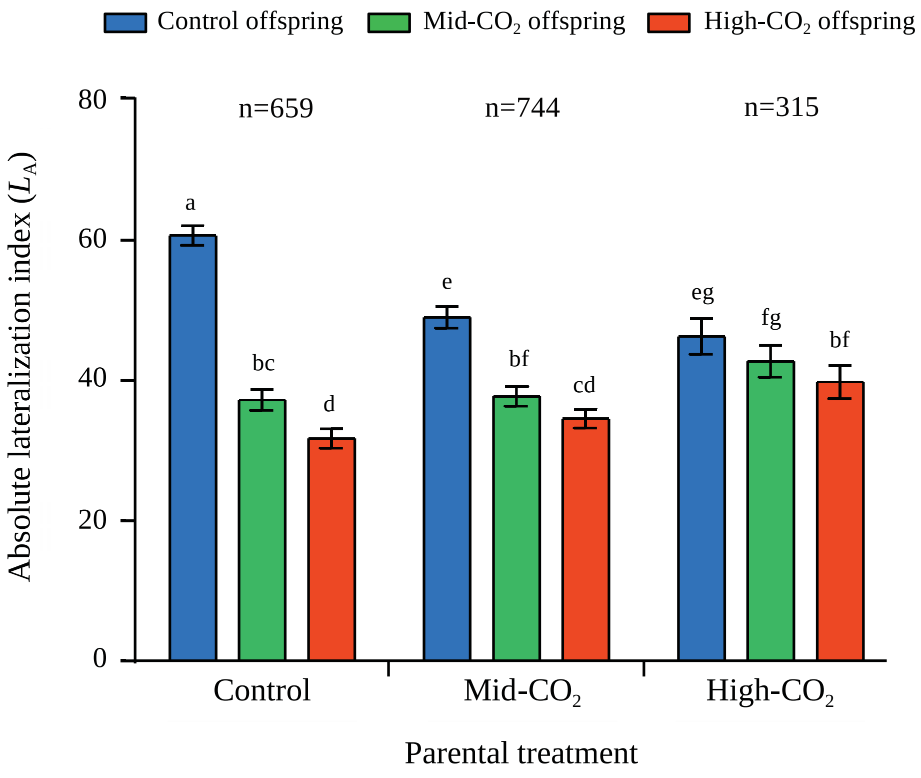

Figure 3.3. Absolute lateralization (L_A) for juvenile fish presented with a T-maze choice chamber. Juvenile fish from control (446 μatm), mid-CO_2 (656 μatm) and high-CO_2 (912 μatm) parental treatments, that had been reared in the same three CO_2 treatments (Figure 3.1), were allowed to choose to turn left or right for a total of 20 turns. The graph shows mean L_A (± SE) calculated from relative lateralization (L_R) values for each individual. Letters above bars represent Tukey's HSD groups. Less breeding occurred at high-CO_2 level, resulting in a smaller sample size of juveniles for this group (Table 3.2).

Further examination of turning preference using relative lateralization (L_R) revealed a left-turn bias was prominent in control-reared juveniles (Figure 3.4a). At higher CO_2, L_R shifted right, towards a value of zero, indicating a less pronounced left-turning preference (Figure 3.4b,c). As observed for L_A, parental exposure to high-CO_2 reduced the magnitude of the effect of elevated CO_2 on L_R in juveniles; nevertheless, there was still a shift in mean L_R towards zero (Figure 3.4g-i), demonstrating that transgenerational acclimation did not restore the bias in turning direction found in present-day conditions. There was also a clear carry-over effect of elevated CO_2, with control-reared juveniles exhibiting a reduced left-turn bias if their parents were exposed to elevated CO_2 (Figure 3.4d,g).

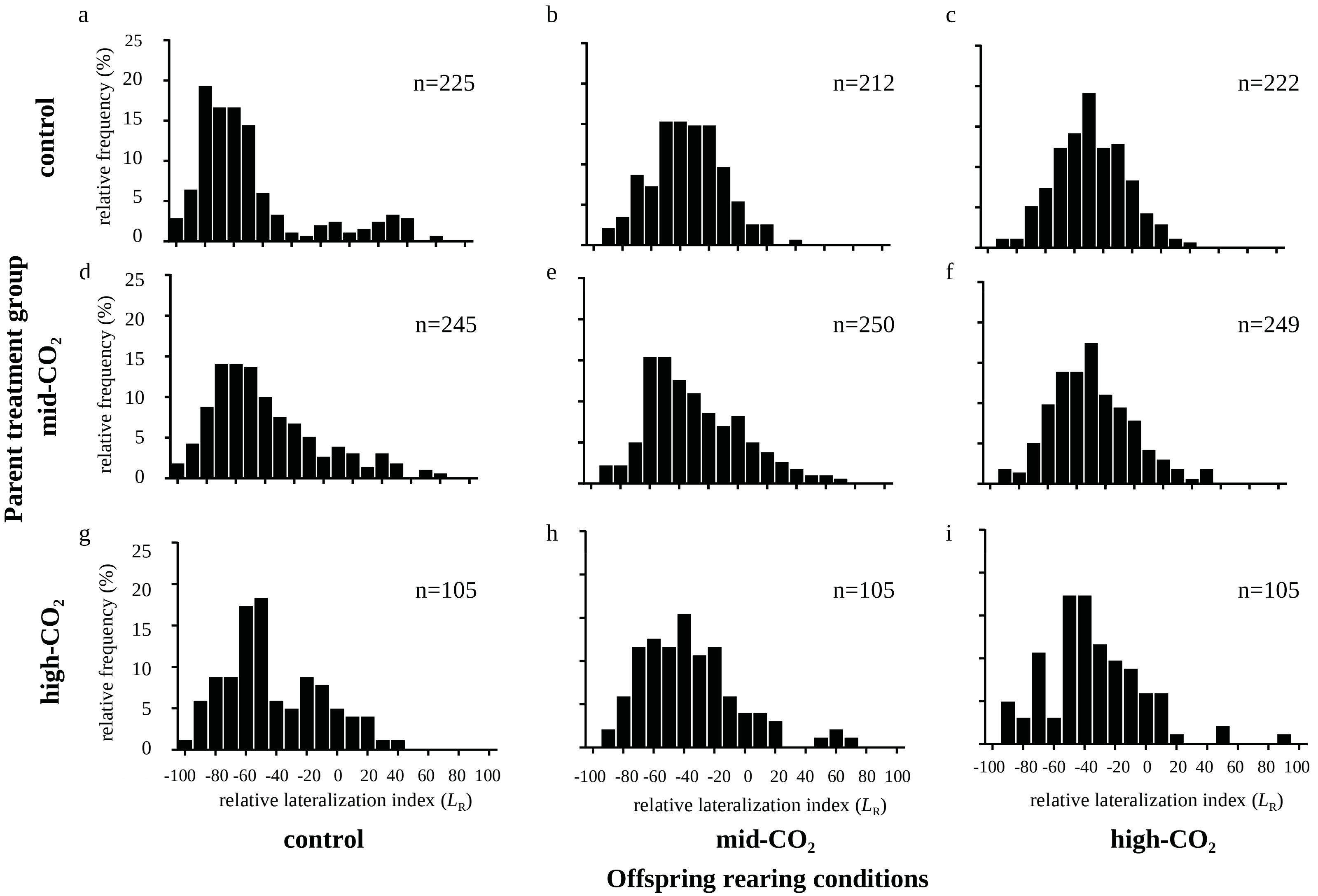

Figure 3.4. Relative lateralization (L_R) for juvenile fish presented with a T-maze choice chamber. Juvenile fish bred from

(Figure 3.4. continued) control (446 μatm), mid-CO_2 (656 μatm) and high-CO_2 (912 μatm) parents and reared under control, mid-CO_2 and high-CO_2 conditions were allowed to choose to turn left or right for a total of 20 turns. Graphs show L_R with positive and negative values indicating right and left turns, respectively. The extreme values of ± 100 indicate fish that turned in the same direction for all 20 turns.

My results show that impaired sensory and cognitive functions in juvenile reef fish caused by a high-CO_2 environment are not reversed or ameliorated by parental exposure to the same high-CO_2 levels as the offspring. Like many reef fish, *A. polyacanthus*, have an innate ability to recognize chemical alarm cues, as well as a tendency to be behaviourally lateralized, but these capabilities are lost when juveniles are exposed to CO_2 levels projected to occur over the next 50-100 years, as previously demonstrated in other reef fish species (Munday et al. 2009a; Dixson et al. 2010; Ferrari et al. 2011a; Domenici et al. 2012). These behaviours are essential for daily survival of a coral reef fish, especially in early life-history stages when predation risk is greatest (Almany and Webster 2006). Impaired behaviours under elevated CO_2 levels suggest increased mortality that could affect population sustainability (Munday et al. 2010). Transgenerational acclimation can restore metabolic and growth rates in reef fish exposed to elevated CO_2 (Miller et al. 2012), but my results show that transgenerational acclimation does not restore key sensory and behavioural traits.

Appropriate predator avoidance behaviour in response to alarm cues is essential for the survival of reef fish, especially young juveniles who are more prone to predation (Lönnstedt et al. 2012). Alarm cues are chemical compounds released into the water when the epidermal tissue is damaged, such as during a predator attack (Chivers and Smith 1998; Brown 2003). Juvenile fish have an innate response to the presence of conspecific CAC, rapidly reducing activity levels and seeking shelter in the presence of even minute traces of CAC. The attraction to conspecific alarm cues observed in CO_2-reared offspring, regardless of parental treatment, could lead to significantly higher mortality among juvenile fishes as CO_2 levels rise in the ocean. Critically, these results indicate that exposure of parents to higher CO_2 levels does not improve the behavioural responses of juveniles to CAC when reared at elevated CO_2. The carry-over effects from high-CO_2 parents for juveniles reared in control conditions further indicate that parental exposure to high-CO_2 does not have a beneficial effect on the behaviour of their offspring. There was no effect of parental breeding pair or clutch number on olfactory responses, suggesting that the responses observed here are widespread and not confined to some breeding pairs or parts of the reproductive season.

Lateralization is another fundamental behaviour for many reef fishes. Gregarious individuals depend on lateralization for group coordination (Brown et al. 2007), especially in defence or escape from a predator. Furthermore, individuals show a higher tendency for lateralization in high-predation locations than those in low-predation locations (Brown et al. 2007). Behavioural lateralization is believed to reduce decision-making time, which is especially important in a life-threatening situation such as a predator encounter. Behavioural lateralization is also closely linked to visual assessment (Sovrano et al. 1999) and cognitive function (Dadda and Bisazza 2006), both of which are affected by elevated CO_2 (Chivers et al. 2014; Chung et al. 2014). I observed a loss of lateralization at projected future CO_2 levels that was not ameliorated across generations, which suggests that predator avoidance might be impaired in a future high-CO_2 environment. *A. polyacanthus* forms loose schools and is not as gregarious as some other marine fishes; consequently, the effects of changed lateralized behaviour may be less for this species than for highly gregarious species. The partial improvement in lateralization for juveniles from high-CO_2 parents that were reared at high-CO_2 is consistent with partial acclimation of some kinematic traits involved in predator-escape responses observed in the only other study (Allan et al. 2014) to examine transgenerational acclimation of behavioural traits to high-CO_2. My results show that while there may be some partial improvement in behavioural traits linked to cognitive performance, such as lateralization, negative effects are not fully ameliorated by parental exposure to elevated CO_2 in this species. Moreover, other highly maladaptive behavioural effects, such as attraction to conspecific alarm cues, exhibit no transgenerational acclimation.

Miller et al. (2012) observed complete restoration of metabolic traits (basic maintenance and activity) in juvenile reef fishes when their parents were exposed to the same high-CO_2 levels. In contrast, I observed no improvement in olfactory-mediated behaviour and only limited improvement in behavioural lateralization when both parents and offspring were exposed to elevated CO_2. This suggests that metabolic traits may have greater potential than behavioural traits for transgenerational acclimation to elevated CO_2. Behavioural changes at elevated CO_2 appear to be a result of the interference with the function of $GABA_A$ neurotransmitter receptors (Nilsson et al. 2012; Chivers et al. 2014; Hamilton et al. 2014). It is possible that the functioning of $GABA_A$ receptors is less plastic (Nilsson et al. 2012) than metabolic pathways in reef fish (Miller et al. 2012), thus limiting the potential for transgenerational acclimation; however, this hypothesis requires further investigation. A further intriguing result was the carry-over effect observed in juveniles from mid- and high-CO_2 parents when reared in control conditions. This could suggest an epigenetic effect due to prior CO_2 exposure, either directly on $GABA_A$ receptor function, or on acid-base regulatory process that control ion gradients across receptor membranes. Because eggs developed in the same CO_2 conditions as parents before juveniles were transferred to control conditions, an epigenetic effect could be developmental or transgenerational. Further studies are needed to determine the mechanisms involved.

In conjunction with the lack of transgenerational acclimation for abnormal olfactory and lateralization responses seen in this study, there is no evidence for within-generation acclimation of behaviour to high-CO_2 in fish (Munday et al. 2014a). Therefore, genetic adaptation will be necessary to overcome these behavioural impairments as CO_2 levels rise in the ocean. Previous studies have reported a bimodal distribution of individual responses to predator odour at intermediate CO_2 levels, with some individuals strongly attracted to the cue whereas others remain repelled (Munday et al. 2010, 2013a). I did not observe a bimodal response of individuals to CAC in either of the elevated CO_2 treatments as found by previous studies; however, there was still some variation among individuals in all the experimental conditions that could be important for future adaptive responses. Selection of CO_2-tolerant behavioural phenotypes has been observed in field-based experiments (Munday et al. 2012), yet whether the phenotypic variation is heritable remains unknown. Determining the heritability of individual variation in CO_2 sensitivity is a priority for future research, as it would provide the opportunity for genetic adaptation to higher CO_2 levels. If reef fish behaviour does not adapt to rising CO_2 levels there could be serious implications for population dynamics and the function of marine ecosystems.

Chapter 4: Transgenerational exposure to ocean acidification affects otolith growth in the coral reef fish, *Acanthochromis polyacanthus*

This chapter is in preparation for submission to Marine Biology

Authors: Megan J. Welch, Sue-Ann Watson, Mark I. McCormick and Philip L. Munday

4.1 Summary

Increasing concentrations of carbon dioxide (CO_2) in the oceans pose a threat to marine organisms that exhibit calcifying properties. Aragonitic ear bones (otoliths) are the main calcified structures in fish, and previous studies have reported changes to the shape and size of otoliths in larval and juvenile fish reared at high CO_2 levels. However, not all species are equally affected and possible transgenerational effects of high CO_2 have rarely been tested. This study examined transgenerational effects on otolith area, perimeter, maximum length and circularity in juveniles of the damselfish *Acanthochromis polyacanthus* under moderate (652 µatm) and high (912 µatm) CO_2 levels predicted for the end of the century. I used a fully cross-factored experiment where parental breeding pairs were maintained under control (446 µatm), moderate CO_2 and high CO_2 conditions, and their offspring were reared for 6 weeks in the same three treatments. Moderate CO_2 exposure affected otolith development, with increased otolith area, perimeter and maximum length in juveniles from control parents, but these effects were absent in juveniles from parents that also experienced elevated CO_2. By contrast, otolith area and perimeter were greater in high CO_2 juveniles from high CO_2 parents. These results show that transgenerational acclimation can mitigate the effects of moderate CO_2 on otolith development in *A. polyacanthus*, but higher CO_2 levels lead to marked effects on otolith development across generations, potentially affecting individual performance such as swimming behaviour.

4.2 Introduction

Anthropogenic carbon dioxide (CO_2) emissions have increased rapidly from preindustrial levels of 280 ppm to over 400 ppm in 2016 (www.esrl.noaa.gov/gmd/ccgg/trends/). The oceans have absorbed approximately one-third of all anthropogenic CO_2 emitted into the atmosphere, leading to a decline in ocean pH through a process called ocean acidification (Caldeira and Wickett 2003). Ocean pH has decreased by 0.1 units and become approximately 30 % more acidic than before the Industrial Revolution. If this rate of emissions continues, atmospheric CO_2 concentrations are predicted to exceed 900 ppm by the end of this century, and ocean pH will further decline by 0.3-0.4 units (Collins et al. 2013). Declining seawater pH is accompanied by a reduction in the carbonate saturation state (Sabine et al. 2004), which reduces the ability of marine organisms to produce calcium carbonate shells and skeletons (Sabine et al. 2004; Kleypas et al. 2006). Consequently, the chemical changes occurring in our oceans due to rising CO_2 levels have had serious implications for marine calcifiers, especially those with aragonitic skeletons such as corals and some molluscs (Feely et al. 2004; Orr et al. 2005; Kleypas et al. 2006).

Fish are not generally considered to be calcifiers, but in fact they precipitate aragonitic ear bones (called otoliths) that sit within the vestibular labyrinth of the inner ear (Popper and Lu 2000). Otoliths are surrounded by a saccular epithelium filled with endolymph (Tohse et al. 2004), and otolith growth is proportional to HCO_3^- concentrations of the environment surrounding the saccular epithelium (Tohse and Mugiya 2001; Checkley et al. 2009). It might be expected that the reduced saturation state of aragonite in more acidic water would affect the precipitation rate of otoliths. However, a general observation is that otoliths tend to increase in size in seawater that has been acidified by CO_2 levels predicted to occur by the end of the century (sea bass at 993 μatm, Checkley et al. 2009; cobia at 800 μatm, Bignami et al. 2013a). When exposed to high CO_2 fish defend against acidosis by increasing HCO_3^- concentrations in their plasma (Heuer and Grosell 2014). The observed increases in otolith size under high CO_2 conditions are therefore likely due to elevated plasma HCO_3^- occurring during acid-base regulation (Heuer and Grosell 2014). Nevertheless, species variation in otolith development under elevated CO_2 is present, with some fish species showing no change in otolith growth at near future CO_2 levels (spiny damsels at 850 μatm, Munday et al. 2011a; clownfish at 1050 μatm, Munday et al. 2011b; clownfish at 900 μatm, Simpson et al. 2011), suggesting that they may be more tolerant to high CO_2.

Otoliths are critical for detection of sound, orientation and acceleration in fish (Popper et al. 2005; Simpson et al. 2011). While fish are approximately the same density as water, the otolith is much denser and therefore moves at a different amplitude than the body (Popper and Lu 2000). The differences in movement result in a bending of the sensory hair cells, enabling the direct detection of sound and stimulating nerves associated with orientation and acceleration (Popper and Lu 2000). Any changes to otolith shape and size could mean that the sensory hairs contact the otolith differently, or not at all, which may affect auditory capacity and swimming performance and ultimately result in important ecological consequences for fish (Lemberget and McCormick 2009). For example, if larger otoliths in larval fish exposed to high CO_2 cause a reduced capacity to orientate toward their settlement location using sound, this could affect recruitment and ultimately, population dynamics. Similarly, changes in swimming performance caused by abnormally large otoliths might affect the chances of attaining prey or avoiding predators. Alternatively, it has been suggested that increased otolith density could improve auditory acuity in larval fish (Bignami et al. 2013b), which could have a positive effect on performance, although this has not been empirically validated. The consequences of variation in otolith size on individual performance is little know and requires further study to determine clear linkages with behaviour (Heuer and Grosell 2014).

To test for biological effects of ocean acidification, most studies have exposed animals to future projected CO_2 levels for periods ranging from days to week or months (Kroeker et al. 2013; Riebesell and Gattuso 2015). However, parental exposure to a changed environment can affect the performance of offspring in that same environment (Marshall and Morgan 2011; Bonduriansky et al. 2012; Donelson et al. 2012; Parker et al. 2012). Parental effects may either be beneficial or detrimental to the offspring (reviewed in Salinas et al. 2013). For example, metabolic rate and growth in juvenile *Amphiprion melanopus* reared at high CO_2 were restored to control levels when the parents also experienced higher CO_2 levels (Miller et al. 2012). In contrast, olfactory behaviours were impaired in juvenile *Acanthochromis polyacanthus* reared in control conditions when their parents had been exposed to elevated CO_2 (Chapter 3). Evidently transgenerational effects can vary amongst species and life history processes, making multi-generational exposure an important component in predicting the future of climate change on marine ecosystems.

In the only study so far to examine transgenerational effects of high CO_2 on otolith development, juvenile three-spined sticklebacks reared in high CO_2 (1167 μatm) had larger otoliths when their parents were also reared in high CO_2 (1013 μatm) (Schade et al. 2014). This suggests that there might be important transgenerational effects on otolith development in other species. While some species, such as the spiny damsel, do not show effects of short-term exposure to elevated CO_2 on otolith development (Munday et al. 2011a), effects could become apparent when several generations are exposed to high CO_2. It is therefore essential to understand potential parental effects of changed environmental conditions on otolith growth and development.

This study tested for transgenerational effects of elevated CO_2 on otolith development in the spiny damselfish, *Acanthochromis polyacanthus*. I used a fully-crossed factorial design where parents and their offspring were reared in control seawater (446 μatm) or acidified seawater matching CO_2 predictions for the end of the century (RCP6: moderate CO_2, 652 μatm, or RCP8.5: high CO_2, 912 μatm) (Meinshausen et al. 2011). Sagittal otoliths were extracted from juveniles reared in all nine combinations of juvenile and parental CO_2 to examine the within and between generation effects of elevated CO_2 on otolith growth. Comparison of otoliths from juvenile fish reared in control and high CO_2 conditions, from parents that had been exposed to control or high CO_2 conditions, allowed me to determine if parental exposure to high CO_2 could mitigate or exacerbate any changes in otolith morphology caused by high CO_2 exposure in juvenile fish. Specifically, I compared otolith area, perimeter, maximum length and circularity among juveniles reared in the nine different treatments.

4.3 Methods

Study species

Wild caught pairs of *A. polyacanthus* from the Orpheus Island region (18.62 °S, 146.49 °E) were housed at James Cook University from September 2012 to May 2013. Breeding pairs were kept in 40 L aquaria where they were given half a terracotta pot as a breeding structure and shelter. Pairs were divided between control, moderate CO_2 and high CO_2 treatments for the breeding season (November- May). CO_2 levels were set at introduction of fish to the systems and remained stable for the acclimation period and duration of the experiment. Temperatures and photoperiod for each system began at winter levels (22.5 °C; 11L:13D) when pairs were introduced in September, and were slowly raised to summer levels (28.5 °C; 13L:11D) over a two-month acclimation period until the start of the breeding season in November. Summer temperatures and photoperiod were maintained for the duration of the experiment.

CO_2 manipulation

Three 8000 L recirculating aquarium systems were used, each set to a different pH and corresponding CO_2 level: a current-day control (446 μatm), moderate CO_2 (656 μatm) and high CO_2 (912 μatm). CO_2 was dosed into a 3000 L sump in each elevated CO_2 system using an Aqua Medic AT Control System (Aqua Medic, Germany), which maintained the desired pH level in each system. Control water was also circulated through a separate 3000 L sump. Equilibrated seawater was then delivered to the holding aquaria at a rate of 1.5 L min^{-1}. Daily pH_{NBS} (pH calibrated to National Bureau of Standards) and temperature measurements were taken using a pH electrode (SevenGo Pro, Mettler Toledo, Switzerland) and temperature probe (Comark C26, Norfolk, UK). Weekly salinity readings were measured using a conductivity sensor (HQ15d; Hach, Loveland, CO, USA). Total alkalinity was measured weekly using Gran Titration (Metrohm 888 Titrando Titrator Metrohm AG, Switzerland) and certified reference material from Dr. A. G. Dickson (Scripps Institution of Oceanography). Carbonate chemistry parameters were calculated using CO2SYS (Pierrot et al. 2006) with the constants K1 from Mehrbach et al. (1973) refit by Dickson and Millero (1987) and Dickson for KHSO4 (Table 4.1). Water chemistry was measured directly in randomly selected aquaria on the relevant timescales.

Table 4.1. Mean (± SD) seawater parameters in the experimental system for adults and juveniles during the breeding season. Temperature, pH, salinity and total alkalinity (TA) were measured directly. pCO$_2$ was estimated from these parameters using CO2SYS. Seawater parameters were consistent for breeding and experimental components of the study.

Treatment	**pH$_{NBS}$**	**Temperature (ºC)**	**Salinity**	**TA (µmol.kg^{-1}SW)**	**pCO$_2$ (µatm)**
Control	8.13 (±0.03)	28.5 (±0.1)	35.6 (±0.9)	2199 (±93)	446 (±40)
Moderate CO$_2$	7.98 (±0.06)	28.5 (±0.1)	35.6 (±0.9)	2125 (±76)	652 (±77)
High CO$_2$	7.85 (±0.03)	28.5 (±0.1)	35.9 (±0.7)	2139 (±113)	912 (±74)

Experimental design

Breeding pairs were checked between 0700 and 0800 h each day for the presence of eggs. Within 12 hours of hatching, larvae were removed from the parent tank and divided equally into 40 L aquaria of control, moderate CO$_2$ and high CO$_2$ treatments. Offspring from control parents allow for examination of developmental effects of elevated CO$_2$, while comparisons with offspring from parents under the elevated CO$_2$ treatments allow for examination of potential transgenerational onset or mitigation of CO$_2$ impacts. Comparisons of all control-reared offspring enabled assessment of carry-over effects of parental CO$_2$ on offspring.

Adults were fed 0.1 g of commercial fish feed pellet (INVE Aquaculture Nutrition NRD 12/20) three times daily during the breeding season (Donelson et al. 2010). Newly hatched larvae were fed live *Artemia nauplii* at 2 nauplii mL^{-1} for one week and then transitioned to INVE Aquaculture Nutrition NRD 2/4 larval pellets for the remainder of the experiment.

Juveniles were reared in their respective treatments for 40-45 days at which point they were sacrificed by anaesthetization with clove oil (Munday and Wilson 1997; JCU Animal Ethics A1828). 20-25 juveniles from each clutch in each treatment were stored in 70 % ethanol.

Otolith analysis

Three breeding pairs in each treatment were randomly selected for otolith measurements from their offspring. Only the first clutch from each breeding pair was selected for otolith analysis. Standard length was measured and otoliths were extracted from a total of 9 juveniles (3 individuals from each treatment) from each family, for a grand total of 81 juveniles. Randomly selected juveniles were removed from the preservative, blotted dry and photographed for length measurements. Standard length was estimated (to the nearest 0.01 mm) using ImageJ analysis. Sagittal otoliths were then removed and stored dry in well plates. The right otolith was selected for analysis and was photographed under a compound microscope (Yamamota and Reinhardt 2003). A calibrated grey-scale image was created in FIJI (Schindelin et al. 2012) and used for morphometric measurements (area, mm^2; perimeter, mm; maximum length, mm; and circularity). Circularity was defined as the square of the otolith perimeter divided by the otolith area.

Statistical analysis

Fish standard length (SL) and otolith morphometric traits were compared among the 9 treatment combinations (3 parent CO_2 treatments x 3 juveniles CO_2 treatments). First, a linear mixed effects (LME) model was used to analyse juvenile SL, with parent CO_2 treatment and juvenile CO_2 treatment as fixed effects in the model, and family as a random effect. Residual analysis was used to examine the assumptions of normality and homogeneity of variance. The model fit was quantified using the Akaike information criterion (AIC).

Because otolith attributes are non-independent, a principal component analysis (PCA) on the correlation matrix was first used to generate an overall measure of otolith shape. The first and second principal components (PC1 and PC2) were then used as composite variables and examined in response to parental and juvenile CO_2 regimes as above using LME models. To determine in greater detail the nature of the effects found by the analysis of the principal components, separate LME analyses were run on the individual otolith variables, with parent CO_2 treatment, juvenile CO_2 treatment and juvenile SL as fixed effects in the model, and family as a random effect. AIC and residual analysis were used to examine model fit and assumptions of analyses.

4.4 Results

Juvenile SL was not affected by CO_2 treatment. While juveniles from moderate CO_2 parents appeared smaller on average than those from control parents, this difference was not significant (Figure 4.1). There was also a trend for greater SL in high CO_2 juveniles from high CO_2 parents, but again this was not significant. However, juvenile SL did influence otolith area, perimeter, maximum otolith length and circularity (all $p < 0.0001$), and was statistically controlled for in each LME model.

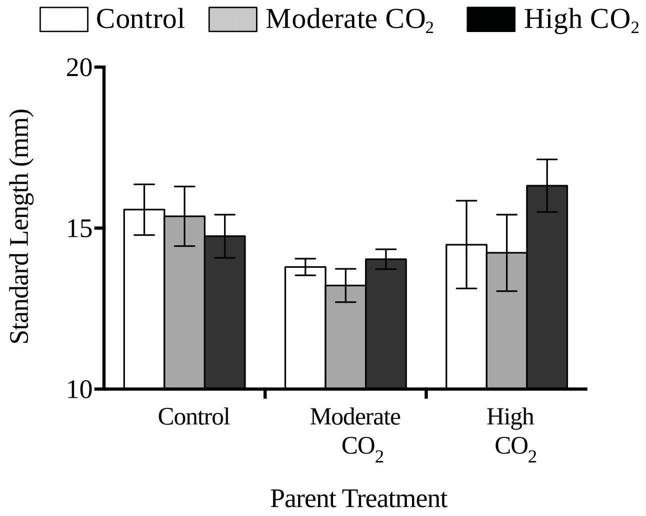

Figure 4.1. Juvenile *Acanthochromis polyacanthus* mean standard length (± SE) from parents in control (446 µatm), moderate CO_2 (652 µatm) or high CO_2 (912 µatm), and reared for 6 weeks under control, moderate CO_2 or high CO_2 conditions. * denotes a significant difference from control offspring hatched from control parents.

The first two components of the PCA explained > 99 % of the variance in otolith morphology (Table 4.2a). PC1 was associated with otolith area, perimeter and length and accounted for approximately 85.78 % of the total variance in otolith morphology. PC2 was associated with otolith circularity and accounted for another 13.80 % variance in otolith morphology. There were significant effects of CO_2 treatment ($F_{8,63} = 23.93$, $p < 0.0001$) and juvenile SL ($F_{1,63} = 678.24$, $p < 0.0001$) on PC1 (Table 4.2b). PC2 only showed a significant effect of CO_2 treatment for control juveniles from high CO_2 parents compared to control juveniles from control parents ($p < 0.05$, Table 4.2c).

Table 4.2. Principle component analysis (PCA) results for otolith morphology (a) and linear mixed effects model (LME) results on principle component (PC) 1 (b) and PC2 (c). Bold values represent a significant difference from present day control offspring (C-C).

(a) Principle components (number of observations: 81)

	Comp. 1	Comp. 2	Comp. 3	Comp. 4
Importance of components:				
Standard deviation	1.852	0.743	0.125	0.033
Proportion of Variance	0.858	0.138	0.004	0.000
Cumulative Proportion	0.858	0.996	0.999	1.000
Loadings:				
Area (mm^2)	0.526	-0.287	-0.576	0.556
Perimeter (mm)	0.536	-0.253	-0.209	-0.803
Otolith max. length (mm)	0.528	-0.245	0.790	0.193
Circularity	-0.397	-0.913		

(b) Linear mixed effects model results for PC1

Treatment	df	t-value	*p*
C-M	63	2.644	**0.010**
C-H	63	0.361	0.719
M-C	63	-0.185	0.854
M-M	63	0.184	0.854
M-H	63	-0.500	0.619
H-C	63	-1.142	0.258
H-M	63	0.354	0.725
H-H	63	1.974	0.053

(c) Linear mixed effects model results for PC2

Treatment	df	t-value	*p*
C-M	63	0.114	0.910
C-H	63	-0.662	0.510
M-C	63	-0.789	0.433
M-M	63	-0.072	0.943
M-H	63	-1.135	0.261
H-C	63	-2.191	**0.032**
H-M	63	-1.084	0.261
H-H	63	-0.360	0.720

Otolith morphology in some moderate and high CO_2 treatments differed from otoliths of fish reared in control conditions (Table 4.3, Figure 4.2). Offspring from control parents that were reared in moderate CO_2 for 6 weeks had significantly larger otolith area and perimeter than their siblings reared in control conditions ($p < 0.01$ for both traits). However, when parents were held under moderate CO_2, otolith area and perimeter for juveniles reared in moderate CO_2 conditions were not different to present-day control juveniles. Juveniles reared in moderate CO_2 from control parents also had significantly longer otoliths than their siblings in control ($p < 0.01$), but there was no effect of CO_2 on maximum otolith length when parents were also under elevated CO_2 (Table 4.3).

Circularity was significantly greater in control juveniles from high CO_2 parents than from control parents ($p < 0.05$). However, the trends for greater circularity in juveniles from moderate CO_2 parents were not significantly different from present-day control juveniles (Table 4.3).

There was a transgenerational effect seen in high CO_2 juveniles from high CO_2 parents, where otolith area and perimeter were again significantly larger compared to present-day control ($p < 0.01$ for both traits). There was a trend for smaller otolith area in juveniles from moderate CO_2 parents, but this was not significant (Table 4.3). Variation around the mean for juvenile SL and otolith morphometrics (area, perimeter, maximum and circularity) was usually highest in offspring from high CO_2 parents (Figure 4.2). This suggests that juveniles, and consequently their otoliths, were less consistent in size than those individuals from control or moderate CO_2 parents.

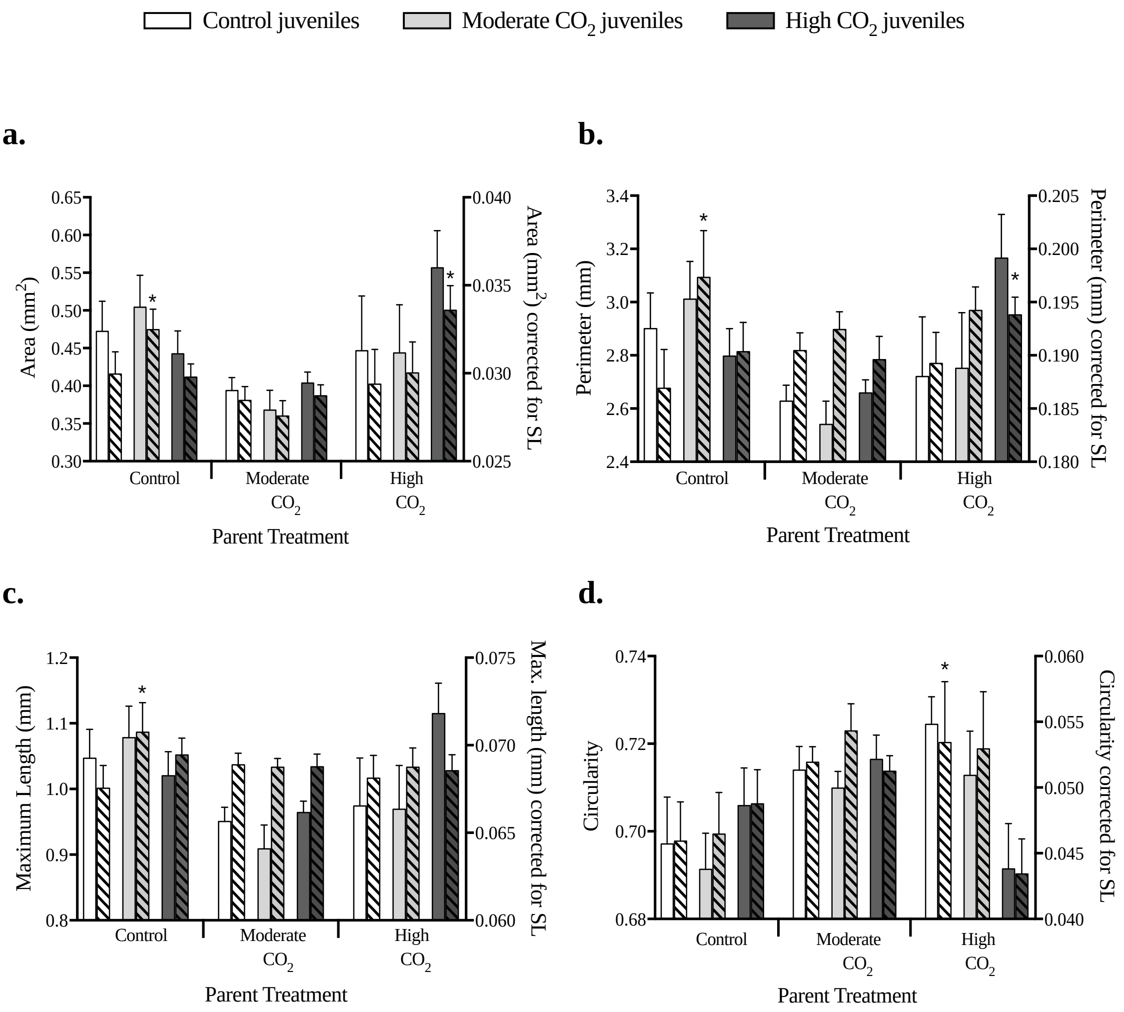

Figure 4.2. Mean (± SE) (a) area, (b) perimeter, (c) maximum length and (d) circularity of otoliths from juvenile *Acanthochromis polyacanthus*. Solid bars are raw means, and crossed bars represent standardized means from LME that are controlled for juvenile standard length as a fixed effect in the model. Juveniles came from parents in control (446 μatm), moderate CO_2 (652 μatm) or high CO_2 (912 μatm), and were reared for 6 weeks under control, moderate CO_2 or high CO_2 conditions. * denotes a significant difference from control offspring hatched from control parents.

Table 4.3. Results for LME on otolith morphometrics for juvenile *Acanthochromis polyacanthus*. Juveniles were hatched from parents held in control (C), moderate CO_2 (M) or high CO_2 (H) (first letter in treatment code), and were then reared in C, M or H (second letter) for 6 weeks. The models account for juvenile standard length. Bold values represent a significant difference from present day control offspring (C-C).

Treatment	df	t-value	*p*
(a) Otolith area			
C-M	63	3.047	**0.003**
C-H	63	0.735	0.465
M-C	63	0.425	0.672
M-M	63	0.499	0.620
M-H	63	0.325	0.746
H-C	63	1.477	0.145
H-M	63	1.998	0.050
H-H	63	2.688	**0.009**
(b) Otolith perimeter			
C-M	63	3.042	**0.003**
C-H	63	0.715	0.477
M-C	63	0.506	0.615
M-M	63	0.642	0.523
M-H	63	0.294	0.770
H-C	63	0.004	0.997
H-M	63	1.545	0.127
H-H	63	2.947	**0.005**
(c) Otolith maximum length			
C-M	63	2.807	**0.007**
C-H	63	1.206	0.232
M-C	63	0.036	0.972
M-M	63	-0.639	0.525
M-H	63	0.079	0.937
H-C	63	-0.884	0.380
H-M	63	-0.295	0.769
H-H	63	1.818	0.074
(d) Otolith circularity			
C-M	63	-0.743	0.460
C-H	63	0.473	0.638
M-C	63	0.709	0.481
M-M	63	0.024	0.981
M-H	63	1.072	0.288
H-C	63	2.089	**0.041**
H-M	63	0.822	0.414
H-H	63	-0.166	0.869

4.5 Discussion

This study found that transgenerational exposure to high CO_2 (912 μatm) had marked effects on otolith growth for juvenile *A. polyacanthus*, with increases seen in otolith area and perimeter. Interestingly, when juveniles were exposed to moderate CO_2 (652 μatm) for 6 weeks, otoliths also had larger areas, perimeters and maximum lengths compared to controls; however, these effects were absent when parents were also exposed to moderate CO_2. These results indicate that otolith development will acclimate to moderate CO_2 levels over generations. However a different transgenerational effect occurred in the high CO_2 treatment, where an effect on otolith area and perimeter was only present when both parents and offspring were both reared at high CO_2. This result is similar to the only other study to examine transgenerational effects of elevated CO_2 on otolith development, where juveniles raised in high CO_2 had larger otoliths when their parents were acclimated to high CO_2 (Schade et al. 2014). These results show that short-term and single generation experiments risk underestimating the effects of high CO_2 on otolith development in marine fishes, which may only become apparent when fishes are exposed to high CO_2 over more than one generation.

Otoliths are the most susceptible calcified structure in fish because of their aragonite composition. The long-term exposure to CO_2 coupled with full reproductive processes in elevated CO_2 demonstrates that aragonite accumulation for otolith formation is in fact altered for the spiny damsel when multiple generations experience ocean acidification. Otoliths are critical for hearing and balance (Popper et al. 2005), and these processes may be affected by changes in otolith morphology. Otolith changes could also alter swimming behaviour, for example, cobia had larger otoliths and exhibited some changes in swimming activity at high CO_2 levels (Bignami et al. 2013a). However, further research is needed to determine the mechanistic relationship between otolith changes and fish behaviour. Importantly, the changes in otolith size and shape in high CO_2 observed in this study are of similar magnitude to the natural variation in these traits among individuals (Campana 1990; Munday et al. 2004). Future studies need to assess the implications of changes to otolith sizes on fish behaviour, and must consider transgenerational exposure to elevated CO_2 as it could differ substantially from the effects seen in short-term exposure.

Moderate CO_2 exposure (652 μatm) generated increased otolith growth for *A. polyacanthus* juveniles whose parents did not experience these CO_2 levels, yet their siblings exposed to high CO_2 showed no changes in otolith development. Importantly, the increases seen in these moderate CO_2 juveniles were lost when parents were exposed to the same, or higher, level of CO_2. This shows that parents are able to ameliorate these effects under this conservative end-of-century CO_2 level. However, exposure to high CO_2 (912 μatm) negates this tolerance, with significant effects on otolith development present when both parents and offspring were reared under high CO_2 conditions. These results are important because they suggest that acclimation potential for this species are restricted to low end-of-century CO_2 projections, and that significant effects on otolith development could occur if CO_2 emissions are not curbed and they reach higher levels projected under a business as usual scenario (RCP8.5).

While otolith characteristics are sensitive to environmental change (Gagliano and McCormick 2004), they are also highly dependent on fish growth (Jones and Hynes 1950). The increasing trends in the otolith size variables closely mirrored the trends in fish size, though SL was not significant across treatments. However, juvenile SL was accounted for in LME models on otolith morphometrics. Otolith morphometrics were not affected by high CO_2 in juveniles hatched from control or moderate CO_2 parents, suggesting tolerance to high CO_2 treatment when there is no family history of high CO_2 exposure. Additionally, juveniles that were reared in control or moderate CO_2 and came from high CO_2 parents showed no changes to otolith area, perimeter, or maximum otolith length. These results therefore suggest that the effects of high CO_2 on otolith growth are due to an interactive effect that comes from both parents and juveniles experiencing a high CO_2 environment.

Previous studies examining effects of similar CO_2 levels on otolith size have found various results, with two studies showing increased otolith size in sea bass (993 μatm) and cobia (800 μatm) (Checkley et al. 2009; Bignami et al. 2013a), and other studies finding no effect in the spiny damsel (850 μatm) (Munday et al. 2011a) and clownfish (900 μatm and 1050 μatm) (Simpson et al. 2011; Munday et al. 2011b). However, these studies involved short-term exposure to CO_2 and did not examine potential long-term effects under the changed environmental conditions. Contrary to Munday et al. (2011a), otolith enlargement was seen after 6 weeks of exposure to moderate CO_2 in *A. polyacanthus* when parents were not exposed to CO_2. However, 6-

week exposure to high CO_2 did not affect otolith development if parents were in a lower CO_2 level than their offspring, similar to *A. polyacanthus* tolerance following 3 weeks of exposure to elevated CO_2 in Munday et al. (2011a). When both parents and offspring were reared in high CO_2, tolerance in otolith growth was obscured. The presence of transgenerational acclimation at moderate CO_2, but not at high CO_2 for *A. polyacanthus*, may indicate that lower levels of elevated CO_2 may be less of a shock to the individuals when they have experienced control conditions for most of their life in the wild. This ‘shock effect’ at high CO_2 is not lessened after one generation, but is ameliorated if juveniles from these high CO_2 adults experience lower levels of CO_2 (present-day control or moderate CO_2). Otolith size was not affected for control or moderate CO_2 juveniles from high CO_2 parents, suggesting that parental high CO_2 exposure does not completely diminish tolerance in otolith development. It is possible that tolerance may be restored after multiple generations experience high CO_2 where any ‘shock effect’ is lessened with each consecutive generation. Further studies will need to verify this hypothesis.

Previous studies have shown that high CO_2 affects auditory preferences in larval fishes (Simpson et al. 2011; Rossi et al. 2015, 2016). However, this change does not appear to be related to any effect on otolith development and instead is related to the effects of high CO_2 on $GABA_A$ neurotransmitter receptors. Simpson et al. (2011) found that larval clownfish reared in moderate and high CO_2 exhibited no avoidance of predator-rich reef sounds, compared to strong avoidance of these sounds by control individuals. There were no changes to otolith growth in the CO_2 levels responsible for these auditory changes. Additionally, preference for acoustic habitat cues was reversed when juvenile barramundi and mulloway were reared in high CO_2 (Rossi et al. 2015; Rossi et al. 2016). Mulloway otoliths were larger in CO_2 juveniles compared to control (Rossi et al. 2016). However, reversal of auditory preferences was consistent for CO_2 juveniles in both control and CO_2 habitats, demonstrating that larger otoliths were not able to ameliorate reversed auditory preferences in high CO_2. GABA is associated with auditory processing frequencies and binaural hearing (Feng and Schellart 1999), and thus impairment of the $GABA_A$ receptor at high CO_2 may be a primary mechanism in altered auditory behaviours.

Greater variation in juvenile SL and otolith morphometrics was seen for juveniles from high CO_2 parents, regardless of juvenile rearing treatments. This suggests that there are high levels of variability in individual sensitivity to CO_2. The larger variation in otolith traits in fish exposed to high CO_2 could promote adaptation of otolith development, potentially eliminating changes in otolith size over time. Further studies to estimate both the heritability in otolith size variation in high CO_2, and the possible selective advantage in maintaining current-day sized otoliths, would be required before the potential for adaptation of otolith size in a high CO_2 environment could be assessed.

This study investigated the effects of elevated CO_2 on otolith development; however, it will be important to consider the possible effects of other stressors that may correlate with lower end-of-century CO_2 predictions in future studies. In particular, the early life history stages of reef fishes may be more susceptible to temperature changes (Munday et al. 2008; Donelson et al. 2010) than to ocean acidification (Munday et al. 2011a), suggesting an earlier onset of altered otolith development when combined with higher temperature. Additionally, there are species differences in behavioural responses (Ferrari et al. 2011a) and in otolith development (Heuer and Grosell 2014) when individuals are acutely exposed to increased CO_2, which will likely contribute to differing effects of CO_2 seen across generations. Further studies are also needed to better understand the mechanistic link between individual performance and otolith size and shape in order predict the consequences of changes in otolith development caused by higher CO_2 levels. This study demonstrated that it will be important to examine this link through multi-generation studies, which best predict the potential future effects of high CO_2 and ocean acidification on fish populations.

Chapter 5: Heritability of behavioural tolerance to high CO_2 in a coral reef fish is masked by non-adaptive phenotypic plasticity

This chapter is prepared for submission to Global Change Biology

Authors: Megan J. Welch and Philip L. Munday

5.1 Summary

Fish behaviour is adversely affected at CO_2 levels predicted for the end of the century. Previous studies have demonstrated limited potential for acclimation for olfactory behaviours in reef fishes under elevated CO_2, indicating that genetic adaptation will be required to maintain behavioural performance in the future. Adaptation depends on the presence of heritable phenotypic variation in the trait, which may differ between populations and environments. Previous studies have shown that some individuals exhibit greater behavioural tolerance to high CO_2 than others, but whether this behavioural tolerance is heritable is unknown. I used parent-offspring regressions to estimate the heritability (h^2) of behavioural tolerance to high CO_2 (754 μatm) in both field and laboratory-reared populations of *Acanthochromis polyacanthus*. The field population of *A. polyacanthus* exhibited high heritability of olfactory behaviour phenotype (father-midoffspring $h^2 = 0.56$) when offspring were acutely exposed to high CO_2 for 4-5 days. The laboratory population exhibited similarly high heritability of olfactory behaviour phenotype ($h^2 = 0.65$) when offspring were acutely exposed to high CO_2. However, this heritability was completely lost when juveniles where chronically exposed to high CO_2 for 6 weeks. Parental exposure to high CO_2 did not alter this relationship between acute and chronic CO_2 treatments; heritability of behavioural phenotype was high where offspring were acutely exposed to high CO_2, but lost when offspring were chronically exposed to high CO_2, regardless of parent treatment (control or high CO_2). The loss of heritability occurred because juveniles that were relatively

tolerant to high CO_2 in the acute treatment lost this tolerance in the chronic CO_2 treatment. This indicates that genetic variation in behavioural tolerance to high CO_2 is obscured by non-adaptive plasticity when offspring are chronically exposed to high CO_2. These results demonstrate that behavioural tolerance to high CO_2 is heritable, but adaptive potential may be constrained by non-adaptive plasticity when a high CO_2 environment is permanently experienced, as will occur due to rising CO_2 levels in the ocean.

5.2 Introduction

Ocean acidification, caused by the uptake of additional CO_2 from the atmosphere (Caldeira and Wickett 2003), will impact many marine species and have far-reaching effects on the ecosystems they inhabit (Gattuso and Hansson 2011). However, some species might be able to adapt to the projected changes in ocean chemistry, which could reduce the impacts on populations and communities (Sunday et al. 2014; Gaylord et al. 2015). Short-term experiments have demonstrated negative effects of future ocean acidification on a wide-range of marine species and ecological processes (Doney et al. 2009; Kroeker et al. 2013), yet few studies have attempted to assess the potential for adaptation. Nevertheless, the limited number of studies conducted to date illustrate that some species exhibit heritable phenotypic variation in response to ocean acidification (Kelly et al. 2013; Malvezzi et al. 2015) and that selection of CO_2 tolerant genotypes can occur (Pespeni et al. 2013; Malvezzi et al. 2015). In some other species, however, there appears to be limited heritable variation of phenotypic traits at high CO_2 (Sunday et al. 2011), or there are genetic correlations with other environmental stressors that could limit adaptive potential (Foo et al. 2014). Assessing the potential for adaptation in a broader range of marine taxa and phenotypic traits affected by ocean acidification is therefore a priority.

Recent studies show that CO_2 levels predicted for the end of the century can have adverse effects on the behaviour of marine fishes (Briffa et al. 2012; Clements and Hunt 2014; Heuer and Grosell 2014) with consequences for key ecological processes such as larval dispersal, habitat selection, competition and predator-prey interactions (Nagelkerken and Munday 2016). Many reef fishes innately recognize ecologically relevant olfactory cues, such as predator odour and chemical alarm cues from injured conspecifics, and they use these cues to avoid danger (Holmes and McCormick 2010; Dixson et al. 2012). Under elevated CO_2, however, reef fish lose the ability to appropriately interpret these cues (e.g. Dixson et al. 2010; Ferrari et al. 2011a, 2012; Nilsson et al. 2012; Chivers et al. 2014). Altered responses to predation threats may have significant population-level effects due to increases in juvenile mortality, which can affect population replenishment (Munday et al. 2010; Ferrari et al. 2011a,b; Chivers et al. 2014). Altered olfactory responses persist for weeks to months in elevated CO_2 conditions (Munday et al. 2013, 2014a), and transgenerational experiments further demonstrate that impaired behaviours are not ameliorated when parents are held under the same elevated CO_2 levels as their offspring (Welch et al. 2014). Nevertheless, previous studies have observed individual variation in olfactory tolerance to elevated CO_2 (Welch et al. 2014), especially at near-future CO_2 levels around 700 μatm (Munday et al. 2010; Ferrari et al. 2011a). Furthermore, selection for CO_2-tolerant behavioural phenotypes has been observed in field-based experiments (Munday et al. 2012). This phenotypic variation and natural selection could be key for future adaptation; however, it is unknown if variation in behavioural tolerance to high CO_2 is heritable in coral reef fishes.

A common observation from evolutionary studies is that heritability is not constant and can vary with environmental conditions (Hoffmann and Merilä 1999). This potentially complicates attempts to assess adaptive potential to climate change and ocean acidification because adaption could be less likely under some environmental conditions than others. Many different hypotheses have been proposed to explain why heritability can vary among environments (reviewed by Hoffmann and Merilä 1999); however, one hypothesis relating to assessing adaptive potential to rapid environmental change is that phenotypic variation may be either increased or decreased by environmental stress (Hoffmann and Hercus 2000). Broad- and narrow-sense heritability have been observed to decline in unfavourable conditions in a variety of animals (Charmantier and Garant 2005; Wilson et al. 2006), suggesting that heritability may also be lower in populations exposed to chronic stress from climate change. On the other hand, heritability may also increase when a stressful environment is encountered (Hoffmann and Parsons 1991; Hoffmann and Hercus 2000). Heritability is likely to be higher in a stressful (or heterogeneous) environment if it leads to greater expression of phenotypic variation compared with less stressful (or homogenous) environments.

Therefore, estimates of heritability may differ in populations exposed to an acute environmental change compared with chronic environmental stress. The effects of acute versus chronic environmental stress are especially relevant to experiments testing adaptive potential to ocean acidification because such experiments often expose juveniles or adults to high CO_2 for just a few days or weeks (i.e. acutely), whereas ocean acidification will result in the permanent (i.e. chronic) exposure to high CO_2 throughout life.

An additional complication of assessing the adaptive potential to high CO_2 is that parental exposure to environmental stress can alter offspring phenotypes independently of genetic variation (Guillaume et al. 2016). Parents can influence the phenotype of their offspring through a range of non-genetic mechanisms that involve the transmission of nutrients, hormones, somatic factors or epigenetic marks (Bonduriansky and Day 2009). Commonly, mothers influence the phenotype of their offspring in different environments through changes in the provisioning of eggs, embryos and juveniles (maternal effects) (Mousseau and Fox 1998; Marshall 2008; Crean and Marshall 2009). There is also increasing evidence that the environmental conditions experienced in one generation can influence future generations through the inheritance of different epigenetic states (epigenetic inheritance) (Jablonka and Lamb 1995; Bonduriansky et al. 2012; Holeski et al. 2012). Consequently, the environment experienced by parents can influence the expression of phenotypic variation in their offspring through a range of non-genetic mechanisms (Day and Bonduriansky 2011; Bonduriansky 2012). Recent studies have shown that parental exposure to high CO_2 can ameliorate the negative effects of high CO_2 on growth and survival of juvenile fish (Miller et al. 2012; Murray et al. 2014) and can also influence the kinematic responses of juveniles to a perceived threat (Allan et al. 2014). Similar beneficial parental effects have not been observed in the average response of juvenile fish to olfactory cues in a high CO_2 environment (Welch et al. 2014). Nevertheless, parental effects could potentially influence the variation in behavioural responses to high CO_2 exhibited by offspring, and thus the heritability of CO_2 tolerance.

There are significant impediments to testing heritability of phenotypic traits in coral reef fishes because: 1) the life cycle of most reef fish species cannot be completed in captivity due to difficulties rearing the small pelagic larva, and 2) those species that can be readily reared in captivity are demersal spawners with paternal egg care, making it practically impossible to cross-fertilize eggs and sperm in a diallel breeding design (Rummer and Munday 2016). The spiny damselfish, *Acanthochromis polyacanthus*, is a monogamous species of reef fish that broods each clutch of offspring for several months after hatching (Kavanagh 2000). Furthermore, this species can be reared with high success in the laboratory. Thus, it is ideally suited to estimating heritability with parent-offspring regressions (Falconer and Mackay 1996; Lynch and Walsh 1998), which is the approach used here.

This study tested for heritability of variation in behavioural responses to chemical alarm cues (CAC) under elevated CO_2 conditions in both wild and laboratory populations of *A. polyacanthus*. Further, it tested for potential differences in heritability associated with acute and chronic exposure to high CO_2 in offspring, and due to high CO_2 exposure in the parental generation. First, I used parent-offspring regressions to estimate broad-sense heritability of behavioural responses to CAC in families of fish collected directly from the reef and exposed to either control (451 μatm) or high CO_2 (754 μatm) conditions for 4-5 days (acute exposure for juveniles and adults, respectively). Behaviour was measured following 4 day exposure to high CO_2, as previous studies have demonstrated that this is sufficient time to induce the full range of behavioural effects of high CO_2 in reef fishes and that longer exposures do not change the mean effects observed (Munday et al. 2010, 2013, 2014a). The sex of wild caught parents was determined after behavioural testing in order to estimate heritability using father-midoffspring regressions and to explore maternal effects on heritability by comparing father-, mother- and midparent-offspring regressions. Following field tests, I formed breeding pairs of *A. polyacanthus* in the laboratory using wild caught adults, and distributed these pairs across control (414 μatm) and high CO_2 (754 μatm) treatments for breeding (Figure 5.1). Clutches of offspring from control and high CO_2 parents were split at hatching and reared under both control or high CO_2 for 6 weeks before undergoing behaviour testing in their respective rearing conditions. As with the wild caught families, heritability of the behavioural response was estimated using father-midoffspring regressions. The laboratory component enabled me to test for heritability of behavioural tolerance to high CO_2 following chronic exposure to high CO_2 from hatching. An additional acute CO_2 treatment was also tested for the laboratory-reared offspring for direct comparison with the chronic CO_2 treatment in the laboratory and with the acute CO_2 treatment in field-caught juveniles. The factorial breeding design in the laboratory further allowed for examination of any effects of parental conditioning to elevated CO_2 on the heritability of behavioural responses to CAC in offspring exposed both acutely and chronically to high CO_2 conditions. Table 5.1 provides a glossary of key heritability terminology.

Table 5.1. Glossary of heritability terms.

Term	**Definition**
Father-midoffspring regression	Regression of the midoffspring phenotypes on the father phenotype for a given family.
Midparent-midoffspring regression	Regression of the midoffspring phenotype on the midparent phenotype for a given family.
Midparent	The average behavioural responses of the mother and father.
Midoffspring	The average behavioural response for a treatment group of related offspring.
Mother-midoffspring regression	Regression of the midoffspring phenotype on the mother phenotype for a given family.
Parent-offspring regression	An estimate of the phenotypic covariance between offspring and parents.

5.3 Methods

Studies species

The spiny chromis, *A. polyacanthus*, are found on reefs throughout the Indo-Australian region. They form long-term monogamous pairs and lay demersal eggs in small caves in the reef (Robertson 1973; Thresher 1983). Pairs typically produce 1-2 clutches of juveniles in a breeding season (Nakazono 1993; Pankhurst et al. 1999). Egg clutches, varying in size from 100 (Robertson 1973) to 550 eggs (Kavanagh 2000), are laid in a single event and cared for by both parents (Robertson 1973; Thresher 1983; Nakazono 1993). Eggs hatch into small, well-developed juveniles that remain with the parents for several months after hatching (Kavanagh 2000).

Experimental Design

Field Collected Fish Experiments: Field data was collected at Lizard Island Research Station (LIRS) on the northern Great Barrier Reef (14°40’ S, 145°28’ E). Twenty adult pairs of *A. polyacanthus* that were brooding offspring were collected from the Lizard Island lagoon during November 2014. Both parents were first collected by placing a barrier net around the nest site, and the offspring were then collected using hand nets. Families were brought into the laboratory where they were housed in 32 L (380 L x 280 W x 300 H mm) aquaria. Parents were separated from their offspring and placed in the high CO_2 treatment (RCP6: 754 µatm). Each clutch of offspring was divided equally so that half were placed in control conditions (452 µatm) and half in high CO_2 treatment (754 µatm). Juveniles were maintained under control and CO_2 conditions for 4 days, and adult pairs were maintained in high CO_2 for 5 days. Temperatures and photoperiod were maintained at summer conditions (27.5 °C; 13L:11D). Adult breeding pairs were fed 0.15 g of commercial fish feed pellet (INVE Aquaculture Nutrition NRD 12/20) twice a day. Juvenile groups were fed 0.05 g of commercial fish feed pellet (INVE Aquaculture Nutrition NRD 5/8) twice a day. Fish were not fed on the morning of their behaviour tests. After the treatment period, all fish were tested for behavioural responses to CAC in a two-channel flume, as described below. Families were returned to their collection sites after testing.

Olfactory responses to CAC in high CO_2 were tested for both parents in the wild caught breeding pairs (N = 20). Behaviour was tested for 20 offspring (10 in control and 10 in high CO_2) from each of these breeding pairs, for a total of 400 wild-caught juveniles.

Laboratory Reared Fish Experiments: Adult *A. polyacanthus* were collected from the northern Great Barrier Reef, Australia and transported to the experimental aquarium facility at James Cook University. Adults were held under high CO_2 (754 µatm) for 7 days, after which they were tested for their response to CAC in a two-channel flume. Adult sensitivity to high CO_2 was categorized by their response in the flume. Individuals that spent ≤ 30% time in the cue were considered to be “tolerant” to high CO_2, whereas individuals that spent ≥ 50% time in the cue were considered “non-tolerant”. Adults were further categorized by size and sex. Breeding pairs were then constructed by pairing individuals of either similar or different behavioural sensitivity:

tolerant male + tolerant female, tolerant male + non-tolerant female, non-tolerant male + tolerant female, non-tolerant male + non-tolerant female (Figure 5.1). This pairing was designed to provide the greatest possible range in parental sensitivities to high CO_2. Assortative pairing can inflate estimates of heritability compared with random pairing (Falconer and Mackay 1996); however, my aim was to generate a maximum possible estimate of heritability (Reeve 1961; Hill 1970). Adult pairs were maintained in 40 L aquaria with control (414 µatm) or high CO_2 (754 µatm) conditions for three months prior to the start of the breeding season. Temperatures and laboratory photoperiod were slowly adjusted during the acclimatization period to reach summer conditions (28.5 °C; 13L:11D) at the start of the breeding season. Pairs were checked daily for the presence of egg clutches once the breeding season commenced. On hatching, clutches were immediately divided and transferred to control and high CO_2 conditions (Figure 5.1). Offspring were reared under the two treatments for 6 weeks, at which point they underwent the same olfactory behavioural testing as their parents. Some offspring from control were moved to high CO_2 (754 µatm) four days before the 6-week testing to create an acute CO_2 treatment group.

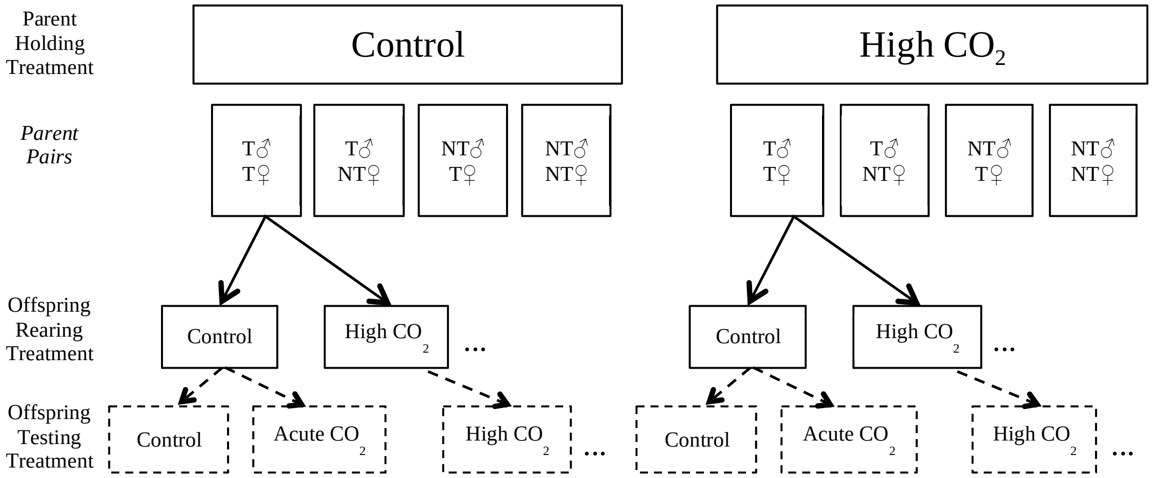

Figure 5.1. Adult breeding and juvenile rearing treatments for laboratory experiments. Parent pairs were constructed based on response to chemical alarm cues, where T = tolerant and NT = non-tolerant. Pair formations are seen in "*Parent Pairs*" boxes and held under their respective treatments, control (414 µatm) and high CO_2 (754 µatm). After hatching, offspring from all parent pairs were divided between control and high

CO_2 conditions. Acute CO_2 refers to offspring reared in control conditions and then held in high CO_2 (754 μatm) 4 days prior to behaviour testing.

A total of 60 breeding pairs were formed for the laboratory experiment, 30 held in control conditions and 30 in high CO_2. Twenty pairs bred in control and 18 pairs bred under high CO_2. Only fish from the first clutch from each breeding pair were used in the experiment. Behavioural response to CAC was tested in 60 juveniles from each clutch (20 in control, 20 in chronic high CO_2 and 20 in acute high CO_2, where possible), for a total of 2,258 laboratory-bred juveniles (752 in control, 751 in chronic high CO_2 and 755 in acute high CO_2).

CO_2 manipulation

Field Collected Fish Experiments: Three header tanks (60 L) fed water into a total of 30 replicate 32 L aquaria where fish were held (N = 10 tanks per system). One header tank was diffused with ambient air, while the other two header tanks were dosed with 100% CO_2 to achieve the desired pH for the high CO_2 treatment (754 μatm). Adult pairs were housed in one high CO_2 system. Clutches of offspring were divided and maintained in both the control and the other high CO_2 system. pH controllers (Aqua-Medic, Germany) maintained the desired pH in the header tanks that supplied the tanks in each system (Table 5.2). Daily pH_{NBS} (National Bureau of Standards) and temperature measurements were taken in each tank using a pH electrode (SevenGo Pro, Mettler Toledo, Switzerland) and temperature probe (Cormark C26, Norfolk, UK). A portable CO_2 equilibrator and infrared sensor (GMP343, Vaisala, Helsinki, Finland) verified seawater pCO_2 (Hari et al. 2008; Munday et al. 2014b). Water samples were taken for the duration of the experiment and used to determine total alkalinity by Gran Titration, within 1% of certified reference material (Prof. A. Dickson, Scripps Institution of Oceanography). Salinity was obtained daily from moorings around Lizard Island that are part of Australia's Integrated Marine Observing System (IMOS). Carbonate chemistry parameters were calculated using CO2SYS (Pierrot et al. 2006) with the constants K1 from Mehrbach et al. (1973) refit by Dickson and Millero (1987), and Dickson for $KHSO_4$ (Table 5.2).

Laboratory Reared Fish Experiments: Two 10,000 L recirculating aquarium systems were set to a different pH and corresponding CO_2 level: a current-day control (414 µatm) and a mid- level end of century CO_2 (754 µatm). CO_2 was dosed into a 3000 L sump using an Aqua Medic AT Control System (Aqua Medic, Germany). This allowed for maintenance of the desired pH level in each system. The equilibrated seawater was then delivered to the holding aquaria at a rate of 1.5 L min^{-1}. Daily pH_{NBS} and temperature measurements were taken using a pH electrode and temperature probe, as conducted for field experiments. Weekly salinity readings were measured using a conductivity sensor (HQ15d; Hach, Loveland, CO, USA). Total alkalinity was estimated weekly using Gran Titration (Metrohm 888 Titrando Titrator Metrohm AG, Switzerland) and using certified reference material from Dr. A.G. Dickson (Scripps Institution of Oceanography). Carbonate chemistry parameters were calculated using CO2SYS (Pierrot et al. 2006) as mentioned above for the field-based experiments (Table 5.2).

Table 5.2. Mean (± SD) seawater parameters in the experimental system for adults and juveniles during the experimental seasons. Temperature, pH, salinity and total alkalinity (TA) were measured directly. pCO_2 was estimated from these parameters using CO2SYS. Seawater parameters were consistent for breeding and experimental components of the study.

Treatment	**pH_{NBS}**	**Temperature (°C)**	**Salinity**	**TA (µmol.kg^{-1} SW)**	**pCO_2 (µatm)**
Field					
Control	8.13 (± 0.03)	27.3 (± 0.5)	35.4 (± 0.02)	2267 (± 30)	452 (± 37)
High CO_2	7.95 (± 0.01)	27.5 (± 0.5)	35.4 (± 0.02)	2281 (± 37)	754 (± 23)
Laboratory					
Control	8.15 (± 0.04)	28.5 (± 0.2)	35.0 (± 1.2)	2146 (± 125)	414 (± 46)
High CO_2	7.94 (± 0.04)	28.5 (± 0.3)	35.1 (± 1.2)	2223 (± 146)	754 (± 92)

Olfaction

Response to CAC was tested in a two-channel choice flume. The size of the chamber differed between adults (30 cm x 13 cm) (Heuer et al. 2016) and juveniles (13 cm x 4 cm) (Gerlach et al. 2007). Individuals were given the choice between two water streams in the flume: seawater containing conspecific CAC versus untreated seawater. Water chemistry in the flume matched the respective treatment for each fish. Water from the two different sources was gravity fed into the choice flume, which is divided down half of its length. A constant flow rate of 450 mL min^{-1} and 100 mL min^{-1} was maintained for the adults and juveniles, respectively. Flow rates were monitored using a flow meter and dye test after every water change.

To produce CAC, control donor fish were euthanized with a quick blow to the head. Donor fish were the same size and approximate age as the fish being flumed in each trial. Superficial cuts were made along the sides of the donor fish. Adult donors were then rinsed with 60 mL of control water, while juvenile donors were rinsed with 15 mL of control water (Ferrari et al. 2011a). The rinse water was collected and immediately mixed with 10 L of treatment water in the tank used to supply CAC to the flume. CAC was replenished after every second fish to ensure a consistent concentration of fresh CAC for the duration of each trial. A ratio of one donor fish to one test fish was used.

For each trial, a single test fish was placed in the centre of a downstream end of the choice flume and given a 2-minute acclimation period. The position of the fish was then recorded every five seconds for a total of 2 minutes. A rest period of 1 minute followed, during which time the water sources were switched and the fish was re-centered in the downstream end of the flume. The entire acclimation and trial process was then repeated to eliminate potential side preference.

Behaviour repeatability

To ensure consistency in phenotypic changes due to elevated CO_2, a total of 20 adults were tested for repeatability of olfactory behaviour: 10 individuals were tested in the field at LIRS and 10 individuals in the laboratory at JCU. An equal number of males and females were tested. Individual olfactory behaviour was measured in the two-channel choice flume, following the protocol above. Individuals were placed into separate holding tanks for 24 hours after the first behavioural trial. The same olfactory behaviour test was then repeated. Percent time spent in the chemical alarm cue was correlated for the first and second trials to estimate trait repeatability.

Juvenile Aging

The age of juveniles tested at LIRS was determined by counting the daily growth increments in the otoliths (ear bones). Sagitta were dissected from two juveniles from each offspring clutch and prepared by standard technique (Fowler 1990). Juveniles were on average 35 days old (± 6 days, SD).

Data analysis

The average time in CAC for each group of offspring (midoffspring value) was used in the parent-offspring regressions (Åkesson et al. 2008). I focused my analysis on the father-midoffspring regression as this should provide the least biased estimate of narrow-sense heritability (Falconer and Mackay 1996); however, I also estimated heritability from mother-midoffspring and midparent-midoffspring regressions as these may provide additional information on maternal and environmental effects when compared with the father-midoffspring regression. Heritability (h^2) was calculated as the slope of the least-squares regression between the midoffspring and midparent values (average of father and mother responses) among families, and two times the slope for father-midoffspring and mother-midoffspring values (Lynch and Walsh 1998).

ANCOVA was used to compare regressions among the different treatment groups, with offspring treatment as the categorical factor and parental olfactory response as the covariate. This analysis allowed us to test if: 1) heritability (the slope of the regression) differed between treatments groups and 2) if the magnitude of the behavioural response to CAC (intercept) differed between fish reared in control versus high CO_2, between acute versus chronic CO_2 treatments and between offspring from control versus high CO_2 parents. A homogeneity of slopes model was used to test for an interaction between the main effects and the covariate, which would indicate that h^2 differed among treatment groups. Where no significant interaction was detected, the model was rerun with the interaction term removed to test for differences in elevation between treatment groups. Separate ANCOVAs were conducted for father-, mother- and midparent-midoffspring regressions.

5.4 Results

Field Collected Fish Experiments

Acute exposure to elevated CO_2 altered juvenile olfactory response to CAC, with 2-4 times more time spent in CAC for CO_2 treated fish compared to sibling fish kept in current-day control seawater (Figure 5.2a; $F_{1,36} = 7.97$, $p < 0.01$). Similar effects of high CO_2 exposure on the response to CAC were present in the mother-midoffspring (Figure 5.2b; $F_{1,36} = 6.33$, $p < 0.05$) and midparent-midoffspring regressions, except that the slopes of the regressions were different in the midparent regression as indicated by a significant interaction (Figure 5.2c; $F_{1,36} = 5.34$, $p < 0.05$).

Heritability in the behavioural response to CAC was high (0.56) in wild-caught fish acutely exposed to high CO_2 (Table 5.3) indicating a large component of additive genetic variation. Heritability was higher in mother-midoffspring regression (0.65) compared with the father-midoffspring regression (0.56), indicating the possible influence of non-genetic maternal effects, although the two values were not significantly different as they overlapped with standard errors of ± 0.23 (Table 5.3). The estimate of heritability from the midparent-midoffspring regression (0.51) was similar to that estimated by the father-midoffspring regression. Interestingly, offspring from three parental pairs exhibited far greater tolerance to high CO_2 than offspring from other parents. Offspring from these three families that had been acutely exposed to high CO_2 exhibited a response to CAC that was more similar to control fish than to other offspring exposed to high CO_2 (Figure 5.2a). The average time in CAC for these high CO_2 offspring was only 2-5% more than their siblings that had been reared and tested in control conditions. Heritability declines to 0.26 if these three families with tolerant offspring responses were removed from the analysis.

Individual repeatability in responses to CAC was very high for wild caught fish with a correlation of r = 0.992 ($p < 0.001$) between the first and second trial for time spent in CAC by adults exposed to high CO_2.

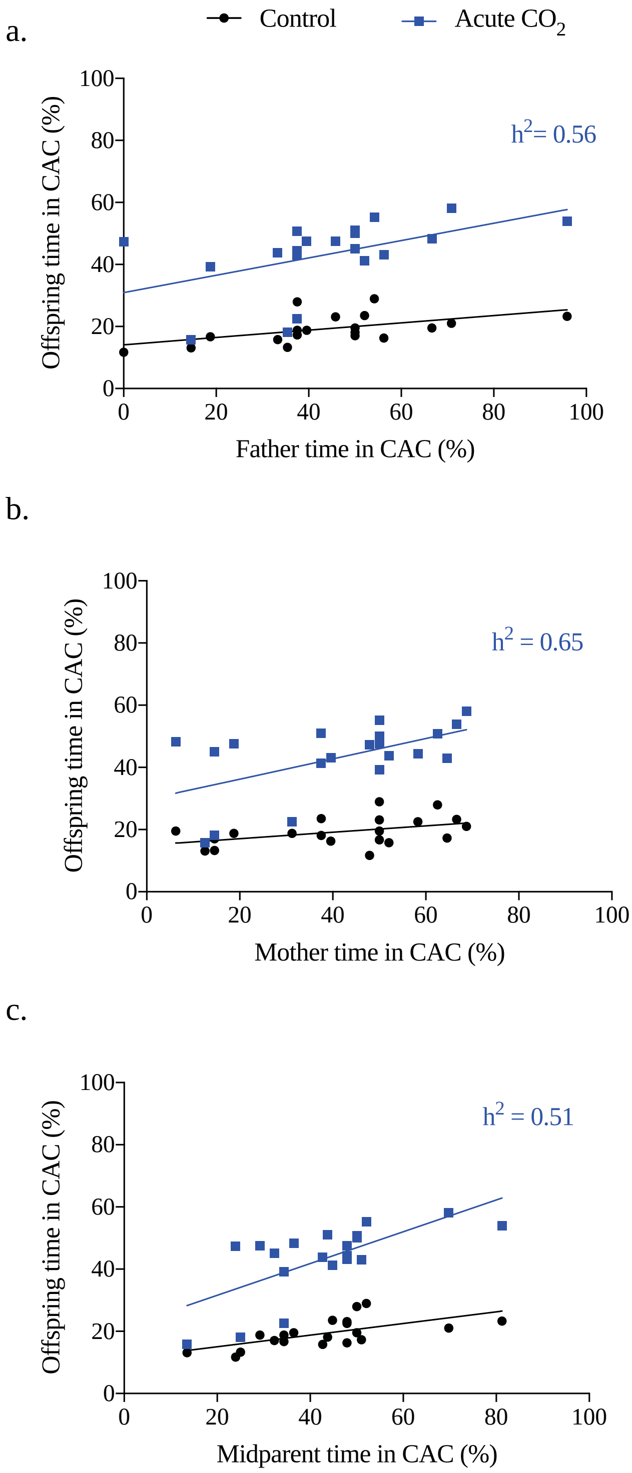

Figure 5.2. Parent-offspring regressions for olfactory preference of fish acutely exposed to high CO_2 in the field. Percent time spent in the chemical alarm cue was measured. (a) Father, (b) mother and (c) midparent responses are plotted along the x-axes. Midoffspring responses are plotted along the y-axes for direct relationships with their parent. Regressions are grouped by offspring treatments: Control (452 µatm) and Acute CO_2 (754 µatm). h^2 is depicted on the graphs.

Laboratory Reared Fish Experiments

For the laboratory-reared fish, I first compared the amount of time in CAC between juveniles that were acutely exposed to high CO_2 and control fish in order to make a comparison with the field data. Similar to the field results, juveniles exposed to high CO_2 spent 2-7 times more time in CAC than sibling fish kept in current-day control seawater (Figure 5.3a; $F_{1,36} = 3.23$, $p > 0.05$). Similar effects of high CO_2 on olfactory responses were present in the mother-midoffspring (Figure 5.3b; $F_{1,36} = 4.75$, $p < 0.05$) and midparent-midoffspring regressions (Figure 5.3c; $F_{1,36} = 0.89$, $p > 0.05$).

Heritability in the behavioural response to CAC was high (0.65) in the laboratory fish that were acutely exposed to high CO_2 (Table 5.3) indicating a large component of additive genetic component. Heritability estimated in the laboratory fish that were acutely exposed to high CO_2 (0.65 ± 0.17 SE) overlapped with the estimate of heritability in the wild caught fish acutely exposed to high CO_2 (0.56 ± 0.23 SE). The mother-midoffspring regression yielded a much lower estimate of heritability (0.18) indicating a strong maternal effect on phenotypic variation among clutches. As expected, the estimate of heritability from the midparent regression was intermediate to the father- and mother-midoffspring regressions (Table 5.3).

In direct contrast to the acutely exposed fish, heritability was very low (0.02) for siblings that had been chronically exposed to high CO_2 from hatching (Table 5.3). With a SE of ± 0.11 the estimate of heritability for fish that were chronically exposed to high CO_2 could not be distinguished from zero. A moderate negative heritability for the mother-midoffspring regression was indicative of strong maternal effects on phenotypic variation among clutches. Similar to the father-midoffspring regression, the estimate of heritability from the midparent-midoffspring regression was very low and could not be distinguished from zero (Table 5.3).

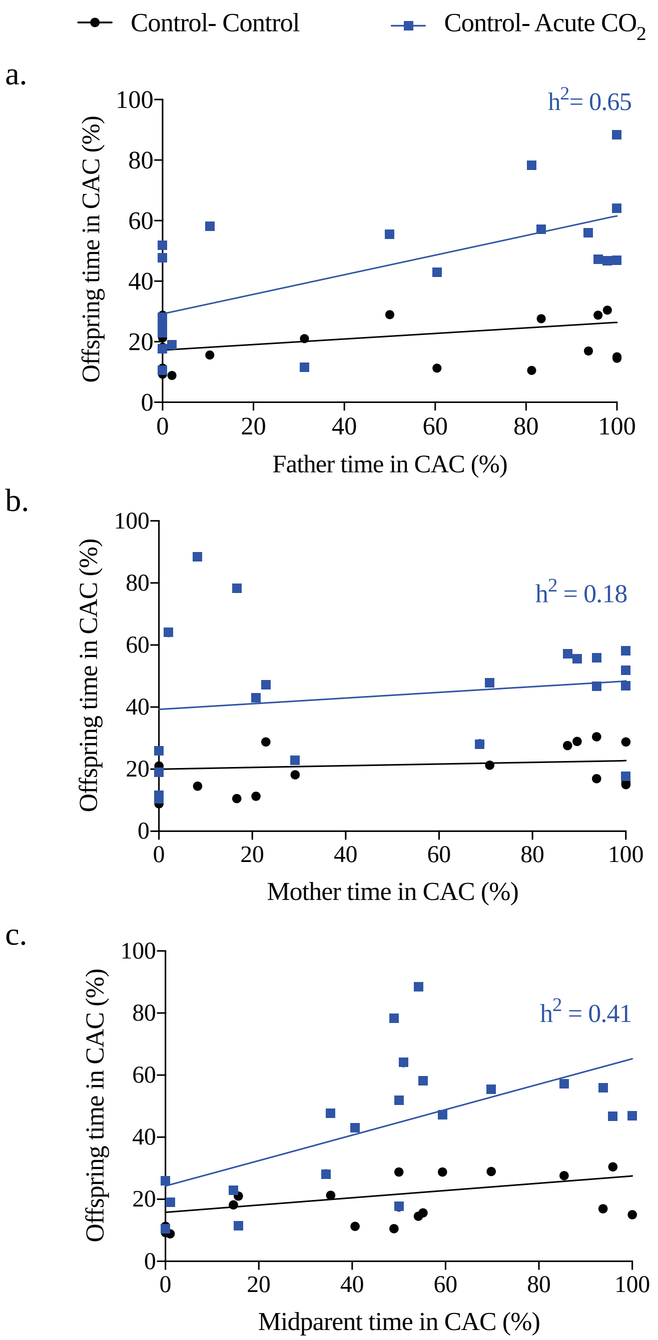

Figure 5.3. Parent-offspring regressions for olfactory responses of fish acutely exposed to high CO_2 in the laboratory. Percent time spent in the chemical alarm cue was measured. (a) Father, (b) mother and (c) midparent responses are plotted along the x-axes. Midoffspring responses are plotted along the y-axes for direct relationships with their parent. Regressions are grouped by offspring rearing treatments, with the first word in the legend indicating parent treatment water (Control, 414 µatm), and the second word in the legend indicating offspring rearing treatment (Control or Acute CO_2, 754 µatm). h^2 is depicted on the graphs.

Table 5.3. Heritability (h^2) of olfactory behaviour for offspring in elevated CO_2 conditions estimated from father-, mother- and midparent-midoffspring regressions. Heritabilities (to the nearest 0.01), standard error (SE) and sample size (N), are shown. * SE of h^2 is twice the standard error of the slope b.

Parent Holding Condition	Juvenile Treatment	Location	Father-Offspring			Mother-Offspring			Midparent-Offspring		
			h^2=2b	SE*	N	h^2=2b	SE*	N	h^2=b	SE	N
Control	Acute CO_2	*Field*	0.56	0.23	20	0.65	0.23	20	0.51	0.13	20
		Laboratory	0.65	0.17	20	0.18	0.23	20	0.41	0.13	20
	Chronic CO_2		0.02	0.11	20	-0.13	0.11	20	-0.05	0.08	20
High CO_2	Acute CO_2		0.28	0.09	18	-0.23	0.11	18	0.05	0.09	18
	Chronic CO_2		-0.01	0.06	18	0.04	0.06	18	0.02	0.05	18

The comparison of regression slopes (Figure 5.4) further demonstrates the effects of acute versus chronic CO_2 exposure on heritability of behavioural tolerance to high CO_2. For offspring from control fathers, there was a significant interaction between the slopes for acute CO_2 offspring and chronic CO_2 offspring (Figure 5.4a; $F_{1,36} = 9.55$, $p < 0.01$). This interaction was due to the positive relationship between fathers and their offspring that were acutely treated with high CO_2, compared with no relationship for the same fathers and sibling offspring that were chronically exposed to high CO_2. Similar interactions between acute and chronic CO_2 treatments were present in the mother-midoffspring (Figure 5.4c; $F_{1,38} = 8.32$, $p < 0.01$) and midparent-midoffspring regressions (Figure 5.4e; $F_{1,36} = 9.31$, $p < 0.01$).

Heritability in the behavioural response to CAC for fish from parents exposed to high CO_2 was moderate (0.28) for offspring acutely exposed to high CO_2 and could not be distinguished from zero for offspring chronically exposed to high CO_2 (Table 5.3). For fish that were acutely exposed to high CO_2, heritability was lower in the group from parents maintained continuously in high CO_2 (0.28) compared with the group from parents that were maintained in control conditions after initial behavioural testing (0.65), indicating a negative effect of parental CO_2 exposure on heritability in the behavioural response to CAC. However, compared with the same offspring treatments from parents exposed to control conditions, there was an overwhelming effect of acute versus chronic CO_2 exposure on heritability and minimal effect of parental CO_2

treatment on heritability. The mother-offspring regressions indicated a strong maternal effect in acutely exposed offspring from high CO_2 parents (-0.23), but not in siblings that were chronically exposed to high CO_2 (0.04). Heritabilities estimated in the midparent-midoffspring regressions could not be distinguished from zero (Table 5.3).

Offspring from high CO_2 fathers were more strongly affected by rearing treatments than by parental CO_2 exposure (Figure 5.4a,b). There was a significant interaction between the slopes for acute CO_2 offspring and chronic CO_2 offspring (Figure 5.4b; $F_{1,32} = 7.29$, $p < 0.05$). This interaction was similar to that seen between offspring from control fathers, resulting from the positive relationship between fathers and their offspring that were acutely treated with high CO_2, but lack of relationship for the same fathers and sibling offspring that were chronically exposed to high CO_2. Similar interactions between acute and chronic CO_2 treatments were present in the high CO_2 mother-midoffspring (Figure 5.4d; $F_{1,32} = 4.89$, $p < 0.05$) and high CO_2 midparent-midoffspring regressions (Figure 5.4f; $F_{1,34} = 4.18$, $p < 0.05$).

As observed in field caught fish, repeatability was very high for laboratory-reared fish with a correlation of $r = 0.997$, $P < 0.001$ between the first and second trial for adults exposed to high CO_2.

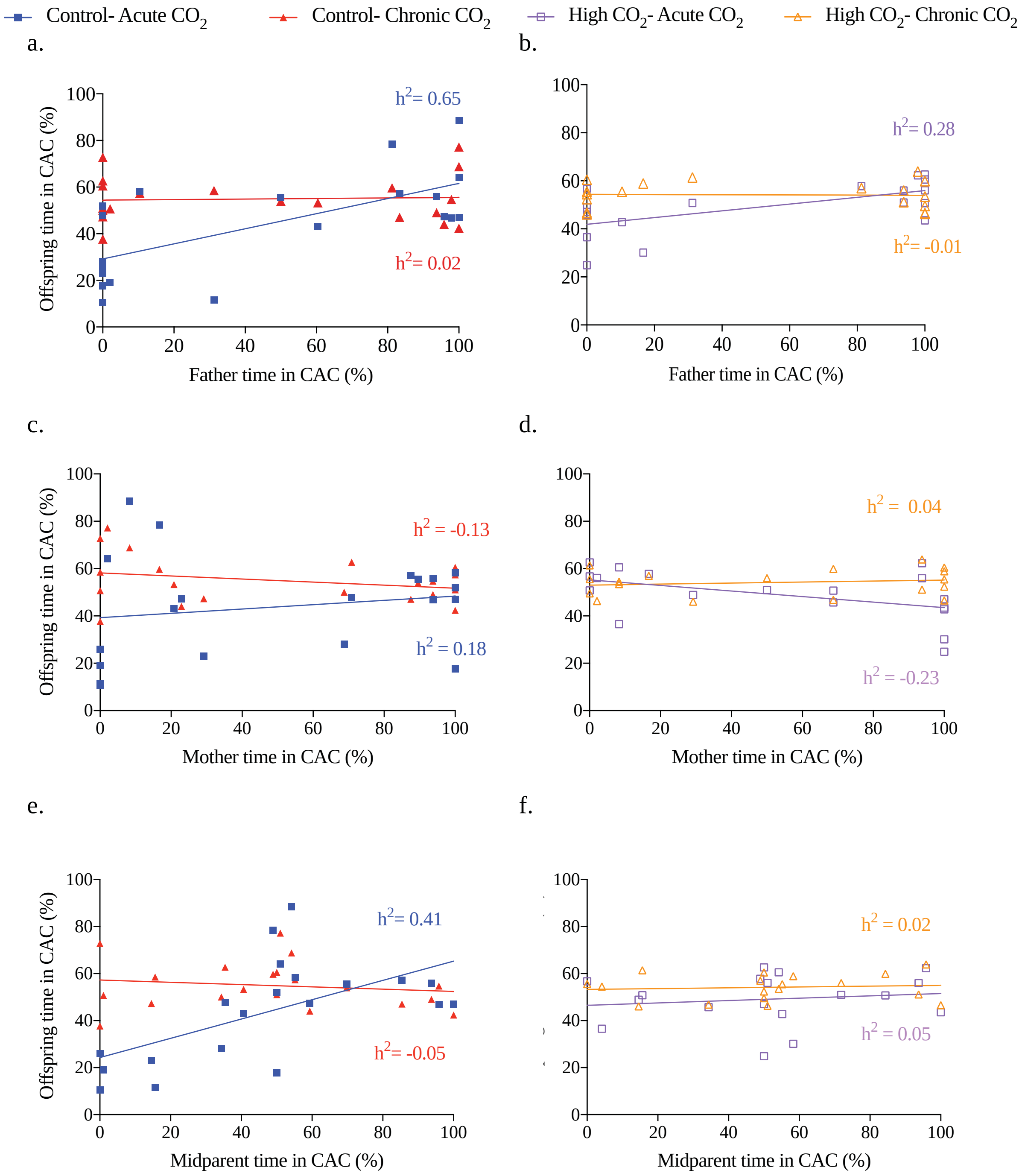

Figure 5.4. Acute CO_2 vs. chronic CO_2 effects on olfactory responses for parent-offspring regressions in the laboratory. Percent time spent in the chemical alarm cue was measured. Father (a,b), mother (c,d) and midparent (e,f) responses are plotted along the x-axes. Midoffspring responses are plotted along the y-axes for direct relationships with their parent. Regressions are grouped by offspring treatments, with the first word in the legend indicating parent treatment water (a,c,e) Control (414 µatm) or (b,d,f) High CO_2 (754 µatm), and the second word in the legend indicating offspring rearing treatment (Acute CO_2 or Chronic CO_2). h^2 is depicted on the graphs.

5.5 Discussion

I found that offspring exposed to elevated CO_2 exhibited impaired anti-predator behaviour, spending an increased amount of time in a water stream containing CAC compared with control fish, consistent with previous findings (e.g. Ferrari et al. 2011a; Welch et al. 2014). Moreover, this impaired behavioural response in offspring was not ameliorated by parental exposure to high CO_2. More importantly, these parent-offspring regressions revealed strong broad-sense heritability of behavioural tolerance to elevated CO_2 in offspring that are acutely exposed to high CO_2, but this heritability was lost in offspring chronically exposed to high CO_2. Parents that were tolerant to elevated CO_2 conditions produced offspring that also showed behavioural tolerance under acute CO_2 treatments, but this effect was not evident in sibling offspring that were reared in high CO_2 from hatching. Parental exposure to high CO_2 also diminished the heritability of behavioural tolerance to high CO_2, but not to the same extent as chronic exposure to high CO_2 in juveniles. These results have important ramifications for understanding the likelihood that fish will be able to adapt to projected future CO_2 levels in the ocean.

These results show that the duration of exposure to high CO_2 can substantially influence the variation in behavioural responses exhibited in juvenile fish, and thus the heritability of behavioural tolerance to high CO_2. In the laboratory population there was high heritability of behavioural response to CAC in offspring that had been acutely exposed to high CO_2, but not in offspring that had been chronically exposed to high CO_2. Importantly, fish in these two treatments were siblings and there was almost no mortality during the six week rearing period that could have led to the selection of less tolerant genotypes; therefore the difference must be due to plasticity, not genetic differences. The reduction in heritability between parents and offspring in chronically exposed offspring occurred due to previously tolerant families (low percentage time in CAC) in acute CO_2 losing this tolerance in the chronically exposed siblings. By contrast, there was no difference in behaviour for juveniles from non-tolerant parents that were acutely or chronically exposed to high CO_2. This suggests that the behavioural phenotype of CO_2 tolerant offspring is modified by non-adaptive plasticity when they are chronically exposed to high CO_2. Innate responses in animals, such as predator and CAC avoidance, tend to occur rapidly and be both favourable to the individual and heritable (Agrawal et al. 1999). However, individual variation in responses may diminish in the longer term due to the convergence of cellular and physiological processes in a common environment (Day and Bonduriansky 2011). Here I observed a reduction in phenotypic variation expressed in juveniles that were chronically exposed to high CO_2 compared with juveniles that were acutely exposed to high CO_2, causing a reduction in the heritability of behavioural tolerance to high CO_2. My results are consistent with reduced heritability in unfavourable conditions in other animals (Charmantier and Garant 2005; Wilson et al. 2006), but they also demonstrate that the mechanism may be non-adaptive plasticity in stressful environments. Critically, these results suggest that the collapse in phenotypic variation under chronically high CO_2 conditions may reduce the potential for adaptation of fish populations to consistently high CO_2 levels in the future.

High heritabilities for acute CO_2 offspring were strongly driven by a few highly tolerant families in both the field and laboratory. Offspring from these highly tolerant families had similar phenotypes in both high CO_2 and control conditions, spending minimal time in CAC. This is consistent with previous observations that some individuals appear to be completely unaffected by this level of CO_2 (700 μatm) and exhibit no change in behaviour compared with controls (Munday et al. 2010). Furthermore, these highly tolerant individuals are selectively favoured in their natural habitat because they suffer lower rates of predation (Munday et al. 2012). These earlier observations suggested that there could be rapid adaption of behavioural tolerance to high CO_2 in fish populations. However, those studies involved juveniles that had been acutely exposed to high CO_2 for the same duration as my acute treatments. These results indicate that these individuals may lack this behavioural tolerance when chronically exposed to high CO_2 from hatching, and this will constrain the adaptive potential of fish populations to high CO_2.

The results for this study showed no potential for transgenerational acclimation, similar to Welch et al. (2014). There was no parental effect on the average response to CAC seen through the increased attraction to the cue for all chronic CO_2 offspring compared to control offspring. However, there was a negative parental effect on the heritability of CO_2 tolerance, with a reduction from 0.65 to 0.28 for control and high CO_2 fathers, respectively. This suggests that transgenerational effects might reduce variation in the behavioural response to high CO_2 and consequently reduce the pace of adaptation. Heritable variation was present in offspring treated with acute CO_2 in the field and laboratory, indicating that adaptation would be expected to occur in instances where individuals experience short-term exposure to high CO_2 levels, as might occur in natural upwelling zones and coastal habitats (Hofmann et al. 2011). However, higher anthropogenic CO_2 levels will be permanent, exposing successive generations of individuals to high CO_2 for extensive periods of time. The chronic CO_2 treatment mirrored this scenario and there was no additive genetic variation observed, regardless of parental treatment.

There was heritable phenotypic variation in behavioural responses of juvenile fish exposed to high CO_2 for 4-5 days, but this variation was lost in fish reared for 6 weeks in high CO_2. However, the rate of decay in heritability through time is unknown. The rate of decay could be important because mortality rates of reef fish from predation are highest in small size classes and diminish rapidly with increasing size and age (Jones and McCormick 2002; Almany and Webster 2006). Therefore, the strongest selection for appropriate behavioural responses to the threat of predation is likely to occur at an early age when juveniles will have had a shorter period of time exposed to high CO_2. It is possible that selection of CO_2 tolerant phenotypes could occur during this critical window of early life, leading to the evolution of more CO_2 tolerant populations, even if that tolerance is obscured by phenotypic plasticity later in life. Further studies assessing the rate of decay in heritability are required to determine the ontogenetic window-of-opportunity for selection to act on genetic variation in the behavioural response to predation threat in a high CO_2 environment.

I used parent-offspring regressions to estimate heritability of variation in the behavioural response of fish to CAC in a high CO_2 environment. Assortative mating can influence estimates of heritability in parent-offspring regressions; in particular, it may generate higher estimates of heritability than random mating (Lynch and Walsh 1998). I assumed that natural breeding pairs in the field were mated randomly and thus my estimates of heritability from the field population are unlikely to be biased by assortative mating. The laboratory breeding pairs, however, were deliberately constructed to include pairs of similar high or low behavioural tolerance to high CO_2. This assortative mating design had the potential to inflate the estimate of heritability. Nevertheless, there was no significant difference in heritability estimated between the field and laboratory populations for the acute CO_2 treatments. The estimate of heritability in acutely exposed fish from the laboratory population (0.65) was higher than from the field population (0.56), but the values were not significantly different due to the relatively large standard errors (± 0.23). It is possible that with a larger sample size these values might have been significantly different. More critical to my findings, however, is that the assortative mating design would have produced the highest estimate of heritability in the fish that were chronically exposed to high CO_2. Despite a possible positive bias, the estimate of heritability in the chronic CO_2 treatment was extremely low with an error range that encompassed zero. Consequently, these results increase confidence that there is negligible heritability of behavioural response to CAC when juvenile fish have been permanently exposed to high CO_2 from hatching, and also when their parents have been chronically exposed to high CO_2.

This study also demonstrated strong maternal effects on the heritability of variation in behavioural tolerance to high CO_2. The difference in heritability estimated by mother- and father-midoffspring regressions is indicative of the magnitude of maternal effects. In this study, heritability calculated from the mother-midoffspring regression was higher than the father-midoffspring regression in the field population, but lower than the father-midoffspring regression in the laboratory population. This suggests that maternal effects increased phenotypic variation in behavioural responses to high CO_2 in the field population, but decreased variation in the laboratory population. These differences may be related to the more variable conditions that mothers experience in the field compared with the laboratory. Laboratory-reared mothers all received the same food ration, and thus may have provisioned their offspring in a similar way. Furthermore, offspring were immediately removed from their parents upon hatching in the laboratory, thus eliminating potential variation in parental care, which might contribute to variation in offspring phenotypes. The strongest influence of maternal effects appeared to occur in the treatment when mothers were reared at high CO_2 and their offspring were reared in control conditions until a few days before behavioural testing, when they were acutely exposed to high CO_2. Heritability estimated by father-midoffspring regression was 0.28 in this treatment, but was reversed to -0.23 in the mother-midoffspring regression. This is consistent with previous observations on negative carry-over effects on behavioural responses to CAC when offspring of high CO_2 parent are reared in control water (Welch et al. 2014). Juvenile fish reared in current-day control conditions are less likely to be repelled by CAC if their parents have been reared in high CO_2. These results suggest that this effect is largely of maternal origin.

While father-midoffspring regressions provide the best possible estimate of narrow sense heritability, they may still include multiple sources of genetic variation (Lynch and Walsh 1998). There may also be non-genetic effects, such as from parental egg care (Robertson 1973), or other environmental factors that could affect phenotypic variation in parents and their offspring. If my estimates of heritability from father-midoffspring regressions contained a large environmental component I would expect to see very different heritability values between field and laboratory conditions due to substantial differences between the field and laboratory environments in general and because the offspring were separated from their parents at hatching in the laboratory, but had ongoing parental care in the wild. Despite the many environmental differences that adults and their offspring experienced in the field compared with the laboratory environment, the estimated heritability of behavioural response to CAC in the field and laboratory populations was remarkably similar. This similarity in heritability estimates, despite the substantial environmental differences between populations supports the presence of a large genetic component to the phenotypic variation in behavioural tolerance to high CO_2 in acutely exposed fish. In another recent study, Schunter et al. (2016) found that patterns of gene expression in the brain of juvenile *A. polyacanthus* exhibited a strong connection to the assignment of their parents as either tolerant or non-tolerant parents, which is also consistent with the presence of genetic variation in CO_2 tolerance for this species.

These results show that heritability in CO_2 tolerance is obscured by non-adaptive phenotypic plasticity when the offspring are permanently exposed to high CO_2. This study underscores the importance of investigating genetic variation in phenotypic traits over relevant time scales. Reef fishes initially display heritable phenotypic variation in behavioural tolerance to high CO_2 that could favour selection of tolerant genotypes during a short ontogenetic window, but this phenotypic variation is lost with longer exposure to high CO_2. A better understanding of the rate of decay in heritability under elevated CO_2 conditions caused by non-adaptive plasticity will provide more insight to the adaptive potential of fish populations in high CO_2 oceans of the future.

Chapter 6: General Discussion

To date, few studies have investigated the potential for acclimation and/or adaptation of marine fishes to ocean acidification. This thesis used a cross-generational approach to explore the potential for coral reef fishes to cope with projected future CO_2 levels in the ocean, from early reproductive processes though to behavioural impairments and otolith development. I found limited potential for acclimation of impaired behaviour, and that exposure to high CO_2 across generations may have significant effects on otolith development that are not apparent in short-term experiments. While heritable phenotypic variation in behavioural response to high CO_2 was detected, this was obscured by non-adaptive phenotypic plasticity with transgenerational CO_2 exposure, demonstrating restricted potential for adaptation of impaired behaviours. Understanding the basis of this variation will be critical for predicting the fate of reef fish populations and communities in a future high CO_2 world.

High CO_2 effects on coral reef fishes

The effects of elevated CO_2 on reef fishes varies among species and the traits examined. Previous studies have reported differences in the effects on life-history processes, otolith development and behaviour (Ferrari et al. 2011a; Heuer and Grosell 2014; Nagelkerken and Munday 2016). Thus, it is important to broaden the selection of traits and species studied to better predict the ecological effects on fish assemblages in a future high CO_2 world.

In **Chapter 2** I demonstrate that the effects of elevated CO_2 on fish reproduction vary among species, even within the same family. Similar to Miller et al. (2013), I found that reproduction is stimulated by high CO_2 in a species of anemonefish, yet there was no stimulation in another species of damselfish. This suggests that the effects of high CO_2 on reproduction are likely to vary greatly among teleost fishes and many more studies will be required to gauge the general impacts on fish populations and communities.

It is not known why reproduction is stimulated by elevated CO_2 in some species and not others; however, $GABA_A$ receptor function could be a possible mechanism. The $GABA_A$ receptor is the main inhibitory neurotransmitter receptor in the vertebrate brain, with high Cl^- and HCO_3^- activity across a gated ion channel. Under high CO_2 exposure, concentrations of Cl^- and HCO_3^- change substantially in the fishes' plasma, which could interfere with the function of the $GABA_A$ receptor (Heuer and Grosell 2014). This interference is believed to be the cause of altered behaviours and impaired sensory performances in fishes at high CO_2 (Nilsson et al. 2012; Hamilton et al. 2014). $GABA_A$ receptors also mediate hormone secretion necessary for normal reproductive output (Trudeau et al. 2000; Henderson 2007; Van Der Kraak 2009; Zohar et al. 2010), and this could explain why reproduction is affected by higher CO_2 levels. Furthermore, the effects of GABA on reproductive hormones seem to vary between inhibition and stimulation depending on reproductive stage of the fish (Trudeau et al. 1993; Khan and Thomas 1999). It is therefore possible that the altered function of the $GABA_A$ receptor in a high CO_2 environment could either enhance or inhibit regular release of reproductive hormones, resulting in stimulated or decreased reproductive outputs seen in different species, or in different studies. Detailed examination of hormonal profiles in fish exposed to high CO_2 is needed to test this hypothesis.

Differences in reproductive output under elevated CO_2 may also be due to species variation during acid-bas regulation. Various strategies may be utilized within and between species in order to cope with hypercapnia, for instance fish may alter their metabolic rates in an attempt to regulate acid-base levels. This metabolic shift may in turn affect energy allocated for reproduction (Foo and Byrne 2016). However, the effects on aerobic scope vary substantially across species (reviewed in Heuer and Grosell 2014), and studies would therefore need to assess metabolic function alongside reproductive behaviour in a given species. Further mechanistic insight across species experiencing hypercapnia is required to determine what drives variation in reproductive behaviours under elevated CO_2.

Reproduction in fish is also strongly affected by temperature. Reproduction occurs within a limited range of temperatures in most fishes (Van Der Kraak and Pankhurst 1997), and this range is much narrower than the overall thermal tolerance range of the species. Rising temperatures can therefore shorten the breeding season (reviewed in Pankhurst and Porter 2003), and can result in decreased reproductive output (Donelson et al. 2010). Furthermore, temperature had a much stronger influence on reproduction in *Amphiprion melanopus* compared to high CO_2 (Miller et al. 2015), indicating that warmer temperatures may offset any potentially positive effects of elevated CO_2 on reproduction. Temperature and pCO_2 levels will rise simultaneously during climate change, thus interactive effects of these two environmental stressors on reproduction will be a key area for future investigation.

Previous studies have predominantly used short-term exposure to elevated CO_2 to explore the effects of projected future CO_2 levels on fish and other marine organisms. However, this 'future shock' exposure (Byrne 2012) to high CO_2 could over- or underestimate species sensitivity to ocean acidification (Dupont et al. 2013). For example, changes in otolith development have been observed in larval and juvenile fishes exposed to high CO_2 for a few days to weeks (Checkley et al. 2009; Bignami et al. 2013a). Yet the effects of long-term exposure on otolith development have not been thoroughly investigated. Behavioural impairments in fishes have also been seen in experiments using short-term exposure to elevated pCO_2 (Munday et al. 2009a; Dixson et al. 2010; Ferrari et al. 2011a; Domenici et al. 2012), threatening individual performance and population sustainability (Munday et al. 2010). However, CO_2 levels will rise over many years and generations, thus the use of multi-generation exposure to elevated CO_2 is important to better predict the long-term consequences of ocean acidification for marine organisms.

Transgenerational effects

Early developmental stages of *Ac. polyacanthus* appear to be highly tolerant to elevated CO_2 levels. Juvenile exposure to elevated CO_2 did not affect survival or growth (Munday et al. 2011a), nor were there effects seen for hatchling size (**Chapter 2**) or juvenile growth (**Chapter 4**). This tolerance was believed to extend to otolith development (Munday et al. 2011a); however, transgenerational exposure caused increased otolith growth when both parents and offspring were reared in high CO_2 (**Chapter 4**). Short-term CO_2 exposure in previous experiments has therefore underestimated the changes in otolith development for *Ac. polyacanthus*. Otolith area and perimeter measurements were larger with transgenerational exposure to high CO_2 (Chapter 4), similar to larger otoliths in juvenile three-spined sticklebacks that experienced transgenerational high CO_2 exposure (Schade et al. 2014). Parental high CO_2 exposure did not affect otolith growth for juveniles reared in control or moderate CO_2, nor were there developmental effects of high CO_2 for juveniles from control parents. Therefore, the effects of high CO_2 on otolith growth in juvenile *Ac. polyacanthus* must depend on an interactive effect that comes from both parents and juveniles experiencing a high CO_2 environment. Changes to otolith shape and size could influence functionality of hearing (Simpson et al. 2011), balance (Popper et al. 2005; Simpson et al. 2011) and swimming behaviour (Bignami et al. 2013a) for reef fishes; however, this has not been experimentally tested. Importantly, the changes in otolith size and shape in high CO_2 are relatively small compared to the natural variation in these traits among individuals. Therefore, the magnitude of any impacts may be small. Future studies need to assess the implications of changes to otolith sizes on individual performance, and must consider transgenerational exposure to elevated CO_2 as it could differ substantially from the effects seen in short-term exposure.

Critical olfactory and cognitive behaviours of juvenile *Ac. polyacanthus* are impaired by high CO_2, and these altered behaviours were not mitigated by transgenerational exposure to future levels of ocean acidification (**Chapter 3**). Fish depend on predator avoidance behaviour, such as appropriate responses to alarm cues (Chivers and Smith 1998) as well as visual and cognitive function (e.g. lateralization), for survival, especially in young juveniles, which are more prone to predation (Lönnstedt et al. 2012). Thus, the inability to acclimate under elevated CO_2 could lead to significantly higher mortality among juvenile fishes as CO_2 levels rise in the ocean. Transgenerational acclimation did not occur for coral reef fish behaviour (**Chapter 3**), but has been observed for growth and metabolic processes in another coral reef fish species (Miller et al. 2012). It is possible that the mechanisms controlling some traits are less plastic than others, thus influencing the potential for transgenerational acclimation to elevated CO_2. For instance, $GABA_A$ receptor function associated with altered behaviours (Nilsson et al. 2012; Chivers et al. 2014; Hamilton et al. 2014) may be less plastic than metabolic pathways (Miller et al. 2012); however, this hypothesis requires further investigation.

The mechanisms responsible for transgenerational plasticity are still not fully understood; however, they can range from nutritional provisioning to the transfer of cytoplasmic factors and epigenetic marks (Bonduriansky 2012; Munday 2014). In

Chapter 3 I observed carry-over effects from parents in elevated CO_2 conditions when offspring were reared in control conditions. *Ac. polyacanthus* egg size was not affected by maternal CO_2 exposure (**Chapter 2**), suggesting that differential egg provisioning was not correlated to these carry-over effects. Alternatively, epigenetic marks (e.g. DNA methylation or modification of histones) from mothers or fathers may be potential mechanisms for transgenerational plasticity (Jablonka and Raz 2009; Munday 2014; Foo and Byrne 2016). Transgenerational epigenetic effects can be very difficult to establish, because eggs develop in mothers experiencing the new environment (e.g. high CO_2), and thus the epigenetic effects may occur within the generation, not across generations. Additional studies should carefully examine the potential mechanisms involved in transgenerational effects. For example, if F1 fish were reared in control conditions through reproduction, any continued carry-over effects present in the F2 generation would demonstrate a transgenerational epigenetic effect.

Adaptation in reef fish behaviour

The lack of transgenerational acclimation of fish behaviour (**Chapter 3**) raises concerns for the performance of populations under future high CO_2 conditions. Genetic adaptation will be necessary to overcome these behavioural impairments as CO_2 levels rise in the ocean. Behaviour studies at intermediate CO_2 levels have reported individual variation in responses to cues (e.g. predator and CAC) (Munday et al. 2010, 2013a; Ferrari et al. 2011a), and selection of CO_2-tolerant behavioural phenotypes has been observed in field-based experiments (Munday et al. 2012). In **Chapter 5** I examined whether this phenotypic variation is heritable to estimate adaptive potential of behavioural impairment under elevated CO_2. Additive genetic variation was present in offspring treated with acute (4 days) CO_2 in both field and laboratory conditions, indicating that adaptation would be expected to occur in instances where individuals experience short-term exposure to high CO_2 levels. However, heritability was lost when offspring were reared under chronic (6 weeks) high CO_2, regardless of parent CO_2 history, indicating that the behavioural phenotype of CO_2 tolerant offspring is modified by non-adaptive plasticity when they are chronically exposed to high CO_2. Higher anthropogenic CO_2 levels will expose multiple generations to high CO_2 for extensive periods of time, as mirrored by chronic and transgenerational CO_2 exposure, therefore the potential for adaptation of fish populations to ocean acidification may be constrained. These results suggest that short-term quantitative genetics studies may overestimate adaptive capacity if non-adaptive plasticity acts to conceal phenotypic variation, as observed in Chapter 5.

The relationship between plasticity and adaptation is an important area of evolutionary research. However, there are divergent opinions on whether plasticity is likely to facilitate or retard evolution (Merilä 2015). Adaptive plasticity could potentially enhance evolution if it leads to the selection on genes associated with the trait (genetic assimilation) (Pigliucci et al. 2006). Alternatively, plasticity could retard genetic adaptation by shifting the mean phenotype closer to the fitness peak, thereby weakening the selection gradient, without changing allelic frequencies. **Chapter 5** further shows that non-adaptive plasticity may constrain evolutionary potential by obscuring the phenotypic variation upon which selection can act.

Although non-adaptive plasticity obscured heritability of behavioural tolerance in fish that were chronically exposed to high CO_2 for many weeks, heritability of behavioural tolerance was very high in fish that were acutely exposed to high CO_2. Reef fish experience the highest predation and mortality rates in their smallest size classes and young age, usually within a few days of settling to the reef (Jones and McCormick 2002; Almany and Webster 2006). It is possible that selection of CO_2 tolerant phenotypes could occur during this early life window when the shortest period of high CO_2 exposure has occurred. This could result in more CO_2 tolerant populations, even if that tolerance is obscured by phenotypic plasticity later in life. The rate of decay in heritability between 4 days and 6 weeks is not known, but would provide additional insight to the likelihood of favourable selection during the early life and the evolution of behaviourally tolerant populations.

The chapters in this thesis focused on the direct effects of high CO_2 on reef fish biology and ecology. However, the rise in CO_2 is causing global warming, which will also affect the biology and ecology of reef fishes (Munday et al. 2008). Future multigenerational studies will need to investigate the interactions between these two stressors. It is possible that warmer temperatures could alter the heritability of behavioural tolerance to high CO_2 observed in Chapter 5, and genetic correlations between variation in response to high temperature and high CO_2 should therefore be assessed. If similar genes are essential for individual performance under multiple stressors (e.g. warming and acidification), there will be a positive genetic correlation (e.g. temperate urchins, Foo et al. 2012). This may enhance the speed of natural selection to warming and acidification. If there is little genetic overlap for performance under the different stressors, the genetic correlation will be closer to zero, indicating that concurrent stressors do not restrict selection (e.g. tropical urchins in warming and acidification, Foo et al. 2014). However, if there is a negative genetic correlation in performance at higher temperature and high CO_2, adaptation will be constrained (Sunday et al. 2014). Future studies will need to address the direction and strength of potential genetic correlations under climate change scenarios of both warming and high CO_2.

Concluding remarks

The results of this thesis illustrate the need for multi-generational studies to understand the full potential for marine fishes to adjust to rising CO_2 levels in the ocean. Transgenerational exposure to elevated CO_2 sustained behavioural impairments, but altered otolith development, compared to short-term exposure for a single generation for *Ac. polyacanthus*. Furthermore, there was variation in the heritability of behavioural tolerance to high CO_2, but this was obscured by non-adaptive plasticity when offspring were chronically exposed to high CO_2 for many weeks. These transgenerational studies varied from previous short-term exposure effects, emphasizing that the full adaptive potential is best estimated when ecologically relevant exposure times (e.g. multiple generations) are considered. The results also indicate that transgenerational acclimation and genetic adaptation are not a panacea to ocean acidification and that adaptive potential may be restricted by complex interactions between these two processes.

Ocean acidification will occur concurrently with multiple stressors, especially rising temperatures. Warming could have opposing effects to high CO_2 (Domenici et al. 2014; Ferrari et al. 2015), therefore effects of ocean acidification, such as impaired behaviours, may be lessened or reversed when warming simultaneously acts on the organism. Temperature effects could also be stronger than CO_2, dominating the extent of impairment on the individual (Miller et al. 2015). Ultimately, the potential for acclimation or adaption may depend on the interactions of multiple stressors, with potential for additive, synergistic or antagonistic effects (Riebesell and Gattuso 2015).

Evolutionary responses may further be constrained or enhanced by genetic correlations (Sunday et al. 2014). Future studies should therefore account for the occurrences of multiple stressors and their genetic implications when predicting evolutionary potential to climate change.

Short-term studies may provide some useful insight to individual stress tolerance, yet do not consider potential prolonged performance in the new environment. Long-term and multigenerational studies may demonstrate contrasting effects that negate or exacerbate findings from short-term exposure to new environments. Long-term studies should further take into account more realistic fluctuations of environmental variables on temporal scales. This study uses fixed levels of elevated CO_2, but natural variation will occur for all environmental stressors as their averages increase with climate change. It is possible that variation in environmental stressors may benefit acclimation and adaptation potential for tolerant individuals; however, more research is needed to determine these effects. Only by examining multiple stressors on marine organisms over more ecologically relevant scales will it be possible to gauge the future for marine population in a rapidly changing environment.

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Appendix 1: Publications not arising from the thesis

Heuer RM, Welch MJ, Rummer JL, Munday PL, Grosell M (2016) Altered brain ion gradients following compensation for elevated CO_2 are linked to behavioural alterations in a coral reef fish. Sci Rep 6:33216.

Munday P, Welch M, Allan B, Watson S-A, McMahon S, McCormick M (2016) Effects of elevated CO_2 on predator avoidance behaviour by reef fishes is not altered by experimental test water. PeerJ 4:e2501.

Schunter C, Welch MJ, Ryu T, Zhang H, Berumen ML, Nilsson GE, Munday PL, Ravasi T (2016) Molecular signatures of transgenerational response to ocean acidification in a reef fish. Nature Clim Change doi:10.1038/nclimate3087.