Relationships Between Behavioral and Physiological Performance Under Elevated CO_2 in Marine Fishes

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Statement on the Contribution of Others

This thesis includes collaborative work conducted with my supervisors, Prof. Philip Munday, Dr. Jodie Rummer, and Prof. Mark McCormick, as well as Michael Jarrold, Dr. Simon Nicol, Dr. Darren Parsons, Stephen Pether, Stephen Pope, and Neville Smith. 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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Declaration of Ethics

The research presented in this thesis was conducted in accordance with the national Health and Medical Research Council (NHMRC) Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 8th Edition (2013) and the Queensland Animal Care and Protection Act (2001). The research received and was conducted under the animal ethics approval from the JCU Animal Ethic Committee Approval numbers A2197, A2357, and A2210.

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General Abstract

Over the past decade there has been a concerted effort to determine how ocean acidification will affect a range of fitness-related traits in marine fishes, with studies often finding negative impacts on either behavioral or physiological performance. Until recently, most studies have focused on the mean responses of the sampled populations to ocean acidification. However, there is a growing recognition of the value in examining individual variation in responses. This can highlight individuals that are best suited to survival in future conditions. Identifying these individuals, however, can be challenging, because performance is not always consistent across all traits. Indeed, correlations can exist between traits that could either help or hinder survival at the individual level, and even affect the ability of marine fishes to adapt to ocean acidification. For instance, if two traits are negatively correlated with respect to the fitness landscape, then selection on one trait will diminish the other, slowing the rate of adaptation, and vice versa. Thus, identifying correlations among key traits is a crucial step towards understanding the potential of marine species to adapt to future climatic conditions. This thesis seeks to identify such correlations by examining the relationship between behavioral and physiological performance in marine fishes and determining how environmental conditions and parental effects might alter this relationship.

Theory predicts that environmental stressors can alter relationships between behavioral and physiological traits, either revealing or masking significant relationships. While both ocean acidification and warming have been found to affect behavioral and physiological performance in marine fishes, they can often interact in complex, non-additive ways, making it difficult to predict their combined impacts on marine fishes. Therefore, in **Chapter 2** I explored the relationship between behavioral and physiological performance in a juvenile reef fish, *Acanthochromis polyacanthus*, reared in a full crossed design of current-day control and predicted future ocean CO_2 and temperature levels. Behaviorally, elevated CO_2, but not elevated temperature, disrupted the fish's response to an alarm odor. Physiologically, aerobic scope was diminished under elevated temperature, but not elevated CO_2. A significant negative correlation was observed between these behavioral and physiological traits in the

combined elevated CO_2 and temperature treatment. These results suggest that correlations between behavior and physiology may only be evident when fish are exposed to multiple stressors. Importantly, the negative correlation between these traits could slow the rate of adaptation to climate change.

Chapter 2 revealed a negative correlation between behavioral and physiological performance in a coral reef fish, but this relationship might not hold for other fishes. It has been hypothesized that different sensitivities of marine fishes to elevated CO_2 may derive from their life styles and the variation in seawater pCO_2 they naturally experience. For example, pelagic fishes could be more susceptible to elevated CO_2 than coral reef fishes due to the relatively stable CO_2 conditions they experience in the open ocean. Therefore, in **Chapter 3** I tested the relationship between behavioral and physiological performance in a large pelagic fish, the yellowtail kingfish *Seriola lalandi*, in a full crossed experimental design of current-day control and predicted future CO_2 and temperature levels. In contrast to the juvenile reef fish, larval kingfish exhibited no behavioral changes in elevated CO_2 conditions. They did, however, exhibit increased resting oxygen uptake ($\dot{M}O_{2Rest}$) at elevated CO_2, and also at higher temperature. Correlations between behavioral and physiological performance were observed, which were inversely related based on the temperature treatment; $\dot{M}O_{2Rest}$ and boldness were negatively correlated at ambient temperature, but positively correlated at elevated temperature. These results show that higher water temperature can alter the relationship between behavioral and physiological performance, potentially altering the direction and pace of adaptation.

Most ocean acidification experiments to date have employed elevated CO_2 treatments that are stable through time. Yet shallow-water habitats such as coral reefs can experience substantial diel cycles in CO_2, and the magnitude of these cycles is predicted to increase as the buffering capacity of the oceans decreases. Diel CO_2 cycles have been shown to reduce the negative effects of elevated CO_2 on behavioral traits in marine fishes, but their effect on physiological traits remains unknown. Nor is it known if diel CO_2 cycles will interact with elevated temperature, or how they might affect relationships between behavioral and physiological performance. In **Chapter 4,** I compared physiological performance of juvenile *A. polyacanthus* under stable elevated CO_2 (1000 μatm) to a diel-cycling elevated CO_2 treatment (1000 ± 500

μatm) at both current-day control and elevated (+2 °C) temperatures. The $\dot{M}O_{2Rest}$ of fish reared at stable elevated CO_2 was higher than that of fish reared in control conditions. By contrast, $\dot{M}O_{2Rest}$ of fish in the diel-cycling elevated CO_2 treatment was comparable to controls, suggesting that diel CO_2 cycles mitigated the negative effect of elevated CO_2. This mitigating effect was not observed at elevated temperature. In the stable elevated CO_2 and temperature treatment, a positive correlation was observed between $\dot{M}O_{2Rest}$ and routine activity. However, as *A. polyacanthus* will likely be subjected to fluctuating, rather than stable, elevated CO_2 in the future, this correlation will not likely influence selection or adaptation. Furthermore, because these fish live in shallow environments exposed to warming, they may not benefit from the mitigating effects of diel CO_2 cycles. These findings highlight the importance of considering the habitats that fishes experience when designing experimental treatments to test the effects of elevated CO_2.

Parental effects can modify the performance of offspring in elevated CO_2, yet it is unknown if they also alter the relationship between behavioral and physiological performance in marine fishes. In **Chapter 5**, I exposed adult pairs of *A. polyacanthus* to either current-day control or elevated CO_2 conditions. I split their offspring equally between control and elevated CO_2 conditions and measured their behavioral and physiological performance (response to an alarm odor for behavior, and aerobic scope for physiology). Offspring exposed to elevated CO_2 displayed an impaired response to alarm odors, regardless of their parental treatment. However, maximal oxygen uptake rates ($\dot{M}O_{2Max}$) were higher in offspring with CO_2-exposed parents, regardless of offspring treatment, and $\dot{M}O_{2Rest}$ and aerobic scope showed significant differences between some treatments. These results demonstrate that parental effects can ameliorate some negative effects of ocean acidification, but not others. There were no correlations observed between behavioral and physiological performance. This result, along with Chapters 2 and 4, suggests that relationships between traits may only arise when fish are exposed to both warmer and more acidic conditions.

This research is among the first to examine the relationship between behavioral and physiological performance of marine fishes in a climate change context. The results demonstrate that correlations between behavioral and physiological performance do exist, but can shift depending on complex interactions between stressors, traits, and

parental effects. Importantly, significant correlations between behavior and physiology were only observed under elevated CO_2 and temperature conditions, which supports the hypothesis that tradeoffs between behavior and physiology can be strengthened under environmental stress. These relationships are important because they have the potential to alter the direction and pace of future adaptation to climate change. Future studies could investigate the causal mechanisms for these relationships and extend this research beyond marine fishes to examine changes to adaptation rates in short-lived organisms. This research underscores the importance of looking beyond the mean to understand individual variation and relationships between different types of performance in order to predict the effects of climate change on marine ecosystems.

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Chapter 1: General Introduction

The scientific advances and technological innovations of the Industrial Revolution ushered in an era of agricultural and economic growth for much of the world. Yet this prosperity came at a cost, cementing the world's dependence upon fossil fuels and dramatically altering the natural environment. Atmospheric concentrations of carbon dioxide have risen from ~280 ppm before the Industrial Revolution to over 400 ppm in 2018 (Dlugokencky & Tans, 2018), the highest concentration in at least the last 800,000 years (Lüthi *et al.*, 2008). These concentrations would be even higher if not for the oceans, which buffer changes to the atmosphere by absorbing approximately one third of anthropogenic CO_2 emissions (Sabine *et al.*, 2004; Zeebe *et al.*, 2008). However, this excess CO_2 changes the chemistry of the water, increasing the concentration of hydrogen and bicarbonate ions and decreasing the concentration of carbonate ions, leading to an overall decrease in oceanic pH through a process known as ocean acidification (Caldeira & Wickett, 2003; Sabine *et al.*, 2004; Doney *et al.*, 2009). Average oceanic pH has already declined by 0.1 pH units since the start of the Industrial Revolution (Rhein *et al.*, 2013), and it is predicted to worsen. If emissions continue at the same rate, oceanic pCO_2 could reach nearly 1,000 µatm by the end of the century (Collins *et al.*, 2013).

As the global scientific community became increasingly aware of ocean acidification in the 1990s and early 2000s, researchers set out to investigate how this changing ocean chemistry could impact marine organisms. Many of these studies focused on calcifying organisms, such corals, mollusks, and coccolithophores (Doney *et al.*, 2009), as the depletion of carbonate ions hinders their ability to form calcium carbonate skeletons and shells (Sabine *et al.*, 2004). However, further research revealed that a wide variety of marine taxa could be negatively affected by ocean acidification (Sabine *et al.*, 2004; Hoegh-Guldberg *et al.*, 2007; Fabry *et al.*, 2008; Doney *et al.*, 2009). For instance, a meta-analysis of 228 studies on marine organisms found decreases in survival, calcification, growth, development, and abundance under ocean acidification conditions (Kroeker *et al.*, 2013). These studies indicate that

ocean acidification threatens the well-being of marine organisms, with negative implications for ecosystem health and function.

Among the studies that have examined the effects of ocean acidification on performance in marine organisms, most have focused on average responses. However, there are advantages to exploring beyond mean responses and investigating the variation in performance between individuals within a species or population. Focusing on variation can reveal whether certain individual are better suited to survival in future conditions than others (Bennett, 1987). Furthermore, examining multiple traits in the same individuals can reveal correlated phenotypic traits (Bennett, 1987; Sunday *et al.*, 2014). Correlations between traits can affect whole-organism performance, helping or hindering survival (Bennett, 1987). Correlated traits also have the potential to affect the capacity of organisms to adapt to adverse environmental conditions like ocean acidification (Munday *et al.*, 2013; Sunday *et al.*, 2014). If traits are heritable, then correlations between them could speed or slow the rate of adaptation, depending on whether the traits are positively or negatively correlated with respect to the fitness landscape (Sunday *et al.*, 2014). Therefore, identifying correlations among key traits is a crucial step towards predicting species persistence in the face of climate change (McBryan *et al.*, 2013; Munday *et al.*, 2013; Sunday *et al.*, 2014).

Effects of Ocean Acidification on Marine Fishes

Initially, it was thought that fishes would be robust to the changes in ocean chemistry associated with acidification compared to calcifying organisms. Fish skeletons are primarily composed of calcium phosphate, not calcium carbonate, and are therefore not directly susceptible to changes in oceanic carbon chemistry (Toppe *et al.*, 2007). Furthermore, it is well known that fishes have a sophisticated internal acid-base regulatory system, and thus it was long assumed that they would unaffected by increasing water pCO_2. When marine fishes are exposed to elevated CO_2 conditions, excess CO_2 accumulates in the blood and other tissues, resulting in a respiratory acidosis (Claiborne *et al.*, 2002). Air-breathing animals can usually compensate for this acidosis through respiratory adjustments (e.g. hyperventilation), but this strategy is ineffective for water-breathers like fishes because the gradient in pCO_2 between their blood and the external environment is much lower than in air-breathers (Heuer &

Grosell, 2014). Rather, fishes primarily compensate for acidosis by accumulating HCO_3^- ions and secreting H^+ ions (Claiborne *et al.*, 2002; Heuer & Grosell, 2014; Esbaugh, 2017). This compensation occurs at time-scales of hours to days, and persists even with continuous exposure to hypercapnic conditions in excess of 10,000 µatm (Heuer & Grosell, 2014). It was this rapid, sustained acid-base compensation that initially led researchers to assume that marine fishes would have broad tolerance to ocean acidification (Pörtner, 2008; Melzner *et al.*, 2009; Kroeker *et al.*, 2010).

However, later work revealed that marine fishes can experience a wide range of physiological and behavioral disturbances at pCO_2 levels between 600-1000 µatm (Heuer & Grosell, 2014; Clements & Hunt, 2015; Nagelkerken & Munday, 2016; Cattano *et al.*, 2018), indicating that even though acid-base compensation restores blood pH, it may come with associated costs. Behavioral perturbations have been consistently observed across a range of sensory systems (Kelley *et al.*, 2018), including olfactory (Munday *et al.*, 2009a; Leduc *et al.*, 2013; Ou *et al.*, 2015; Porteus *et al.*, 2018), auditory (Simpson *et al.*, 2011; Castro *et al.*, 2017), and visual (Forsgren *et al.*, 2013; Chung *et al.*, 2014) discrimination. Fishes have also exhibited impaired general cognitive function, which affects activity levels and boldness (Munday *et al.*, 2010; Ferrari *et al.*, 2011a; Jutfelt *et al.*, 2013; Hamilton *et al.*, 2014), lateralization (Domenici *et al.*, 2012; Jutfelt *et al.*, 2013; Lopes *et al.*, 2016; Schmidt *et al.*, 2017), and learning ability (Ferrari *et al.*, 2012; Chivers *et al.*, 2014). These sensory and cognitive impairments can affect important ecological processes such as predator-prey interactions (Ferrari *et al.*, 2011b; Allan *et al.*, 2013), competition (McCormick *et al.*, 2013), dispersal (Munday *et al.*, 2009a), and settlement (Devine *et al.*, 2012). A handful of studies have even demonstrated that fishes exposed to elevated CO_2 in laboratory conditions have increased mortality rates in the field (Munday *et al.*, 2010; Ferrari *et al.*, 2011a; Chivers *et al.*, 2014). Together, these effects on ecological processes suggest that behavioral impairments can have far-reaching influences on patterns of recruitment and population sustainability (Ferrari *et al.*, 2011a), as well as shifts in community structure and ecological function (Nagelkerken & Munday, 2016).

Given the breadth of neurological disturbances produced by elevated CO_2 exposure, it was hypothesized that CO_2 affects central neural processing rather than individual

sensory systems (Heuer & Grosell, 2014). Nilsson and colleagues (2012) proposed that these disturbances stem from interference with $GABA_A$ neuroreceptor function. The $GABA_A$ receptor is the primary inhibitory neuroreceptor in the vertebrate brain. Under control conditions, the inflow of Cl^- and HCO_3^- ions causes hyperpolarization and inhibition of the neuron. However, under elevated CO_2 conditions, the concentrations of HCO_3^- and Cl^- change to restore blood pH. This could reverse the flow of ions across the $GABA_A$ receptors, causing depolarization and excitation of the neuron, and thus the cascading behavioral abnormalities observed at elevated CO_2. This hypothesis was supported by experiments in which gabazine, a $GABA_A$ antagonist, reversed the behavioral changes associated with elevated CO_2 (Nilsson *et al.*, 2012; Chivers *et al.*, 2014; Hamilton *et al.*, 2014; Lai *et al.*, 2015; Lopes *et al.*, 2016; Regan *et al.*, 2016). Further work by Heuer (2016) measured brain ion gradients in control and CO_2-exposed fish, and found that behaviorally-impaired CO_2-exposed fish had increased intra- and extra-cellular HCO_3^- ion concentrations, providing further evidence for CO_2 compensation. Recently, Porteus *et al.* (2018) have augmented the $GABA_A$ hypothesis, finding that the olfactory system itself experiences changes under elevated CO_2. By using electrophysiological recordings of peripheral olfactory nerves, they were able to isolate changes to the olfactory system from changes to central brain processing. They found that exposure to elevated CO_2 reduced the magnitude of nerve activity, and required higher doses of olfactory cues to produce a response. This result, combined with prior work on $GABA_A$ receptors, suggests that both central neural processing and sensory function contribute to the behavioral impairments observed in marine fishes under elevated CO_2 conditions.

The increases in acid-base regulatory processes required to maintain internal pH under elevated CO_2 conditions could be energetically costly, resulting in shifts to energy budgets or metabolic traits (Ishimatsu *et al.*, 2008; Strobel *et al.*, 2012). Indeed, metabolic traits, as approximated by oxygen uptake rates, have been a primary focus of elevated CO_2 experiments due to their association with fitness-related traits (Pörtner & Knust, 2007; Farrell *et al.*, 2008; Pörtner & Peck, 2010). Aerobic scope represents the total aerobic capacity available to an organism after accounting for basic maintenance, and is calculated as the difference between resting and maximal oxygen uptake rates. Aerobic scope is often regarded as an indicator of individual performance, as it links to a fish's ability to perform essential activities

such as activity, growth, development, and reproduction (Pörtner & Farrell, 2008; Pörtner & Peck, 2010). It has been hypothesized that elevated CO_2 could reduce aerobic scope via increases to resting oxygen uptake rates, as constant acid-base regulation demands more energy at rest (Pörtner & Farrell, 2008). However, studies to date show that elevated CO_2 has mixed effects on aerobic scope, and can increase, decrease, or have no effect on aerobic scope in a range of marine fishes (Lefevre, 2016; Cattano *et al.*, 2018; Hannan & Rummer, 2018). Similarly, other physiological metrics such as growth, mortality, and reproduction have exhibited highly variable responses to elevated CO_2 conditions across species (Heuer & Grosell, 2014; Cattano *et al.*, 2018). These varied results suggest that the physiological cost of acid-base regulation may differ between species, and that some species may have adapted to these conditions.

Ocean Warming and Acidification

While ocean acidification represents a significant threat to marine ecosystems, it will not occur in isolation. Rising atmospheric CO_2 concentrations are also causing the oceans to become warmer, as the oceans stores 90% of the excess heat that accumulates in our climate system due to climate change (Collins *et al.*, 2013). If emissions continue unchecked, average sea surface temperatures are projected to increase by between 2-3 °C by the end of the century (Collins *et al.*, 2013).

Like most marine organisms, fish are ectotherms, which means their body temperature is strongly influenced by the external environment. Consequently, individual performance is tightly linked to environmental temperature in fishes, because temperature is a major determinant of physiological processes such as metabolism (Farrell, 2002), growth (Atkinson, 1994), cardiac output (Eliason *et al.*, 2011), and muscle development (Hanel *et al.*, 1996). The mechanisms that underpin these changes are complex, and include whole-animal processes like balancing ATP generation with consumption, as well as cellular-level processes such as the temperature-dependent speed of chemical reactions and enzyme efficiencies (Cossins & Bowler, 1987). Additionally, elevated temperatures increase the energetic cost of maintaining cellular function and thus the basic cost of living under warmer conditions (Brett, 1971; Clarke & Johnston, 1999).

Metabolic traits have been a particular focus in efforts to understand how climate change will impact marine fishes, because it has been hypothesized that the capacity for oxygen uptake, transport and delivery at higher temperatures can constrain the performance of marine species (Pörtner & Knust, 2007; Pörtner & Farrell, 2008). Thermal performance curves are commonly used to describe the effect of temperature on oxygen consumption, as a proxy for metabolic rates (Schulte *et al.*, 2011). These curves generally begin at a species' minimum critical temperature, increase exponentially with temperature, peak at the thermal optimum, and then decrease rapidly towards the maximum critical temperature. However, the shape of these curves can vary between species (Schulte *et al.*, 2011). For instance, while resting oxygen uptake rates ($\dot{M}O_{2Rest}$) generally increase exponentially with temperature in marine fishes (Clarke & Johnston, 1999), maximal oxygen uptake rates ($\dot{M}O_{2Max}$) can show greater variation. In some species, $\dot{M}O_{2Max}$ reaches its optimum at a lower temperature than $\dot{M}O_{2Rest}$, resulting in a decline in aerobic scope (Schulte, 2015). However, other species exhibit $\dot{M}O_{2Rest}$ and $\dot{M}O_{2Max}$ that both increase exponentially with temperature, resulting in a broader aerobic scope curve (Gräns *et al.*, 2014; Norin *et al.*, 2014). Understanding the mechanistic underpinnings of these curves is a complex undertaking, given that processes like adaptation and acclimation can act separately on $\dot{M}O_{2Rest}$ and $\dot{M}O_{2Max}$, altering the shape of aerobic scope curves between species (Schulte, 2015). Still, these curves are useful for describing how aerobic performance in marine fishes responds to elevated temperature. For instance, many tropical marine fishes have shown decreased aerobic scope with only small temperature increases, indicating that they are likely living close to their thermal optima (Nilsson *et al.*, 2009; Johansen & Jones, 2011; Rummer *et al.*, 2014; Habary *et al.*, 2017). This pattern suggests that the warming associated with climate change could have negative consequences for reef fish populations (Pörtner & Peck, 2010).

In addition to the physiological changes that result from increasing temperatures, marine fishes have also shown changes in some behavioral traits. Indeed, these behavioral changes are not unexpected, given that physiological and biochemical mechanisms underpin behavioral responses. Warming has been shown to affect behaviors such as activity (Biro *et al.*, 2010; Johansen *et al.*, 2014; Davis *et al.*, 2017; Schmidt *et al.*, 2017), boldness (Biro *et al.*, 2010; Davis *et al.*, 2017), predator-prey

interactions (Grigaltchik *et al.*, 2012; Allan *et al.*, 2015), and risk assessment (Lienart *et al.*, 2014) in marine fishes. The ecological implications of these behavioral changes are nuanced, and dependent on other environmental factors such as predator abundance and food availability. For example, while higher levels of activity can increase foraging success (O'Brien, 1979), increased activity and boldness as well as impaired risk assessment can make fish more susceptible to predation (Lima & Dill, 1990; Werner & Anholt, 1993; Biro *et al.*, 2003a). Still, it is clear that elevated temperature can affect both behavioral and physiological performance in a variety of marine fishes.

While great efforts have been dedicated to determining the effects of both elevated CO_2 and temperature on marine organisms, most studies have focused on a single stressor at a time (Kroeker *et al.*, 2013; Riebesell & Gattuso, 2015). However, researchers are increasingly calling for experiments that incorporate multiple climate change stressors into their treatments. Not only do multifactorial experiments more accurately reflect future conditions, but they can also reveal non-additive interactions between stressors on performance (McBryan *et al.*, 2013; Todgham & Stillman, 2013; Riebesell & Gattuso, 2015). Thus, extrapolations based on single stressor responses could lead to inaccurate predictions about organismal responses to future climate conditions (McBryan *et al.*, 2013; Todgham & Stillman, 2013; Gaylord *et al.*, 2015).

Elevated CO_2 and temperature have been shown to interact both synergistically and antagonistically on performance in marine fishes, with great variation between species and traits (Kroeker *et al.*, 2013; Cattano *et al.*, 2018). For instance, while a meta-analysis revealed that elevated temperature tends to have a greater effect than elevated CO_2 on resting oxygen uptake rates in marine fishes (Lefevre, 2016), both synergistic (Enzor *et al.*, 2013) and antagonistic (Munday *et al.*, 2009b) interactions have been observed. Similar interactions have been demonstrated in behavioral traits. For instance, elevated temperature reduced the effect of elevated CO_2 on lateralization in damselfish *Pomacentrus wardi* (Domenici *et al.*, 2014), and elevated CO_2 reversed the effect of elevated temperature on food consumption and foraging activity in anemonefish *Amphiprion melanopus* (Nowicki *et al.*, 2012). However, temperature dominated the effect of elevated CO_2 on prey escape responses in damselfish *Pomacentrus wardi* (Allan *et al.*, 2017), demonstrating that interactions do not always

occur. Interestingly, the interactions between stressors can vary within a species, depending on the treatment or the trait being measured. For example, elevated CO_2 and temperature had different interacting effects on behavioral traits of dottyback *Pseudochromis fuscus*, interacting antagonistically on predator selectivity, but synergistically on predation rate (Ferrari *et al.*, 2015). A similar shift was observed in the physiological performance of two species of cardinalfishes, where elevated CO_2 and temperature acted antagonistically on aerobic scope at a moderately elevated temperature, but became additive at a high temperature (Munday *et al.*, 2009b). Given the complexity and unpredictable nature of interactions that have been observed, it will be necessary to conduct multi-stressor experiments to best predict the impacts of future climate change on performance in marine organisms.

Correlations Between Behavior and Physiology

While there have been extensive investigations into the mean effects of elevated CO_2 and temperature on marine fishes, the variation in responses between individuals has usually been overlooked (Pistevos *et al.*, 2011; Sunday *et al.*, 2011). This oversight is not unique to climate change studies; in 1987, Bennett (1987) outlined how the field of physiology had fallen prey to the “tyranny of the Golden Mean”, treating individual variation as noise or measurement errors rather than useful information. Since then, there has been a renewed effort to describe patterns of individual variation in animal species (Biro & Stamps, 2010; Sih *et al.*, 2015), but rarely has this effort extended to climate change studies. Considering individual variation in responses can improve predictions for future scenarios, particularly if previous predictions were based on average population responses (Dhillon & Schulte, 2011; Pistevos *et al.*, 2011). Individual variation can become even more useful when variation in multiple traits is considered. Both behavioral (Sih *et al.*, 2004; Roche *et al.*, 2016) and physiological traits (Burton *et al.*, 2011) display broad and consistent variation between individuals, and there has been a recent push to describe patterns of covariation between behavioral and physiological traits to better understand the mechanisms underpinning these relationships (Careau *et al.*, 2008; Davis *et al.*, 2017, 2018; Biro *et al.*, 2018). In the context of climate change, correlations between behavioral and physiological performance can help or hinder survival at the individual

level, and even have implications for the ability of organisms to adapt to climate change (Munday *et al.*, 2013; Sunday *et al.*, 2014).

It has long been known that heritable phenotypic variation is the raw material on which selection acts to shape evolutionary trajectories (Darwin, 1859). Therefore, to predict the potential for species to adapt to climate change, researchers looks for heritable phenotypic variation in traits, as well as whether selection acts on this variation (Falconer & Mackay, 1996). In marine fishes, there is evidence for significant heritability in fitness-related traits that are influenced by elevated temperatures (Muñoz *et al.*, 2015; Munday *et al.*, 2017) and elevated CO_2 (Malvezzi *et al.*, 2015; Welch & Munday, 2017; Tasoff & Johnson, 2018). Together, these studies on marine fishes suggest that for certain traits, there is considerable adaptive potential to elevated CO_2 and temperature conditions. However, correlations between traits can limit adaptive potential (Kelly & Hofmann, 2013; Munday *et al.*, 2013; Sunday *et al.*, 2014). If two traits are positively correlated, then selection will be able to act unimpeded on the population, as selection is acting in the same direction as the most variation in the population (Munday *et al.*, 2013, Figure 1.1A). However, if the traits display a negative correlation, then selection will be acting orthogonally to the direction of the most variation in the population (Figure 1.1B). As a result, selection will likely be limited in its ability to act on this population, as selection for improvement of either trait will decrease performance in the other (Sunday *et al.*, 2014). Describing this relationship between traits can therefore aid in predicting whether selection can act freely on a population, or will be constrained, limiting species' ability to adapt to future conditions. Thus, identifying correlations among key traits is an important step in predicting species persistence in the face of climate change (Kelly & Hofmann, 2013; Munday *et al.*, 2013; Sunday *et al.*, 2014; Healy *et al.*, 2018).

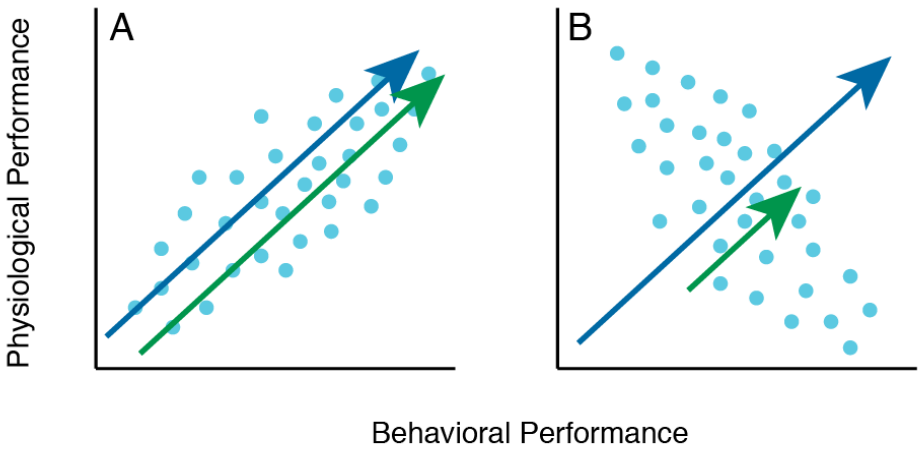

Figure 1.1. Schematic representation of two possible relationships between behavioral and physiological traits, and how the relationship would be predicted to affect the population-level response to selection. The blue arrows show the direction and magnitude of selection acting on the mean of the population, and the green arrows show the direction and magnitude of the response to selection. In (A), there is unimpeded evolution because selection acts in the same direction as most of the variation in the trait. In (B), the evolutionary response is minimal because selection acts orthogonally to the direction of the most variation in the trait. Adapted from Munday *et al.* (2013).

This thesis focuses on the relationship between behavioral and physiological performance, as these traits are essential for individual survival and have been observed to co-vary in an array of taxa (Careau *et al.*, 2008; Biro & Stamps, 2010; Davis *et al.*, 2017, 2018). Importantly, environmental stressors have been shown to alter the relationship between behavioral and physiological traits (Killen *et al.*, 2013). In particular, it has been hypothesized that moderate stressors act to reveal or amplify relationships between behavior and physiology, while severe stressors mask or dampen these relationships (Killen *et al.*, 2013). For instance, a positive correlation was observed between resting metabolic rate and risk-taking behavior in juvenile European sea bass *Dicentrarchus labrax* when fish were exposed to hypoxia, but this correlation was not observed under control conditions (Killen *et al.*, 2012). While the mechanism underpinning these changes to correlations under different environmental stressors is not known, it has been suggested that stressors can amplify the intraspecific phenotypic variation in a population due to differing sensitivities

between individuals (Stearns *et al.*, 1991; Hoffmann & Merilä, 1999; Hoffmann & Hercus, 2000; Sgrò & Hoffmann, 2004). Given that correlations between behavior and physiology can shift between environments and have the potential to limit adaptive capacity, measurements of correlations under control conditions may not be representative of correlations under stressful conditions. This could lead researchers to misestimate the capacity for organisms to adapt to climate change conditions. Thus, it will be important to examine relationships between behavior and physiology under multiple climate change-relevant scenarios.

Susceptibility of Pelagic Fishes

Interestingly, while acid-base compensation is a consistent response of virtually all marine fishes to elevated pCO_2, fishes display a wide variation in behavioral and physiological responses to elevated CO_2 across species (Heuer & Grosell, 2014; Cattano *et al.*, 2018). It has been suggested that this variation might be attributed to differential lifestyles, which can either confer tolerance or impose disadvantages on responses to elevated CO_2 (Melzner *et al.*, 2009; Kroeker *et al.*, 2013; Cattano *et al.*, 2018). For instance, the effects of elevated CO_2 can differ between habitats. The open ocean experiences relatively stable pCO_2 through time (Doney *et al.*, 2009). By contrast, coastal and shallow-water marine ecosystems can experience changes to pCO_2 over a variety of spatial and temporal scales (Duarte *et al.*, 2013). Therefore, it has been hypothesized that organisms living in the pelagic environment might be more susceptible to increasing CO_2 levels than coastal species, as pelagic species evolved in a relatively more stable CO_2 environment (Munday *et al.*, 2008a; Pörtner, 2008). A recent meta-analysis of 320 contrasts of 42 marine fish species supports this hypothesis, showing that under elevated CO_2, benthic and benthopelagic species had lower mortality rates and unaffected growth rates, while pelagic species exhibited increased mortality rates and decreased growth rates (Cattano *et al.*, 2018). Unfortunately, due to insufficient data on the responses of pelagic species, similar comparisons of behavioral and metabolic traits cannot be performed (Cattano *et al.*, 2018). Therefore, more ocean acidification experiments on pelagic species are needed to better understand patterns of tolerance and susceptibility to elevated CO_2.

Many pelagic fishes are active, rapid swimmers with high metabolic and growth rates compared to benthic species (Dickson, 1995). Continuous swimming and fast growth rates are energetically demanding, and so many pelagic fishes have evolved mechanisms for efficient and rapid swimming (Dickson, 1995; Brill, 1996). For instance, pelagic fishes generally have a higher proportion of slow-twitch red muscle to fast-twitch white muscle, allowing them to swim for prolonged periods of time (Boddeke *et al.*, 1959; Videler, 1993). Additionally, large pelagic fishes such as tunas have proportionally larger hearts that increase blood flow and dense capillary structures that increase oxygen delivery to the tissues (Korsmeyer & Dewar, 2001). These high physiological demands and resulting adaptations that have improved swimming performance for pelagic fishes may play a role in the diverging responses of pelagic versus benthic species to elevated CO_2 conditions. Only a handful of studies have examined large pelagic fishes' behavioral and physiological responses to elevated CO_2 (e.g. Bignami *et al.*, 2013, 2014, 2016; Pimentel *et al.*, 2014, 2016; Munday *et al.*, 2015), and thus further research is needed to develop this hypothesis. Importantly, given the vastly different physiologies of pelagic versus benthic fishes, we could expect that these fishes exhibit different relationships between behavior and physiology under both control and elevated CO_2 and temperature conditions. This, in turn, could affect their adaptive potential under future climatic conditions. Therefore, it will be critical to examine differences between active pelagic fishes and sedentary benthic fishes in response to elevated CO_2 and temperature to better understand patterns of sensitivity to these stressors, as well as potential limitations to adaptation.

CO_2 Fluctuations

Most ocean acidification experiments to date have employed control and elevated CO_2 treatments that are stable through time (Kroeker *et al.*, 2013; Heuer & Grosell, 2014; Cattano *et al.*, 2018). This is because projections for future ocean acidification are based on data collected from the open ocean, an environment that experiences relatively stable pCO_2 (Doney *et al.*, 2009). However, many shallow-water marine ecosystems experience natural fluctuations in pCO_2 on a variety of temporal scales (Duarte *et al.*, 2013). Thus, there is concern that these stable pCO_2 experiments may have limited relevance for shallow-water organisms (McElhany & Busch, 2013; Wahl *et al.*, 2016). On coral reefs, a particularly prominent pattern of CO_2 fluctuations is

diel CO_2 cycles (Shaw *et al.*, 2012). These cycles are primarily driven by the net consumption of CO_2 by photosynthesis during the day, and the net production of CO_2 by respiration at night (Falter *et al.*, 2013). Currently, coral reefs experience fluctuations in pCO_2 that range from ± 50 to 600 µatm around the mean, which in some cases can exceed average end-of-century CO_2 predictions (Kayanne *et al.*, 1995; Manzello, 2010; Shaw *et al.*, 2012; Albright *et al.*, 2013; Kline *et al.*, 2015). Furthermore, the magnitude of these fluctuations is predicted to increase over time, as the buffering capacity of the oceans decreases as more CO_2 is absorbed (Shaw *et al.*, 2012; McNeil & Sasse, 2016). Consequently, as ocean acidification progresses, shallow-water organisms will likely be exposed to more variability in pCO_2 conditions.

There has been a recent push for more experiments to incorporate CO_2 fluctuations into their treatments to better model future conditions and assess the likely impacts on shallow-water marine organisms (McElhany & Busch, 2013; Wahl *et al.*, 2016; Vargas *et al.*, 2017). A number of such studies have emerged in recent years, primarily focused on calcifying organisms. For example, three species of coral had improved performance in growth and calcification rates under diel CO_2 cycles compared to a stable elevated CO_2 (Dufault *et al.*, 2012; Comeau *et al.*, 2014; Chan & Eggins, 2017). However, results from other taxa are more varied. The mussels *Mytilus californianus* and *Mytilus galloprovincialis* showed fewer negative developmental effects when reared under variable pH conditions compared to stable pH conditions (Frieder *et al.*, 2014) and blue mussel *Mytilus edulis* exhibited improved calcification under variable pH conditions (Wahl *et al.*, 2018). However, *Mytilus edulis* was also found to have a higher metabolic rate, antioxidant enzyme activity, and lipid peroxidation under variable pH conditions compared to stable pH, indicating that variable pH conditions are energetically expensive for this species (Mangan *et al.*, 2017). Diel CO_2 cycles appear to have no effect on some species, as in two species of bivalves that had survival, growth, and development unaffected by diel CO_2 cycles (Clark & Gobler, 2016) and one species of barnacle that had unaffected growth rates in stable versus fluctuating pH (Eriander *et al.*, 2016). Thus, it appears that the effects of CO_2 fluctuations may be trait- or species- specific.

In fishes, relatively little is known about how CO_2 fluctuations affect performance at higher average CO_2 levels. Physiologically, both pink salmon *Oncorhynchus gorbuscha* and European eel *Anguilla anguilla* showed improved metabolic performance under diel CO_2 cycles compared to a stable elevated CO_2 (Methling *et al.*, 2013; Ou *et al.*, 2015). However, CO_2 cycles had no effect on growth, survival, or development in two coral reef fishes (Jarrold & Munday, 2018a). Behavioral studies have also shown mixed effects, with diel CO_2 cycles reducing or eliminating behavioral abnormalities that were observed under stable elevated CO_2 in two coral reef fishes (Jarrold *et al.*, 2017), but having no effect on behavioral responses of blacksmith *Chromis punctipinnis* (Kwan *et al.*, 2017). Importantly, many of the aforementioned studies employed cycling CO_2 treatments that had a different mean to the stable elevated CO_2 treatments, which could make it difficult to determine if the observed improvements and impairments were due to the fluctuations themselves or differing mean CO_2 treatments. Nevertheless, these studies suggest that diel CO_2 cycles can modify behavioral and physiological responses to elevated CO_2, but may have differing effects between species or traits. If diel CO_2 cycles could improve performance in some or all traits, this could change the distribution of phenotypic variance within a population, affecting the relationship between behavior and physiology. Thus, relationships between behavior and physiology should be compared between stable and diel-cycling CO_2 conditions to more accurately understand potential constraints on adaptation for shallow-water organisms.

Parental Effects and Ocean Acidification

Many of the ocean acidification experiments that have found negative effects of elevated CO_2 have focused on a single generation of organisms (Kroeker *et al.*, 2013; Calosi *et al.*, 2016). However, these studies risk over- or under-estimating the effects of ocean acidification, as the environment experienced by one generation has the potential to influence performance in later generations via parental effects (Salinas *et al.*, 2013; Munday, 2014; Donelson *et al.*, 2018). Parental effects differ from adaptive evolution in that they do not influence the phenotype of offspring through the selection of genotypes across generations; rather, parental effects encompass a variety of non-genetic mechanisms for phenotypic change in offspring. These mechanisms can include changes in nutritional provisioning of eggs and embryos as well as the

transmission of hormones, proteins, or other cytoplasmic factors (Mousseau & Fox, 1998). In some instances, mothers and fathers can also transmit epigenetic factors that alter gene expression in offspring (Bonduriansky *et al.*, 2012; Metzger & Schulte, 2016; Torda *et al.*, 2017). Importantly, through these mechanisms, parents can influence the performance of their offspring in environmental conditions that are either the same or different to those they experience (Marshall & Uller, 2007; Donelson *et al.*, 2010; Salinas *et al.*, 2013; Burgess & Marshall, 2014).

There is evidence that parental effects can partially or completely ameliorate the negative effects of elevated CO_2 on offspring in a wide range of marine organisms (Ross *et al.*, 2016; Donelson *et al.*, 2018). For instance, larval Sydney rock oyster *Saccostrea glomerata* had slower growth and development rates when they were reared under elevated CO_2 as compared to control conditions, but parental exposure to elevated CO_2 ameliorated these negative effects, resulting in larger and more developed larvae (Parker *et al.*, 2012). However, parental effects are not always beneficial for offspring (Uller, 2008; Burgess & Marshall, 2014; Kronholm & Collins, 2016). Parental exposure to stressful conditions can have negative impacts on offspring performance. For example, when adult sea urchin *Strongylocentrotus droebachiensis* were exposed to elevated CO_2 for 4 months, larval survival decreased (Dupont *et al.*, 2013). However, the duration of adult exposure can affect whether parental effects are helpful or detrimental to offspring performance. In the aforementioned study on sea urchins, 16 months of exposure eliminated the negative effect of elevated CO_2, restoring larval survival to control levels (Dupont *et al.*, 2013). Therefore, it is important to consider that parental effects can have both positive and negative outcomes for offspring, and furthermore that these effects can be context-dependent.

In marine fishes, there is some evidence that parental exposure to elevated CO_2 can ameliorate the negative effects of ocean acidification. Physiological traits in particular have shown a variety of positive parental effects. In Atlantic silverside *Menidia menidia*, parental exposure to elevated CO_2 ameliorated the negative effects of elevated CO_2 on growth and survival (Murray *et al.*, 2014). A similar study on cinnamon clownfish *Amphiprion melanopus* showed that parental effects mitigated the cost of elevated CO_2 on growth, survival, and resting oxygen uptake rate (Miller

et al., 2012). By contrast, behavioral traits have exhibited a greater variety of responses to parental exposure to elevated CO_2. Parental exposure to elevated CO_2 did not mitigate the negative effect of elevated CO_2 on antipredator responses to alarm odors in juvenile spiny damselfish *Acanthochromis polyacanthus* (Welch *et al.*, 2014), nor on reductions in feeding strikes in the presence of a predator odor in juvenile orange clownfish *Amphiprion percula* (McMahon *et al.*, 2018). However, kinematic responses to a startle stimulus in juvenile *A. melanopus* were partially restored when parents and offspring were both exposed to elevated CO_2 (Allan *et al.*, 2014). These disparate results suggest that effects of parental exposure to elevated CO_2 on offspring may differ between species or traits. Importantly, it is not currently known how parental effects might alter relationships between behavioral and physiological traits. Given that parental effects have had more consistently positive effects on physiological performance under elevated CO_2 in marine fishes than behavioral performance, this may result in shifts to physiological, but not behavioral performance at the population level when offspring and parents both experience elevated CO_2 conditions. This could change correlations between behavior and physiology, impacting individual performance maxima and even adaptive potential. Thus, incorporating parental effects will be essential to predicting the long-term implications of ocean acidification on marine fish performance.

Aims and Thesis Outline

This thesis investigates the relationship between behavioral and physiological performance under elevated CO_2 in marine fishes. While there is extensive literature examining average responses of organisms to elevated CO_2, correlations between traits have remained largely unexplored. Understanding these relationships may improve predictions about species persistence, reveal correlations that could hinder survival for individuals, and provide information on the factors that affect adaptive potential. To investigate this broad goal, I examined relationships between behavioral and physiological performance in marine fishes through four lenses, each of which contextualized the relationship within a broader theme.

In **Chapter 2**, I examined how the relationship between behavioral and physiological performance might change under multiple stressors, specifically elevated CO_2 and

temperature, in a coral reef fish. To this end, I reared spiny damselfish *Acanthochromis polyacanthus* from hatching to the juvenile stage under a full crossed design of elevated CO_2 and temperature representing projected future conditions for the year 2100 under a high-emissions scenario (RCP 8.5, Collins *et al.*, 2013). *A. polyacanthus* is a highly abundant coral reef fish with a wide geographic distribution in the western Pacific. The species is readily reared in captivity, which has made it a model species for climate change experiments (e.g. Donelson *et al.*, 2012; Welch *et al.*, 2014). In this study I measured the response of juvenile spiny damselfish to an alarm odor using a feeding assay, and aerobic scope using intermittent-flow respirometry. I also tracked individual fish through both assays to be able to describe the relationship between these traits under all four experimental treatments. I hypothesized that there would be no correlation between traits under control conditions, but there would be correlations under elevated CO_2, elevated temperature, or both, as previous work suggests that stressors can reveal or amplify relationships between behavioral and physiological traits (Killen *et al.*, 2013). The results of this chapter shed light on the relationship between behavioral and physiological performance, and how it can shift under different climate change stressors.

In **Chapter 3,** I examined a similar relationship in a species with very different lifestyle traits. Large pelagic fishes are highly active, with stronger demands on their aerobic physiology compared with relatively sedentary reef fishes. Pelagic fishes also live in the open ocean, which experiences relatively stable pCO_2, while shallow-water ecosystems like reefs can experience wide fluctuations in pCO_2. Therefore, in this chapter I examined the relationship between behavioral and physiological performance under elevated CO_2 and temperature in juvenile yellowtail kingfish *Seriola lalandi*, a large pelagic fish with a circumglobal distribution (Bray, 2018). As in Chapter 2, I reared fish under a full crossed design of elevated CO_2 and temperature. I measured behavioral performance using activity and boldness, and physiological performance using aerobic scope as well as resting and maximal oxygen uptake rates. I hypothesized that there would be correlations between behavior and physiology under elevated temperature, as this stressor could amplify a relationship between the traits. However, I also hypothesized that there would be a weak or nonexistent correlation between the traits under elevated CO_2, as it has been suggested that pelagic fishes are highly susceptible to elevated CO_2 because they

evolved in a relatively stable CO_2 environment (Munday *et al.*, 2008a; Pörtner, 2008), and theory suggests that severe stressors can mask or dampen relationships between behavior and physiology (Killen *et al.*, 2013). This study explores how relationships between behavioral and physiological performance might differ between relatively sedentary reef fishes and more active pelagic species.

Chapter 4 expanded upon Chapter 2 by examining how diel CO_2 cycles might modify the relationship between behavior and physiology. Reef fishes can experience daily fluctuations in CO_2 that have been shown to modify behavioral responses to elevated CO_2, but the effects of these diel CO_2 cycles on physiological performance are not known. Furthermore, diel CO_2 cycles may interact with elevated temperature in unpredictable ways and affect the relationship between behavioral and physiological performance. I reared juvenile spiny damselfish *A. polyacanthus* from hatching under five different stable and fluctuating CO_2 treatments crossed with current-day and elevated temperature. I quantified behavioral performance using measures of routine activity, and physiological performance using metabolic traits. I hypothesized that diel CO_2 cycles would weaken the relationship between behavior and physiology, because diel CO_2 cycles have been shown to ameliorate the negative effects of elevated CO_2 on a variety of behavioral and physiological traits (Methling *et al.*, 2013; Ou *et al.*, 2015; Jarrold *et al.*, 2017). This could reduce the variation in these traits, thereby weakening the relationship between them. The results of this chapter demonstrate how diel CO_2 cycles can modulate mean responses to elevated CO_2, as well as relationships between behavior and physiology.

Finally, in **Chapter 5** I considered how parental effects can modify fish behavioral and physiological performance, as well as correlations between these traits. Parental exposure to elevated CO_2 has shown mixed results in ameliorating the negative effects of CO_2 on marine fishes, and its effect on correlations between traits remains unexplored. Adult *A. polyacanthus* were exposed to elevated CO_2 or control conditions prior to the breeding season, and then offspring were evenly split between elevated CO_2 and control conditions. I measured behavioral and physiological performance using the same traits as in Chapter 2 (i.e. reduction in feeding strikes following an alarm odor and metabolic traits). Because parental effects have been shown to ameliorate negative effects on metabolic traits (Miller *et al.*, 2012) but not

response to alarm odors (Welch *et al.*, 2014; McMahon *et al.*, 2018), I hypothesized that parental exposure to elevated CO_2 might decrease the variation in metabolic traits, but not response to alarm odors, thereby shifting the relationship between behavior and physiology. This study highlights the importance of considering parental effects when determining how behavioral and physiological performance might be related.

Together, these four chapters advance our knowledge of the impacts of projected future CO_2 levels on behavioral and physiological performance, as well as correlations between these traits. Importantly, negative correlations between behavioral and physiological performance could decrease individual performance maxima and adaptive potential. Therefore, understanding these relationships will be crucial to accurately predicting how marine fishes will respond to a rapidly changing environment.

Chapter 2: A negative correlation between behavioral and physiological performance under ocean acidification and warming

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2.1 Summary

Many studies have examined the average effects of ocean acidification and warming on phenotypic traits of reef fishes, finding variable, but often negative effects on behavioral and physiological performance. Yet the presence and nature of a relationship between these traits is unknown. A negative relationship between phenotypic traits could limit individual performance and even the capacity of populations to adapt to climate change. Here, we examined the relationship between behavioral and physiological performance of a juvenile reef fish under elevated CO_2 and temperature in a full factorial design. Behaviorally, the response to an alarm odor was negatively affected by elevated CO_2, but not elevated temperature. Physiologically, aerobic scope was significantly diminished under elevated temperature, but not under elevated CO_2. At the individual level, there was no relationship between behavioral and physiological traits in the control and single-stressor treatments. However, a statistically significant negative relationship was detected between the traits in the combined elevated CO_2 and temperature treatment. Our results demonstrate that trade-offs in performance between behavioral and physiological traits may be revealed when multiple climate change stressors are considered, and suggest that this negative relationship could limit adaptive potential to climate change.

2.2 Introduction

A major goal of climate change research is to determine whether species will withstand environmental change. A wealth of studies has assessed biological responses to projected future conditions in a variety of taxa, providing an indicator of large-scale trends in response to climate change conditions (Pörtner & Farrell, 2008; Kroeker *et al.*, 2013; Wittmann & Pörtner, 2013). However, it can be advantageous to look beyond mean responses and examine the variation in performance between individuals within a species or population. Once disregarded and considered to be noise or error between measurements, individual variation is now recognized as a metric worth investigating in its own right (Careau *et al.*, 2008; Williams, 2008). In particular, a surge of interest has grown around variation in behavioral and physiological traits for their potential to identify patterns of trait covariation (Biro & Stamps, 2008; Careau *et al.*, 2008), determine the mechanistic underpinnings of these covariations (Williams, 2008; Biro & Stamps, 2010; Metcalfe *et al.*, 2016), and predict their evolutionary implications (Lande & Arnold, 1983; Careau & Garland, 2012).

The relationship between behavioral and physiological performance could be especially important in the context of climate change. Examining this type of variation can highlight the individuals that are best suited to survival in future climate conditions, as well as reveal correlations between types of performance that can either help or hinder survival at the individual level (Munday *et al.*, 2013). Furthermore, correlations between traits could have implications for the potential of organisms to adapt to climate change (McBryan *et al.*, 2013; Munday *et al.*, 2013). In particular, if the behavioral and physiological traits of interest are heritable, then correlations between them could either accelerate or decelerate adaptive evolution (Lande, 1979; Lande & Arnold, 1983). If the traits are negatively correlated, selection on one would diminish the other, decreasing the rate of adaptation, and vice versa. Thus, identifying correlations among key traits is an important step in predicting species persistence in the face of climate change (McBryan *et al.*, 2013; Munday *et al.*, 2013; Sunday *et al.*, 2014). Importantly, environmental stressors have been shown to alter the relationship between behavioral and physiological traits, either amplifying or masking significant correlations (Killen *et al.*, 2013). Therefore, to improve our understanding of the

effects of climate change on fish, it will be necessary to evaluate these relationships not only under current-day conditions, but also under different climate change-relevant scenarios.

Ocean acidification and warming are two of the primary environmental stressors in marine ecosystems (Doney *et al.*, 2009; Collins *et al.*, 2013). As both are driven by increasing carbon dioxide emissions, they will likely increase in tandem, forcing marine organisms to contend with both stressors simultaneously (Hoegh-Guldberg & Bruno, 2010; Doney *et al.*, 2012). The majority of climate change studies on marine organisms have focused on single stressors, although there has been an increasing recognition of the importance of a multi-stressor approach (Kroeker *et al.*, 2013; Riebesell & Gattuso, 2015). Multi-stressor experiments more accurately capture likely future scenarios, and can reveal unexpected interactions between stressors. For instance, ocean acidification and warming have been shown to interact both synergistically and antagonistically on the responses of marine organisms (Kroeker *et al.*, 2013; Riebesell & Gattuso, 2015). Thus, multi-stressor experiments are crucial, as single-stressor studies could lead to inaccurate predictions about organismal responses to future climate conditions (Gaylord *et al.*, 2015; Riebesell & Gattuso, 2015).

Reef fishes can be sensitive to the effects of both elevated CO_2 and temperature. Elevated CO_2 levels projected for the end of the century have been documented to impact a range of behavioral traits in reef fishes such as lateralization, activity, homing ability, learning, anxiety, and olfactory and auditory discrimination (reviewed by Clements & Hunt, 2015; Nagelkerken & Munday, 2016). These behavioral changes can make fishes more vulnerable to predation and have been linked to higher mortality rates (Munday *et al.*, 2010; Ferrari *et al.*, 2011b; Allan *et al.*, 2013). Elevated CO_2 has also been documented to affect physiological traits, such as aerobic scope (Munday *et al.*, 2009b; Rummer *et al.*, 2013a), escape performance (Allan *et al.*, 2013), and reproduction (Miller *et al.*, 2013; Welch & Munday, 2016) in some reef fishes, but the physiological effects of CO_2 are generally more variable across species than behavioral effects (Lefevre, 2016; Hannan & Rummer, 2018). By contrast, temperature is well known to impact fish physiological performance through its effect on rates of biochemical reactions (Fry, 1971). Elevated temperatures predicted for the end of the century have been shown to alter aerobic scope (Nilsson

et al., 2009; Rummer *et al.*, 2014; Habary *et al.*, 2017), body size (Munday *et al.*, 2008b), reproductive output (Donelson *et al.*, 2010; Pankhurst & Munday, 2011), and swimming ability (Johansen & Jones, 2011) in reef fishes. Behaviorally, both boldness and activity levels have been shown to change in response to temperature increases (Biro *et al.*, 2010; Johansen *et al.*, 2014; Lienart *et al.*, 2014).

In this study, we investigated the effects of elevated CO_2 and temperature on behavioral and physiological performance of juvenile spiny chromis, *Acanthochromis polyacanthus*. We used a full factorial design which consisted of a current-day ambient CO_2 level (~500 μatm) and average summer temperature for the study location (29 °C), crossed with elevated CO_2 (~1000 μatm) and temperature (32 °C) based on projections for the end of the century under RCP 8.5 (Meinshausen *et al.*, 2011; Collins *et al.*, 2013). The behavioral trait we measured was the percent reduction in feeding strikes after exposure to damage-released olfactory cues for a conspecific (i.e. alarm odors). Conspecifics display an innate aversion to these reliable indicators of predation risk, typically reducing activity and seeking shelter (Ferrari *et al.*, 2010). However, previous studies have found that when fishes are reared under elevated CO_2 levels, they no longer reduce their activity or stop feeding in the presence of these odors (Ferrari *et al.*, 2011a; Welch *et al.*, 2014). The inability to use alarm odors under elevated CO_2 conditions makes prey more vulnerable to predators and can lead to higher mortality at key life-history stages (Ferrari *et al.*, 2011a; Chivers *et al.*, 2014). The physiological trait we determined was aerobic scope. Aerobic scope refers to the total capacity for aerobic activity available to an organism, after accounting for basic maintenance (Fry, 1947, 1971), and is calculated as the difference between the maximal and resting oxygen uptake rates of an organism. Aerobic activities such as growth, reproduction, and swimming are essential life-history processes; therefore, a reduction in aerobic scope could reduce individual performance and fitness (Brett, 1964; Pörtner & Farrell, 2008; Pörtner & Peck, 2010). Both elevated CO_2 and elevated temperature have been predicted to decrease aerobic scope in fishes (Pörtner & Knust, 2007; Pörtner & Farrell, 2008). However, when tested, elevated temperature often decreases aerobic scope in coral reef fishes (Nilsson *et al.*, 2009; Rummer *et al.*, 2014), while elevated CO_2 has had more variable effects (Lefevre, 2016; Hannan & Rummer, 2018).

By tracking the responses of individual fish when tested for their behavioral and physiological performance, we were able to determine the relationship between these traits at the individual level. We then compared this relationship between all four of the treatment groups to determine how the relationship between these traits might change under different environmental stressors, and ultimately, how adaptation to climate change conditions might be either facilitated or hindered by this relationship.

2.3 Methods

Study species, Broodstock Collection and Maintenance

The spiny chromis, *Acanthochromis polyacanthus*, is a tropical damselfish from the western Pacific Ocean. The species has direct development (i.e. eggs hatch into juveniles), and both parents care for the eggs and offspring for up to 45 days post-hatch (Kavanagh, 2000). Without a pelagic larval phase, the species is easily bred in captivity. Due to its wide geographic distribution, abundance, and amenability to laboratory experimentation, *A. polyacanthus* has become a model species for studying the effects of climate change on coral reef fishes (e.g. Donelson *et al.*, 2010; Welch *et al.*, 2014).

Adult *A. polyacanthus* were collected using hand nets from the Bramble Reef area (site 1: 18°22’S, 146°40’E; site 2: 18°25’S, 146°40’E) on the Great Barrier Reef in July 2015. Physical and chemical water parameters from a similar mid-shelf reef in the central Great Barrier Reef can be seen in Appendix A. Fish were transported to James Cook University (Townsville, Australia), where they were sorted into breeding pairs and housed in 60 L aquaria. Pairs were provided with half a terracotta pot for shelter and as a suitable artificial breeding site. Aquaria were checked daily for the presence of newly laid clutches. Pairs were fed *ad libitum* on commercial fish feed pellets (INVE Aquaculture Nutrition NRD 12/20) once per day outside the breeding season (July–October) and twice per day during the breeding season (November–May). Starting in October, water temperatures were increased at a rate of 0.5 °C per week until the summer breeding temperature of 29 °C was reached during the first week of November 2015.

Offspring were fed freshly hatched *Artemia* spp. nauplii for the first two days post-hatch (dph), then a combination of *Artemia* spp. nauplii and weaning fish feed (INVE Aquaculture Nutrition Wean-S 200-400 µm) daily for the following three days. They were fed the weaning fish feed from 6-21 dph and then switched to a small pellet fish feed (INVE Aquaculture Nutrition NRD 5/8) at 22 dph.

Carbonate Chemistry

Water for this experiment was supplied via two 8000 L recirculating aquarium systems. The ambient pCO_2 level (~500 µatm) in one system was used as the current-day control. The other system was dosed with CO_2 to match the end-of-century projection for surface ocean pCO_2 under RCP 8.5 (~1000 µatm). The pCO_2 level was controlled by an Aqua Medic AT Control System (Aqua Medic, Germany), which dosed CO_2 into a 3000 L sump connected to the system whenever the pH in the system rose above the set point. An identical 3000 L sump on the current-day control was not dosed with additional CO_2. Temperature was maintained at a current-day control of 29 °C by circulating seawater through a SolarWise heater/chiller (Brisbane, Queensland, Australia) on each system. The equilibrated seawater from each system was then either delivered directly into the aquaria, or passed over Toyesi 2.5-kW inline heaters (Prospect, New South Wales, Australia) to raise the temperature to the elevated treatment of 32 °C. Water was delivered into fish aquaria at a rate of 1.5 L min^{-1} in a temperature-controlled room.

The pH_{NBS} and temperature for each system were recorded daily using a pH electrode (SevenGo Pro, Mettler Toledo, Switzerland) and temperature probe (Cormark C26, Norfolk, UK). The pH_T was measured weekly with a spectrophotometer following standard operating procedures (Dickson *et al.*, 2007) using the indicator dye meta/*m*-cresol purple (*m*-cresol purple sodium salt 99%, non-purified, Acros Organic).

Total alkalinity was also 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). All water quality parameters were measured in randomly selected aquaria. The pCO_2 was calculated as a function of pH_T, temperature, salinity, and total alkalinity in CO_2SYS using the

constants K1 from Mehrbach *et al.* (1973) refit by Dickson & Millero (1987), and $KHSO_4$ from Dickson (1990) (Table 2.1).

Table 2.1. Seawater parameters for the experimental period (21 Jan to 6 May 2016). Values are means ± SD.

Treatment	**Temperature (°C)**	**Salinity (ppt)**	**pH_{total}**	**Total alkalinity (µmol kg^{-1} SW)**	***p*CO_2 (µatm)**
Control	29.0 ± 0.2	34.9 ± 1.4	7.94 ± 0.3	2101.4 ± 129.2	486.7 ± 35.6
Elevated CO_2	29.0 ± 0.2	35.6 ± 0.5	7.68 ± 0.3	2111.8 ± 100.8	966.8 ± 74.5
Elevated Temperature	31.9 ± 0.2	34.9 ± 1.4	7.89 ± 0.3	2101.4 ± 129.2	545.5 ± 39.9
Elevated CO_2 & Temperature	31.9 ± 0.2	35.6 ± 0.5	7.64 ± 0.3	2111.8 ± 100.8	1078.1 ± 82.3

Experimental Design

One clutch of offspring from each of four different parental pairs was used for this experiment. At 1 dph, offspring in each clutch were divided into each of the four CO_2 x temperature treatment groups: control, elevated CO_2, elevated temperature, and elevated CO_2 and temperature (Table 2.1), representing a 2 x 2 factorial design. Behavioral trials were performed at 60-66 dph, and physiological trials were performed on the same individuals at 62-68 dph, allowing at least one day rest between trials. To track individual fish between trials, immediately following the behavioral trial, individuals were placed into labeled PVC pipes (8 cm diameter, 5 cm length) that were covered at both ends by a thin plastic mesh to allow for flow-through of water, and then placed into treatment tanks. All trials were performed during daylight hours only (09:00-18:00) in the fish's respective treatment water. Research was carried out under approval of the James Cook University animal ethics committee (permit: A2197) and according to the University's animal ethics guidelines.

Behavioral Assay

The percent change in feeding strikes has been widely used to test the behavioral responses of fishes to conspecific alarm cues (Larson & Mccormick, 2005; Mitchell *et*

al., 2011; Lienart *et al.*, 2014; Chivers *et al.*, 2016), and this method has been used in previous ocean acidification experiments (Ferrari *et al.*, 2011a, 2012; Chivers *et al.*, 2014). Using percent change in feeding strikes rather than the absolute feeding rate can account for differences in activity or feeding rate between individuals. The change in feeding strikes in response to alarm odors was tested using methods similar to those described by Ferrari *et al.* (2011b). Trials were conducted in 13 L (36×21×20 cm) flow-through aquaria. Each tank contained a small shelter made of half a PVC pipe (8 cm diameter) at one end of the tank, and an airstone at the opposite end. Attached to the airstone was an injection tube for adding food and alarm odors to the tank. The end of the tube was located just above the airstone to ensure rapid dispersal of food and alarm odors throughout the tank. The tank was surrounded on three sides with white plastic to visually isolate the fish, and a black plastic curtain with a small flap separated the tank from the observer and other external stimuli.

Single juvenile *A. polyacanthus* were placed into tanks filled with their treatment water to habituate overnight (15 hours). Each tank received a continuous flow of the relevant treatment water at 0.6 L min^{-1}. A maximum of fourteen fish were tested per day. Ten minutes prior to a trial, the flow-through system to the tank was turned off to prevent the alarm odor from washing out of the tank. At this point, the flap in the observation curtain was opened and a camera placed into the opening, to habituate the fish to the camera. The fish was unable to see the observer, but the fish could be viewed on the camera's screen. Immediately prior to a trial, 20 mL of water were drawn from the injection tube and discarded to remove any stagnant water that might have collected in the tube. A further 60 mL were drawn from the tube for flushing alarm odors into the tank.

Alarm odors were freshly prepared during the first 10 minutes of each trial, as these cues have been shown to lose potency after 20 minutes when kept at room temperature (Chivers *et al.*, 2013). One juvenile *A. polyacanthus* donor was used for each test fish. The donor fish was euthanized with a quick blow to the head. The alarm odor was then prepared by making eight superficial vertical cuts along each side of the body with a scalpel blade. The cuts were rinsed well with 10 mL of seawater, and the cue water was passed through a filter to remove any solid material or scales (Ferrari *et al.*, 2011a).

To start a trial, the camera (Canon Powershot G15, Canon Powershot G16, or Canon Powershot GX9) was turned on, and the fish was recorded for five minutes to ensure it did not exhibit any abnormal behaviors. Next, 2.5 mL of *Artemia* solution (containing ~250 individuals per mL) was slowly flushed into the tank with 20 mL of seawater to allow the fish to establish a stable feeding rate. After five minutes, another 2.5 mL of *Artemia* solution was flushed into the tank with 20 mL of seawater. Finally, after another five minutes, 2.5 mL of *Artemia* solution, followed by 10 mL of alarm odor, followed by 20 mL of seawater were flushed into the tank. The trial ended five minutes after the addition of the alarm odor, with each trial totaling 20 minutes. The entire trial was filmed for analysis. Following trials, fish were returned to their rearing tanks. Between 8 and 14 individuals from each of four family groups were tested per treatment, for a grand total of 38-47 fish per treatment.

Videos were analyzed by a single researcher (T.L.) to determine the feeding rate of the fish before and after the addition of the alarm odor. Video file names were scrambled so that the viewer was unaware of the treatment. Feeding strikes were counted for four minutes in each time period, allowing 30 seconds after the addition of food or alarm odor to ensure a steady feeding rate, and that the alarm odor had fully permeated the tank. The percent change in feeding strikes after the addition of the alarm odor was then calculated.

Physiological Assay

To estimate aerobic scope, the maximum ($\dot{M}O_{2Max}$) and resting ($\dot{M}O_{2Rest}$) oxygen uptake rates were measured using intermittent flow respirometry, based on standard respirometry methods (Roche *et al.*, 2013; Rummer *et al.*, 2016). Fish were starved for 24 hours prior to testing to ensure a post-absorptive state (Niimi & Beamish, 1974). As *A. polyacanthus* is a coral reef fish with a relatively sedentary lifestyle, $\dot{M}O_{2Max}$ was measured following a chase to exhaustion (3 min.) and a brief (1 min.) air exposure, as this method has been shown to reliably capture $\dot{M}O_{2Max}$ (Roche *et al.*, 2013). Following the chase and air exposure, fish were then immediately placed into darkened glass respirometry chambers (38 mL total volume including tubing) that were submerged in aquaria containing the fish's treatment water. Submersible pumps fitted to each chamber supplied a continuous flow (20 mL min^{-1}) of water to the

chambers from the surrounding water bath. A digital relay timer (SuperPro Hydroponics Recycling Timer, Xiamen, China) was used to stop water flow for five minutes and then resume flushing for 10 minutes, continuously, for the duration of the trial. Water flow was stopped for five minutes to ensure that O_2 did not fall below 80% saturation. Fish remained in chambers for four hours to recover to $\dot{M}O_{2Rest}$. While adult fish typically remain in chambers for up to 24 hours (Clark *et al.*, 2013), smaller fish recover much more quickly from exhaustive exercise and are commonly measured for only 2-3 hours to minimize stress and the risk of starvation (Chapter 3; McLeod *et al.*, 2013; Killen *et al.*, 2014; Ferrari *et al.*, 2015; Hess *et al.*, 2017). The temperature-compensated oxygen concentration (mg L^{-1}) of the water in each chamber was continuously recorded every 2 seconds (0.5 Hz) using oxygen-sensitive REDFLASH dye on contactless spots (2 mm) adhered to a glass tube in line with the chamber, and linked to a Firesting Optical Oxygen Meter (Pyro Science e. K., Aachen, Germany) with 2 m fiber-optic cables.

Oxygen uptake rates were calculated using linear least squares regression using LabChart version 7.2.5 (ADInstruments, Colorado Springs, CO, USA). Background microbial respiration was subtracted from total chamber respiration to determine the oxygen uptake rate of the fish, as per Rummer *et al.* (2016). The value of $\dot{M}O_{2Max}$ was determined to be the maximum slope (30 second intervals) immediately following the exhaustive chase. The value of $\dot{M}O_{2Rest}$ was calculated as the average of the lowest 10% of slopes during the trial, excluding outliers above or below 2 SD. Aerobic scope was calculated as the difference between $\dot{M}O_{2Max}$ and $\dot{M}O_{2Rest}$. At the end of each trial, fish were euthanized using an overdose of clove oil. Any excess water was removed by blotting with a paper towel, and the fish's mass (0.5783 ± 0.1433 g; mean ± SD) and standard length (26.5 ±0.3 mm; mean ± SD) were recorded. Between trials, the water bath, chambers, and pumps were cleaned with a 10% bleach solution and freshwater to minimize bacterial growth. Between 6 and 14 individuals from each of four family groups were tested per treatment, for a grand total of 37-38 fish per treatment.

Statistical Analyses

Linear mixed-effects models (LME, "nlme" package in R) were used to determine the effect of CO_2 and temperature on behavioral and physiological traits. For percent

reduction in feeding strikes, the CO_2 and temperature treatments were fixed effects, with fish mass and time of day as covariates, allowing for interactions between CO_2 treatment, temperature treatment, mass, and time of day. Family was included as a random factor to account for the possibility that sibling responses were more similar to each other than non-sibling responses. Mass and time of day were mean-centered to help with the interpretation of model intercepts. For physiological traits (aerobic scope, $\dot{M}O_{2Rest}$, and $\dot{M}O_{2Max}$), CO_2 and temperature treatments and mean-centered mass were fixed effects, and family was a random effect. Assumptions of normality and homogeneity of residuals were visually assessed with Q-Q plots and frequency distributions. When the variance of the model residuals increased as the fitted values increased, a power variance function was used to reduce heteroscedasticity. Parameters were estimated using restricted maximum-likelihood. Covariates and interactions between the fixed factor and covariates were dropped when not significant for model simplification and fit. P-values were calculated from the "nlme" package in R, and results were considered statistically significant at $P<0.05$. *Post hoc* multiple comparisons were done using the R function "glht" in the package "multcomp" using Tukey's HSD contrasts for unequal sample sizes.

The relationship between behavioral and physiological performance was modeled using a linear mixed effects model for each treatment. Here we were interested in describing the relationship between traits, and not necessarily assigning causation. We used linear mixed effect models in order to incorporate additional random effects. For each linear mixed effect model, aerobic scope was the dependent variable and percent reduction in feeding strikes was the independent variable. Mean-centered mass was included as a covariate, and family was included as a random factor. When there was a significant relationship observed, linear regressions were used to determine if there were differences between families, with aerobic scope as the dependent variable and percent reduction in feeding strikes, mass, and family as covariates. All analyses were conducted using R version 3.1.3 (R Core Team, 2014).

2.4 Results

Behavior

There was a highly significant effect of CO_2 treatment on percent reduction in feeding strikes following the addition of the alarm odor (t_{160}=−3.69, P<0.001, Figure 2.1). Fish from the two current-day control CO_2 treatments exhibited a 43.3% greater reduction in feeding strikes than fish from the two elevated CO_2 treatments. Temperature treatment did not affect the reduction in feeding strikes (t_{160}=−0.03, P=0.98). There was no interaction between CO_2 and temperature on percent reduction in feeding strikes (t_{160}=−0.23, P=0.82).

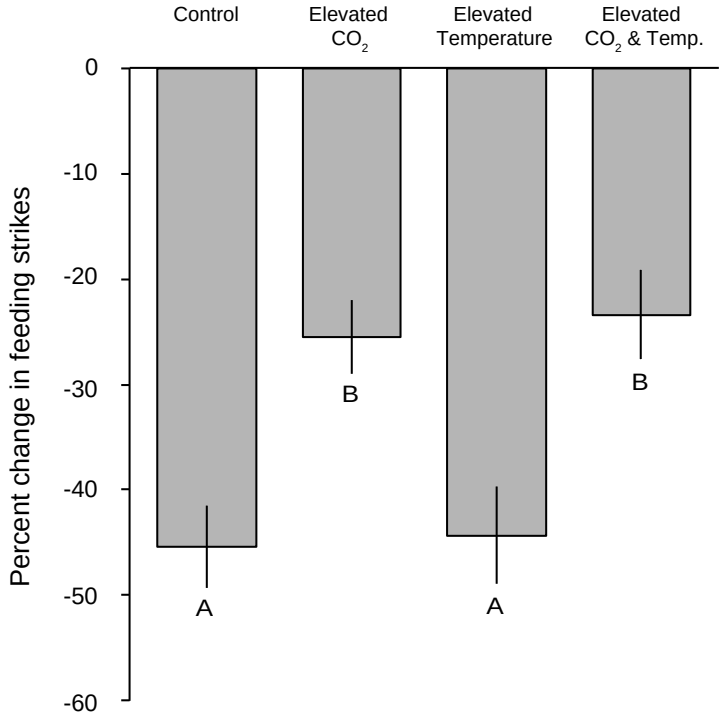

Figure 2.1. The effect of elevated CO_2 and temperature on percent change in feeding strikes in juvenile spiny chromis damselfish following the addition of an alarm odor. Values are means ± SE. Letters represent Tukey's HSD groups. N = 38–47 per treatment.

Physiology

There was a significant interaction between CO_2 and temperature treatments on aerobic scope (t_{134}=2.15, P=0.03; Figure 2.2A). Tukey's post-hoc tests revealed that

there was a significant difference in aerobic scope between the control and elevated temperature treatments held at control CO_2 (z=−4.03, P=0.001), but this was not the case when CO_2 was elevated (pairwise interactions, P>0.05). Despite the significant interaction, there was a strong effect of temperature evident in the data, which was supported by a significant main effect of temperature (t_{134}=−3.73, P<0.001). Fish from the two elevated temperature treatments exhibited a 20% lower aerobic scope than fish from the two control temperature treatments. There was no main effect of CO_2 treatment on aerobic scope (t_{134}=−1.43, P=0.15), while mass had a significant effect on aerobic scope (t_{134}=−5.19, P<0.001).

There was a significant interaction between CO_2 treatment and temperature treatment on $\dot{M}O_{2Max}$ (t_{140}=3.47, P<0.001; Figure 2.2B). Tukey's post-hoc tests revealed that juveniles reared in the current-day control and in the combined elevated CO_2 and temperature treatments had a significantly higher $\dot{M}O_{2Max}$ than juveniles from the elevated temperature treatment (control: z=3.58, P=0.002; elevated CO_2 and temperature: z=3.61, P=0.002), while juveniles from the elevated CO_2 treatment had a $\dot{M}O_{2Max}$ which was not significantly different from the other three treatments (pairwise interactions, P>0.05). As was the case with aerobic scope, there a significant main effect of temperature treatment on $\dot{M}O_{2Max}$ (t_{140}=−3.58, P<0.001). Fish from the two elevated temperature treatments had an 18.2% lower $\dot{M}O_{2Max}$ than fish from the two control temperature treatments. There was no main effect of CO_2 treatment on $\dot{M}O_{2Max}$ (t_{140}=−1.29, P=0.20) and mass had a highly significant effect on $\dot{M}O_{2Max}$ (t_{140}=−3.32, P=0.001).

There was a significant interaction between CO_2 treatment and temperature treatment on $\dot{M}O_{2Rest}$ (t_{136}=3.45, P<0.001; Figure 2.2B). Post-hoc Tukey's tests revealed that juveniles reared in the current-day control, elevated temperature, and elevated CO_2 treatments all had similar values for $\dot{M}O_{2Rest}$ (pairwise interactions, P>0.05), whereas juveniles reared in the elevated CO_2 and temperature treatment had a significantly higher $\dot{M}O_{2Rest}$ (pairwise interactions, P<0.05). Neither CO_2 treatment nor mass had a significant effect on $\dot{M}O_{2Rest}$ (t_{136}=−0.40, P=0.69 and t_{136}=0.05, P=0.96, respectively).

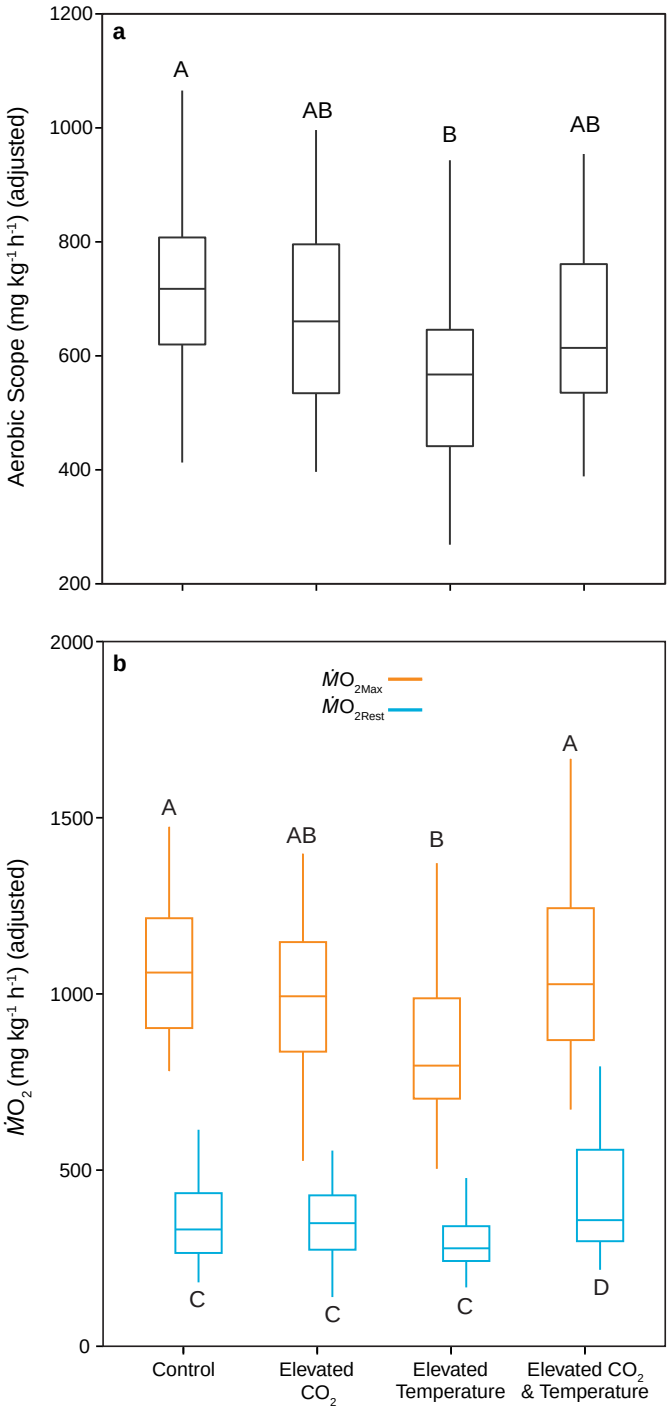

Figure 2.2. The effect of elevated CO_2 and temperature treatments on resting and maximal oxygen uptake rates and aerobic scope in juvenile spiny chromis damselfish. Boxplots show median and inter-quartile range for (**A**) absolute aerobic scope ($\dot{M}O_{2Max}$ - $\dot{M}O_{2Rest}$); and (**B**) resting ($\dot{M}O_{2Rest}$; blue boxes) and maximal oxygen uptake rates ($\dot{M}O_{2Max}$; orange boxes). Letters represent Tukey's HSD groups. N = 32–38 per treatment.

Relationship between Behavioral and Physiological Performance

There was no significant relationship between percent reduction in feeding strikes and aerobic scope in the juveniles reared in the current-day control (t_{27}=−0.29, P=0.77, Figure 2.3), elevated CO_2 (t_{26}=1.72, P=0.10), or elevated temperature (t_{27}=0.22, P=0.83) treatments. However, there was a significant negative relationship between percent reduction in feeding strikes and aerobic scope in juveniles reared at elevated CO_2 and temperature (t_{27}=−3.09, P=0.005). This relationship was consistent across all family groups (ANOVA of LM, Family x Reduction in Feeding Strikes interaction, P>0.05, Figure 2.4).

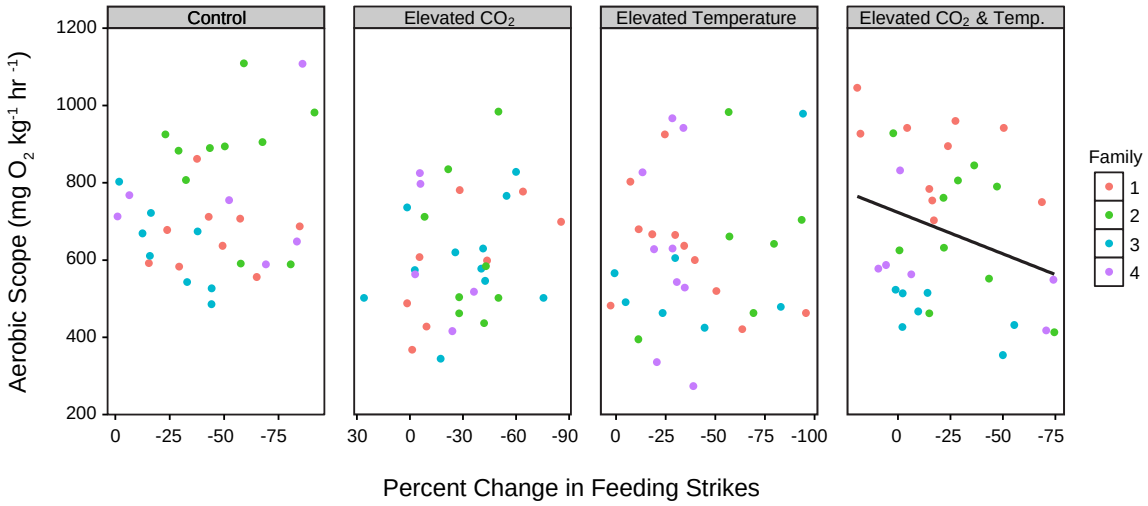

Figure 2.3. The relationship between percent change in feeding strikes and aerobic scope. Panels represent different treatments, and colours represent different family groups. Trend lines are shown as derived from linear mixed effect models. The relationship is statistically significant for the elevated CO_2 and temperature treatment.

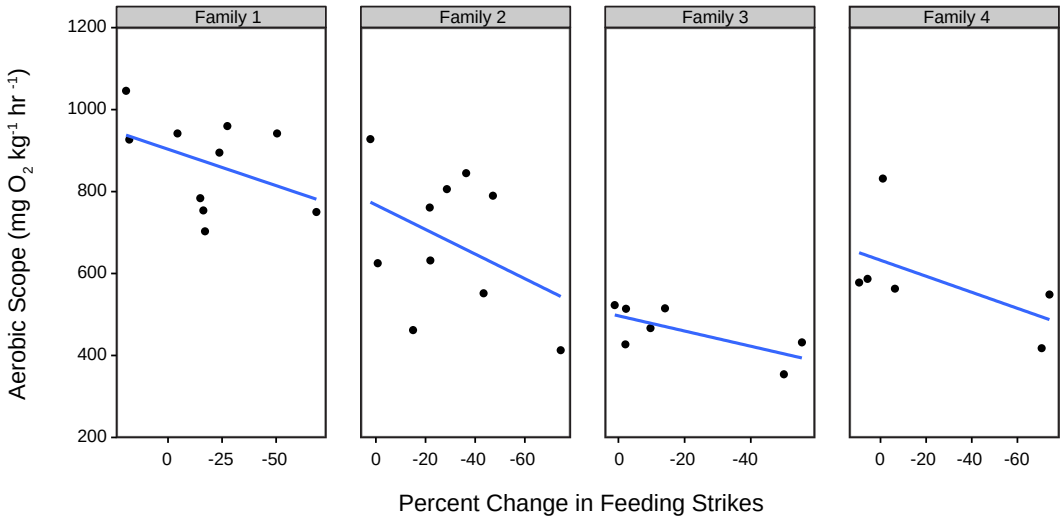

Figure 2.4. The relationship between percent change in feeding strikes and aerobic scope for the elevated CO_2 and temperature treatment. Panels represent different family groups. Trend lines are shown as derived from linear models.

2.5 Discussion

Our study found a negative relationship between changes in feeding strikes in response to alarm odor and aerobic scope, but only when fish were reared in elevated CO_2 and temperature conditions. Our results indicate that when exposed to two climate change stressors, there are no winner and loser individuals– rather, each individual seems constrained along a maximal performance ridge (Sunday *et al.*, 2014), such that an individual could maintain a relatively high aerobic scope, or an ecologically appropriate response to alarm cue, but not both. Furthermore, the relationship was consistent across family groups, suggesting that certain families do not hold a distinct advantage over others in dealing with this limitation. This negative correlation could have implications for the adaptive potential of this species. Selection for improved performance of either trait could decrease performance in the other, slowing adaptation. Given the rapid pace at which the global environment is changing, any factor that slows or limits selection for improved performance could have serious implications for individual performance and ultimately, population success.

We detected a significant correlation between traits only in the multi-stressor treatment. This result aligns well with theory that environmental stress can act as a revealing or amplifying factor on correlations between behavior and physiology (Killen *et al.*, 2013). The mechanism for this pattern is not known, but may be explained by differing sensitivities to the stressors between individuals, which can increase the intraspecific phenotypic variation in the traits (Hoffmann & Hercus, 2000; Killen *et al.*, 2012). Additionally, the increased demands on performance that are imposed by stressors can emphasize the importance of certain traits, making links between behavior and physiology more evident under stressful conditions (Killen *et al.*, 2013). Indeed, since we observed behavioral performance to be most affected by elevated CO_2 and physiological performance to be most affected by elevated temperature, it is intuitive that the presence of both stressors might be necessary to observe this relationship. Unfortunately, ocean waters will likely become both warmer and more acidic in the future, meaning that future conditions may elicit this negative correlation between behavioral and physiological performance.

This study investigated phenotypic, not genotypic correlations between traits. While phenotypic correlations are often indicative of genetic correlations (Lynch & Walsh, 1998), we did not specifically test the genetic basis of the phenotypic traits considered here. Correlations between phenotypic traits might enhance or hinder selection, but ultimately adaptation can only be influenced by genetic covariation between traits. This means that the observed phenotypic variation must be heritable for adaptation to occur. Recent studies demonstrate that aerobic scope has high heritability under elevated temperatures (Munday *et al.*, 2017), and response to alarm odor is heritable, at least under acute exposure to elevated CO_2 (Schunter *et al.*, 2016; Welch & Munday, 2017). These studies suggest that these traits have a significant amount of additive genetic variation, indicating that the correlations we have detected could indeed affect adaptation.

Our behavioral performance trials demonstrated that juveniles reared under elevated CO_2 conditions did not reduce their feeding strikes after the addition of an alarm odor to the same extent as juveniles reared under current-day control conditions. This is consistent with previous studies that have shown elevated CO_2 to negatively impact a range of behavioral and sensory traits in reef fishes (Clements & Hunt, 2015;

Nagelkerken & Munday, 2016). A likely mechanism underpinning the behavioral changes we observed is a disruption to neurotransmitter receptor function. Fishes have robust acid-base regulatory systems that help them to maintain a stable internal pH (Heuer & Grosell, 2014). However, the changed concentration of acid-base relevant ions that is required to maintain internal pH under elevated CO_2 conditions can interfere with the function of $GABA_A$ neurotransmitter receptors, resulting in altered behavior and impaired olfactory responses (Nilsson *et al.*, 2012; Heuer *et al.*, 2016). It is important to note that our behavioral assay does not differentiate between fish with impaired olfactory preferences versus those with increased activity and boldness; rather, the assay encompasses both responses in one ecologically-relevant test. Because ocean acidification has been shown to alter a broad range of cognitive functions in marine fishes, it is likely that it affects central neural processing rather than individual behaviors or sensory systems (Nilsson *et al.*, 2012; Heuer & Grosell, 2014). Thus, changes to both boldness and olfactory preference likely stem from the same disruption to $GABA_A$ functioning, allowing them to be assessed simultaneously in our assay. Through this method, we were able to portray a real-life scenario involving both foraging and anti-predator response in which a fish must respond appropriately to an alarm odor.

In our physiological performance trials, we documented that the aerobic scope of juvenile *A. polyacanthus* decreased under elevated temperature. This negative effect of elevated temperature on aerobic performance has been previously shown in a range of coral reef fishes (Nilsson *et al.*, 2009; Rummer *et al.*, 2014; Habary *et al.*, 2017), although the mechanism by which temperature affects aerobic performance in ectotherms is still not fully understood (Schulte, 2015). There was also a non-significant trend toward decreased aerobic scope in fish maintained under elevated CO_2 conditions. Elevated CO_2 has been shown to have mixed effects on aerobic scope in a range of marine fishes, with elevated temperature typically having a greater effect than elevated CO_2 (Lefevre, 2016; Hannan & Rummer, 2018), which aligns well with our results. However, our results contrast with a 38% increase in aerobic scope that was observed in adult *A. polyacanthus* exposed to similar CO_2 levels (Rummer *et al.*, 2013a). We hypothesize that the differing results primarily stem from the different life-stages of the tested fish (i.e. juvenile vs. sexually-mature adults). It has been suggested that early life stages of fish are more sensitive to changes in pH due to their

high surface area-to-volume ratio (Rombough, 1997; Melzner *et al.*, 2009), which could explain this discrepancy. Our results further underscore the importance of considering life stage when determining species responses to elevated CO_2.

This study indicates that there is a negative correlation between behavioral and physiological performance in juvenile damselfish exposed to elevated CO_2 and temperature. This relationship could reduce individual performance maxima, as well as limit the potential for adaptive evolution in the population. However, it is important to note that the extent to which this relationship is detrimental will depend upon other factors, such as food availability and predator abundance, and the relative benefits of a higher aerobic scope versus stronger anti-predator response in these environments. Juvenile coral reef fishes are generally considered to live in a high-risk environment, as up to 55% of settlement-stage juveniles are estimated to be consumed within days of reaching a reef (Almany & Webster, 2006), suggesting that effective anti-predator responses would be under strong selection. Similarly, a high aerobic scope is thought to be beneficial when food is abundant, but less helpful when food is scarce (Burton *et al.*, 2011), and thus its importance will depend upon food availability. Still, in reef fishes there is evidence for heritability of phenotypic variation in physiological and behavioral traits under elevated CO_2 and temperature (Schunter *et al.*, 2016; Munday *et al.*, 2017; Welch & Munday, 2017), so it is not unreasonable to expect that adaptation can and will act on these traits under future climate conditions.

While we saw a clear correlation in these traits under elevated CO_2 and temperature conditions, the proximal cause for the relationship remains unknown. Linkages among metabolic traits and behavioral traits have been well explored (Biro & Stamps, 2010; Sih *et al.*, 2015; Metcalfe *et al.*, 2016), but proximal causes are generally more difficult to identify. For instance, the observed negative correlation might be caused by genetic linkages between traits, but could also be explained by a shared hormonal feature (Jacobson, 2005), or a trade-off in energetics between internal pH regulation and thermal tolerance (Kelly & Hofmann, 2013). This work thus represents an important first step in identifying this correlation, and opens an avenue for future research to identify the mechanistic basis of this relationship.

Chapter 3: Correlated Effects of Ocean Acidification and Warming on Behavioral and Metabolic Traits of a Large Pelagic Fish

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3.1 Summary

Ocean acidification and warming are co-occurring stressors, yet their effects on early life stages of large pelagic fishes are not well known. Here, we determined the effects of elevated CO_2 and temperature at levels projected for the end of the century on activity levels, boldness, and metabolic traits (i.e. oxygen uptake rates) in larval kingfish (*Seriola lalandi*), a large pelagic fish with a circumglobal distribution. We also examined correlations between these behavioral and physiological traits measured under different treatments. Kingfish were reared from the egg stage to 25 days post-hatch in a full factorial design of ambient and elevated CO_2 (~500 µatm and ~1000 µatm) and temperature (21 °C and 25 °C). Activity levels were higher in fish from the elevated temperature treatment compared with fish reared under ambient temperature. However, elevated CO_2 did not affect activity, and boldness was not affected by either elevated CO_2 or temperature. Both elevated CO_2 and temperature resulted in increased resting oxygen uptake rates compared to fish reared under ambient conditions, but neither affected maximum oxygen uptake rates nor aerobic scope. Resting oxygen uptake rates and boldness were negatively correlated under ambient temperature, but positively correlated under elevated temperature. Maximum oxygen uptake rates and boldness were also negatively correlated under ambient temperature. These findings suggest that elevated temperature has a greater impact on behavioral and physiological traits of larval kingfish than elevated CO_2. However, elevated CO_2 exposure did increase resting oxygen uptake rates and interact with

temperature in complex ways. Our results provide novel behavioral and physiological data on the responses of the larval stage of a large pelagic fish to ocean acidification and warming conditions, demonstrate correlations between these traits, and suggest that these correlations could influence the direction and pace of adaptation to global climate change.

3.2 Introduction

The oceans are becoming more acidic due to increased uptake of anthropogenic carbon dioxide from the atmosphere (Collins *et al.*, 2013). The dissolution of additional CO_2 into oceanic surface waters causes a decrease in oceanic pH through a process known as ocean acidification (Doney *et al.*, 2009). There is now an extensive body of literature assessing biological responses to ocean acidification across a wide range of marine species (Hendriks *et al.*, 2010; Kroeker *et al.*, 2010; Wittmann & Pörtner, 2013). These single-stressor studies provide useful indications of large-scale trends in response to acidification, as well as the mechanisms underlying these responses. However, marine organisms are subject to multiple co-occurring stressors, which might interact in diverse ways. Ocean warming is an ecologically-relevant stressor that co-occurs with acidification (Doney *et al.*, 2012); yet, predicting the combined impacts of ocean acidification and warming is not straightforward, as elevated CO_2 and temperature have been found to interact both synergistically and antagonistically on marine organisms (Kroeker *et al.*, 2013; Riebesell & Gattuso, 2015).

Previous studies have investigated the effects of ocean acidification on marine fishes, finding effects on metabolic rate, behavioral performance, reproductive output, otolith growth, and sensory responses in some species, but not others (Heuer & Grosell, 2014; Clements & Hunt, 2015; Lefevre, 2016; Cattano *et al.*, 2018). More recently, studies have begun to investigate the interacting effects of ocean acidification and warming on marine fishes, finding they may mitigate, reverse, or enhance the effects of elevated CO_2. For instance, Domenici *et al.* (2014) found that elevated CO_2 and temperature have different and interacting effects on behavioral lateralization in reef fishes, whereas Munday *et al.* (2009) found that elevated CO_2 and temperature have additive effects on metabolic rates in two species of cardinalfishes. Other studies

point towards temperature having a greater overall effect on ontogenetic development, swimming ability (Watson *et al.*, 2018), and the outcome of predator-prey interactions (Allan *et al.*, 2017) compared with elevated CO_2. Therefore, it is necessary to consider the interacting effect of warming to properly understand the effects of ocean acidification on fishes and predict outcomes for the future.

Among the existing experimental research on the effects of ocean acidification and warming on marine fishes, large pelagic fishes have been relatively understudied. This represents a critical knowledge gap, as large pelagic fishes are both ecologically and economically important. As abundant top predators, they can impact the structure and functioning of the marine ecosystem, and have strong top-down influences on marine food webs (Frank *et al.*, 2005; Casini *et al.*, 2009). They are also a critical food source for millions of people in coastal regions worldwide, and constitute a large proportion of wild-caught fisheries (FAO, 2016). Furthermore, large pelagic fishes are hypothesized to be more susceptible to ocean acidification and warming due to the relatively stable environments they experience in open waters (Munday *et al.*, 2008a; Pörtner, 2008) when compared to the highly fluctuating temperature and pH conditions of coastal and shallow water habitats (Hofmann *et al.*, 2011; Waldbusser & Salisbury, 2014). Thus, their absence from the literature represents a critical avenue for climate change research, and motivated our study.

We focused on the larval stages of a large pelagic fish. The larval stage is one of the most vulnerable, yet critically important, stages in the development of marine fishes. Larval fishes are subject to high mortality rates due in part to predation, as well as environmental effects on growth (Houde, 1989). The dynamics of the larval phase can influence patterns of population replenishment and connectivity in adult populations (Chambers & Trippel, 1997; Cowen & Sponaugle, 2009). Additionally, larval fishes are presumed to be more vulnerable to changes in temperature and pH than adults, possibly due to their larger surface area-to-volume ratio, which makes them more susceptible to environment perturbations (Rombough, 1997; Melzner *et al.*, 2009). Therefore, understanding how elevated CO_2 and temperature affect larval pelagic fishes could have broader implications for how adult populations might be affected by climate change.

Here, we tested the effects of elevated CO_2 and temperature on key behavioral and physiological traits during the larval stage in yellowtail kingfish, *Seriola lalandi*, from New Zealand. We used a cross-factored experiment that comprised current-day ambient CO_2 levels (~500 μatm) and average summer temperature for the study location (21 °C), crossed with elevated CO_2 (~1000 μatm) and temperature (25 °C) based on projections for the open ocean by the end of the century under RCP 8.5 (Meinshausen *et al.*, 2011; Collins *et al.*, 2013). The specific traits we focused on were routine activity, boldness, and metabolic performance, and we also examined correlations between these traits.

Routine activity is a commonly examined behavioral trait in ocean acidification studies of larval fishes. Ecologically, activity levels are relevant because increased activity has been shown to increase feeding and growth rates in fishes and other animals, but decrease survivorship due to higher incidences of predation (Werner & Anholt, 1993; Biro *et al.*, 2003a, 2003b). In previous studies, the routine activity of large pelagic fishes has been mostly unaffected by elevated CO_2 levels ranging from 800 to 2100 μatm (Bignami *et al.*, 2013, 2014, 2016; Munday *et al.*, 2015). However, one species, mahi mahi *Coryphaena hippurus*, did exhibit a decrease in swimming duration upon exposure to 1600 μatm CO_2 (Pimentel *et al.*, 2014), and a decrease in maximum swimming velocity upon exposure to 1460 μatm CO_2 (Bignami *et al.*, 2014). By contrast, temperature is well known to affect activity in marine fishes (Fukuhara, 1990; Biro *et al.*, 2010). The mechanism for this relationship is not well known, though it has been suggested that temperature influences activity indirectly through its effect on metabolic rate (Biro & Stamps, 2010; White & Kearney, 2014).

We also measured boldness, or the propensity to take risks, which is another commonly measured behavioral trait in fishes (Wilson *et al.*, 1994; Brown *et al.*, 2005; Ariyomo & Watt, 2012). As with increased activity, increased boldness in fishes has been linked to decreased survivorship through higher rates of predation (Biro *et al.*, 2003a; Munday *et al.*, 2010), and also higher likelihood of being captured by fishing gear (Biro & Dingemanse, 2009). Boldness has not been specifically studied with respect to CO_2 effects on early life stages of large pelagic fishes, but other ocean acidification studies on marine fishes have found boldness to either increase (Munday *et al.*, 2010) or decrease (Jutfelt *et al.*, 2013) under elevated CO_2.

Temperature has also been shown to increase boldness in marine fishes (Biro *et al.*, 2010; Lienart *et al.*, 2014). As with activity, the relationship between temperature and boldness has been suggested to be driven by the direct impact of temperature on metabolic rate (Biro & Stamps, 2008; Biro *et al.*, 2010).

Our physiological trait of interest was metabolic performance, which we approximated by measuring maximal and resting oxygen uptake rates, and calculating aerobic scope, or the difference between these two values. Metabolic traits reveal the energetic requirements of organisms, and are therefore assumed to underpin a range of fitness-related traits (Burton *et al.*, 2011). For instance, maximal and resting oxygen uptake rates have been correlated with swimming performance and foraging success in fishes (Metcalfe *et al.*, 2016; Norin & Clark, 2016). Studies to date show mixed effects of elevated CO_2 on maximum and resting oxygen uptake rates in marine fishes (Couturier *et al.*, 2013; Lefevre, 2016; Hannan & Rummer, 2018). While few studies have examined the effects of elevated CO_2 on metabolic performance in larval pelagic fishes, Pimentel *et al.* (2014) did find a reduction in oxygen uptake rates of mahi mahi upon exposure to 1600 μatm CO_2. By comparison, temperature has been well established to affect metabolic traits in marine fishes through its effect on rates of biochemical reactions (Fry, 1971). Furthermore, a recent meta-analysis (Lefevre, 2016) indicates that elevated temperature generally has a greater effect than elevated CO_2 on metabolic traits of marine fishes.

In addition to documenting mean trends across activity, boldness, and metabolic performance, we also tracked individual fish through all assays to determine whether correlations exist between traits at the individual level. Historically, individual variation has often been treated as noise around the population mean (Careau *et al.*, 2008). However, there has been a recent surge of interest in studying individual variation in both behavioral and physiological traits (Sih *et al.*, 2004; Burton *et al.*, 2011), and whether these traits are correlated (Careau *et al.*, 2008; Biro & Stamps, 2010; Metcalfe *et al.*, 2016). It has been proposed that environmental stressors can alter the relationship between behavioral and physiological traits, either revealing or masking these relationships (Killen *et al.*, 2013). Importantly, correlations between traits could have implications for the capacity to adapt to environmental change. For instance, if the behavioral and physiological traits of interest are heritable, then

correlations between them could either increase or decrease the rate of adaptive evolution, depending on whether the traits are positively or negatively correlated with respect to the fitness landscape (Sunday *et al.*, 2014). If traits are positively correlated, then selection on one trait will enhance the other, accelerating the rate of adaptation, and vice versa. Thus, examining correlations between behavioral and physiological traits, as well as how those correlations shift under different environmental conditions, can reveal the evolutionary implications of climate change.

3.3 Methods

Study Species, Broodstock, Egg and Larval Maintenance

We chose the yellowtail kingfish, *Seriola lalandi*, because it is one of the few species of large pelagic fishes that can be reliably reared in captivity (Sicuro & Luzzana, 2016). The yellowtail kingfish has a circumglobal distribution in subtropical and temperate waters (Bray, 2018). Kingfish inhabit open coastal waters, where they form large shoals around deep reefs, pinnacles, or rocky outcrops (Kailola *et al.*, 1993). Adults can reach up to 1.93 m in length and weigh over 58 kg (Roberts *et al.*, 2015). Kingfish are an important recreational and commercial fishery in subtropical countries such as Australia, New Zealand, Peru, Chile, USA, South Africa, and Japan, and have been a target for aquaculture in some of these countries as well (Bray, 2018).

This study was conducted at the National Institute of Water and Atmospheric Research (NIWA) Northland Marine Research Centre in Ruakaka, New Zealand. Wild-caught, adult yellowtail kingfish were maintained as broodstock in six 20 m^3 circular tanks (Figure 3.1). Each tank contained up to six fish, with approximately equal sex ratios in each tank. The fish had been maintained at NIWA for up to nine years. Filtered (10 μm) seawater was supplied to the tanks at 130 L min^{-1}, and each tank was exposed to an ambient photoperiod and ambient ocean temperatures (maximal seasonal range of 13–24 °C). The broodstock were fed a mixture of pilchard (*Sardinops sagax*) and squid (*Notodarus* spp.). For further details, all broodstock, egg, and larval maintenance protocols followed Watson *et al.* (2018).

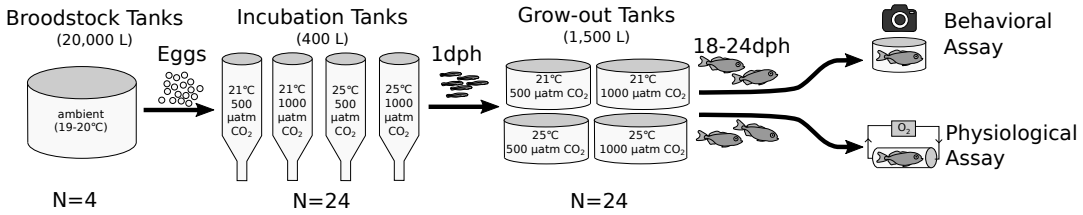

Figure 3.1. A schematic diagram of the broodstock, egg, and larval maintenance of yellowtail kingfish prior to behavioral and physiological testing. Illustration by Erin Walsh.

The offspring used for this experiment were collected from a spawning event on the night of 23 January 2017. Spawning occurred within the last 2 hours of daylight across four broodstock tanks, containing a total of nine females, nine males, and one fish of unknown sex. Eggs were collected from all four tanks to maximize genetic variation. Ambient water temperatures ranged from 19–20 °C in the week prior to spawning, but dropped to 18.2 °C on the night of spawning. Eggs were collected on the morning of 24 January 2017, approximately 12 hours after fertilization using an external egg collector as described by Moran *et al.* (2007). Eggs were collected in approximately equal proportions from each tank and mixed together. They were rinsed with oxygenated seawater for 5 minutes, disinfected with Tosylchloramide (chloramine-T) at 50 ppm for 15 minutes, then rinsed a second time in seawater. The eggs were then transferred to 24 conical 400-L incubation tanks at 12:45 h, with an average concentration of 101,778 ± 9860 (SD) eggs per tank.

The incubation tanks were exposed to a 14:10 light to dark photoperiod. Flow-through seawater was supplied at 4 L min^{-1}, and the tanks were aerated with a weighted 4 mm airline. Tanks were at ambient ocean temperature (18.2 °C) at stocking, after which the heating was turned on and tank temperatures rose to either 21 or 25 °C overnight, which resulted in hatching after three or two days, respectively. At one day post-hatch (dph), larvae were transferred from incubator tanks to 24 reciprocal 1500 L circular grow-out tanks (black interior, sloped bottoms) at an average concentration of 44,227 ± 2152 (SD) larvae per tank. Tanks were supplied with seawater at a flow rate of 3 L min^{-1} and, as with incubation tanks, exposed to a 14:10 light to dark photoperiod and aerated with a weighted 4 mm airline. Larvae were fed with enriched rotifers up to 4 times per day.

Carbonate Chemistry

Seawater was pumped continuously from the ocean, sand and particle (5 μm) filtered, and UV (150 mW cm^{-2}) sterilized before reaching large header tanks. Inside the header tanks, foam fractionators removed any additional organic matter, and oxygen diffusers ensured a minimum dissolved oxygen concentration of 100% saturation. Water from the header tanks was then gravity-fed into eight 100 L sumps. These sumps were treated to create a fully crossed 2 × 2 experimental design of CO_2 and temperature, with CO_2 at either ambient (~500 μatm) or elevated levels (~1000 μatm), and temperature at either an ambient (21 °C) or elevated (25 °C) level. There were two replicate sumps for each treatment, totaling eight sumps. Each sump supplied water to three rearing tanks, meaning that there were six replicate experimental tanks for each CO_2 × temperature treatment throughout the duration of the experiment. Each sump contained two aquarium pumps; one (HX-6540, Hailea, Guangdong, China) delivered water from the sump to the experimental rearing tanks, while the second (Maxi 103, Aqua One, Ingleburn, NSW, Australia) ensured even mixing of the water within the sump. The second pump was also the site of CO_2 dosing for the elevated CO_2 treatments. A pH computer (Aqua Medic, Bissendorf, Germany) and needle valve were used to slowly dose CO_2 into the pump inlet, which ensured a slow, steady stream of CO_2 that was immediately mixed by the pump impeller.

The temperature and pH_{total} of each rearing tank were measured daily using a pH electrode (SG8 SevenGo Pro, Mettler Toledo, Switzerland). The pH electrode was calibrated using Tris buffers obtained from A.G. Dickson (Scripps Institution of Oceanography, La Jolla, CA, USA, batch number 26). Water samples for carbonate chemistry analysis were collected from all rearing tanks at the start, middle, and end of the experiment, and immediately poisoned with a saturated solution of mercuric chloride at 0.05% of the sample volume. The samples were later analyzed for total alkalinity (TA) at the University of Otago Research Centre for Oceanography (Dunedin, New Zealand). See Watson *et al.* (2018) for full details of the water sample analysis. Salinity was measured for each sample bottle using a YSI Pro30 salinity probe. Temperatures reported by the pH electrode were cross-checked each day with a calibrated 3 decimal point digital thermometer (FSH15-077-8 Digital thermometer,

Fisherbrand™ Traceable™ Digital Thermometer, Thermo Fisher Scientific, Waltham, MA, USA).

Carbonate chemistry parameters in each tank were calculated in CO_2SYS using the measured values of pH_{total}, salinity, temperature, and TA and the constants K1 and K2 from Mehrbach *et al.* (1973), refit by Dickson & Millero (1987) and Dickson for $KHSO_4$ (1990). Seawater carbonate chemistry parameters are displayed in Table 3.1.

Table 3.1. Experimental water chemistry. Mean (± SD) temperature, salinity, pH_{total}, total alkalinity, and pCO_2 in experiments with yellowtail kingfish (*Seriola lalandi*) eggs and larvae. Water chemistry in broodstock tanks was measured in the week prior to spawning. Temperature, salinity, pH_{total}, and total alkalinity were measured directly, while pCO_2 was estimated from these parameters in CO_2SYS.

CO_2 Treatment	**Temperature Treatment**	**Temperature (°C)**	**Salinity**	**pH_{total}**	**Total alkalinity (µmol kg^{-1} SW)**	**pCO_2 (µatm)**
Broodstock - ambient	Broodstock - ambient	19.2 (0.6)	35.6 (0.1)	7.906 (0.024)	2329.6 (6.1)	589.4 (38.0)
Control	21 °C	21.1 (0.1)	35.6 (0.1)	7.995 (0.025)	2318.8 (7.2)	462.0 (42.8)
Control	25 °C	24.8 (0.4)	35.6 (0.1)	7.938 (0.011)	2319.9 (7.7)	538.3 (15.6)
Elevated	21 °C	21.1 (0.1)	35.6 (0.2)	7.718 (0.028)	2319.0 (3.8)	959.8 (57.3)
Elevated	25 °C	24.9 (0.4)	35.6 (0.1)	7.700 (0.012)	2320.0 (6.2)	1010.6 (30.4)

Experimental Design

Behavioral and metabolic traits were assessed from 18–24 dph during daylight hours only (08:00–19:00). Each morning, the fish to be tested that day were sampled randomly from the experimental rearing tanks. The fish were placed individually into labeled sample jars (10 cm diameter, 10 cm height) maintained at the fish's respective treatment conditions. A total of 137 fish were used in the study. Fish were only tested in a single assay, except for 45 individuals that were tracked through both the behavioral and physiological assays to examine correlations between traits. All experiments were conducted in each fish's respective treatment water. After the final

assay, fish were euthanized using an overdose of clove oil. Any excess water was removed by blotting with a paper towel, and the fish's mass (0.028 ± 0.008 g; mean ± SD) and standard length (9.74 ± 1.07 mm; mean ± SD) were recorded. Research was carried out under approval of the James Cook University animal ethics committee (permit: A2357) and according to the university's animal ethics guidelines.

Behavioral Assay

Routine activity and boldness were determined using an open field test (Burns, 2008; Ariyomo & Watt, 2012). The test arena consisted of a round, white plastic bucket (19 cm diameter, 7 cm height) placed inside a white plastic bin (52 cm length, 32 cm width, 34 cm height), which was opaque to minimize visual disturbance for the fish, but allowed light through for filming. A sheet of white corflute was fitted to the top of the plastic bin, with a small circular hole cut into its center, where a video camera (HC-V160, Panasonic Australia, Macquarie Park, NSW, Australia) was placed. To begin a trial, a fish was placed into the center of the arena by gently transferring it with a beaker to minimize stress. The lid was immediately fit to the plastic bin, the camera was turned on, and the fish was filmed for 17 minutes. At the end of a trial, the fish was removed from the test arena with a beaker, returned to its respective treatment water, and the test arena was rinsed with seawater.

All videos were analyzed blind to treatment using Lolitrack software (v4.1.0 Loligo Systems, Tjele, Denmark), which tracked and quantified the movements of the fish. The first and last minute of each video were discarded to allow for the researcher to enter and exit the arena area. Before each video analysis, a circular arena was drawn within the test arena, with the same central point, but which was 13 cm in diameter, or approximately three body lengths away from the edges of the test arena. This "inner zone" was used to quantify boldness. The open field test has been commonly used in fishes to determine boldness based on the idea that a novel, open field is considered dangerous, and that venturing into the inner zone represents boldness, or the willingness to undertake risk (Burns, 2008; Ariyomo & Watt, 2012). Therefore, we quantified boldness as time spent in the inner zone. The parameters quantified by the software were: total distance moved (cm), average swimming velocity (cm s^{-1}), time active (defined as time spent moving) (s), and time spent in the inner zone (s). Between 29 and 38 individuals were tested per treatment.

Physiological Assay

Oxygen uptake rates ($\dot{M}O_2$) of fish were determined using intermittent flow respirometry, based on standard respirometry methods (Roche *et al.*, 2013; Rummer *et al.*, 2016). Fish were starved for 20 hours prior to testing to ensure a post-absorptive state (Niimi & Beamish, 1974). To measure maximal oxygen uptake ($\dot{M}O_{2Max}$), fish were chased (3 minutes) in a circular container (20 cm diameter, 9 cm height) and then exposed to air (1 minute). This chase protocol was determined in pilot trials to be sufficient for all fish to reach exhaustion. Immediately following the chase and air exposure, fish were gently placed into individual darkened glass respirometry chambers (15 mL total volume including tubing) submerged in a water bath containing the fish's respective treatment water. The water bath received a continuous flow of treatment water to ensure the temperature and CO_2 of the water remained constant. Fish remained in chambers while recovering back to their resting oxygen uptake rates ($\dot{M}O_{2Rest}$) over four hours. Although adult fish typically remain in chambers for 24 hours (Clark *et al.*, 2013), larvae and small juvenile fish recover much more quickly from exhaustive exercise, and are commonly measured for only 2–3 hours to minimize stress and the risk of starvation (McLeod *et al.*, 2013; Killen *et al.*, 2014; Ferrari *et al.*, 2015; Hess *et al.*, 2017). Flush pumps supplied the chambers with clean, well-oxygenated water for 2 minutes every 8 minutes, ensuring that O_2 levels within chambers did not fall below 80% air saturation. This flush pattern was controlled using a custom-built timer which turned power to the flush pumps on and off via a programmed timing sequence. The temperature-compensated oxygen concentration (mg L^{-1}) of the water in each chamber was continuously recorded (once every two seconds) using oxygen-sensitive REDFLASH dye on contactless spots (2 mm) adhered to a glass tube in line with the chamber, and linked to a Firesting Optical Oxygen Meter (Pyro Science e. K., Aachen, Germany) with 2 m fiber-optic cables. Between 11 and 14 individuals were tested per treatment.

Oxygen uptake rates were calculated using linear least squares regression in LabChart version 7.2.5 (ADInstruments, Colorado Springs, CO, USA). Background microbial respiration was subtracted from total chamber respiration to determine the oxygen uptake rate of the fish, as per Rummer *et al.* (2016). The $\dot{M}O_{2Max}$ was taken to be the highest oxygen uptake rate (over 2 minute intervals) and usually occurred during the first measurement cycle. The $\dot{M}O_{2Rest}$ was estimated as the average of the lowest 10%

of values, excluding outliers above or below 2 SD. Aerobic scope was calculated as the difference between $\dot{M}O_{2Max}$ and $\dot{M}O_{2Rest}$.

Statistical Analyses

All analyses were conducted using R version 3.1.3 (R Core Team, 2014). Linear mixed-effects models (LME, "nlme" package in R) were used to determine the effect of CO_2 and temperature treatment on behavioral and metabolic traits. Total distance traveled (in body lengths) and velocity (in body lengths per second) were standardized by body length to facilitate comparisons between treatments where fish were differently sized. For these dependent variables, the CO_2 and temperature treatments were fixed effects, with time of day as a covariate, allowing for interactions between CO_2 treatment, temperature treatment, and time of day. Time of day was mean-centered to help with the interpretation of model intercepts. For time spent active and time spent in the inner zone, similar linear mixed effects models were used, with the addition of mean-centered mass as a covariate, since these measures were not standardized by fish length, but distance traveled and velocity were. Time spent in the inner zone was square-root transformed to achieve normal distribution errors. For aerobic scope, maximum oxygen uptake, and resting oxygen uptake, CO_2 and temperature treatments and mean-centered mass were fixed effects. For all linear mixed effect models, tank was included as a random effect. Assumptions of normality and homogeneity of residuals were visually assessed with Q-Q plots and frequency distributions. When the variance of the model residuals increased as the fitted values increased, a power variance function was used to allow for heteroscedasticity. Parameters were estimated using restricted maximum-likelihood. Covariates and interactions between the fixed factor and covariates were dropped when not significant for model simplification and fit. Correlations between behavioral and physiological traits were calculated using linear models (LM), with mass as a covariate, and separate analyses were undertaken between ambient vs. elevated CO_2 treatments and ambient vs. elevated temperature treatments.

3.4 Results

Behavior

Elevated temperature significantly affected the distance traveled in body lengths (t_{103}=6.08, P<0.0001; Figure 3.2A). Fish maintained at 25 °C swam 138% further on average than fish maintained at 21 °C, whereas CO_2 treatment did not affect the distance traveled (t_{103}=0.41, P=0.68; Figure 3.2A), and there was no significant interaction between CO_2 and temperature treatments. Elevated temperature also significantly affected the average velocity of fish in body lengths per second (t_{103}=4.47, P<0.0001; Figure 3.2B), with fish maintained at 25 °C swimming 59% faster than fish maintained at 21 °C. Again, CO_2 treatment did not affect average velocity (t_{103}=−0.34, P=0.74; Figure 3.2B), and there was no interaction between CO_2 and temperature. Elevated temperature also affected the time that fish spent active (t_{103}=5.95, P<0.0001; Figure 3.2C), but CO_2 had no effect (t_{103}=0.59, P=0.56; Figure 3.2C), and there was no interaction between CO_2 and temperature. Fish maintained at 25 °C were, on average, 55% more active than fish maintained at 21 °C. There were no significant main effects of either CO_2 (t_{103}=−1.13, P=0.26; Figure 3.3) or temperature (t_{103}=−0.88, P=0.38; Figure 3.3) on time spent in the inner zone.

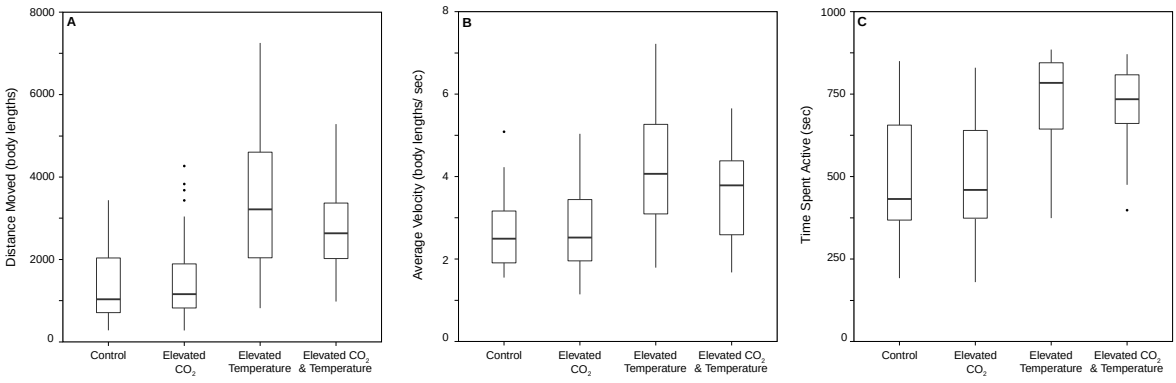

Figure 3.2. The effect of elevated CO_2 and temperature treatments on: (A) the total distance moved, standardized by body length; (B) average velocity, standardized by body lengths; and (C) the time spent active during a 15 min open field test of larval yellowtail kingfish. Boxplots show median and inter-quartile range. N = 29, 38, 29, and 34, respectively.

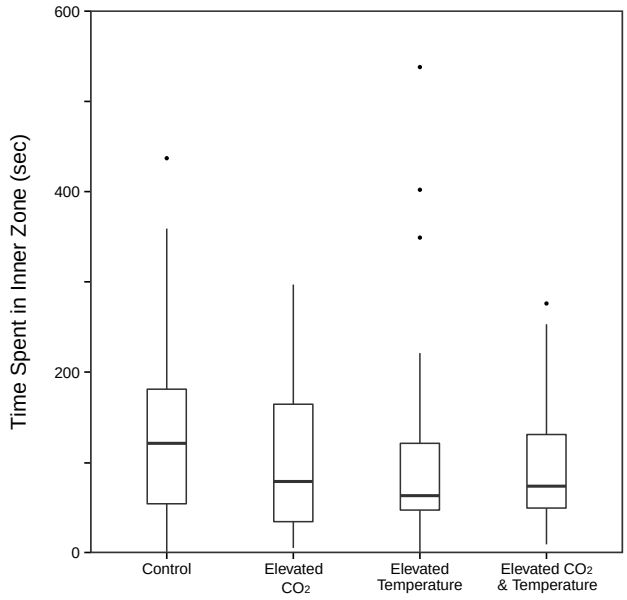

Figure 3.3. The effect of elevated CO_2 and temperature treatments on the time spent in the inner zone during a 15 min open field test in larval yellowtail kingfish. Boxplots show median and inter-quartile range. N = 29, 38, 29, and 34, respectively.

Physiology

Both elevated CO_2 (t_{18}=2.59, P=0.02; Figure 3.4A) and temperature (t_{18}=2.15, P=0.04; Figure 3.4A) significantly affected $\dot{M}O_{2Rest}$. Fish maintained under elevated CO_2 exhibited a 21% increase in $\dot{M}O_{2Rest}$ compared to fish maintained under ambient CO_2 levels, and those maintained at 25 °C showed a 20% increase in $\dot{M}O_{2Rest}$ compared to fish maintained at 21 °C. There was a trend toward a negative interaction between CO_2 and temperature (t_{18}=−2.00, P=0.06; Figure 3.4A). Neither elevated CO_2 nor temperature significantly affected $\dot{M}O_{2Max}$ (t_{18}=0.83, P=0.42 and t_{18}=0.15, P=0.88, respectively; Figure 3.4A), and there was no interaction between treatments. Aerobic scope was significantly affected by fish mass (t_{24}=−3.46, P=0.002; Figure 3.4B), but not elevated CO_2 or temperature (t_{18}=−0.48, P=0.64 and t_{18}=−0.27, P=0.79, respectively; Figure 3.4B), and there was no interaction between treatments.

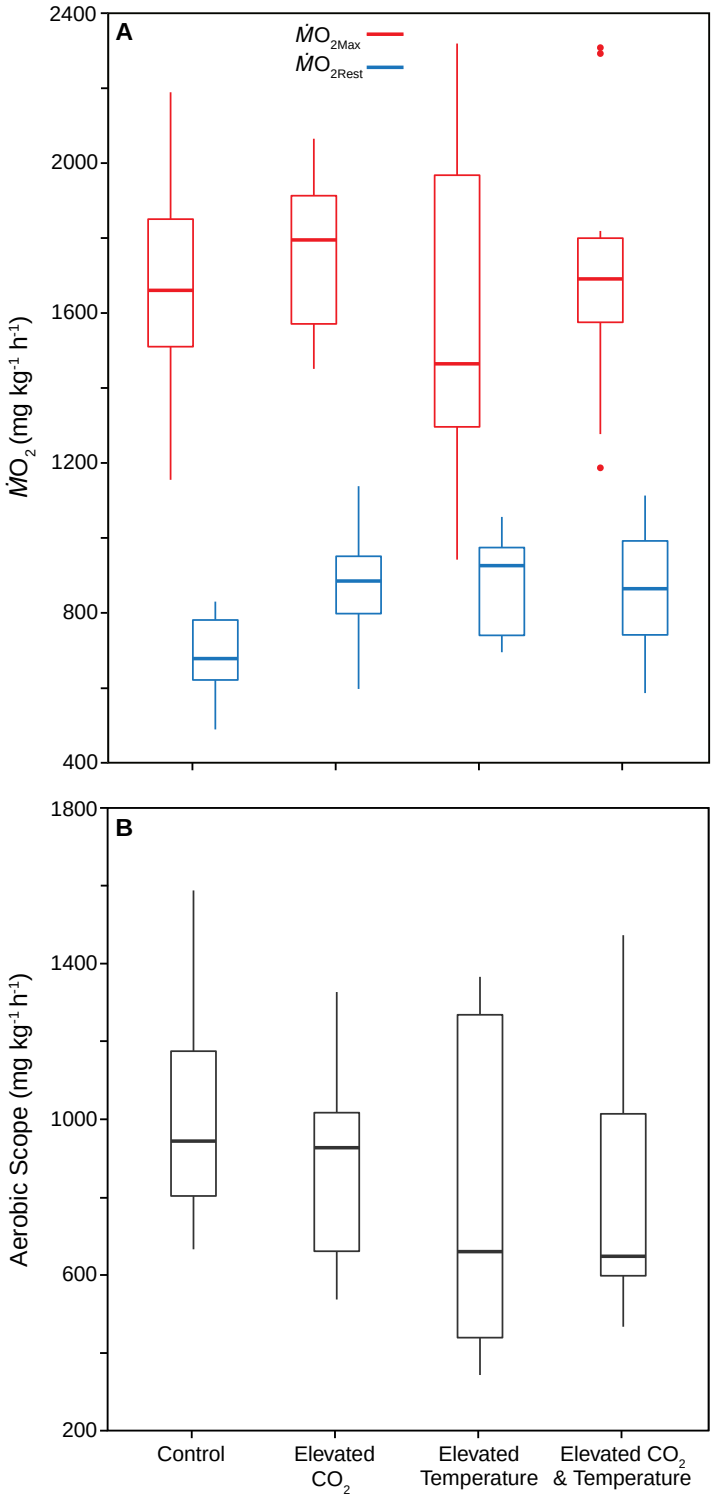

Figure 3.4. The effect of elevated CO_2 and temperature treatments on: (A) resting and maximal oxygen uptake rates ($\dot{M}O_{2Rest}$ and $\dot{M}O_{2Max}$), and (B) aerobic scope ($\dot{M}O_{2Max}$ − $\dot{M}O_{2Rest}$) of larval yellowtail kingfish. Boxplots show median and inter-quartile range. N = 14, 13, 14, and 11, respectively.

Correlations

There were a number of correlations between behavioral and physiological traits. When comparing across temperature treatments, there was a significant positive relationship between $\dot{M}O_{2Rest}$ and time spent in the inner zone (i.e. boldness) in fish maintained at 25 °C (LM t=2.99, slope estimate=2.22, P=0.009; Figure 3.5A). By contrast, there was a negative relationship between $\dot{M}O_{2Rest}$ and boldness in fish maintained at 21 °C (LM t=−2.95, slope estimate=−0.80, P=0.008; Figure 3.5A). Within each temperature treatment, these trends were consistent across CO_2 treatments (ANOVAs of LMs, CO_2 treatment × Time spent in inner zone interaction, P>0.05). Fish maintained at 21 °C also exhibited a significant negative relationship between boldness and $\dot{M}O_{2Max}$ (LM t=−2.61, slope estimate=−1.50, P=0.02; Figure 3.5B), but this relationship was absent in fish maintained at 25 °C. These trends were also consistent across CO_2 treatments (ANOVAs of LMs, CO_2 treatment × Time spent in inner zone interaction, P>0.05).

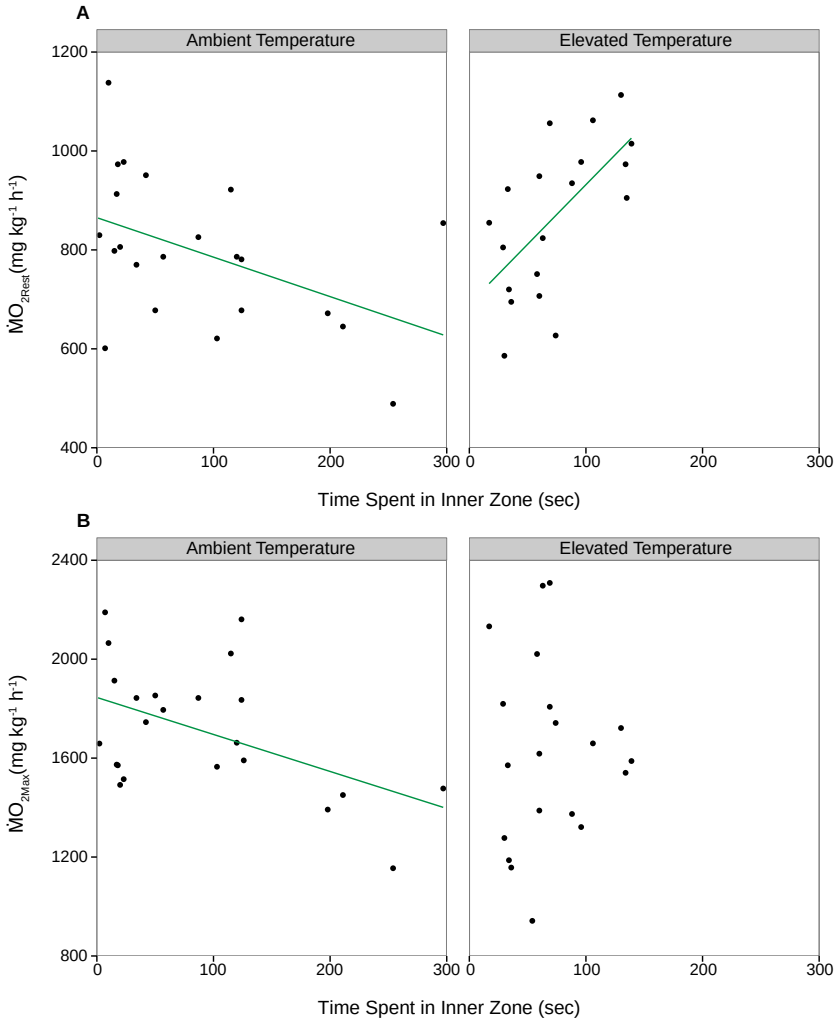

Figure 3.5. The relationship between (A) time spent in the inner zone of the arena and $\dot{M}O_{2Rest}$ and (B) time spent in the inner zone of the arena and $\dot{M}O_{2Max}$ in larval yellowtail kingfish. Panels represent ambient (21 °C) and elevated (25 °C) temperature treatments. Trend lines are shown as derived from linear models, and are only displayed for statistically significant relationships.

3.5 Discussion

Our results indicate that, while elevated temperature alone affected behavioral traits, elevated CO_2 and temperature had an equal effect on metabolic traits in larval yellowtail kingfish. Fish maintained under elevated temperature traveled further, had a higher mean velocity, and spent more time active than fish maintained under ambient temperature. By contrast, elevated CO_2 had no effect on these traits. Both elevated CO_2 and temperature increased resting oxygen consumption rates. There was also evidence of a non-additive interaction, as the combined elevated CO_2 and temperature treatment did not have a significantly different $\dot{M}O_{2Rest}$ from the single-stressor treatments. Boldness, aerobic scope, and $\dot{M}O_{2Max}$ were not affected by elevated CO_2 or temperature. Finally, we found that temperature influenced the relationship between boldness and $\dot{M}O_{2Rest}$ / $\dot{M}O_{2Max}$, which is important because it could influence natural selection and consequently adaptive potential to ocean acidification and warming.

Kingfish reared under elevated temperature exhibited higher activity levels than individuals reared under ambient temperature. This aligns well with other studies that have observed increased activity at higher temperature (e.g. Bignami *et al.,* 2016; Pimentel *et al.*, 2014). It has been proposed that variation in activity level may be attributed to differences in $\dot{M}O_{2Rest}$, because fish with higher $\dot{M}O_{2Rest}$ have higher energetic demands, causing them to become more active and seek food (Biro & Stamps, 2010). Alternatively, fish with higher activity levels might develop a higher $\dot{M}O_{2Rest}$ to cope with the increased energetic demands of a highly active lifestyle (White & Kearney, 2014). Regardless of the mechanism, our results support a link between temperature and activity.

Increased activity under elevated temperature could have either positive or negative effects on larval kingfish. Higher activity rates are likely to increase foraging success (O'Brien, 1979), which could help fish to meet the high energetic demands of their elevated $\dot{M}O_{2Rest}$. Conversely, increased activity could also make fish more vulnerable to predation, particularly in the larval and early juvenile stages (Werner & Anholt, 1993; Biro *et al.*, 2003a, 2003b). Mortality in the early life stages of pelagic fishes can have significant effects on recruitment patterns in adult populations (Houde, 1989). The relative effects of increased foraging success versus increased mortality due to predation will thus largely depend on the abundance and distribution of both predators and prey, which are themselves subject to temperature-induced changes (Llopiz *et al.*, 2014).

In contrast to temperature, elevated CO_2 levels did not affect any of the activity metrics. The effect of elevated CO_2 on activity of larval pelagic fishes appears to be highly variable, with some species showing decreased activity (Bignami *et al.*, 2014; Pimentel *et al.*, 2014) and others experiencing no change in activity (Bignami *et al.*, 2013, 2014, 2016; Munday *et al.*, 2015). Coral reef fishes have shown similar variability, with both increases (Munday *et al.*, 2010) and no changes (Ferrari *et al.*, 2012; Sundin & Jutfelt, 2016) in activity under elevated CO_2. These results suggest some degree of inter-species variation in response to elevated CO_2, although the variation in results could also be attributed to different experimental methodologies and CO_2 levels. Nevertheless, our results indicate that larval kingfish will likely not experience changes in activity levels due to elevated CO_2 under relevant ocean acidification conditions.

Boldness of larval yellowtail kingfish, as measured by time spent in the inner zone of the test arena, did not vary regardless of CO_2 or temperature treatment. Boldness has not been previously assessed for the larval stages of large pelagic fishes, but studies on other fish species have found both an increase (Munday *et al.*, 2010) and a decrease (Jutfelt *et al.*, 2013) in boldness under elevated CO_2. As with activity levels, it appears that larval kingfish are resilient to changes in boldness due to elevated CO_2. Boldness has been linked with elevated temperature previously (Biro *et al.*, 2010; Lienart *et al.*, 2014), but notably, prior studies have predominantly examined temperature changes on time-scales of hours to days. We exposed kingfish to elevated

temperature from the egg stage to 18–24 dph, which may have conferred some benefits in mitigating the effects of elevated temperature. Alternatively, we may not have found any differences in boldness between treatments because the kingfish were well fed. Previous work has shown that fish adopt more risky behaviors when food is scarce (Biro *et al.*, 2003a), and that elevated temperature can exacerbate risky behaviors under low food conditions (Lienart *et al.*, 2014). It is likely that food will be less abundant in nature; therefore, future work could cross food availability with long-term temperature exposure to tease out the relationship between boldness and temperature.

A significant increase in $\dot{M}O_{2Rest}$ was observed in fish maintained at both elevated CO_2 and temperature. The positive correlation between temperature and $\dot{M}O_{2Rest}$ in marine fishes has been well established due to the influence of temperature on biochemical reactions, and our results are consistent with these findings (Houde, 1989; Rombough, 1997). The effects of CO_2 on $\dot{M}O_{2Rest}$ have been more varied; recent meta-analyses indicate that, on average, elevated CO_2 has no effect on $\dot{M}O_{2Rest}$ (Lefevre, 2016; Hannan & Rummer, 2018), although both increases (Munday *et al.*, 2009b; Enzor *et al.*, 2013) and decreases in $\dot{M}O_{2Rest}$ (Pimentel *et al.*, 2014) under elevated CO_2 have been observed. This inter-species variability suggests that the effect of CO_2 on metabolism is species-specific, and underscores the importance of studying the responses of many species of different lifestyles to elevated CO_2 (Hannan & Rummer, 2018). The observed increase in $\dot{M}O_{2Rest}$ at both elevated CO_2 and temperature is indicative of a higher cost of living under end-of-century climatic conditions. With respect to elevated CO_2, this increase in $\dot{M}O_{2Rest}$ suggests an increased metabolic cost of acid-base regulation. Importantly, oceanic pH is relatively uniform in comparison with the strong latitudinal gradient of temperature, indicating that a geographical shift in distribution will not ameliorate the costs of elevated CO_2. By comparison, adult kingfish have a peak distribution at 22.5 °C in Australian waters and display seasonal shifts away from warmer waters (Brodie *et al.*, 2015), suggesting that at present, they avoid warmer temperatures and the higher metabolic costs they incur. Nonetheless, an overall increase in average temperature is predicted for the end of the century, which would force even greater distribution shifts to avoid an increased metabolic cost.

In contrast to $\dot{M}O_{2Rest}$, neither $\dot{M}O_{2Max}$ nor aerobic scope of larval kingfish was significantly affected by elevated CO_2 or temperature. In other marine fishes, the effects of CO_2 on $\dot{M}O_{2Max}$ and aerobic scope have been diverse, with many species showing no effect (Hannan & Rummer, 2018), but some increases (Couturier *et al.*, 2013) and decreases (Munday *et al.*, 2009b) have been observed. Therefore, our results are consistent with many previous studies. By contrast, temperature tends to increase $\dot{M}O_{2Max}$ (Norin & Clark, 2016), although decreases in $\dot{M}O_{2Max}$ have been observed in some tropical fish species, likely because they live closer to their thermal limits than temperate species (Nilsson *et al.*, 2009). Aerobic scope has shown a strong species-dependence in response to elevated temperature as well (Nilsson *et al.*, 2009; Lefevre, 2016). It is possible that we did not detect an effect of elevated temperature on $\dot{M}O_{2Max}$ or aerobic scope because there was higher individual variation in $\dot{M}O_{2Max}$ in the elevated temperature treatment as compared with controls, which may have masked a significant effect. This high variability could represent true inter-individual differences in $\dot{M}O_{2Max}$, but could also be indicative of differential recovery times from the exhaustive chase. Still, our results suggest that elevated CO_2 and temperature are unlikely to have a meaningful impact on $\dot{M}O_{2Max}$ or aerobic scope in yellowtail kingfish, which is consistent with findings for many other species of marine fishes (Lefevre, 2016).

The increase in $\dot{M}O_{2Rest}$ in conjunction with the lack of change in $\dot{M}O_{2Max}$ and aerobic scope suggests that, while there are higher maintenance costs under elevated CO_2 and temperature conditions, this does not diminish the capacity of fish to perform aerobic activities. However, while the overall capacity for aerobic activity did not diminish, $\dot{M}O_{2Rest}$ comprises a larger proportion of the aerobic scope at higher CO_2 and temperature conditions than under ambient conditions. Thus, a smaller fraction of the metabolic scope is available for aerobic activities such as growth, development, and reproduction. This is relevant because fish in this experiment were fed ad libitum with highly nutritious food, meaning that they could easily meet the higher energy requirements of fast growth. In nature, food distribution is more patchy and unreliable, making it more difficult for fish to meet higher energetic demands. Therefore in food-scarce environments, we might expect to see a decline in aerobic scope at higher temperatures (McLeod *et al.*, 2013).

We observed opposing relationships between boldness and $\dot{M}O_{2Rest}$ under elevated versus ambient temperature treatments. Under elevated temperature, bolder individuals had a higher $\dot{M}O_{2Rest}$, while under ambient temperature, bolder individuals had a lower $\dot{M}O_{2Rest}$. Correlations between boldness and $\dot{M}O_{2Rest}$ have been observed previously (Biro & Stamps, 2010), and two models have been proposed to explain these patterns. The "performance model" posits that bolder individuals will consume more energy, and thus require a higher resting oxygen uptake rate to support their higher metabolic needs (Careau *et al.*, 2008). The performance model predicts a positive relationship between $\dot{M}O_{2Rest}$ and boldness. Conversely, the "allocation model" is based on the idea that organisms have a finite supply of energy and must balance their energy budget between $\dot{M}O_{2Rest}$ and boldness-related activities (Careau *et al.*, 2008). This model predicts a negative relationship between $\dot{M}O_{2Rest}$ and boldness. The performance model has broader support from experimental evidence than the allocation model (Biro & Stamps, 2010), and our results support the performance model under elevated temperature. However, we observed a negative relationship between $\dot{M}O_{2Rest}$ and boldness under ambient temperature conditions. It is possible that the allocation model holds for this population under ambient temperature, and that the performance model applies under elevated temperature. Indeed, it has been suggested that environmental stressors such as temperature can alter the relationship between behavioral and physiological traits (Killen *et al.*, 2013). Still, the proximal cause for this shift from a positive to a negative relationship is not clear, and represents a fruitful avenue for future research.

Importantly, our results show that boldness and $\dot{M}O_{2Rest}$ have opposing relationships under different thermal, but not CO_2, regimes. The implication of this pattern is that different thermal regimes could have opposing influences on selection, driving changes in rates of adaptation to environmental change. We cannot predict with certainty the direction that selection will take, as the relative benefits of having a higher $\dot{M}O_{2Rest}$ or boldness will depend upon environmental factors such as food availability and predator density. Still, both boldness (Brown *et al.*, 2007; Ariyomo *et al.*, 2013) and aerobic scope (Munday *et al.*, 2017) have shown heritability in fishes, suggesting that their correlation could indeed influence the rate and direction of adaptation.

A similar relationship between $\dot{M}O_{2Max}$ and boldness was also detected, with bolder individuals having a lower $\dot{M}O_{2Max}$ at ambient temperature, but the relationship disappeared under elevated temperature. The links between $\dot{M}O_{2Max}$ and behavioral traits have been relatively understudied, and there are no models to explain the underlying causes of such patterns. However, $\dot{M}O_{2Rest}$ and $\dot{M}O_{2Max}$ have shown correlations in fishes (Norin & Malte, 2012), suggesting that the similar relationships that we saw between $\dot{M}O_{2Rest}$ and $\dot{M}O_{2Max}$ with boldness are plausible.

In conclusion, this study indicates that elevated temperature has a greater effect than elevated CO_2 levels on the behavior, physiology, and correlations between behavior and physiology of larval kingfish. At elevated temperature, larval kingfish displayed elevated activity levels and $\dot{M}O_{2Rest}$, indicating a higher cost of living. However, the overall effect of these traits will ultimately depend upon the distribution and abundance of predators and food sources for the larval fish. It is likely that as ocean warming progresses, kingfish will shift their distributions polewards to avoid incurring the metabolic and behavioral costs associated with warmer waters, as has been documented in a range of marine species (Pecl *et al.*, 2017). Unlike temperature, pCO_2 does not have a strong latitudinal gradient, and thus rising CO_2 levels cannot be avoided through range shifts. This implies that kingfish may still experience increased metabolic costs in future climatic conditions, unless they can adapt to elevated CO_2. Further work is needed to determine whether variation in $\dot{M}O_{2Rest}$ to elevated CO_2 is heritable and thus whether adaptation is possible. Our results also showed correlations between $\dot{M}O_{2Rest}$ and boldness that were temperature-dependent. These opposing correlations could influence the rate and direction of future adaptation, but their consequences will depend on additional factors such as predation risk and food availability. Future work could include additional stressors to determine the relative benefits of boldness and $\dot{M}O_{2Rest}$, revealing the evolutionary implications of climate change. Our findings provide novel insights into the behavioral and physiological impacts of future climate conditions on the early life stages of a large pelagic fish, a critical knowledge gap in climate change research.

Chapter 4: Beneficial effects of diel CO_2 cycles on reef fish metabolic performance are diminished under elevated temperature

This chapter is prepared for submission to Coral Reefs

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4.1 Summary

Elevated CO_2 levels affect metabolic performance in some coral reef fishes. However, all studies to date have employed stable elevated CO_2 levels, whereas reef habitats can experience substantial diel fluctuations in pCO_2. Additionally, past studies have often investigated the effect of elevated CO_2 in isolation, despite the fact that ocean temperatures will increase in tandem with CO_2 levels. Here, we tested the effects of stable versus diel-cycling elevated CO_2 conditions and elevated temperature on metabolic traits of juvenile spiny damselfish, *Acanthochromis polyacanthus*. We also examined correlations between metabolic traits and measures of routine activity. Resting oxygen uptake rates ($\dot{M}O_2$) were higher in the stable elevated CO_2 treatment compared with the control, but were restored to control levels under diel CO_2 fluctuations. However, the benefits of diel CO_2 fluctuations were diminished at elevated temperature. Factorial aerobic scope showed a similar pattern, but neither maximal $\dot{M}O_2$ nor absolute aerobic scope was affected by CO_2 or temperature. At stable elevated CO_2 and temperature, resting $\dot{M}O_2$ was positively correlated with routine activity, but no correlations were detected in other treatments. Our results suggest that diel CO_2 cycles can ameliorate the increased metabolic cost associated with elevated CO_2, but elevated temperature diminishes the benefits of diel CO_2 cycles. Thus, previous studies may have misestimated the effect of ocean acidification on the metabolic performance of reef fishes by not accounting for environmental CO_2 fluctuations. Our findings provide novel insights into the interacting effects of diel CO_2 fluctuations and temperature on the metabolic performance of reef fish.

4.2 Introduction

Increased uptake of carbon dioxide (CO_2) from the atmosphere is driving a decrease in global oceanic pH through a process known as ocean acidification (Doney *et al.*, 2009). Average sea surface pH is predicted to decrease by between 0.3 and 0.4 units over the next century if anthropogenic emissions of CO_2 remain unchecked (Collins *et al.*, 2013). Ocean acidification projections are based on data collected from open ocean environments that have a relatively stable pCO_2 through time (Doney *et al.*, 2009). By contrast, many shallow-water marine ecosystems experience fluctuations in pCO_2 on a variety of temporal scales, which in some instances can exceed end-of-century CO_2 predictions (Hofmann *et al.*, 2011; Duarte *et al.*, 2013). More specifically, in many habitats such as coral reefs, seagrass meadows, and kelp forests, pCO_2 fluctuates on a daily cycle, falling during the day as CO_2 is taken up by photosynthesis, and peaking at night as CO_2 is released by respiration (Frieder *et al.*, 2012; Shaw *et al.*, 2012; Challener *et al.*, 2016). Additionally, CO_2 fluctuations are predicted to increase in amplitude with ongoing ocean acidification due to the associated change in seawater buffering capacity caused by increased CO_2 uptake (Shaw *et al.*, 2013; McNeil & Sasse, 2016). Thus, organisms living in shallow-water environments will have to contend with periods of even higher CO_2 in the future, while also experiencing a greater range of CO_2 levels.

A host of experiments have been conducted to gauge the effects of ocean acidification on a range of shallow-water marine taxa (Hendriks *et al.*, 2010; Kroeker *et al.*, 2010; Wittmann & Pörtner, 2013; Nagelkerken & Munday, 2016). However, most studies have employed stable elevated CO_2 levels, and thus there is concern regarding their ecological relevance (McElhany & Busch, 2013; Wahl *et al.*, 2016). Indeed, a number of studies that have incorporated CO_2 fluctuations into their experimental designs have shown that these fluctuations can significantly modify biological responses to ocean acidification (Cornwall *et al.*, 2013; Comeau *et al.*, 2014; Frieder *et al.*, 2014; Ou *et al.*, 2015; Clark & Gobler, 2016; Eriander *et al.*, 2016; Jarrold *et al.*, 2017; Mangan *et al.*, 2017; Enochs *et al.*, 2018; Wahl *et al.*, 2018). These results have spurred an interest in including CO_2 fluctuations in ocean acidification research to

better model future conditions and assess the likely impacts on shallow-water marine organisms (McElhany & Busch, 2013; Wahl *et al.*, 2016).

In addition to increasing acidification, the oceans are also facing increasing warming due to rising atmospheric CO_2 concentrations (Doney *et al.*, 2012). Average oceanic surface temperatures are projected to increase between 2-3 °C by the year 2100 if emissions continue unchecked (Collins *et al.*, 2013). Thus, marine organisms will be exposed to both ocean acidification and warming in the near future (Doney *et al.*, 2012). There is a growing recognition of the importance of studying ocean acidification and warming in tandem, as their combined effects on marine organisms are not always additive, but instead may be synergistic or antagonistic (Kroeker *et al.*, 2013; Riebesell & Gattuso, 2015). Thus, incorporating both warmer and more acidic conditions into experimental treatments means that results can be used to better predict the future impacts of climate change.

An extensive body of literature has grown over recent years to assess the effects of ocean acidification and warming on marine fishes. Initially it was thought that marine fishes would be resilient to changes in ocean pCO_2 because of their robust acid-base regulatory systems (Ishimatsu *et al.*, 2008; Melzner *et al.*, 2009). However, experimental trials have shown that acid-base regulation can come at a cost (Strobel *et al.*, 2013), altering physiological and behavioral traits in some species (Heuer & Grosell, 2014; Cattano *et al.*, 2018). Physiologically, metabolic traits have been a key focus in ocean acidification studies, as they describe the energetic requirements of organisms that may underpin a range of fitness-related traits (Farrell *et al.*, 2008; Biro & Stamps, 2010; Eliason *et al.*, 2011). Recent meta-analyses indicate that elevated CO_2 tends to increase resting oxygen uptake rates ($\dot{M}O_{2Rest}$) in marine fishes (Cattano *et al.*, 2018), but there is no consistent effect on maximal oxygen uptake rates ($\dot{M}O_{2Max}$) or aerobic scope (Lefevre, 2016), though both increases and decreases in these traits have been observed (Hannan & Rummer, 2018). Elevated CO_2 has also been shown to alter a range of sensory and behavioral responses in marine fishes such as activity, boldness, olfaction, lateralization, and escape responses in some species, but not others (reviewed in Clements & Hunt, 2015; Nagelkerken & Munday, 2016; Cattano *et al.*, 2018).

Compared with the effects of stable elevated CO_2, relatively little is known about how CO_2 fluctuations affect the performance of marine fishes at higher average CO_2 levels. Because physiological and behavioral impairments in fishes caused by elevated CO_2 are often concentration-dependent (Cattano *et al.*, 2018), it has been hypothesized that diel CO_2 cycles might either mitigate or exacerbate the effects of elevated CO_2 on these traits (Shaw *et al.*, 2013). Indeed, diel CO_2 cycles reduced or eliminated behavioral impairments in two species of coral reef fishes as compared to stable elevated CO_2 (Jarrold *et al.*, 2017). In contrast, diel CO_2 cycles did not affect behavioral responses in juvenile blacksmith (Kwan *et al.*, 2017). With regards to physiological performance, diel CO_2 cycles were shown to alter metabolic performance in pink salmon larvae and European eels (Methling *et al.*, 2013; Ou *et al.*, 2015). However, it is important to note that both of these studies employed fluctuating CO_2 treatments that peaked at the same level as their stable CO_2 treatments, making it difficult to determine whether the observed changes were due to the fluctuations themselves or differing mean CO_2 treatments. Thus, it remains uncertain how diel CO_2 cycles will affect metabolic traits in fishes.

The effects of temperature on marine fishes are more consistent. Elevated temperature is well known to affect metabolic traits in marine fishes through its effect on rates of biochemical reactions (Fry, 1971). This typically results in higher rates of $\dot{M}O_{2Rest}$ and $\dot{M}O_{2Max}$, although decreases in $\dot{M}O_{2Max}$ can sometimes be seen outside a species thermal window (Fry & Hart, 1948; Rombough, 1997). Aerobic scope, by contrast, has shown a strong species-dependence in response to elevated temperature (Lefevre, 2016). A range of behavioral changes in marine fishes have also been observed at elevated temperature, such as altered activity, boldness, risk assessment, escape responses, and competitive interactions (Biro *et al.*, 2010; Lienart *et al.*, 2014; Warren *et al.*, 2016; Allan *et al.*, 2017; Schmidt *et al.*, 2017). The mechanism underpinning these behavioral changes is unknown, but it has been suggested that temperature can influence behavior indirectly through its effect on metabolic rate (Biro & Stamps, 2010; Biro *et al.*, 2010).

The interacting effects of elevated CO_2 and temperature are often complex, and differ between species and traits. For instance, the combined effects of elevated CO_2 and temperature on $\dot{M}O_{2Rest}$ were antagonistic in larval kingfish *Seriola lalandi* (Chapter

3), but synergistic in bald notothen *Pagothenia borchgrevinki* (Enzor *et al.*, 2013). Behaviorally, elevated temperature reduced the effect of elevated CO_2 on lateralization in a damselfish *Pomacentrus wardi* (Domenici *et al.*, 2014). Interestingly, the nature of interactions between elevated CO_2 and temperature can shift within a species depending on the treatment or the trait being measured. For instance, elevated CO_2 and temperature acted antagonistically on aerobic scope at a moderately elevated temperature (i.e. +2 °C above ambient), but became additive at a high temperature (i.e. +3 °C above ambient) in two species of cardinalfishes (Munday *et al.*, 2009b). For the dottyback *Pseudochromis fuscus*, elevated CO_2 and temperature acted antagonistically on predator selectivity, but synergistically on predation rate (Ferrari *et al.*, 2015). These studies highlight the unpredictable nature of the interacting effects of elevated CO_2 and temperature, and therefore underscore the need to incorporate multiple stressors into experiments to accurately assess the effects of future climate change on marine fishes. To date no studies have considered how elevated temperature may affect the interactive effects of elevated CO_2 and diel CO_2 cycles on metabolic performance in marine fishes.

While many climate change studies focus on the mean effects of environmental stressors on performance, fewer consider individual variation in responses. Not only can a focus on variation highlight the individuals best suited for survival in future conditions, but it can also reveal correlated phenotypic traits (Bennett, 1987; Sunday *et al.*, 2014). Correlations between traits can help or hinder survival at the individual scale, and can even have implications for the capacity of populations to adapt to climate change. If the traits of interest are heritable, then correlations between them could either accelerate or decelerate adaptive evolution, depending on whether the traits are positively or negatively correlated with respect to the fitness landscape (Sunday *et al.*, 2014). If the traits are negatively correlated, selection on one would diminish the other, decreasing the rate of adaptation, and vice versa (Lande, 1979; Lande & Arnold, 1983). It is particularly important to investigate correlations in the context of climate change, as environmental stressors have been shown to alter the relationship between behavioral and physiological traits, either revealing or masking these relationships (Killen *et al.*, 2013). Thus, identifying correlations among traits, and how they shift under different environmental conditions, is a key step in

predicting species persistence in the face of climate change (McBryan *et al.*, 2013; Munday *et al.*, 2013; Sunday *et al.*, 2014).

To test the interacting effects of elevated CO_2, diel CO_2 fluctuations, and elevated temperature on reef fish physiology, we reared juvenile damselfish *Acanthochromis polyacanthus* for 11 weeks from hatching under a series of CO_2 and temperature treatments. Coral reefs are highly dynamic systems, and currently experience diel CO_2 fluctuations ranging from ± 50 to 600 μatm around the mean (Kayanne *et al.*, 1995; Manzello, 2010; Shaw *et al.*, 2012; Albright *et al.*, 2013; Kline *et al.*, 2015). The magnitude of these fluctuations is projected to increase by the end of the century (Shaw *et al.*, 2013). Thus, we employed three CO_2 treatments: a stable elevated CO_2 (1000 μatm), a fluctuating elevated CO_2 (1000 ± 500 μatm), and a stable control (450 μatm). We did not employ a fluctuating control CO_2 treatment because our primary goal was to test how CO_2 cycles affect physiological performance of reef fishes at the higher average pCO_2 conditions projected for the end of the century. We maintained these three treatments at the summer average temperature for the study location, 29 °C. To test the interacting effect of elevated temperature, we also reared fish at 31 °C at both elevated CO_2 treatments (stable and fluctuating) to represent projected end-of-century temperature and CO_2 conditions (RCP 8.5, Collins *et al.*, 2013). We tested the physiological performance of the fish by measuring their metabolic traits, specifically $\dot{M}O_{2Rest}$ and $\dot{M}O_{2Max}$ as well as absolute aerobic scope (AAS) and factorial aerobic scope (FAS). In addition to determining the mean effects of fluctuating elevated CO_2 and temperature on metabolic traits, we also measured routine activity in the same individuals to investigate whether there were correlations between behavioral and metabolic traits. Specifically, we used video tracking software to quantify active time and average velocity during an open field test. The mean results of these tests are reported in Jarrold & Munday (2018b); here, we focus on correlations between these measures of activity and metabolic traits at the individual level because of their potential to affect individual performance and adaptive potential.

4.3 Methods

Study Species and Broodstock Maintenance

The spiny chromis damselfish, *Acanthochromis polyacanthus*, is common on coral reefs throughout the western-Pacific region. *A. polyacanthus* is a demersal spawner, laying its eggs in small caves or crevices in the reef. The species has direct development, meaning that eggs hatch directly into juveniles without a pelagic larval phase, and both parents provide care to the offspring for up to 45 days post-hatching (Kavanagh, 2000). Because it can be bred and reared in captivity with a high success rate, *A. polyacanthus* has become a model species for the study of ocean acidification and warming in coral reef fishes (e.g. Munday *et al.*, 2008a, 2011, Donelson *et al.*, 2010, 2012; Welch *et al.*, 2014; Heuer *et al.*, 2016).

Adult *A. polyacanthus* were collected using hand nets from Bramble Reef (site 1: 18°22'S, 146°40'E; site 2: 18°25'S, 146°40'E) on the Great Barrier Reef in July 2015. Physical and chemical water parameters from a similar mid-shelf reef in the central Great Barrier Reef can be seen in Appendix A. The fish were transported to an environmentally controlled aquarium research facility at James Cook University (Townsville, Australia) where they were housed in 60 L aquaria as breeding pairs. Each pair was provided with half a terracotta pot for use as a shelter and spawning site. Water pCO_2 was maintained at a stable, ambient level (~490 μatm). Water temperatures were increased at a rate of 0.5°C per week until the summer breeding temperature of 29 °C was reached during the first week of December 2016. Aquaria were checked daily for the presence of newly laid clutches. Pairs were fed *ad libitum* on commercial fish feed pellets (INVE Aquaculture Nutrition NRD 12/20) once daily outside the breeding season and twice daily during the breeding season.

Carbonate Chemistry

This study was conducted at the National Sea Simulator (SeaSim) facility at the Australian Institute of Marine Science (AIMS) (Cape Cleveland, Australia).
The experimental setup consisted of 10 independent flow-through systems, with 2 systems per CO_2 x temperature treatment. Each system supplied ultra-filtered seawater (0.04 μm), at either 29 or 31 °C, to three custom-built 50 L tanks at the rate of 50 L h^{-1}. Thus, there were 6 tanks per treatment, for a total of 30 tanks. To ensure

temperature stability to ± 0.1 °C, the experimental tanks were placed in individual temperature-controlled water baths. The treatments and tank replicates were positioned randomly in the experimental room.

A custom-designed system controlled the pCO_2 and temperature levels in each system. A Model Predictive Control logic running on a programmable logic controller (PLC) (Series S7-1500, Siemens, Australia) was integrated into the general SeaSim control system, which allowed for SCADA (Siemens WinCC) accessibility and data archiving. Non-dispersive infrared measurements were used to feedback tank pCO_2 into each system line. On each system, tank water was delivered to an equilibrator (SeaSim, AIMS design, custom built) by an in-tank submersible pump (Universal Pump 1260, EHEIM, Deizisau, Germany). Membrane contactors (Membrana Liqui-Cel 2.5x8 Extra-Flow. 3M, USA) dissolved CO_2 into the flow-through water. The CO_2 was delivered through Gas Mass Flow Controllers (GFC17 series, Aalborg, Orangeburg, USA) as directed by the treatment plan, as well as a feedback signal from the experimental tanks. There was also an NDIR CO_2 analyzer (Telaire T6613, Amphenol, Australia) in the equilibrator to provide live feedback of the pCO_2 of the air in the chambers to the PLC. The CO_2 analyzers were calibrated each month using certified calibration gas mixtures at 0, 600 and 2000 ppm. For the duration of the experiment, the incoming coastal seawater had a pCO_2 of between 500-550 µatm. To decrease the control pCO_2 level, membrane contactors (Membrana Liqui-Cel 4x28 Extra-Flow) were used to remove CO_2, using CO_2–depleted air as a sweep gas. 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). Mean values for each of these seawater parameters are presented in Table 4.1. Figure 4.1 shows the shape of the CO_2 treatments.

Table 4.1. Experimental water chemistry. Values are mean ± SD of daily average, minimum, and maximum pCO_2, temperature, salinity, and total alkalinity (TA). The pCO_2 values are based on readings taken every ten minutes. The temperature values are based on readings taken every hour. The salinity and TA values are based on measurements taken weekly.

CO_2 treatment	**Temperature treatment**	**Mean pCO_2 (µatm)**	**Min pCO_2 (µatm)**	**Max pCO_2 (µatm)**	**Temperature (°C)**	**Salinity**	**TA (µmol kg^{-1})**
450	29	435 ± 38	425 ± 36	477 ± 163	28.8 ± 0.2	35.4 ± 0.4	2325 ± 33
1000	29	992 ± 19	956 ± 74	1035 ± 95	28.8 ± 0.2	35.4 ± 0.4	2325 ± 32
1000	31	981 ± 8	958 ± 42	1002 ± 23	31.1 ± 0.3	35.4 ± 0.4	2310 ± 48
1000 ± 500	29	1060 ± 34	521 ± 43	1544 ± 80	28.8 ± 0.2	35.4 ± 0.4	2323 ± 32
1000 ± 500	31	1079 ± 49	556 ± 64	1570 ± 118	31.1 ± 0.3	35.4 ± 0.4	2314 ± 39

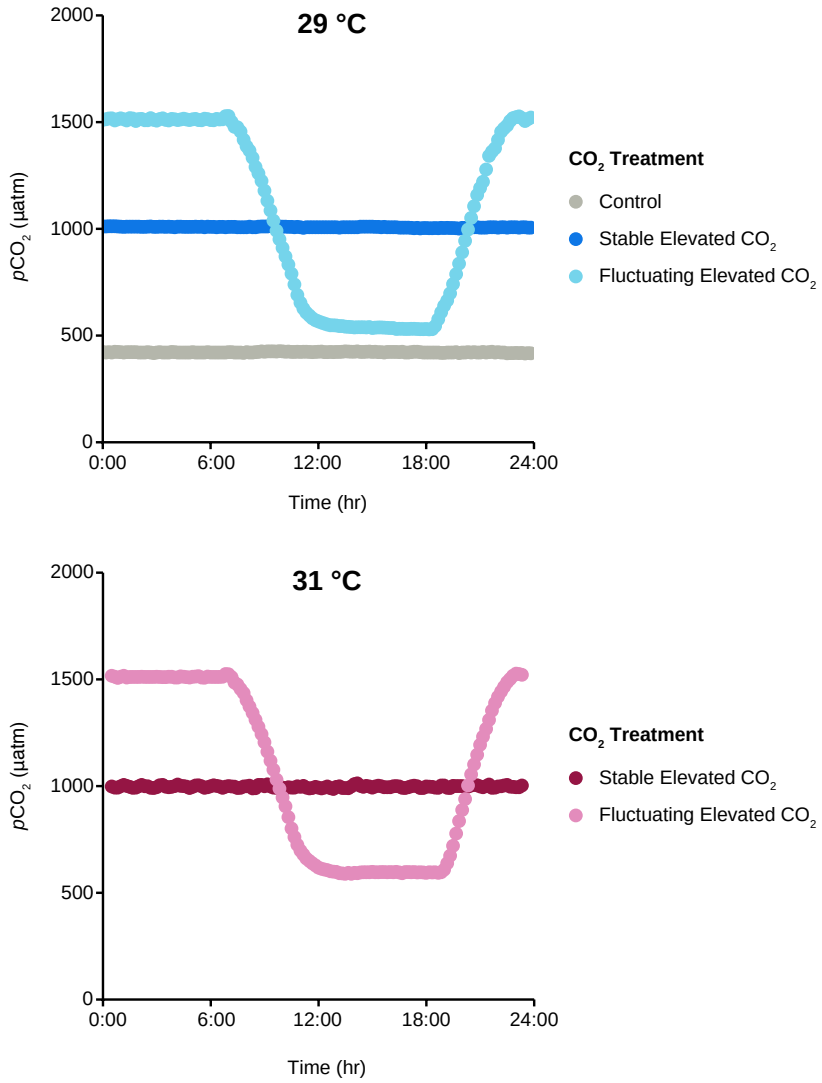

Figure 4.1. Plots of daily stable and fluctuating CO_2 for both temperature treatments. CO_2 measurements were taken every ten minutes. Data are taken from a representative 24-hour period.

Experimental Design

Two breeding pairs supplied a total of three clutches of offspring for the experiments. Whenever a clutch hatched, they were transferred at one day post hatch (dph) to the experimental system, and split evenly between the five treatments, with two tanks per treatment. There were three treatments at 29 °C, with CO_2 treatments of control (450 µatm), stable elevated CO_2 (1000 µatm) and fluctuating elevated CO_2 (1000 ± 500 µatm), and two treatments at 31 °C, with CO_2 treatments of stable elevated CO_2 (1000 µatm) and fluctuating elevated CO_2 (1000 ± 500 µatm). Offspring were fed 6 mL of freshly hatched *Artemia* nauplii (approx. 4000 mL^{-1}) for the first four dph. From 5-28 dph they were fed 0.12 g of a weaning fish feed (INVE Aquaculture Nutrition Wean-S 200–400 µm) daily. From 29-42 dph they were fed 0.15 g of a small pellet fish feed (INVE Aquaculture Nutrition NRD 5/8) daily, which was increased to 0.2 g after 42 dph, and to 0.35 g after 70 dph.

Research was carried out under approval of the James Cook University animal ethics committee (permit: A2210) and according to the University's animal ethics guidelines.

Behavioral Assay

Routine activity was assessed at 63-70 dph during daylight hours only (9:00-17:00). Routine activity was determined using an open field test using methods similar to those described by Laubenstein *et al.* (Chapter 3). The test arena consisted of a round, white plastic arena (26 cm diameter, 6 cm height) placed inside a white plastic bin (52 cm length, 32 cm width, 34cm height), which was opaque to minimize visual disturbance for the fish, but allowed light through for filming. Two wooden planks (60 cm length, 2 cm width, 9 cm height) were laid across the top of each bin, and a sheet of white corflute was placed on top of the planks. The corflute had a small circular hole cut into its center, where a video camera (Casio EX-ZR2000) was placed. All fish were tested in their respective treatment water at a depth of 5 cm. A

trial began when a fish was placed into the center of the arena by gently transferring it with a beaker to minimize stress. The camera was turned on, and the fish was filmed for 15 minutes.

At the end of a trial, the fish was removed from the test arena with a beaker, tagged with a small colored elastomer tag (Northwest Technologies) injected under the skin to keep track of individuals between tests, and returned to its respective treatment water. Fish from each pCO_2 treatment were tested at random times throughout the day to account for any possible time of day effects in the fluctuating treatments. The test arena was rinsed with seawater between trials.

Routine activity was determined using Lolitrack software (v4.1.0 Loligo Systems, Tjele, Denmark), which tracked and quantified the movements of the fish. The videos were analyzed blind to treatment by scrambling the video file names prior to analysis. The first five minutes of each video were discarded to allow for the fish to habituate to the arena. The software quantified the following parameters: time active (seconds) and average swimming velocity (cm s^{-1}). Between 26 and 29 individuals were tested per treatment.

Physiological Assay

Metabolic traits were assessed at 66-76 dph, giving individuals at least two days to recover from any effects of handling during the routine activity trial. The oxygen uptake rates ($\dot{M}O_2$) of fish were measured using an intermittent-flow respirometry system, based on standard respirometry methods (Roche *et al.*, 2013). Prior to respirometry trials, fish were fasted for 24 hours to ensure a post-absorptive state (Niimi & Beamish, 1974). To obtain maximal oxygen uptake rates ($\dot{M}O_{2Max}$), fish were chased for 3 minutes in a circular container (20 cm diameter, 9 cm height), and then exposed to air for 1 minute, as this chase protocol has been previously determined to be sufficient for *A. polyacanthus* to reach exhaustion (Chapter 2). Following the chase and air exposure, fish were immediately placed into darkened glass respirometry chambers (28 mL total volume, including tubing) that were submerged in a water bath containing the fish's respective treatment water. The water bath received a continuous flow-through of treatment water, such that fish in the respirometry chambers experienced the same CO_2 fluctuation patterns as they had in

the rearing stage. The respirometry chambers were supplied with treatment water from the water bath by flush pumps, for one minute every five minutes. This pattern ensured that O_2 levels within the chambers did not fall below 80% air saturation. The temperature-compensated oxygen concentration (mg L^{-1}) of the water in each chamber was continuously recorded (once every two seconds) using oxygen-sensitive REDFLASH dye on contactless spots (2 mm) adhered to a glass tube in line with the chamber, and linked to a Firesting Optical Oxygen Meter (Pyro Science e. K., Aachen, Germany) with 2 m fiber-optic cables. Fish remained in the respirometry chambers for 20 hours while they recovered to their resting oxygen uptake rates ($\dot{M}O_{2Rest}$). At the end of each trial, fish were removed from the chambers and their mass (0.376 ± 0.100 g; mean ± SD) was recorded. Between 25 and 29 individuals were tested per treatment.

Individual oxygen uptake rates were calculated using linear least squares regression in LabChart version 7.2.5 (ADInstruments, Colorado Springs, CO, USA). Background microbial respiration was subtracted from total chamber respiration to determine the oxygen uptake rates of the fish as per Rummer *et al.* (2016). The $\dot{M}O_{2Max}$ was taken to be the highest oxygen uptake rate (over 4 minute intervals) and usually occurred during the first measurement cycle. The $\dot{M}O_{2Rest}$ was estimated as the average of the lowest 10% of values, excluding outliers above or below 2 SD. Absolute aerobic scope (AAS) was calculated as the difference between $\dot{M}O_{2Max}$ and $\dot{M}O_{2Rest}$, and factorial aerobic scope (FAS) was calculated as the quotient of $\dot{M}O_{2Max}$ and $\dot{M}O_{2Rest.}$

Statistical Analyses

Separate linear mixed-effects models (LME, “nlme” package in R) were used to determine the effect of CO_2 and temperature on $\dot{M}O_{2Rest}$, $\dot{M}O_{2Max}$, FAS and AAS. Treatment was a main effect with five levels, as the design was not fully factorial. Mass was included as a fixed effect in each model and was mean-centered to aid with the interpretation of model intercepts. Clutch was included as a random effect. Initially, fully interactive models were run. Then non-significant interaction terms were removed, with the final model choice confirmed based on Akaike information criterion (AIC) (Appendix B). Thus, all models used were additive (Mass + Treatment). Assumptions of normality and homogeneity of residuals were visually assessed with Q-Q plots and frequency distributions of residuals, and met for all

models. Parameters were estimated using restricted maximum-likelihood. *Post hoc* multiple comparisons between treatments were done using the R function “glht” in the package “multcomp” using Tukey’s HSD contrasts.

The relationships between activity and metabolic traits were calculated using linear mixed effect models. Metabolic traits ($\dot{M}O_{2Rest}$, $\dot{M}O_{2Max}$, FAS, AAS) were used as the response variables, and measures of activity (time spent active, average swimming velocity) were the predictor variables. Mean-centered mass was included as a covariate, and clutch was included as a random effect. As previously, fully interactive models were run initially, then models were simplified when interactions were not significant and confirmed by AIC (Appendix B). Thus, all models were additive (Mass + Metabolic Trait). Separate analyses were undertaken for each treatment to discern different relationships that might have emerged under different conditions. All analyses were conducted using R version 3.1.3 (R Core Team, 2014).

4.4 Results

The resting oxygen uptake rates ($\dot{M}O_{2Rest}$) of juvenile fish were significantly different between CO_2 and temperature treatments (Figure 4.2A). Fish reared at stable elevated CO_2 exhibited a significantly higher $\dot{M}O_{2Rest}$ than fish reared at stable control conditions (Tukey contrasts, z=4.98, P<0.001). However, the $\dot{M}O_{2Rest}$ of fish reared at the fluctuating elevated CO_2 treatment was fully restored to control levels, being significantly lower than fish reared at stable elevated CO_2 (Tukey contrasts, z=−2.85, P=0.03) and not significantly different from control fish (Tukey contrasts, z=1.89, P=0.32). When fish were reared at elevated temperature, their $\dot{M}O_{2Rest}$ was significantly higher than that of control fish (Tukey contrasts, stable elevated CO_2: z=5.75, P<0.001; fluctuating elevated CO_2: z=5.05, P<0.001), but without any differences between the stable and fluctuating elevated CO_2 treatments (Tukey contrasts, z=−0.71, P=0.95). Mass significantly affected the $\dot{M}O_{2Rest}$ of fish (t_{121}=−3.41, P<0.001).

Neither maximal oxygen uptake rates ($\dot{M}O_{2Max}$) nor absolute aerobic scope (AAS) showed any significant differences between CO_2 and temperature treatments (Tukey contrasts, min. P=0.20, Figures 4.2B and 4.3B). Mass significantly affected both

$\dot{M}O_{2Max}$ and AAS of the fish (t_{124}=−4.70, P<0.001 and t_{116}=−3.83, P<0.001, respectively). However, factorial aerobic scope (FAS) did show significant differences between treatments (Figure 4.3A). Fish reared at stable elevated CO_2 had a significantly lower FAS than fish reared at control conditions (Tukey contrasts, z=−3.62, P=0.003). Fish reared in the fluctuating elevated CO_2 treatment had an intermediate FAS, which was not significantly different from either the control or stable elevated CO_2 groups (Tukey contrasts, control: z=−2.03, P=0.25; stable elevated CO_2: z=1.42, P=0.61). Fish reared at elevated temperature in both CO_2 treatments had the lowest FAS, which were both significantly different from the control group (Tukey contrasts, stable elevated CO_2 and temperature: z=−5.09, P<0.001; fluctuating elevated CO_2 and temperature: z=−5.15, P<0.001) and the fluctuating elevated CO_2 group (Tukey contrasts, stable elevated CO_2 and temperature: z=−2.81, P=0.04; fluctuating elevated CO_2 and temperature: z=−2.89, P=0.03), but not from the stable elevated CO_2 group (Tukey contrasts, stable elevated CO_2 and temperature: z=−1.51, P=0.56; fluctuating elevated CO_2 and temperature: z=−1.48, P=0.57).

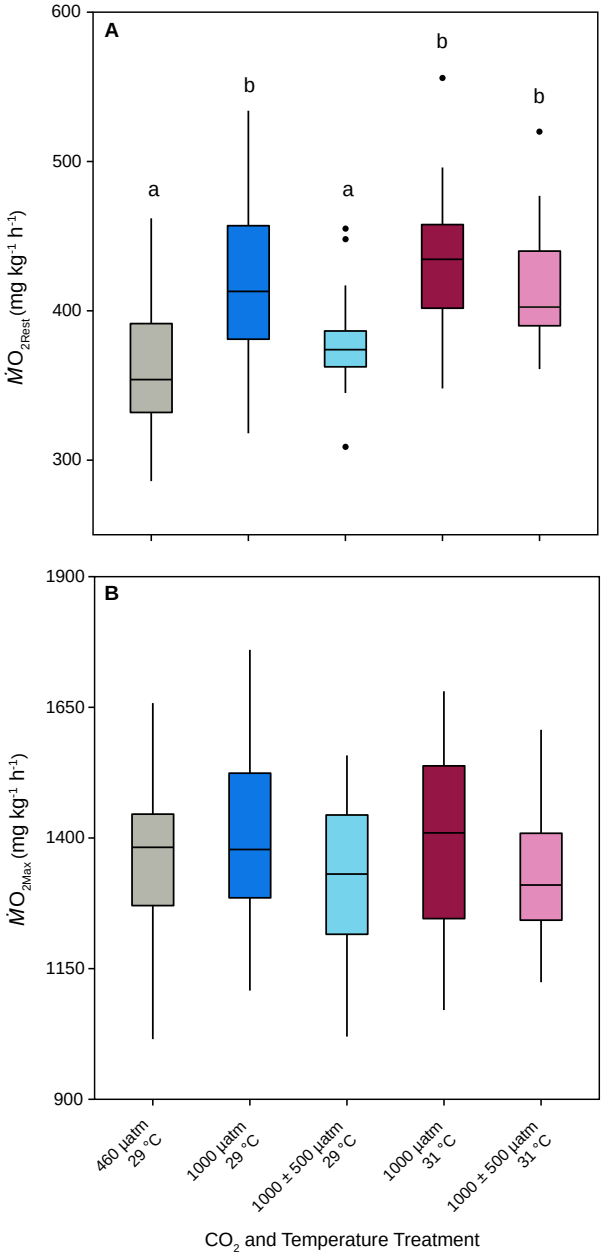

Figure 4.2. The effect of stable versus fluctuating elevated CO_2 and temperature treatments on: (A) resting oxygen uptake rate ($\dot{M}O_{2Rest}$), and (B) maximum oxygen uptake rate ($\dot{M}O_{2Max}$) of juvenile spiny damselfish. Boxplots show median and inter-quartile range. Letters represent Tukey's HSD groups (sig. differences at P=0.05). N_{Rest}= 27, 25, 23, 26, 28; N_{Max}=28, 26, 24, 26, 28, respectively.

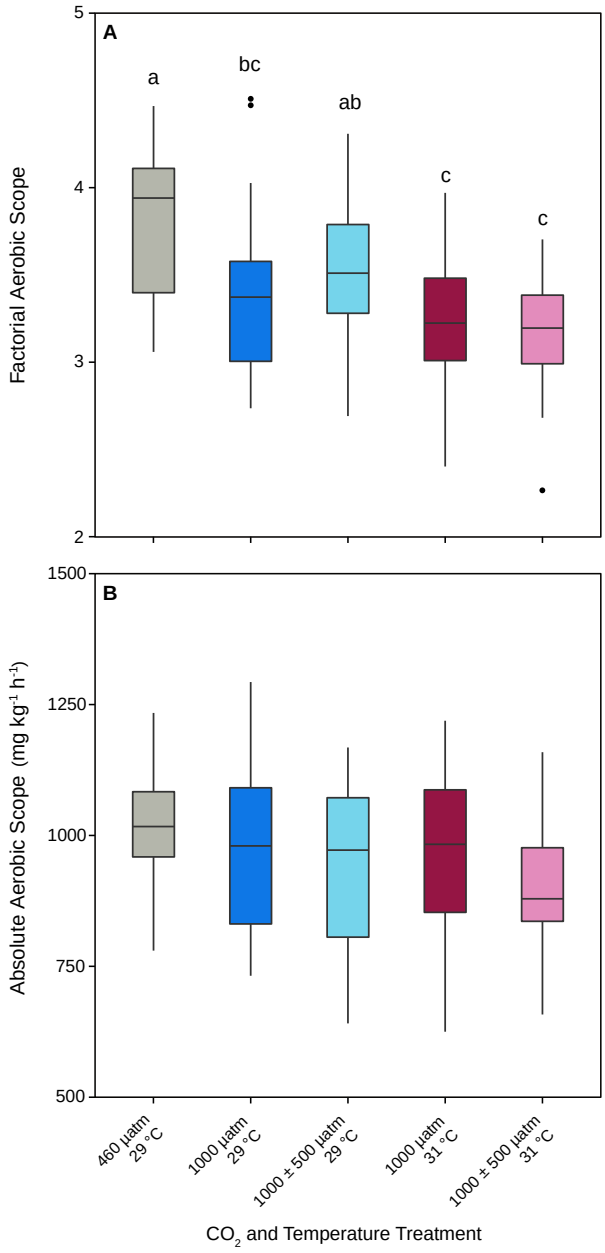

Figure 4.3. The effect of stable versus fluctuating elevated CO_2 and temperature treatments on: (A) factorial aerobic scope ($\dot{M}O_{2Max}/\dot{M}O_{2Rest}$), and (B) absolute aerobic scope ($\dot{M}O_{2Max}$ - $\dot{M}O_{2Rest}$) of juvenile spiny damselfish. Boxplots show median and inter-quartile range. Letters represent Tukey's HSD groups (sig. differences at P=0.05). N= 26, 25, 22, 24, 27, respectively.

There was a significant positive relationship between $\dot{M}O_{2Rest}$ and time spent active in fish reared in the stable elevated CO_2 and temperature treatment (t_{20}=2.98, P=0.007, Figure 4.4). This relationship was consistent between clutches (ANOVA Clutch x Time spent active, P=0.65). There were no significant correlations between other metabolic traits and time spent active or average swimming velocity in any of the other treatments (min. P=0.1).

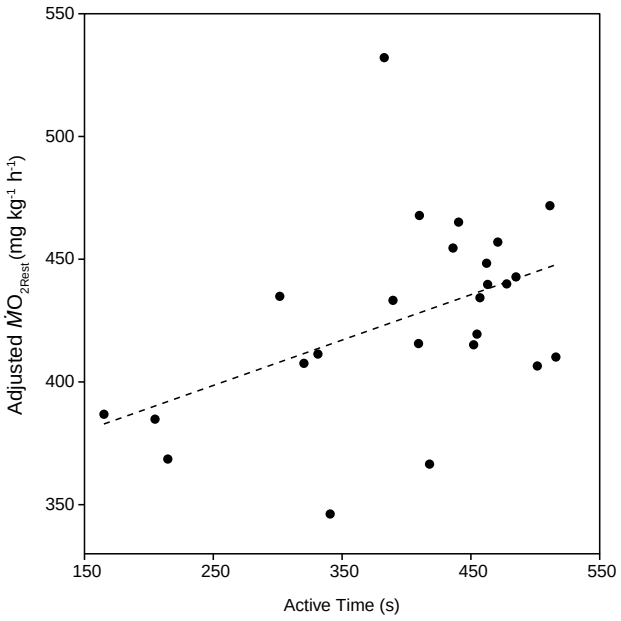

Figure 4.4. The relationship between active time in an open field test and $\dot{M}O_{2Rest}$ in juvenile spiny damselfish reared at 1000 µatm and 31 °C. Because mass significantly affected the relationship between these traits, the $\dot{M}O_{2Rest}$ has been adjusted to account for fish mass, and represents the residuals of a generalized least squares linear model with mass as a covariate, added to the predicted value for a fish of average mass. The trend line is shown as derived from a linear model. N=25.

4.5 Discussion

Here, we demonstrate that diel CO_2 cycles can modify metabolic traits of marine fish, and for the first time, that elevated temperature has an antagonistic effect on these changes. At ambient temperature, both resting oxygen uptake rates ($\dot{M}O_{2Rest}$) and factorial aerobic scope (FAS) were significantly altered by a stable elevated CO_2 treatment, but diel CO_2 cycles restored them back to control levels. However, at elevated temperature, diel CO_2 cycles did not alter $\dot{M}O_{2Rest}$ or FAS. Neither maximal

oxygen uptake rates ($\dot{M}O_{2Max}$) nor absolute aerobic scope (AAS) were affected by CO_2 or temperature treatment. These results highlight the importance of considering natural CO_2 variation when designing ocean acidification studies and suggest that some prior studies may have misestimated the effects of elevated CO_2 on fish metabolic traits because they did not include diel CO_2 cycles. Our results also underscore the complexity of temperature interactions with elevated CO_2, as the benefits of diel CO_2 cycles were diminished at elevated temperature.

Damselfish reared at stable elevated CO_2 conditions had a higher $\dot{M}O_{2Rest}$ than damselfish reared at stable control conditions. Across marine fish species, the effect of stable elevated CO_2 shows great species variation, with many species showing no change in $\dot{M}O_{2Rest}$ (Lefevre, 2016; Hannan & Rummer, 2018), but some showing increases (Chapter 3; Munday *et al.*, 2009; Enzor *et al.*, 2013) and some showing decreases (Rummer *et al.*, 2013a; Pimentel *et al.*, 2014, 2015). In a recent meta-analysis, Cattano *et al.* (2018) found that in 64 ocean acidification experiments, fishes exposed to 1000 μatm CO_2 showed, on average, an increase in resting metabolic rate. Our results indicate that for juvenile spiny damselfish, elevated CO_2 conditions come at a metabolic cost, as observed in the Cattano *et al.* (2018) meta-analysis; however, diel CO_2 cycles completely mitigated this cost, restoring $\dot{M}O_{2Rest}$ to control levels. To our knowledge, only two other studies to date have investigated the effect of stable versus fluctuating CO_2 on metabolic traits in marine fishes. Methling *et al.* (2013) showed that CO_2 fluctuations decreased $\dot{M}O_{2Rest}$ and increased $\dot{M}O_{2Max}$, AAS, and FAS in European eel *Anguilla anguilla*. By contrast, Ou *et al.* (2015) demonstrated that CO_2 fluctuations increased $\dot{M}O_{2Max}$, but did not affect $\dot{M}O_{2Rest}$ in larval pink salmon *Oncorhynchus gorbuscha*. However, in both papers, the fluctuating CO_2 treatments had a different mean to the stable elevated CO_2 treatment, making it difficult to determine whether the observed changes were due to the fluctuations themselves or differing mean CO_2 treatments. This highlights the importance of controlling for mean CO_2 when investigating the impact of diel CO_2 cycles on the sensitivity of marine organisms to ocean acidification (Boyd *et al.*, 2016). Nevertheless, taken together, our results and the two earlier studies suggest that the effects of fluctuating CO_2 treatments on metabolic traits may differ among species, potentially depending on the patterns of CO_2 variations they naturally experience, and that species likely adapt to these patterns.

The mechanism underpinning decreased $\dot{M}O_{2Rest}$ under diel CO_2 cycles compared with stable elevated CO_2 is unknown. It has been hypothesized that elevated CO_2 induces a metabolic cost in fish because of increased acid-base regulation (Pörtner & Knust, 2007; Strobel *et al.*, 2013). If this is the reason we observed elevated $\dot{M}O_{2Rest}$ in our stable elevated CO_2 treatment, then the decrease in $\dot{M}O_{2Rest}$ observed during fluctuations could be explained by preexisting adaptations to diel CO_2 cycles. Fish on coral reefs experience diel CO_2 fluctuations that range from ± 50 up to ±600 µatm around the mean in some habitats, and thus may already have some local adaptations to fluctuations in CO_2 that ameliorate the metabolic cost of these cycles. Further studies that compare populations that experience diel CO_2 cycles to populations that do not could help determine if there is local adaptation to CO_2 fluctuations.

Interestingly, the benefits of diel CO_2 cycles on $\dot{M}O_{2Rest}$ were not evident when fish were reared at elevated temperature. This suggests that, although the fish are responsive to diel CO_2 cycles, ultimately elevated temperature is the more dominant driver of $\dot{M}O_{2Rest}$. Indeed, a recent review demonstrated that temperature tends to play a larger role than elevated CO_2 on $\dot{M}O_{2Rest}$ in marine fish (Lefevre, 2016). It is not clear why elevated temperature had this effect on $\dot{M}O_{2Rest}$ in the cycling CO_2 treatment, but it is possible that elevated temperature and CO_2 acted synergistically on $\dot{M}O_{2Rest}$, as has been previously observed in spiny damselfish (Chapter 2), which acted to mask the positive effects of diel CO_2 cycles on $\dot{M}O_{2Rest}$. The implication of these results is that diel CO_2 cycles may provide some respite for reef fishes from the increased cost of living under elevated CO_2 conditions, but these benefits will be diminished under elevated temperature. Given that ocean warming and acidification are co-occurring stressors, these fish may not experience the benefits of diel CO_2 cycles in future warmer conditions.

In contrast to $\dot{M}O_{2Rest}$, neither $\dot{M}O_{2Max}$ nor AAS were significantly affected by elevated CO_2 or temperature. This result is consistent with previous work, which shows that elevated CO_2 tends not to affect $\dot{M}O_{2Max}$ or AAS in marine fish (Hannan & Rummer, 2018), though there are some exceptions (Munday *et al.*, 2009b; Couturier *et al.*, 2013). $\dot{M}O_{2Max}$ generally increases exponentially with temperature in marine fishes, until it reaches a limit that may be set by structural or functional constraints on

oxygen use and delivery (McBryan *et al.*, 2013). Our results indicate that juvenile spiny damselfish may be close to this limit, as we did not see changes in $\dot{M}O_{2Max}$ with temperature, which is similar to trends seen in other tropical reef fish species (Munday *et al.*, 2009b; Nilsson *et al.*, 2009; Johansen & Jones, 2011). Thus, our results indicate that elevated CO_2, temperature, and diel CO_2 cycles are unlikely to have a meaningful impact on $\dot{M}O_{2Max}$ or AAS in juvenile spiny damselfish, which is consistent with findings for many other species of marine fishes (Lefevre, 2016).

The increase in $\dot{M}O_{2Rest}$ combined with the lack of change in $\dot{M}O_{2Max}$ and AAS suggests that, while there are higher maintenance costs under elevated CO_2 and temperature conditions, this may not diminish the capacity of fishes to perform key aerobic activities such as growth, development, and reproduction. However, FAS showed a similar pattern to $\dot{M}O_{2Rest}$, with the exception of the fluctuating elevated CO_2 treatment being intermediate between the control and stable elevated CO_2 treatments at control temperature. This similarity in patterns is not surprising, given that FAS represents the ratio of $\dot{M}O_{2Max}$ to $\dot{M}O_{2Rest}$, and as such can be sensitive to changes in the denominator. Still, this pattern indicates that $\dot{M}O_{2Rest}$ constitutes a larger proportion of the total aerobic capacity at elevated CO_2 and temperature conditions, but not when fishes are exposed to diel CO_2 cycles at control temperature. This could lead to diminished growth in stable elevated CO_2 conditions, depending on the fish's capacity to meet increased energetic demands. Indeed, a concurrent study demonstrated that fish reared under diel cycling elevated CO_2 weighed more than fish reared under stable elevated CO_2 conditions (Jarrold & Munday, 2018b).

It is worth noting that *A. polyacanthus* does not have a pelagic larval stage, and instead develops directly into a reef-associated juvenile. Even though we observed a mitigating effect of diel CO_2 cycles on $\dot{M}O_{2Rest}$ and FAS of juvenile *A. polyacanthus* at higher average CO_2 levels, other reef fishes may still experience increased metabolic costs of elevated CO_2 in the future during their pelagic larval duration. This is a time when fishes spend a period of several weeks to months in the open ocean, an environment with a relatively stable CO_2 profile. Larval fishes are predicted to be more susceptible to elevated CO_2 than older life stages because of their high surface area-to-volume ratio and less-developed acid-base regulatory mechanisms (Ishimatsu *et al.*, 2008; Melzner *et al.*, 2009). High survival rates during the larval phase are

critical for shaping patterns of connectivity and population replenishment in adult populations (Chambers & Trippel, 1997; Cowen & Sponaugle, 2009), and thus some reef fishes may still be affected by projected future CO_2 levels as a result of their pelagic larval stage, despite the benefits of diel CO_2 cycles on the reef-associated juvenile stage.

We observed a significant positive relationship between activity and $\dot{M}O_{2Rest,}$ but only in the most extreme treatment with both stable elevated CO_2 and temperature. It has been proposed that environmental stressors can reveal or amplify relationships between behavioral and physiological traits (Killen *et al.*, 2013). This revealing effect may be caused by differential individual sensitivity to stressors, or increased demands on performance brought on by stressors that accentuate links between behavior and physiology (Killen *et al.*, 2013). Thus, these relationships may exist in a weaker or less obvious state in less stressful conditions, but could be amplified in more stressful conditions.

Links between $\dot{M}O_{2Rest}$ and activity have been observed in a variety of animal taxa (Careau *et al.*, 2008). Two competing models have been proposed to predict the direction of this relationship. The "allocation model" predicts a negative correlation between $\dot{M}O_{2Rest}$ and activity, based on the concept that animals have a limited energy budget and must allocate resources to either $\dot{M}O_{2Rest}$ or activity (Careau *et al.*, 2008). Conversely, the "performance model" predicts a positive correlation between $\dot{M}O_{2Rest}$ and activity. This model suggests that underlying physiological differences drive behavioral differences, or vice versa (Careau *et al.*, 2008). That is, it may be that animals with a higher $\dot{M}O_{2Rest}$ require more energy to sustain themselves, leading them to become more active to seek food (Biro & Stamps, 2010). It is also possible that more active animals need more energy to sustain their active lifestyles, causing them to develop larger-than-average organs that consume a greater $\dot{M}O_{2Rest}$ (White & Kearney, 2014). Regardless of the mechanism, our results support the performance model.

The correlation observed between activity and $\dot{M}O_{2Rest}$ could drive changes in rates of adaptation to environmental change, but it is difficult to predict with accuracy the direction that selection might take. This will depend on the relative benefits of higher

or lower $\dot{M}O_{2Rest}$ and activity levels in a given environment, which are influenced by environmental factors such as predator abundance and food density. For instance, highly active fishes with high $\dot{M}O_{2Rest}$ would likely struggle in an environment with many predators and scarce food resources, as they would either have difficulty meeting their energetic demands or expose themselves to higher rates of predation, while fishes with low activity levels and $\dot{M}O_{2Rest}$ would fare better in such an environment. Both metabolic traits (Munday *et al.*, 2017) and activity (Bell, 2005) can be heritable in fishes, suggesting that this correlation could influence adaptive potential to climate change. However, this correlation was only observed under stable elevated CO_2 and temperature conditions, which does not represent the most ecologically-relevant future conditions. Thus, it is not likely that this correlation will affect adaptive potential to future elevated CO_2 and temperature conditions, unless this relationship persists in the species that experience a pelagic larval duration where stable elevated CO_2 and temperature conditions could be experienced.

In conclusion, we determined that diel CO_2 cycles can alleviate the negative effects of elevated CO_2 on metabolic traits in juvenile spiny damselfish. However, these benefits are diminished when juveniles also experience elevated temperature, as will occur as a result of global climate change. These results highlight the importance of considering natural CO_2 fluctuations when evaluating the responses of marine fishes to ocean acidification. Indeed, the addition of diel CO_2 fluctuations to our study suggests that some previous work may have overestimated the effects of elevated CO_2 on reef fish metabolic traits. Importantly, though, these effects are lessened at elevated temperatures, indicating that reef fishes will likely still need to contend with higher metabolic costs in the future. We also found a significant positive relationship between $\dot{M}O_{2Rest}$ and activity in stable elevated CO_2 and temperature conditions. However, this particular relationship is not likely to have an influence on the adaptation of juvenile reef fishes to climate change, as these fishes will experience fluctuating, not stable, elevated CO_2 conditions for most of their lives. Although we did not observe a significant correlation between traits in fluctuating elevated CO_2 and temperature conditions, this does not preclude the existence of such correlations between metabolic traits and other aerobic activities, such as reproduction. Future work could examine a greater variety of traits to determine how behavioral and

physiological traits are related, and thus how the relationship could influence the direction and pace of future adaptation to climate change.

Chapter 5: Parental effects alter physiological but not behavioral performance under elevated CO_2 in a coral reef fish

5.1 Summary

Parental effects can ameliorate the negative effects of ocean acidification on some behavioral and physiological traits in marine fishes. Correlations between these traits can limit individual performance and even constrain adaptive potential, yet it is not known if parental effects modulate these correlations. Here, we examined the relationship between behavioral and physiological performance of a juvenile reef fish in a full factorial design of parent and offspring exposure to elevated CO_2. Behavioral performance, as measured by response to alarm odor, was impaired by offspring exposure to elevated CO_2. Parental exposure to elevated CO_2 did not mitigate these negative effects. By contrast, maximal oxygen uptake rates were higher in offspring when parents were exposed to elevated CO_2, regardless of juvenile CO_2 exposure, and resting oxygen uptake rates and aerobic scope exhibited significant differences between some treatments. There were no significant relationships between behavioral and physiological performance in offspring, regardless of parental environment. Our results demonstrate that parental effects are important to incorporate into future ocean acidification research for their potential to affect mean behavioral and physiological performance in offspring, but we found no correlations between these types of performance that would affect individual performance or hinder adaptive evolution to ocean acidification. However, it will be important to test the generality of these results for other species, traits, and environmental drivers, as correlations may be observed in these cases.

5.2 Introduction

The pH of the world's oceans is decreasing due to uptake of additional carbon dioxide (CO_2) from the atmosphere, in a process known as ocean acidification (Doney *et al.*, 2009). If anthropogenic emissions of CO_2 are not curtailed, atmospheric CO_2 concentrations could reach nearly 1,000 µatm by the year 2100, and the oceans would

experience a corresponding drop in average pH of 0.3-0.4 units (Collins *et al.*, 2013). These predicted end-of-century CO_2 conditions have been shown to negatively impact performance in a variety of marine organisms (Kroeker *et al.*, 2010, 2013; Przeslawski *et al.*, 2015; Cattano *et al.*, 2018) which could have implications for population replenishment, community structure and ecosystem function. However, these experimental results must be interpreted with caution, as the majority of experiments that found negative effects of elevated CO_2 were focused on a single generation (Kroeker *et al.*, 2013). Ocean acidification will occur over multiple generations for most species, which could allow them to acclimate or adapt to future projected CO_2 levels (Kelly & Hofmann 2013; Sunday *et al.*, 2014). Importantly, the environment experienced by one generation can affect the performance of subsequent generations (Mousseau & Fox, 1998; Salinas *et al.*, 2013), potentially leading to improved performance under future climatic conditions. Therefore, it is crucial to take these effects into account to predict how marine organisms will respond to predicted future ocean acidification conditions (Sunday *et al.*, 2014; Donelson *et al.*, 2018).

Parents can influence the performance of their offspring under altered environmental conditions by a variety of mechanisms, including changes to nutritional provisioning of eggs and embryos, transmission of hormones, proteins or other cytoplasmic factors, and in some cases, through epigenetic processes that influence gene expression in the offspring (Badyaev & Uller 2009; Bonduriansky *et al.*, 2012; Torda *et al.*, 2017). Recent studies have shown that parental exposure to elevated CO_2 can partially or completely mitigate the negative impacts of elevated CO_2 on offspring across a variety of marine taxa (Munday, 2014; Putnam & Gates 2015; Ross *et al.*, 2016). For instance, larval Sydney rock oyster *Saccostrea glomerata* that were reared in elevated CO_2 conditions had slower rates of growth and development, but if their parents were exposed to elevated CO_2, larvae were larger and developed faster (Parker *et al.*, 2012). Parental effects will likely play an important role in helping organisms to cope with a rapidly changing environment, as they can take effect in a single generation (Gienapp *et al.*, 2008), whereas genetic adaptation will take multiple generations (Merilä, 2012; Sunday *et al.*, 2014). However, parental effects do not always have a positive effect on offspring performance (Uller, 2008; Burgess & Marshall, 2014; Kronholm & Collins, 2016). For instance, if parents experience a stressful environment, this can decrease the performance of their offspring, regardless of the

environment they experience (e.g. Dupont *et al.*, 2013; Welch *et al.*, 2014; Parker *et al.*, 2017). Furthermore, the nature of parental effects can depend upon the length of exposure to a stressor that the adults experience. For example, larval survival in sea urchin *Strongylocentrotus droebachiensis* was decreased when adults were exposed to elevated CO_2 for 4 months, but larval survival increased back to control levels when adults had 16 months exposure to elevated CO_2 (Dupont *et al.*, 2013). Thus, the benefits of parental effects can be context-dependent.

Ocean acidification can negatively impact the performance of marine fishes across a range of behavioral and physiological traits (Nagelkerken & Munday, 2016; Cattano *et al.*, 2018). Yet recent evidence indicates that marine fishes can also exhibit positive parental effects under ocean acidification. This is especially the case for physiological traits, with parental exposure to elevated CO_2 mitigating the negative effects of elevated CO_2 on growth and survival in Atlantic silverside *Menidia menidia* (Murray *et al.*, 2014) and growth, survival, and resting oxygen uptake rate in cinnamon clownfish *Amphiprion melanopus* (Miller *et al.*, 2012). Behavioral traits, however, have shown mixed responses to parental exposure to elevated CO_2. Juvenile spiny damselfish *Acanthochromis polyacanthus* exposed to elevated CO_2 demonstrated impaired antipredator responses to alarm odors, regardless of the CO_2 exposure their parents received (Welch *et al.*, 2014). Similarly, parental exposure to elevated CO_2 did not alleviate the negative effect of offspring CO_2 exposure on reductions in feeding strikes in the presence of a predator odor in juvenile orange clownfish *Amphiprion percula* (McMahon *et al.*, 2018). By contrast, juvenile *A. melanopus* displayed partial restoration of kinematic responses to a startle stimulus when parents and offspring were both exposed to elevated CO_2 (Allan *et al.*, 2014). Given the variable responses of fishes to parental exposure to elevated CO_2, it appears that positive parental effects may be trait- or species-specific.

While the effects of ocean acidification on behavioral and physiological performance have been explored independently, there is a growing interest in understanding the relationship between these types of performance (Biro & Stamps, 2010; Careau & Garland, 2012). Examining links between behavior and physiology can not only reveal patterns of trait covariation (Careau *et al.*, 2008; Biro & Stamps, 2010), but also reveal the mechanisms that underpin these covariations (Williams, 2008; Careau

& Garland, 2012) and even predict the evolutionary implications of such patterns (Careau & Garland, 2012; Sih *et al.*, 2015). The linkages between behavioral and physiological performance will be particularly important in the context of ocean acidification, given that impairments to both types of traits have been observed in marine fishes under ocean acidification (Heuer & Grosell, 2014; Cattano *et al.*, 2018). Thus, by evaluating behavioral and physiological performance within individual fish, we can identify correlated traits that might help or hinder fish survival under ocean acidification (Sgrò & Hoffmann, 2004; Killen *et al.*, 2013). Correlations between traits could also affect the potential of fishes to adapt to ocean acidification, if the traits of interest are heritable (Munday *et al.*, 2013; Sunday *et al.*, 2014). If traits are negatively correlated relative to the fitness landscape, then selection for improved performance on one trait would diminish the other, slowing the rate of adaptation (Lande, 1979; Lande & Arnold, 1983). Thus, examining relationships between behavioral and physiological performance can be a powerful tool for predicting individual and population persistence in the face of ocean acidification.

Importantly, environmental stressors can alter the relationship between behavioral and physiological traits, amplifying or diminishing significant correlations between them (Killen *et al.*, 2013). Therefore, we may observe correlations between behavioral and physiological performance under current-day conditions that are not evident under future acidified conditions, or vice versa. Parental effects may further alter the relationship between behavioral and physiological performance. Because parental effects have been shown to mitigate a variety of physiological traits affected by elevated CO_2 in fish (Miller *et al.*, 2012; Murray *et al.*, 2014), but fewer behavioral traits (Allan *et al.*, 2014; Welch *et al.*, 2014; McMahon *et al.*, 2018), we may expect to see a shift in physiological, but not behavioral, performance when parents and their offspring are both exposed to elevated CO_2. This could change correlations between behavior and physiology under ocean acidification conditions. Therefore, it is necessary to examine these relationships in offspring with and without parental exposure to elevated CO_2 in order to fully understand the effects of ocean acidification on fish populations.

In this study, we investigated if parental effects alter the relationship between behavioral and physiological performance in juvenile spiny damselfish,

Acanthochromis polyacanthus. We used a full factorial design, in which adult breeding pairs were exposed to either a current-day control CO_2 level (~480 µatm) or an elevated CO_2 level (~1000 µatm) consistent with projections for the end of the century under RCP 8.5 (Collins *et al.*, 2013), for 3 months prior to the breeding season. Once offspring hatched, they were split between control and elevated CO_2 conditions and reared for 60 days in their respective conditions, at which point their behavioral and physiological performance were tested. For behavioral performance, we examined the percent reduction in feeding strikes after exposure to damage-released olfactory cues (i.e. alarm odors). Alarm odors are a reliable indicator of risk, and fish display an innate aversion to the alarm odors produced by conspecifics (Brown, 2003; Ferrari *et al.*, 2010). However, fish exposed to elevated CO_2 conditions do not respond appropriately to these cues (Chapter 3; Ferrari *et al.*, 2011; Ou *et al.*, 2015; Welch *et al.*, 2014), increasing their susceptibility to predation (Ferrari *et al.*, 2011a; Chivers *et al.*, 2014). For physiological performance, we measured metabolic traits, specifically maximal ($\dot{M}O_{2Max}$) and resting ($\dot{M}O_{2Rest}$) oxygen uptake rates, as well as aerobic scope ($\dot{M}O_{2Max}$ - $\dot{M}O_{2Rest}$). Metabolic traits have been a key focus of climate change studies because they describe the energetic requirements of organisms that may underpin a range of fitness-related traits (Farrell *et al.*, 2008; Biro & Stamps, 2010; Eliason *et al.*, 2011). We also tracked individual fish between behavioral and physiological trials to determine the relationship between these traits at the individual level. This allowed us to compare this relationship across all four treatment groups to determine how parental effects might alter this relationship.

5.3 Methods

Broodstock and Offspring Maintenance

Adult *A. polyacanthus* were collected using hand nets from the Bramble Reef area at two sites on the Great Barrier Reef in July 2015 (site 1: 18°22’S, 146°40’E; site 2: 18°25’S, 146°40’E). Physical and chemical water parameters from a similar mid-shelf reef in the central Great Barrier Reef can be seen in Appendix A. The fish were transported to James Cook University (Townsville, Australia) where they were sorted into breeding pairs. Pairs were housed in 60L aquaria, with half a terracotta pot for shelter and as a breeding site. Pairs were fed *ad libitum* on commercial fish feed pellets (INVE Aquaculture Nutrition NRD 12/20) once daily before the breeding

season (July-October) and twice daily during the breeding season (November-May). Beginning in October, water temperatures were increased by 0.5°C per week until the summer temperature (29 °C) was reached in the first week of November.

During the breeding season, pairs were checked daily for the presence of egg clutches. Newly hatched offspring were fed *Artemia* spp. nauplii for the first two days post-hatch (dph), then a combination of *Artemia* spp. nauplii and weaning fish feed (INVE Aquaculture Nutrition Wean-S 200-400 µm) for the following three days. They were fed the weaning fish feed from 6-21 dph, and a small pellet fish feed (INVE Aquaculture Nutrition NRD 5/8) from 22 dph onward.

Carbonate Chemistry

Water was supplied to tanks by two 8000 L recirculating aquarium systems, one maintained at ambient CO_2 (~480 µatm) and the other dosed with additional CO_2 to achieve the desired elevated CO_2 level (~1000 µatm). The elevated CO_2 treatment was controlled by an Aqua Medic AT Control System (Aqua Medic, Germany), which slowly dosed CO_2 into a 3000 L sump connected to the system whenever the pH rose above the set point. An identical 3000 L sump on the current-day control system was not dosed with CO_2. The temperature was maintained at 29 °C by circulating seawater through a Solarwise heater/ chiller (Brisbane, Queensland, Australia) on each system. Water was delivered to the aquaria at a rate of 1.5 L min^{-1} in a temperature-controlled room.

The pH_{NBS} and temperature for each system were recorded daily using a pH electrode (SevenGo Pro, Mettler Toledo, Switzerland) and temperature probe (Cormark C26, Norfolk, UK). The pH_T was measured weekly by spectrophotometry (Shimadzu, UV mini 1240) and salinity was measured weekly with a conductivity probe (Hach HQ40d meter, IntelliCAL CDC401 probe). Total alkalinity was estimated weekly by Gran titration (Metrohm 888 Titrando Titrator Metrohm AG, Switzerland) to within 1% of certified reference material (Prof. A. G. Dickson, Scripps Institution of Oceanography, batch #135). All water quality parameters were measured in randomly selected aquaria. The pCO_2 was calculated as a function of pH_T, temperature, salinity, and total alkalinity in CO_2SYS using the constants K1 from Mehrbach *et al.* (1973) refit by Dickson & Millero (1987), and $KHSO_4$ from Dickson (1990) (Table 5.1).

Table 5.1. Mean (±SD) seawater chemistry parameters for *Acanthochromis polyacanthus* adults and juveniles held under control and elevated CO_2. Juvenile measurements were taken from the day the first clutch was laid to the last day of assays. Parental measurements were taken from the start of CO_2 treatments until the last clutch was laid, with the exception of temperature data which was taken when tanks reached the summer average of 29 °C.

Treatment	**Temperature (°C)**	**Salinity (ppt)**	**pH_T**	**Alkalinity (µmol kg^{-1} SW)**	***p*CO_2 (µatm)**
Juvenile Control CO_2	29.0 ± 0.1	34.9 ± 1.3	7.95 ± 0.04	2131 ± 154	486 ± 34
Juvenile Elevated CO_2	28.9 ± 0.2	35.6 ± 0.7	7.68 ± 0.03	2136 ± 113	967 ± 75
Parent Control CO_2	29.1 ± 0.3	35.1 ± 1.5	7.98 ± 0.06	2389 ± 267	484 ± 36
Parent Elevated CO_2	28.9 ± 0.3	36.0 ± 1.3	7.71 ± 0.04	2454 ± 335	1031 ± 94

Experimental Design

This study used a full crossed design to test if parental effects modify the relationship between behavioral and physiological performance. Four breeding pairs were maintained at control CO_2 and five breeding pairs were maintained at elevated CO_2. The morning after offspring hatched, they were split equally between control and elevated CO_2 conditions. Behavioral trials were conducted at 60-66 dph and physiological trials were conducted at 62-68 dph, allowing at least one day rest between trials. Individuals were tracked between trials by placing them into labeled PVC pipes (8 cm diameter, 5 cm length) that were covered at both ends with a thin plastic mesh to allow for flow-through of water, which were placed into treatment tanks. All trials were performed during daylight hours only (09:00-18:00) in the fish's respective treatment water. Research was carried out under approval of the James Cook University animal ethics committee (permit: A2197) and according to the University's animal ethics guidelines.

Behavioral Assay

The ability to detect and respond appropriately to alarm odors is critical for survival, particularly for larval and juvenile fishes that are highly susceptible to predation (Almany & Webster, 2006). The percent change in feeding strikes is a commonly-used method to measure fish responses to chemical cues (Larson & Mccormick, 2005; Mitchell *et al.*, 2011; Chivers *et al.*, 2014), and has been frequently used in ocean

acidification experiments (Ferrari *et al.*, 2011a, 2012; Chivers *et al.*, 2014). The percent change in feeding strikes rather than an absolute change was used to account for any differences in activity levels or feeding rates between individuals. The percent change in feeding strikes was assessed using methods similar to those described by Laubenstein *et al.* (Chapter 2). Trials were conducted in 13 L flow-through aquaria, which each contained half a small PVC pipe (8 cm diameter) for shelter and an airstone. An injection tube, which was used to add food and alarm odors into the tank, was attached to the airstone to ensure food and odors dispersed quickly. The tank was surrounded on three sides with white plastic, and the fourth side had a black plastic curtain with a small flap to separate the tank from external stimuli.

Juvenile *A. polyacanthus* were habituated to observation tanks overnight (15 hours). Treatment water was supplied to the tanks at 0.6 L min^{-1}. The flow-through system to tanks was turned off ten minutes prior to a trial to prevent the alarm odor from washing out of the tank. At the same time, the camera was positioned in front of the tank to habituate the fish to the camera. 20 mL of water were drawn from the injection tube and discarded to remove any possible stagnant water, and a further 60 mL were drawn for flushing food into the tank. Alarm odors were prepared as described by Laubenstein *et al.* (Chapter 2) during the first ten minutes of each trial to prevent the odors losing potency.

To start a trial, the camera (Canon Powershot G15, Canon Powershot G16, or Canon Powershot GX9) was turned on, and the fish was recorded for five minutes to ensure normal behavior. Then 2.5 mL of *Artemia* solution (containing ~250 individuals per mL) was slowly flushed into the tank with 20 mL of seawater to allow the fish to establish a stable feeding rate. After five minutes, another 2.5 mL of *Artemia* solution and 20 mL of seawater were flushed into the tank. Finally, after five minutes, 2.5 of *Artemia* solution, 10 mL of alarm odor, and 20 mL of seawater were flushed into the tank. The trial ended five minutes after the alarm odor was added, totaling 20 minutes for a trial. Between 39 and 47 fish were tested per treatment. Videos were analyzed blind to treatment to determine the feeding rate of the fish before and after the addition of the alarm odor. Feeding strikes were counted for four minutes in each time period, which began 30 seconds after the addition of food or alarm odor to ensure a steady feeding rate, and that the alarm odor had permeated the tank. These feeding

rates were used to calculate the percent change in feeding strikes. Between 39 and 47 individuals were tested per treatment.

Physiological Assay

Oxygen uptake rates ($\dot{M}O_2$) were measured using intermittent flow respirometry based on standard methods (Roche *et al.*, 2016; Rummer *et al.*, 2016). Fish were starved for 24 hours before testing to ensure a post-absorptive state (Niimi & Beamish, 1974). Immediately before fish were placed into respirometry chambers, they were chased in a circular container (20 cm diameter, 9 cm height) for 3 minutes and then exposed to air for 1 minute, as this chase protocol has been previously determined to be sufficient for *A. polyacanthus* to reach exhaustion (Chapter 2). Fish were then placed into darkened glass respirometry chambers (38 mL total volume including tubing) that were submerged in a water bath containing the fish's treatment water. The chambers were supplied with treatment water from the water bath by flush pumps, for ten minutes every fifteen minutes. This flush pattern, which was controlled by a digital relay timer (SuperPro Hydroponics Recycling Timer, Xiamen, China), ensured that oxygen did not fall below 80% saturation. The temperature-compensated oxygen concentration (mg L^{-1}) of the water in each chamber was continuously recorded every 2 seconds (0.5 Hz) using oxygen-sensitive REDFLASH dye on contactless spots (2 mm) adhered to a glass tube in line with the chamber, and linked to a Firesting Optical Oxygen Meter (Pyro Science e. K., Aachen, Germany) with 2 m fiber-optic cables. Fish remained in chambers for four hours while they recovered to their resting oxygen uptake rate ($\dot{M}O_{2Rest}$). Although adult fish are typically measured for up to 24 hours, juvenile fish recover much more quickly from exhaustive exercise, and are frequently measured for only 2-3 hours to minimize stress and the risk of starvation (Chapter 2; McLeod *et al.*, 2013; Killen *et al.*, 2014; Ferrari *et al.*, 2015; Hess *et al.*, 2017).

Oxygen uptake rates were calculated using linear least squares regression using LabChart version 7.2.5 (ADInstruments, Colorado Springs, CO, USA). Background microbial respiration was subtracted from total chamber respiration to determine the oxygen uptake rate of the fish, as per Rummer *et al.* (2016). The $\dot{M}O_{2Max}$ was taken to be the highest slope (30 second intervals) immediately following the exhaustive chase. The $\dot{M}O_{2Rest}$ was estimated as the average of the lowest 10% of slopes during

the trial, excluding outliers above or below 2 SD. Aerobic scope was calculated as the difference between $\dot{M}O_{2Max}$ and $\dot{M}O_{2Rest}$. At the end of each trial, fish were euthanized using an overdose of clove oil. Any excess water was removed by blotting with a paper towel, and the fish's mass (0.6484 ± 0.1310 g; mean ± SD) and standard length (27.8 ± 0.2 mm; mean ± SD) were recorded. The water bath, chambers, and pumps were cleaned between trials with a 10% bleach solution and freshwater to minimize bacterial growth. Between 30 and 38 individuals were tested per treatment.

Statistical Analyses

Linear mixed-effects models (LME, "nlme" package in R) were used to determine the effect of CO_2 exposure in parents and offspring on behavioral and physiological performance of the offspring. For percent reduction in feeding strikes, offspring CO_2 treatment and parental CO_2 treatment were fixed effects, with fish mass and time of day as covariates, allowing for interactions between fixed effects and covariates. Mass and time of day were mean-centered to aid in the interpretation of model intercepts. Family was included as a random factor to account for the possibility that sibling responses were more similar to each other than non-sibling responses. For aerobic scope, $\dot{M}O_{2Rest}$, and $\dot{M}O_{2Max}$,the fixed effects were offspring CO_2 treatment, parental CO_2 treatment, and fish mass, and family was included as a random effect. Initially, fully interactive models were run. Then non-significant interaction terms and covariates were gradually removed, with the final model choice confirmed based on Akaike information criterion (AIC) (Appendix B). Assumptions of normality and homogeneity of residuals were visually assessed with Q-Q plots and frequency distributions of residuals, and met for all models. Parameters were estimated using restricted maximum-likelihood. P-values were calculated from the "nlme" package in R, and results were considered statistically significant at $P<0.05$. When required, *post-hoc* multiple comparisons were done using the R function "glht" in the package "multcomp" using Tukey's HSD contrasts for unequal sample sizes.

Relationships between behavioral and physiological performance were calculated using linear mixed effect models for each treatment. Metabolic traits ($\dot{M}O_{2Rest}$, $\dot{M}O_{2Max}$, aerobic scope) were used as the response variables, and percent change in feeding strikes was the predictor variable. Mean-centered mass was included as a covariate, and family was included as a random effect. As previously, fully interactive

models were run initially, then models were simplified when interactions were not significant and confirmed by AIC (Appendix B). All analyses were conducted using R version 3.1.3 (R Core Team, 2014).

5.4 Results

Behavior

There was a highly significant effect of offspring CO_2 treatment (t_{160}=−3.97, P<0.001), but not parental CO_2 treatment (t_7=−1.89, P=0.10) on the percent reduction in feeding strikes following the addition of the alarm odor. Fish reared in elevated CO_2 reduced their feeding strikes significantly less than fish reared at control conditions, regardless of their parental CO_2 exposure (Figure 5.1). There was no interaction between offspring and parental CO_2 treatments (t_{160}=0.61, P=0.54). There was a trend for offspring from elevated CO_2 parents to have a lower reduction in feeding strikes when reared in control conditions compared with offspring from control parents (Figure 5.1), which would indicate a negative carryover effect of elevated CO_2, but this was not statistically significant (Tukey contrasts, z=−1.89, P=0.23).

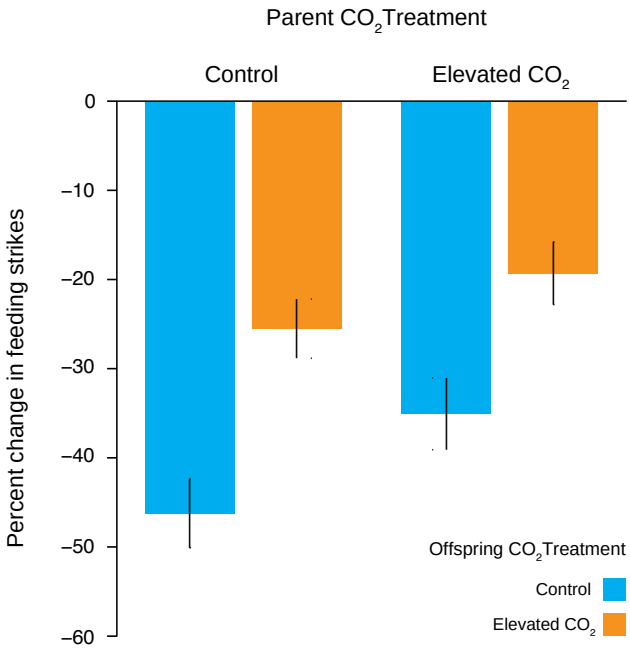

Figure 5.1. The effect of parental and offspring CO_2 exposure on percent change in feeding strikes of juvenile spiny damselfish following the addition of an alarm odor. Values are means ± SE.

Physiology

There was a significant effect of parental CO_2 treatment (t_7=3.19, P=0.02), but not offspring CO_2 treatment (t_{129}=−1.55, P=0.12) on $\dot{M}O_{2Max}$. Fish from CO_2 exposed parents exhibited a higher $\dot{M}O_{2Max}$ than fish from control parents, regardless of the offspring CO_2 treatment (Figure 5.2A). There was no interaction between offspring and parental CO_2 treatments on $\dot{M}O_{2Max}$ (t_{129}=0.72, P=0.47). Mass had a significant effect on $\dot{M}O_{2Max}$ (t_{129}=−3.46, P<0.001).

There was a significant effect of parental CO_2 treatment (t_7=2.76, P=0.03), but not offspring CO_2 treatment (t_{123}=−0.33, P=0.74) on $\dot{M}O_{2Rest}$. There was no interaction between offspring and parental CO_2 treatments (t_{123}=−0.47, P=0.64). Post-hoc analyses revealed that control-reared offspring from CO_2-exposed parents had a significantly higher $\dot{M}O_{2Rest}$ than both offspring treatments from control parents (Tukey contrasts, control offspring & control parents: z=2.76, P=0.03; control offspring & CO_2-exposed parents: z=2.95, P=0.02, Figure 5.2A). CO_2-reared offspring from CO_2-exposed parents had an intermediate $\dot{M}O_{2Rest}$ (Tukey contrasts, control offspring & control parents: z=2.12, P=0.14; control offspring & CO_2-exposed parents: z=2.32, P=0.09; CO_2-reared offspring & control parents: z=−0.99, P=0.75).

There was a significant effect of parental CO_2 treatment (t_7=2.39, P=0.04), but not offspring CO_2 treatment (t_{123}=−1.43, P=0.15) on aerobic scope, and there was not a significant interaction (t_{123}=1.05, P=0.29). Mass had a significant effect on aerobic scope (t_{123}=−5.48, P<0.001). Post-hoc analyses revealed that offspring from CO_2-exposed parents exhibited a higher aerobic scope than CO_2-reared offspring from control parents (Tukey contrasts, control offspring & CO_2-exposed parents: z=3.47, P=0.003; CO_2-reared offspring & CO_2-exposed parents: z=3.64, P=0.002, Figure 5.2B). Control offspring from control parents had an intermediate aerobic scope (Tukey contrasts, CO_2-reared offspring & control parents: z=−1.43, P=0.47; control offspring & CO_2-exposed parents: z=2.39, P=0.08; CO_2-reared offspring & CO_2-exposed parents: z=2.54, P=0.06).

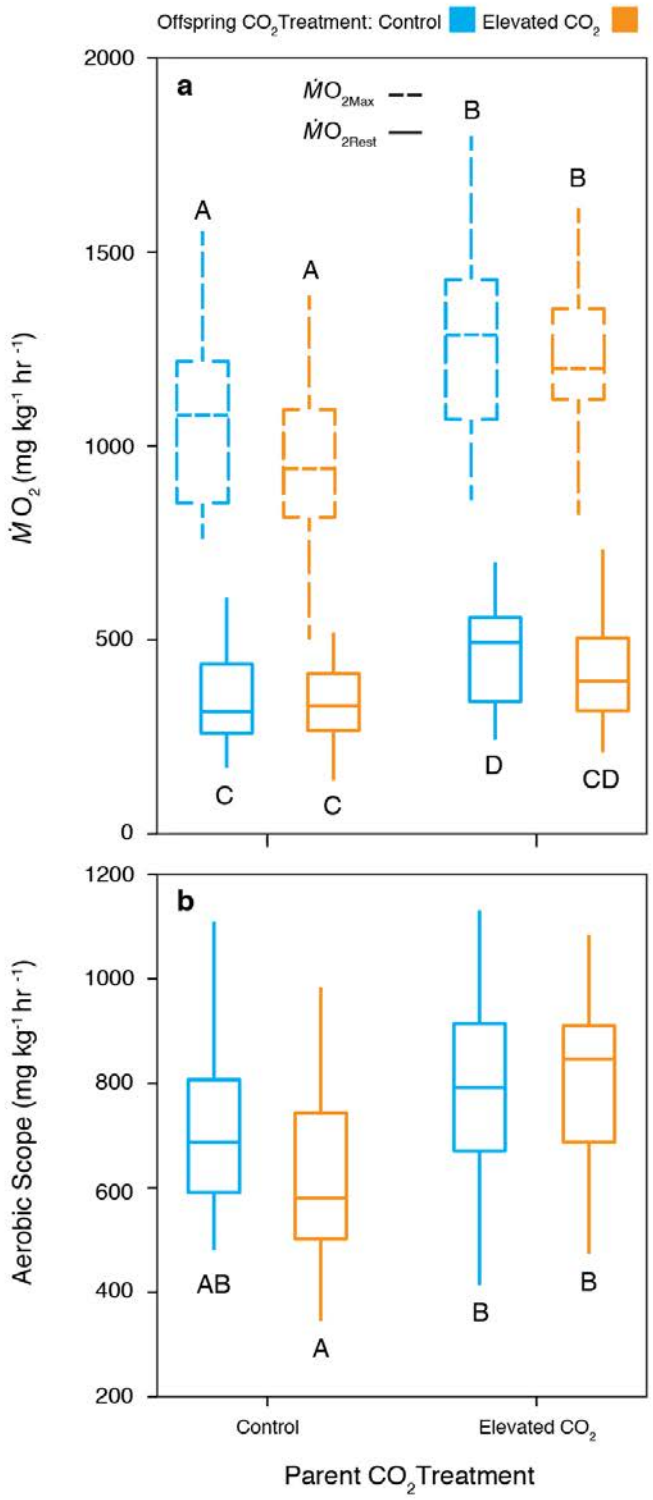

Figure 5.2. The effect of parental and offspring CO_2 exposure on resting and maximal oxygen uptake rates and aerobic scope of juvenile spiny damselfish. Boxplots show median and inter-quartile range for (A) resting ($\dot{M}O_{2Rest}$; solid lines) and maximal oxygen uptake rates ($\dot{M}O_{2Max}$; dashed lines); and (B) absolute aerobic scope ($\dot{M}O_{2Max}$ - $\dot{M}O_{2Rest}$).

Correlations

Across all treatments, there were no statistically significant relationships between percent change in feeding strikes and aerobic scope, $\dot{M}O_{2Max}$, or $\dot{M}O_{2Rest}$ (all $P>0.05$).

5.5 Discussion

Parental effects can mitigate some of the negative effects of environmental stressors on their offspring. Our study demonstrated that parental exposure to elevated CO_2 can have positive effects on certain traits in a coral reef fish, but other traits are not affected. While parental exposure to elevated CO_2 altered metabolic performance, impaired antipredator behaviors were observed in offspring reared under elevated CO_2 regardless of their parental treatment. We also examined the relationship between behavioral and physiological performance, as negative correlations between traits can limit individual performance, and even influence the potential for adaptive evolution to environmental change. However, there were no correlations between behavioral and physiological performance in juvenile spiny damselfish under control or elevated CO_2 conditions, regardless of parental exposure to elevated CO_2. Overall, our results indicate that parental effects are important to incorporate into future ocean acidification research for their potential to affect mean performance in some traits, but they did not influence the relationship between traits, at least for the traits measured here.

In the behavioral trials, offspring reared under elevated CO_2 displayed an impaired antipredator response by not reducing their feeding strikes when exposed to alarm odors to the same extent as fish reared in control conditions, as has been observed previously (e.g. Chapter 2; Ferrari *et al.*, 2011). Furthermore, parental exposure to elevated CO_2 did not improve antipredator responses, as fish reared in elevated CO_2 with parents held at elevated CO_2 showed similar responses to fish reared in elevated CO_2 with parents held at control conditions. This aligns with previous studies, which have also found that impaired olfactory responses in coral reef fishes show no or little improvement with parental exposure to elevated CO_2 (Welch *et al.*, 2014; McMahon *et al.*, 2018). The mechanism underpinning behavioral abnormalities in fish under elevated CO_2 has been linked to a disruption to neurotransmitter receptor function. Under elevated CO_2 conditions, fish use their robust acid-base regulatory system to

prevent tissue and blood acidosis (Heuer & Grosell, 2014). This process changes the concentration of HCO_3^- and Cl^- ions, altering their gradient across $GABA_A$ neuroreceptors and thereby affecting neuroreceptor function (Nilsson *et al.*, 2012; Heuer *et al.*, 2016). It is possible that parental exposure to elevated CO_2 cannot alleviate the negative effects of offspring CO_2 exposure due to limited plasticity in $GABA_A$ receptor functioning (Nilsson *et al.*, 2012), although a recent molecular study suggests otherwise. Schunter *et al.* (2018) found that juvenile spiny damselfish exposed to elevated CO_2 had upregulated expression for GABAergic genes, including genes involved in GABA production, GABA secretion, and all of the $GABA_A$ receptor subunits. However, if the fish had parents that were also exposed to elevated CO_2, these gene expression levels returned to baseline levels. This indicates that there is capacity for parental effects to act on the expression of key GABAergic genes, despite the evidence from experiments that have shown that parental exposure to elevated CO_2 does not improve behavioral responses in offspring (Welch *et al.*, 2014; McMahon *et al.*, 2018). More research is needed to explain the seeming contradiction between these results. Additionally, previous studies have found negative carry-over effects of parental exposure to elevated CO_2 when offspring are reared in control conditions (Welch *et al.*, 2014). Although we saw a trend towards this pattern, as offspring reared at control conditions from CO_2-exposed parents seemed to have a lower reduction in feeding strikes than control offspring from control parents, this difference was not statistically significant, indicating that parental exposure to elevated CO_2 neither helped nor harmed offspring's behavioral performance. Overall, our work underscores the inability of fishes to acclimate behavioral responses to elevated CO_2 though parental effects, but the reason for this inflexibility remain unknown.

In contrast to the behavioral trials, parental effects were clearly evident in the metabolic responses of juvenile spiny damselfish. There was a significant increase in $\dot{M}O_{2Max}$ in offspring of CO_2-exposed parents, regardless of their rearing conditions. This pattern of increased $\dot{M}O_{2Max}$ from parental exposure to elevated CO_2 has not been previously documented in marine fishes, but could have a positive effect on offspring because cardiac output and swimming performance have been shown to correlate with $\dot{M}O_{2Max}$ (Claireaux *et al.*, 2005). Mechanistically, this increase in $\dot{M}O_{2Max}$ could result from an increase in carbonic anhydrase activity that helps to

enhance oxygen transport (Rummer *et al.*, 2013b; Hannan & Rummer, 2018), though further work is needed to test this hypothesis. We also observed that $\dot{M}O_{2Rest}$ was significantly higher in control-reared offspring from CO_2-exposed parents compared to both control and CO_2-reared offspring from control parents. This is indicative of a higher cost of living for control-reared offspring from CO_2-exposed parents. This result is unique, as few studies have examined the effects of parental exposure to elevated CO_2 on metabolic traits in marine fishes using a full factorial design. Future studies could test for increased energetic demands in offspring of CO_2-exposed parents. Combined, our results suggest that parental exposure to elevated CO_2 results in a metabolic cost (i.e. higher $\dot{M}O_{2Rest}$), at least for offspring in current-day conditions, but also increases $\dot{M}O_{2Max}$, which could increase some aspects of individual performance in both control and elevated CO_2 conditions.

Given that both $\dot{M}O_{2Rest}$ and $\dot{M}O_{2Max}$ were affected by parental exposure to elevated CO_2, it is not surprising that there were also effects on aerobic scope. In particular, offspring of CO_2-exposed parents had significantly higher aerobic scope than CO_2-reared offspring from control parents, because the overall effect on $\dot{M}O_{2Max}$ was greater than the effect on $\dot{M}O_{2Rest}$. This demonstrates, for the first time in a marine fish, that parental exposure to elevated CO_2 can have an overall positive effect on the aerobic scope of offspring. This increase in aerobic scope could improve individual performance, as aerobic scope has been linked to a fish's capacity for essential activities such as swimming, growth, development, and reproduction (Pörtner & Farrell, 2008; Pörtner & Peck, 2010). However, the benefits of an increased aerobic scope will depend upon the frequency and extent to which a fish uses the full range of its aerobic scope, which has not yet been determined for reef fishes, including *A. polyacanthus*. Nevertheless, given that other climate change relevant stressors such as elevated temperature have been shown to decrease aerobic scope in some tropical marine fishes (Chapter 2; Nilsson *et al.*, 2009; Johansen & Jones, 2011; Rummer *et al.*, 2014; Habary *et al.*, 2017), a positive effect of parental CO_2 exposure could potentially help to offset these decreases in aerobic scope.

Here we found that for $\dot{M}O_{2Max}$ and aerobic scope, parental exposure to elevated CO_2 had a similar effect on the performance of offspring, regardless of whether they were reared under elevated or control CO_2 conditions. This suggests that there is a

fundamental effect of elevated CO_2 during the parental phase that alters offspring development. Beneficial parental effects are predicted to occur when there is high predictability between parental and offspring environments (Marshall & Uller, 2007; Bonduriansky *et al.*, 2012). *A. polyacanthus* offspring are not dispersive, instead remaining with their parents for up to 45 days post-hatching (Kavanagh, 2000). Thus, we might expect there to be good predictability between parental and offspring environments in this species, leading to the evolution of an adaptive parental effect. The aforementioned work by Schunter *et al.* (2016) supports this hypothesis, as juvenile spiny damselfish from parents exposed to control versus elevated CO_2 conditions showed differential regulation of key genes and proteins, suggesting an epigenetic effect. However, it is also possible that the observed changes to metabolic traits could derive from embryonic exposure to elevated CO_2. Spiny damselfish are demersal spawners, and parents care for eggs until hatching (Kavanagh, 2000). Thus, both control-reared and CO_2-reared offspring that had CO_2-exposed parents experienced elevated CO_2 during the embryonic stage until hatching. It could have been this exposure during early development that affected metabolic traits, rather than an inherited effect from parents. Disentangling early developmental effects from true parental effects is a challenge for these types of studies, particularly for species that brood or provide parental care to their offspring (Torda *et al.*, 2017; Donelson *et al.*, 2018). Still, regardless of the underlying mechanism, our results indicate that parental or early developmental exposure to elevated CO_2 can be highly effective in mitigating the negative impacts of ocean acidification on physiological performance in a coral reef fish.

Across all offspring and parental CO_2 treatments, no correlations were observed between behavioral and physiological performance. This is consistent with previous studies on trait correlations, which found no significant correlations between behavior and physiology when only one stressor was present, either elevated CO_2 or temperature (Chapter 2; Chapter 4). However, significant correlations between behavioral and physiological performance were detected when fish were exposed to both elevated CO_2 and elevated temperature (Chapter 2; Chapter 4). This pattern suggests that significant correlations between behavioral and physiological performance may only occur when fish are exposed to multiple stressors. Indeed, it has been hypothesized that stressors can act to amplify relationships between behavior

and physiology (Killen *et al.*, 2013). While the underlying cause for this pattern is not known, it may be caused by individuals having different sensitivities to the stressors, which can increase the intraspecific phenotypic variation in the traits (Hoffmann & Hercus, 2000; Sgrò & Hoffmann, 2004). Thus, elevated CO_2 in isolation may not represent a great enough stressor to markedly increase intraspecific variation in performance and elicit a significant relationship between traits. In marine fishes, elevated temperature has been shown to have a greater effect on metabolic traits than elevated CO_2 (Lefevre, 2016; Hannan & Rummer, 2018). Therefore, elevated CO_2 may act to increase intraspecific variation in behavioral performance, while elevated temperature increases variation in physiological performance. Furthermore, there could be correlations between other traits we did not measure here, and future work could examine a greater variety of traits to determine if other tradeoffs in performance might exist. Ultimately, though, ocean acidification will co-occur with a number of other stressors, such as ocean warming (Doney *et al.*, 2012), and the negative relationship between behavior and physiology that occurs when both of these stressors are present could have a negative effect on individual performance, and ultimately on adaptive potential to the combined effects of warming and acidification (Chapter 2; Chapter 4). Future research should examine how parental effects could modulate correlations between behavior and physiology under scenarios of multiple climate change stressors.

In conclusion, this study shows that parental exposure to elevated CO_2 can have different effects on behavioral versus physiological performance in a coral reef fish. Parental exposure to elevated CO_2 affected $\dot{M}O_{2Rest}$, $\dot{M}O_{2Max}$, and aerobic scope, but failed to mitigate impaired antipredator responses to alarm cues under elevated CO_2. The proximal cause for these diverging effects of parental exposure to elevated CO_2 is unknown, and represents a promising avenue for future research. Our work also suggests that parental effects do not have a strong influence on correlations between behavioral and physiological performance, at least under elevated CO_2 alone. This is particularly salient in light of previous findings, which suggest that parental effects do not ameliorate the behavioral impairments associated with elevated CO_2. Thus, fishes will likely need to rely on adaptation to cope with increasing CO_2 exposure in the future. However, adaptation might still be constrained under multiple climate change stressors (Chapter 2; Chapter 4), and thus considering the role of tradeoffs between

behavior and physiology in facilitating or constraining adaptation will be crucial to predicting the future of marine organisms under climate change.

Chapter 6: General Discussion

To date, most ocean acidification experiments have focused on the mean responses of marine organisms to elevated CO_2. Yet individual variation in responses to elevated CO_2 could affect relationships between behavioral and physiological performance, and consequently impact individual success as well as adaptive potential. In this thesis, I tracked individual fish through multiple assays under different projected future conditions, investigating how relationships between behavioral and physiological performance shift due to multiple stressors, life-history traits, fluctuating CO_2, and parental effects. I found a number of significant correlations between behavioral and physiological performance, but crucially, only one of these relationships, a negative correlation between aerobic scope and reduction in feeding strikes, has clear negative implications for the fish. Furthermore, elevated temperature played a critical role in shaping relationships between behavior and physiology across CO_2 treatments, which underscores the importance of conducting multistressor studies. These findings provide novel evidence of tradeoffs between different types of performance under different environmental conditions, and suggest that future studies should incorporate and account for these relationships in order to most accurately predict the fate of marine fishes in a high CO_2 future.

In addition to the primary findings on relationships between behavior and physiology, I also demonstrated a variety of average population-level responses to elevated CO_2 and temperature. For example, I found that temperature had a greater effect than CO_2 on the behavioral performance of kingfish, while CO_2 dominated the effect of temperature on behavior in spiny damselfish. For metabolic traits, elevated CO_2 and temperature interacted in non-additive ways on both spiny damselfish and kingfish. Together, these studies highlight the complexity of interactions between elevated CO_2 and temperature, as well as similarities and differences in the responses of shallow-water and pelagic fishes to these stressors. I also found that diel CO_2 cycles mitigated the negative impacts of elevated CO_2 on metabolic traits, but these benefits were diminished at elevated temperature. This is a novel finding in marine fishes, which demonstrates the importance of using appropriate treatments in experiments on shallow-water organisms, as well as the masking effect that higher temperature can

have on the benefits of diel CO_2 cycles. Finally, I found parental exposure to elevated CO_2 altered physiological, but not behavioral performance in spiny damselfish. This mirrors previous work on parental effects in other fishes, and suggests that fish populations will need to rely on adaptation to overcome the behavioral impairments associated with elevated CO_2. Together, these results broaden the scope of ocean acidification research on fish and suggest that future experiments should incorporate these multigenerational and multistressor treatments to more accurately represent the breadth of processes that affect acclimation and adaptation to elevated CO_2 in marine fishes.

Relationships between Behavior and Physiology

An emerging field of research seeks to understand relationships between behavioral and physiological performance: under what circumstances do they covary, what underpins their covariation, and what implications do these covariations have for evolutionary potential? My thesis adapted this research to understand how relationships between behavior and physiology might change under different climate change stressors. Across four data chapters, I observed a number of significant correlations between behavioral and physiological performance. In spiny damselfish, I found that response to an alarm cue was negatively correlated with aerobic scope, but only when fish were reared under elevated CO_2 and temperature conditions. I also observed a positive relationship between activity and $\dot{M}O_{2Rest}$ in spiny damselfish when fish were reared under stable elevated CO_2 and temperature. In kingfish, I found a correlation between $\dot{M}O_{2Rest}$ and boldness that was positive when fish were reared at elevated temperature, but negative when fish were reared at ambient temperature.

Together, my four data chapters support the hypothesis that relationships between behavior and physiology can shift under different environmental stressors (Killen *et al.*, 2013). In spiny damselfish, significant correlations were only observed when fish were reared under both elevated CO_2 and temperature conditions, suggesting that these stressors acted to amplify the relationship between behavior and physiology. In kingfish, the correlations reversed sign depending on the fish's thermal environment. These shifts may be caused by different individual sensitivity to stressors, or increased demands on performance that accentuate links between behavior and

physiology (Killen *et al.*, 2013), but further research is needed to confirm this hypothesis. Interestingly, Killen and colleagues hypothesized that moderate stressors would cause an amplification of the relationship between behavior and physiology, while severe stressors would cause a weakening of this relationship (Killen *et al.*, 2013). Thus, while elevated CO_2 and temperature represented the most stressful conditions that fish experienced in these experiments, they acted like moderate stressors in terms of their ability to strengthen, rather than weaken, the relationship between behavioral and physiological performance.

The observed correlations could affect individual performance and the potential for adaptive evolution in the population. However, across all four data chapters, only one relationship has clear negative consequences for the fish. Spiny damselfish reared under elevated CO_2 and temperature were constrained along a maximal performance ridge, such that an individual could maintain a relatively high aerobic scope, or an ecologically appropriate response to alarm cue, but not both. Importantly, this relationship was consistent across family groups, suggesting that certain families do not hold an advantage over others in dealing with this limitation. Because this correlation was observed under both elevated CO_2 and temperature, which are likely to co-occur as climate change proceeds, future conditions may elicit this negative relationship. This correlation could impact the adaptive potential of spiny damselfish, as selection for improved performance of either trait could decrease performance in the other, slowing adaptation. Knowing that global climate change is proceeding rapidly, correlations that slow or limit selection for improved performance could have serious implications for individual performance, and ultimately, population success.

While the aforementioned relationship between traits could have negative implications for spiny damselfish and potentially other coral reef fishes under future conditions, it is less obvious how the other correlations I observed might affect selection. These correlations were observed between measures of activity/boldness and $\dot{M}O_{2Rest}$, so the direction that selection will take is dependent upon the relative benefits of higher activity/boldness versus $\dot{M}O_{2Rest}$ under future conditions. These benefits will not be consistent across environments, and will instead depend on additional factors such as predator abundance and food availability. For instance, bolder and more active fish are likely to have increased foraging success (O'Brien,

1979) and therefore may outcompete less bold or active fish in a low-food environment. Yet the benefits of being bold and active could diminish in an environment with many predators, as these traits increase susceptibility to predation, particularly in larval and juvenile fishes (Werner & Anholt, 1993; Biro *et al.*, 2003a, 2003b). On the other hand, an elevated $\dot{M}O_{2Rest}$ requires a higher energy supply, meaning that fish might struggle to meet energetic demands in food-poor, high predation environments. Thus, to understand which phenotypes will be favored in the future, further research is needed to determine how patterns of food and predator distribution will change. Importantly, though, while relationships between activity/ boldness and $\dot{M}O_{2Rest}$ were observed in both spiny damselfish and kingfish, they will likely only become relevant for kingfish in the future. This is because for spiny damselfish, the significant correlation between traits was only observed under stable elevated CO_2 and temperature conditions, and not the more ecologically-relevant diel cycling elevated CO_2 and temperature. For kingfish, temperature is a key factor that will determine the direction that selection takes. This is especially salient in light of the southward range-shifts that have been already observed in kingfish populations (Champion *et al.*, 2018) and are predicted for the future as ocean warming continues (Champion *et al.*, 2019). It may be possible that if some kingfish shift to cooler waters, but others remain in warmer waters, selection could proceed in opposing directions on these different populations. Ultimately, though, further work is needed to determine the relative benefits of boldness, activity, and $\dot{M}O_{2Rest}$ by better modeling future patterns of food availability and predator density.

An important caveat for my thesis is that I measured phenotypic, not genotypic correlations. Phenotypic correlations are often indicative of genetic correlations (Lynch & Walsh, 1998), but ultimately the observed phenotypic variation must be heritable for adaptation to occur. Metabolic traits (Munday *et al.*, 2017), responses to alarm odor (Welch & Munday, 2017), activity patterns (Bell, 2005), and boldness (Brown *et al.*, 2007; Ariyomo *et al.*, 2013) have all shown heritability in marine fishes. This suggests that these traits have a significant amount of additive genetic variation, and thus that the correlations observed in this thesis could indeed affect adaptation. Future work could determine the extent to which genetic variation underpins the observed phenotypic variation using methods similar to Healy *et al.* (2018), by using multilocus association mapping to identify single nucleotide

polymorphisms that underpin a proportion of the observed phenotypic variation. Alternatively, to estimate the genetic components of phenotypic traits, researchers could employ diallel breeding designs (Munday *et al.*, 2013; Tasoff & Johnson 2018) or use the animal model (Kruuk, 2004) to compare phenotypic similarity among individuals that have a known relationship to each other in a multigenerational pedigree (e.g. Malvezzi *et al.*, 2015; Munday *et al.*, 2017).

While this thesis provides novel evidence for correlations between behavioral and physiological performance in marine fishes, it cannot elucidate the proximal cause for these relationships. From an evolutionary biology perspective, we might predict that the observed phenotypic correlations are underpinned by a genetic correlation between the traits (Sunday *et al.*, 2014). Genetic correlations can arise from pleitropy, when a gene influences multiple traits, or linkage disequilibrium, when there is non-random association of alleles at different loci (Falconer & Mackay, 1996). They could even result from correlations of both traits with a third, unmeasured trait (Clark, 1987). While evolutionary biologists are primarily interested in detecting and describing the magnitude and direction of correlations between phenotypic traits (Travisano & Shaw 2012), physiologists seek out mechanistic explanations that underpin these associations (Hofmann & Todgham 2010; Somero, 2010; Kelly & Hofmann 2013). These goals can be thought of as "two sides of the same coin", as physiological connections should be observable as genetic correlations (Careau & Garland, 2012). Thus, while evolutionary biologists might focus on pleiotropic genes, physiologists would want to identify the downstream processes affected by those genes, and how they could cause correlations between traits. For instance, physiologists have proposed a number of hypotheses to understand how behavior and physiology might be linked. One such hypothesis has been termed the "allocation model", which suggests that organisms have a finite amount of energy to expend on competing resources (Careau *et al.*, 2008). This model predicts a negative relationship between $\dot{M}O_{2Rest}$ and behavioral traits like activity or boldness, and has some support in the literature. For instance, juvenile rockcod *Trematomus bernacchii* exposed to elevated temperature exhibited increased oxygen uptake rates that were accompanied by decreased activity, providing evidence for tradeoffs between energetically-demanding behavioral and physiological processes (Davis *et al.*, 2017). Conversely, the "performance model" posits that behavioral traits should correlate positively with

metabolic traits, as a higher sustained energy output via increased activity would require larger or stronger organs to convert food into energy, resulting in a higher $\dot{M}O_{2Rest}$ (Careau *et al.*, 2008; Biro & Stamps, 2010). In kingfish, I observed both positive and negative correlations between $\dot{M}O_{2Rest}$ and boldness, depending on the fish's rearing temperature. In spiny damselfish, I observed a positive relationship between $\dot{M}O_{2Rest}$ and activity when fish were reared under elevated CO_2 and temperature. Thus, in similar studies, and even within the same species, I observed both "allocation" and "performance"-type relationships between behavioral and physiological traits.

Importantly, I measured different behavioral traits in different experiments; two focused on response to an alarm odor, while the other two measured activity and boldness. While I observed significant correlations between behavioral and physiological traits across both sets of experiments, it is likely that these relationships have different mechanistic underpinnings. Ultimately, research on relationships between behavior and physiology is growing into a vibrant field of study. A suite of potential underlying mechanisms has been identified, but a single universal mechanism to describe these relationships is not yet evident, and indeed may not exist. Thus, my thesis provides new examples of relationships between behavior and physiology, as well as how they shift under different climate change-relevant stressors, which can be built upon to better understand the proximal causes for these relationships.

Ocean Warming and Acidification

Elevated temperature is known to affect some physiological and behavioral traits in marine fishes, but it can also interact with elevated CO_2 in complex and unpredictable ways. Of the behavioral traits I measured, I found that temperature affected measures of routine activity in yellowtail kingfish, but did not affect response to an alarm odor in spiny damselfish. Higher rates of activity with increasing temperature have been previously observed in a number of marine fish species (Fukuhara, 1990; Biro *et al.*, 2010; Mccormick & Meekan, 2010; Pimentel *et al.*, 2014; Bignami *et al.*, 2016), although decreases in activity at higher temperatures have also been observed (Johansen *et al.*, 2014; Jarrold & Munday, 2018b). These differences are likely tied to

the thermal performance curves for each species; if the fishes were tested at temperatures within their preferred thermal range, then increased metabolic costs would cause the fish to become more active to forage for food and meet their higher energetic needs (Biro & Stamps, 2010). By contrast, fishes that were tested beyond their optimal thermal range might exhibit decreased activity as an energy-saving strategy (Johansen *et al.*, 2014). The consequences of increased activity in kingfish at higher temperatures are nuanced, as increased activity is linked both to increased foraging success (O'Brien, 1979) and vulnerability to predation (Werner & Anholt, 1993; Biro *et al.*, 2003a, 2003b). Thus, the net positive or negative effect of increased activity will depend upon additional factors, such as predator abundance and food availability. By contrast, temperature did not affect response to an alarm odor. Impaired olfactory responses have been primarily observed under elevated CO_2 conditions (Ferrari *et al.*, 2011a; Chivers *et al.*, 2014; Welch *et al.*, 2014; Ou *et al.*, 2015), but an interaction between elevated CO_2 and temperature on this trait has not been previously explored. While temperature likely influenced activity indirectly through its effect on metabolic rates, impairments in olfaction are thought to be driven by disruption to $GABA_A$ neuroreceptor function (Nilsson *et al.*, 2012) and/or olfactory nerve function (Porteus *et al.*, 2018). My results indicate that these processes are not affected by temperature changes. Overall, my results suggest that elevated CO_2 has a greater impact on behavioral responses that involve decision-making, such as response to an alarm odor, while elevated temperature has a greater impact on behavioral responses that are dictated by energetic constraints, such as activity.

In contrast to the relatively simple relationship between temperature and elevated CO_2 observed in behavioral traits, physiological traits showed much more complex interactions that differed between species. For the spiny damselfish, there were significant interactions between CO_2 and temperature on aerobic scope, $\dot{M}O_{2Max}$, and $\dot{M}O_{2Rest}$. For the kingfish, while there were no main effects of CO_2 or temperature on aerobic scope or $\dot{M}O_{2Max}$, there was a significant interaction between CO_2 and temperature on $\dot{M}O_{2Rest}$. Finally, elevated temperature diminished the beneficial effects of diel CO_2 cycles on $\dot{M}O_{2Rest}$ in spiny damselfish. Combined, these results suggest that interactions between temperature and CO_2 on metabolic traits are unpredictable and often non-additive. A recent meta-analysis found that these

stressors most frequently interact additively and antagonistically on aerobic scope and $\dot{M}O_{2Rest}$ in marine ectotherms, with synergistic interactions being more rare (Lefevre, 2016). In this thesis, I observed temperature and CO_2 to act antagonistically on $\dot{M}O_{2Rest}$ in kingfish, but synergistically in spiny damselfish. Some of these different interactions might be ascribed to acclimation or adaptation of the species to these stressors. For instance, in a previous experiment, juvenile spiny damselfish that were shock-exposed to elevated temperature had an increased $\dot{M}O_{2Rest}$, but when they were allowed to develop under elevated temperature conditions, $\dot{M}O_{2Rest}$ returned to control levels (Donelson *et al.*, 2012). This indicates that spiny damselfish have a capacity for developmental acclimation to elevated temperature. However, when spiny damselfish were reared under both elevated temperature and CO_2, $\dot{M}O_{2Rest}$ was elevated, indicating that the combined effects of both stressors were too high for the fish to overcome through developmental acclimation. Ultimately, the mechanisms underpinning antagonistic and synergistic interactions between temperature and CO_2 are still unknown (Lefevre, 2016), and therefore reliable predictions about the effects of these stressors cannot be made. This underscores the need for a variety of multi-stressor experiments to aid in understanding underlying mechanisms for non-additive interactions and improving predictions about species persistence in the future.

Susceptibility of Pelagic Fishes

It has been hypothesized that pelagic fishes are more susceptible to ocean acidification than shallow-water fishes due to the stable CO_2 conditions they experience in the open ocean (Munday *et al.*, 2008a; Pörtner, 2008). In this thesis, a reef fish was both behaviorally and physiologically affected under elevated CO_2, while a pelagic fish only displayed an increased $\dot{M}O_{2Rest}$ under elevated CO_2. These results do not support the hypothesis that pelagic fishes are more susceptible to ocean acidification. Instead, the pelagic fish experienced similar physiological impairments to the reef fish and no behavioral impairments. There have been few studies examining the effects of elevated CO_2 on behavioral performance in pelagic fishes, and their results do not always coincide. For instance, in studies of three species of large pelagic fishes, all but one species demonstrated unchanged activity levels under elevated CO_2 conditions (Bignami *et al.*, 2013, 2014, 2016; Pimentel *et al.*, 2014; Munday *et al.*, 2015). Furthermore, for the one species that did experience altered

activity, changes were only observed when CO_2 was in excess of 1400 µatm (Pimentel *et al.*, 2014), which surpasses end-of-century predictions for the open ocean under a high-emissions scenario (Collins *et al.*, 2013). By contrast, ocean-phase coho salmon exhibited impaired responses to an alarm odor under elevated CO_2 (Williams *et al.*, 2018), and freshwater-phase larval pink salmon exposed to elevated CO_2 showed a similar impairment in responding to alarm odors (Ou *et al.*, 2015). Thus, the behavioral responses of pelagic fishes to elevated CO_2 appear to be varied and potentially species-specific, similar to patterns observed for demersal species (Cattano *et al.*, 2018).

There are a number of possible explanations for the discrepancy between what has been hypothesized and what has been observed with respect to the behavioral responses of pelagic fishes to elevated CO_2. One possibility is that the fishes were tested too early in their development to observe behavioral impairments under elevated CO_2. Given that behavioral impairments are thought to derive from disruptions to $GABA_A$ neuroreceptor function as a result of internal acid-base regulation (Nilsson *et al.*, 2012), fish would need to have fully developed and functioning acid-base regulatory systems to observe behavioral impairments. However, this is an unlikely explanation, as ion exchange primarily occurs at the gills in juvenile fishes, and the kingfish had functional gills at the age they were tested, indicating that they likely had full capacity for branchial acid-base regulation (Claiborne *et al.*, 2002; Brauner, 2008). Instead, it is more likely that pelagic fishes are no more susceptible to elevated CO_2 than many demersal species. The reason that demersal species previously seemed to be highly susceptible to elevated CO_2 is that experiments often employed stable elevated CO_2 treatments. Yet, coastal habitats often experience substantial natural CO_2 variability, and new studies show that the effects of elevated CO_2 on the behavioral performance of shallow-water fishes are mitigated by diel CO_2 cycles (Jarrold *et al.*, 2017). These new finding suggest that the behavioral effects of elevated CO_2 may have been overestimated in shallow-water species because the importance of natural CO_2 cycles has been overlooked. Shallow-water species might not be markedly different to pelagic species in their behavioral susceptibility to elevated CO_2 after incorporating appropriate CO_2 variation. Ultimately, to deepen our understanding of species differences in behavioral sensitivity to elevated CO_2, more studies are needed that incorporate a wide variety of

pelagic and demersal fishes, taking into account the variation in pCO_2 they naturally experience. It would also be informative to compare changes in gene expression under elevated CO_2 in pelagic fishes to demersal fishes to help to discern differences in the mechanisms underpinning behavioral responses, as per Schunter *et al.* (2018).

Although no behavioral perturbations were observed under elevated CO_2 in kingfish, $\dot{M}O_{2Rest}$ was elevated compared to controls. This result contrasts with the one other study on metabolic performance in larval pelagic fishes under elevated CO_2, which found that mahi mahi *Coryphaena hippurus* had reduced $\dot{M}O_{2Rest}$ when exposed to 1600 µatm CO_2 (Pimentel *et al.*, 2014). However, this result does align with finding for other marine fishes, which tend to show elevated $\dot{M}O_{2Rest}$ under elevated CO_2 (Cattano *et al.*, 2018). Elevated $\dot{M}O_{2Rest}$ is indicative of a higher cost of living under elevated CO_2 conditions, and is likely due to an increased cost of acid-base regulation. My results also indicate that kingfish cannot overcome the metabolic cost of elevated CO_2 through developmental acclimation. Developmental acclimation involves permanent phenotypic responses to the environment that are established during early ontogeny (Angilletta, 2009) and can arise due to changes in gene expression that result from hormonal and epigenetic processes (Beldade *et al.*, 2011). The kingfish were exposed to elevated CO_2 starting from a few hours post-fertilization until they were tested at 18-24 days post-hatching, and thus had the opportunity to, but did not experience, these developmental shifts. Given that developmental acclimation was not able to ameliorate the increased metabolic costs of elevated CO_2 in kingfish, other processes such as parental effects or adaptation will be required for these fish to avoid increased metabolic costs in future conditions.

Diel CO_2 Cycles

Fluctuations in pCO_2 have been hypothesized to affect the responses of shallow-water marine organisms to elevated CO_2, potentially mitigating or exacerbating its effect (Shaw *et al.*, 2013). I demonstrated that diel CO_2 cycles alleviated the negative effects of elevated CO_2 on $\dot{M}O_{2Rest}$ in juvenile spiny damselfish, but this benefit was not evident when fish were also exposed to elevated temperature. Previous studies have found diel CO_2 cycles to alter metabolic performance in pink salmon *Oncorhynchus gorbuscha* (Ou *et al.*, 2015) and European eel *Anguilla anguilla* (Methling *et al.*,

2013). However, these studies employed fluctuating CO_2 treatments with lower mean CO_2 than the stable elevated CO_2 treatments, meaning that the observed changes to metabolic performance could be attributed to either the fluctuations themselves or the lower mean CO_2 level. Thus, my results are novel for isolating the effect of diel CO_2 cycles on metabolic performance in marine fishes.

My results suggest that previous studies may have misestimated the effects of elevated CO_2 on physiological performance in shallow-water fishes. In particular, the observed increase in $\dot{M}O_{2Rest}$ at stable elevated CO_2 indicates a higher cost of living, which could have negative effects on energy-demanding processes such as growth. However, diel CO_2 cycles eliminated this negative effect, which would theoretically also reduce the negative effects of CO_2 on processes like growth. A concurrent study supports this hypothesis, as spiny damselfish that were reared under diel CO_2 cycles had a significantly higher mass than fish reared under stable elevated CO_2 (Jarrold & Munday, 2018b). The proximal cause for this improved performance under diel CO_2 cycles is unknown, and warrants further investigation. It may be the case that reef fishes have developed adaptations to CO_2 fluctuations that mitigate their metabolic cost, as they already experience diel CO_2 cycles in their natural habitat (Kayanne *et al.*, 1995; Manzello, 2010; Shaw *et al.*, 2012; Albright *et al.*, 2013; Kline *et al.*, 2015). In this case, stable elevated CO_2 conditions could be stressful for the fish, causing an increase in $\dot{M}O_{2Rest}$. Future experiments could investigate the mechanisms that enable the spiny damselfish to maintain $\dot{M}O_{2Rest}$ in cycling CO_2 conditions using methods similar to McKenzie *et al.* (2002), by exposing fish to elevated CO_2 conditions, with and without diel CO_2 cycles, and taking regular measurements of critical blood parameters, such as pH, pCO_2 and HCO^-_3, over the course of an entire CO_2 cycle to track how these metrics change or show resilience to elevated CO_2. Still, regardless of the mechanism, these results demonstrate that diel CO_2 cycles can play a crucial role in modifying a reef fish's physiological performance under elevated CO_2, and therefore should be incorporated into future ocean acidification experiments on shallow-water organisms to better predict their responses to future elevated CO_2 conditions.

Importantly, the benefits of diel CO_2 cycles on $\dot{M}O_{2Rest}$ were diminished at elevated temperature. No previous studies have demonstrated how diel CO_2 cycles and

elevated temperature interact on metabolic performance in marine fishes, but my results indicate that elevated temperature is a dominant stressor to elevated CO_2 for $\dot{M}O_{2Rest}$. This aligns well with the results of a recent meta-analysis, which found that $\dot{M}O_{2Rest}$ in marine fishes tends to be more strongly affected by elevated temperature than by elevated CO_2 (Lefevre, 2016). Given that CO_2 and temperature will likely rise in tandem due to global climate change, these results suggest that reef fishes will still be subjected to elevated metabolic costs under future conditions, even in the presence of diel CO_2 cycles. It is not clear why elevated temperature diminished the benefits of diel CO_2 cycles on metabolic performance, but this pattern might be explained by a synergistic interaction between elevated temperature and CO_2 on $\dot{M}O_{2Rest}$, as has been previously observed in spiny damselfish (Chapter 2). However, a critical avenue for future research is to consider how diel temperature cycles might interact with diel CO_2 cycles. The temperatures of shallow-water ecosystems fluctuate on a daily cycle, driven by patterns of solar heating, currents, tides, and wind (Crabbe, 2008). Furthermore, climate change is predicted to increase the amplitude of diel temperature fluctuations (Wang & Dillon, 2014). Yet, as with ocean acidification experiments, many ocean warming experiments do not incorporate diel temperature cycles in their treatments. Diel temperature cycles have been shown to impact fish performance both positively and negatively, increasing growth in some species (Biette & Geen, 1980; Cooke *et al.*, 2003; Rodgers *et al.*, 2018) while decreasing growth in others (Schaefer & Ryan, 2006; Dhillon & Fox, 2007; Imholt *et al.*, 2011). Importantly, diel temperature cycles peak during the day, while diel CO_2 cycles peak at night, which could complicate predictions as to how they will interact on fish performance. Thus, future research should combine cycling treatments of both stressors to better predict species responses to future climatic conditions.

Parental Effects

The environment experienced by parents can shape offspring phenotypes, either helping or hindering performance. I found that parental exposure to elevated CO_2 increased both aerobic scope and $\dot{M}O_{2Max}$ in spiny damselfish offspring, but did not mitigate the impaired response to an alarm odor. This aligns well with studies on other marine fishes, which have found that parental exposure to elevated CO_2 does not improve behavioral responses to alarm cues and predator cues under elevated CO_2

(Welch *et al.*, 2014; McMahon *et al.*, 2018), but can improve growth, survival, and resting oxygen uptake rates (Miller *et al.*, 2012; Murray *et al.*, 2014). These results provide hope that the negative effects of elevated CO_2 on offspring metabolic traits can be ameliorated by parental exposure to elevated CO_2. However, appropriate antipredator behaviors like responses to alarm odors are crucial for survival in juvenile fishes (Ferrari *et al.*, 2011a; Chivers *et al.*, 2014). If fish cannot acclimate to elevated CO_2 either developmentally or via parental effects, then they will have to rely on adaptation to overcome these behavioral impairments. It is not clear why parental effects can restore performance in physiological, but not behavioral traits. One possibility is that the mechanisms underpinning behavioral versus physiological traits have different capacities for plasticity, i.e. the $GABA_A$ neuroreceptors that affect behavioral impairments may be less plastic than metabolic pathways, although, a recent study by Schunter *et al.* (2018) suggests otherwise. Schunter *et al.* (2018) found evidence for parental effects on gene expression that would affect $GABA_A$ neuroreceptor function, counter to experimental studies that have shown no improvement in behavioral responses to elevated CO_2 after parental exposure (Chapter 5, Welch *et al.*, 2014; McMahon *et al.*, 2018). A natural follow-on from Schunter and colleagues' work would be to compare offspring phenotypes for both behavioral and physiological performance with changes in gene expression, as there could be differential responses to parental exposure to elevated CO_2 between individuals. Indeed, Schunter and colleagues found differential gene expression in offspring from parents that were deemed tolerant or sensitive to elevated CO_2, suggesting that both parental phenotype and CO_2 exposure play an important role in shaping offspring performance.

In contrast to behavioral performance, parental exposure to elevated CO_2 significantly increased offspring aerobic scope and $\dot{M}O_{2Max}$, irrespective of the offspring CO_2 treatment. This suggests that there is a fundamental effect of elevated CO_2 during the parental phase that alters offspring performance. It is difficult to discern the underlying mechanism for this pattern. Parental effects can include nutritional provisioning, transmission of hormones or other cytoplasmic factors, and epigenetic processes such as DNA methylation and modification of histones (Mousseau & Fox, 1998; Jablonka & Raz, 2009; Bonduriansky *et al.*, 2012). As mentioned previously, molecular studies on spiny damselfish have shown that parental exposure to elevated

CO_2 can result in differential regulation of specific genes and proteins, which would suggest an epigenetic mechanism (Schunter *et al.*, 2018). Yet it is also possible that the improved performance derived not from the parents themselves, but developmental acclimation in the embryonic stage. Egg clutches were laid and developed in the parental CO_2 treatment, and transferred to the offspring treatment at one day post-hatching. Thus, this early exposure to control or elevated CO_2 conditions could have influenced metabolic performance in juveniles, rather than parental effects. Distinguishing parental effects from developmental acclimation is both difficult and necessary for understanding how offspring performance can improve under adverse environmental conditions. Future research could include experiments which span two to three generations to provide evidence for grandparent effects (Torda *et al.*, 2017; Donelson *et al.*, 2018) or studies that look for patterns of gene expression in control versus parentally-exposed offspring (Schunter *et al.*, 2018). Additionally, future studies should incorporate multiple stressors. In marine fishes, transgenerational experiments have demonstrated how parental exposure to elevated CO_2 (Miller *et al.*, 2012; Murray *et al.*, 2014; Welch *et al.*, 2014; McMahon *et al.*, 2018) and temperature (Donelson *et al.*, 2012; Salinas & Munch, 2012; Shama *et al.*, 2014; Le Roy *et al.*, 2017) affect offspring performance in isolation, but it is not known how interactions between these stressors could shape parental effects and offspring responses. Thus, experiments that span multiple generations and include multiple stressors, while challenging, are needed to provide a full picture of modes of parental effects on marine fishes under climate change.

Concluding Remarks

In this thesis, I observed correlations between behavior and physiology in marine fishes that have the potential to affect individual performance maxima and adaptive potential to future climate conditions. Thus, in order to fully understand the implications of future climate change beyond its effects on mean performance, it will be useful to design future studies to measure multiple performance traits in individuals. Similarly, because none of the correlations between behavior and physiology were consistently observed across environments, it will be important to measure relationships under multiple, ecologically-relevant climate change stressors. In addition to the findings on relationships between behavior and physiology, this

thesis has also demonstrated the importance of temperature on these traits. While this thesis primarily concerns elevated CO_2, temperature played a major role, such as when it dominated the effect of elevated CO_2 on behavioral traits in kingfish, diminished the benefits of diel CO_2 cycles on $\dot{M}O_{2Rest}$, and interacted synergistically and antagonistically with elevated CO_2 on $\dot{M}O_{2Rest}$. These findings underscore the need for multistressor studies, which can reveal unpredictable and unexpected interactions that organisms will likely experience in natural settings. Indeed, this thesis also emphasizes the importance of striving to re-create real-world conditions in a laboratory setting, as diel CO_2 cycles significantly impacted metabolic traits, and parental exposure to elevated CO_2 improved physiological performance in offspring. Finally, this thesis explored differences in responses between reef and pelagic fishes, with results that contradict predictions about the sensitivity of pelagic fishes, but ultimately improve our understanding of these trends. Together, these results provide a comprehensive analysis of individual and population-level responses to elevated CO_2 in marine fishes.

There are a number of avenues for future research that can build on the results from this thesis. Measuring a wider variety of behavioral and physiological traits could help to determine a mechanistic basis for their covariation. Similarly, measuring these traits in a wider variety of fishes with different lifestyles can broaden our understanding of the consistency of these relationships. The next generation of experiments should also strive to incorporate as many ecologically-relevant factors as possible, including natural and predicted future variations in pCO_2 and temperature, and parental and multigenerational effects. With all of these factors in mind, future research should also turn to genomics (Nielsen *et al.*, 2009; Brennan *et al.*, 2018) and transcriptomics (Connon *et al.*, 2018; Schunter *et al.*, 2018) to determine the patterns of genes and gene expression underpinnings these correlations. Together, these methods can help to create a more holistic understanding of how marine fishes will respond to and cope with future climate change.

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Appendix A: Conditions at Davies Reef

Table A.1. Seasonal averages (mean ± SD) and ranges of measured and calculated physical and chemical water parameters at Davies Reef, central GBR during the period 17–27 January 2012 in austral summer and 29 July–6 August 2012 in austral winter. Adapted from Albright *et al.* (2013).

	Temperature (°C)	**Salinity (ppt)**	**pH_T**	**Alkalinity (µmol kg^{-1})**	**pCO_2 (µatm)**
Summer					
Mean	28.5 ± 0.2	35.0 ± 0.1	8.03 ± 0.03	2276 ± 16	404 ± 40
Range	28.1 – 28.9	34.9 – 35.1	7.92 – 8.10	2213 – 2304	325– 542
Winter					
Mean	22.3 ± 0.1	35.6 ± 0.1	8.09 ± 0.02	1985 ± 19	348 ± 24
Range	22.1 – 22.7	35.5 – 35.7	8.03 – 8.17	1887 – 2028	275 – 420

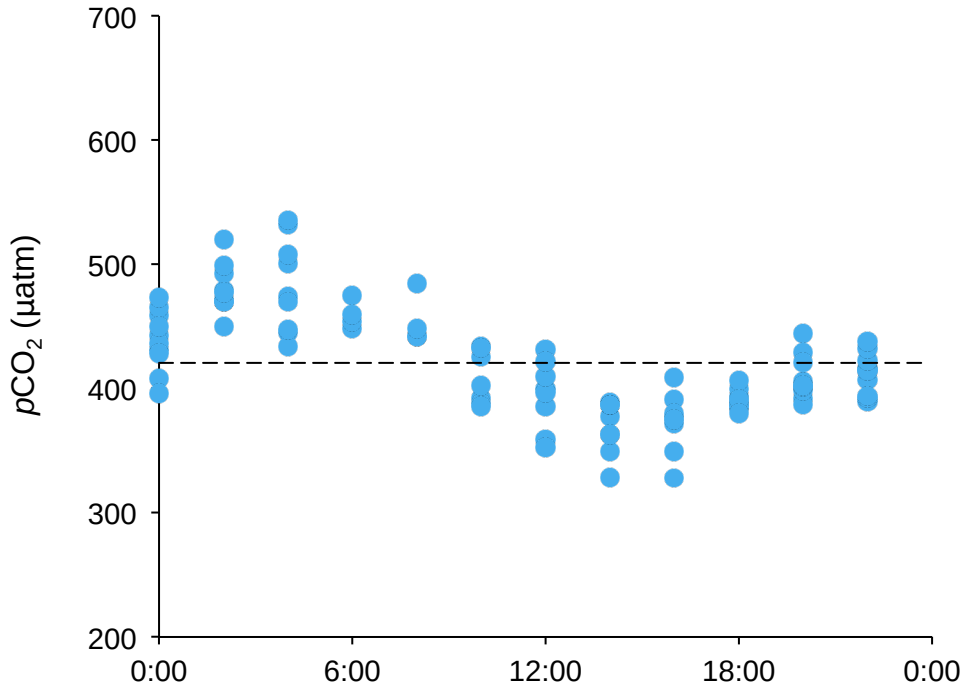

Figure A.1. A composite diel pCO_2 curve for the Davies Reef flat from 10 consecutive days in January 2012, during the austral summer. Dashed horizontal line represents average daily conditions. Adapted from Albright *et al.* (2013).

Appendix B: AIC Tables

Chapter 4

Table B4.1. AIC comparison table for $\dot{M}O_{2Rest}$. Display of two statistical models, both linear mixed effect models (LME) with Family as a random effect. Selected model shown in bold.

Model	df	AIC	BIC
Mass * Treatment	12	1338.03	1372.34
Mass + Treatment	**8**	**1335.52**	**1358.40**

Table B4.2. AIC comparison table for $\dot{M}O_{2Max}$. Display of two statistical models, both linear mixed effect models (LME) with Family as a random effect. Selected model shown in bold.

Model	df	AIC	BIC
Mass * Treatment	12	1679.53	1714.12
Mass + Treatment	**8**	**1676.61**	**1699.67**

Table B4.3. AIC comparison table for absolute aerobic scope (AAS). Display of two statistical models, both linear mixed effect models (LME) with Family as a random effect. Selected model shown in bold.

Model	df	AIC	BIC
Mass * Treatment	12	1565.21	1599.05
Mass + Treatment	**8**	**1562.72**	**1585.29**

Table B4.4. AIC comparison table for factorial aerobic scope (FAS). Display of two statistical models, both linear mixed effect models (LME) with Family as a random effect. Selected model shown in bold.

Model	df	AIC	BIC
Mass * Treatment	12	139.61	173.45
Mass + Treatment	**8**	**134.87**	**157.43**

Table B4.5. AIC comparison table for relationship between active time and $\dot{M}O_{2Rest}$ in the stable elevated CO_2 and temperature treatment. Display of two statistical models, both linear mixed effect models (LME) with Family as a random effect. Selected model shown in bold.

Model	df	AIC	BIC
Mass * Active time	6	257.16	264.47
Mass + Active time	**5**	**255.39**	**261.48**

Chapter 5

Table B5.1. AIC comparison table for percent change in feeding strikes. Display of three statistical models, all linear mixed effect models (LME) with Family as a random effect. Selected model shown in bold.

Model	df	AIC	BIC
Time of Day * Mass * Offspring CO_2 * Parent CO_2	18	14.15	70.7
Mass * Offspring CO_2 * Parent CO_2	10	7.38	38.79
Offspring CO_2 * Parent CO_2	**6**	**4.42**	**23.27**

Table B5.2. AIC comparison table for $\dot{M}O_{2Rest}$. Display of two statistical models, all linear mixed effect models (LME) with Family as a random effect. Selected model shown in bold.

Model	df	AIC	BIC
Mass * Offspring CO_2 * Parent CO_2	10	1687.4	1716.4
Mass + Offspring CO_2 * Parent CO_2	**7**	**1683.2**	**1703.6**

Table B5.3. AIC comparison table for $\dot{M}O_{2Max}$. Display of two statistical models, all linear mixed effect models (LME) with Family as a random effect. Selected model shown in bold.

Model	df	AIC	BIC
Mass * Offspring CO_2 * Parent CO_2	10	1906.4	1935.9
Mass + Offspring CO_2 * Parent CO_2	**7**	**1904.7**	**1925.3**

Table B5.4. AIC comparison table for aerobic scope. Display of two statistical models, all linear mixed effect models (LME) with Family as a random effect. Selected model shown in bold.

Model	df	AIC	BIC
Mass * Offspring CO_2 * Parent CO_2	10	1738.0	1767.1
Mass + Offspring CO_2 * Parent CO_2	**7**	**1733.4**	**1753.8**