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Effects of diel pCO$_2$ fluctuations on coral reef fishes now and into the future

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General Abstract

The uptake of anthropogenic carbon dioxide from the atmosphere has increased the partial pressure of carbon dioxide ($p\text{CO}_2$) and decreased the pH of the oceans, termed ocean acidification (OA). The majority of the models predicting future OA conditions are based on the open ocean, where $p\text{CO}_2$ and pH are relatively stable. However, $p\text{CO}_2$ fluctuates at a variety of temporal scales in coastal ecosystems. Coral reefs possess a daily cycle, where the $p\text{CO}_2$ is elevated at night and lower during the day, mainly due to the photosynthesis, respiration, and calcification cycle of benthic reef organisms. Moreover, the magnitude of $p\text{CO}_2$ fluctuations is projected to increase in the future as OA advances. To date, most studies on the effects of elevated $p\text{CO}_2$ on reef fishes have used stable CO_2 treatments, which may not accurately represent physiological responses under predicted future fluctuation in $p\text{CO}_2$. In this thesis, I aim to examine fluctuations of $p\text{CO}_2$ on coral reefs and how fishes will be affected in the future by these conditions.

While coral reef $p\text{CO}_2$ is known to fluctuate on a daily cycle, much less is known about $p\text{CO}_2$ variation at the microhabitat scale that animals occupy. Therefore, in **Chapter 2** I investigate the pH/$p\text{CO}_2$ variability of three different reefs and three coral reef microhabitats (hard coral, soft coral, and sand). I found typical diel variation in $p\text{CO}_2$ associated with photosynthesis and respiration cycles. These fluctuations differed between reefs more than between the microhabitats within a reef. The variation was likely influenced by water flow and wind speed. These results fall within the normal range of coral reef diel variation and suggest that it is important to consider physical and hydrodynamic factors when projecting future $p\text{CO}_2$ variation. Data from Chapter 2 on the current-day natural fluctuations of $p\text{CO}_2$ in the environment can be used to inform ecologically relevant $p\text{CO}_2$ treatments to measure how fishes and other coral reef organisms will be affected by OA in the future.

Elevated pCO_2 has the potential to negatively impact fishes due to the increased costs of acid-base regulation. In **Chapter 3**, I explored the effects of an 8 h exposure to one of four pCO_2 treatments, two stable (ambient and stable elevated) or two fluctuating levels of pCO_2 (increasing, and decreasing) on two species of damselfishes, *Acanthochromis polyacanthus* and *Amblyglyphidodon curacao*. *Acanthochromis polyacanthus* required more energy upon exposure to both stable pCO_2 treatments during the 8 h trial compared to fish exposed to the fluctuating pCO_2 treatments. However, there was no effect of pCO_2 treatment on the swimming or oxygen uptake rates of *A. curacao*. This suggests that, for some species of coral reef fishes, performing under fluctuating pCO_2 conditions may be less energetically-costly than performing under stable pCO_2 conditions. Moreover, these results highlight the importance of ecologically relevant pCO_2 treatments when testing how fishes will perform in future OA conditions.

In order to examine how fishes utilizing different activity periods will be affected by longer exposures to stable and fluctuating elevated pCO_2, in **Chapter 4** I exposed four species of coral reef fishes – *Lutjanus fulviflamma, Caesio cuning, Abudefduf whitleyi,* and *Cheilodipterus quinquelineatus* – to three different treatments of pCO_2 (ambient, fluctuating elevated, and stable elevated) for 9-11 d. Aerobic scope and swimming speeds increased in *L. fulviflamma, C. cuning,* and *A. whitleyi* upon exposure to fluctuating elevated pCO_2 when compared to ambient conditions. In contrast, aerobic scope decreased in *C. quinquelineatus* upon exposure to fluctuating elevated pCO_2 when compared to ambient conditions. Thus, this chapter provides further evidence that some species of coral reef fishes may benefit from exposure to ecologically relevant elevated fluctuating pCO_2 conditions; however, other species, such as the cardinalfish studied here, or maybe nocturnal species more broadly, may be more sensitive to future OA conditions.

Overall, Chapters 3 and 4 provided evidence that some species of coral reef fish benefit upon exposure to elevated $p\text{CO}_2$ in terms of increased oxygen uptake and swimming performance. It is possible that the unique oxygen transport system of teleosts facilitates maintained oxygen delivery and swimming performance of coral reef fishes exposed to OA conditions. Teleost fishes possess Root effect haemoglobins, which are extremely pH sensitive when compared to the haemoglobins of other vertebrates, such as humans. This means that small changes in pH can result in large releases of O_2 to the tissues, which could be further magnified during a generalised acidosis, such as ocean acidification conditions. Plasma accessible carbonic anhydrase (paCA) at select tissues can re-acidify red blood cells causing an increased release in O_2 to those tissues (e.g., muscle, heart) by taking advantage of the pH sensitive Root effect haemoglobins. This mechanism has been confirmed in salmonids, but has not been tested in coral reef fishes. Therefore, in **Chapter 5** I exposed *A. polyacanthus* to three different $p\text{CO}_2$ treatments (ambient, elevated fluctuating, or stable elevated) for 12 d and then either sham-injected or injected fish with a paCA inhibitor prior to an exercise challenge. Maximum oxygen uptake rates were unaffected in *A. polyacanthus* that had been exposed to elevated $p\text{CO}_2$ conditions and sham-injected; yet, in *A. polyacanthus* where paCA was inhibited, $M\text{O}_{2\ max}$ decreased. This suggests that paCA is involved in maintaining oxygen uptake rates during exposure to stable elevated $p\text{CO}_2$. Additionally, there was evidence that fish exposed to stable elevated $p\text{CO}_2$ switched to anaerobic metabolism earlier and took longer to recover from the exhaustive exercise challenge when paCA was inhibited. This suggests that stable elevated $p\text{CO}_2$ is more stressful than ecologically relevant elevated fluctuating $p\text{CO}_2$. Moreover, stable elevated $p\text{CO}_2$ conditions may be creating a need for the Root effect and paCA mechanism to aid in maintaining performance, but this may be less important during ecologically relevant fluctuating elevated $p\text{CO}_2$ conditions.

The research presented here is among the first to measure pCO$_2$ fluctuations at the microhabitat scale, examine how predicted future pCO$_2$ fluctuations affect the physiological performance of coral reef fishes, and examine the possible mechanisms underpinning changes in physiological performance under elevated and fluctuating pCO$_2$ conditions. My findings emphasize that exposure to OA relevant pCO$_2$ fluctuations can affect the performance of coral reef fishes differently than traditionally used stable elevated pCO$_2$ conditions. Overall, this thesis advances our understanding of how future ocean acidification conditions will affect coral reef fishes and displays evidence of a mechanism fish employ to cope with these conditions. These data can be used to identify how fishes will fair in the face of ocean acidification; however, future work is needed to define the ecological consequences of combined stressors on coral reef fish populations to gain a more complete picture of how fish communities will be affected in the future.

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Chapter 1:   General introduction

1.1   Ocean acidification

Since the Industrial Revolution, carbon dioxide (CO_2) concentrations in the atmosphere are rising at an unprecedented rate as a result of increased anthropogenic inputs (Doney and Schimel, 2007). Atmospheric CO_2 has increased by ~40%, from ~280 ppm during pre-industrial times to levels consistently >400 ppm since 2016 (Dlugokencky and Tans, 2015; Tian et al. 2016). If the current emissions trajectory is maintained, atmospheric CO_2 will exceed 900 ppm by 2100 (IPCC, 2019). Approximately one fourth of the anthropogenic CO_2 released into the atmosphere is absorbed by the oceans (Fabricius et al., 2020). As atmospheric CO_2 concentrations increase, the partial pressure of CO_2 (pCO_2) in the oceans increases as well (Doney, 2010). The increased uptake of CO_2 by the oceans is causing a shift in the relative concentrations of carbonate and bicarbonate ions and a reduction in seawater pH, a process referred to as ocean acidification (Doney, 2010). The average pH of the oceans has declined by 0.1 unit since the Industrial Revolution (Pörtner et al., 2014) and is predicted to decrease by as much as another 0.4 units by 2100 (Pörtner et al., 2014). Ocean acidification has traditionally been expected to adversely affect fish physiology, behaviour, and ecology. Indeed, a meta-analysis of the effects of ocean acidification relevant pCO_2 treatments found increased mortality and calcification, as well as negative impacts to the behaviour of fishes (Cattano et al., 2018).

1.2 pCO$_2$ fluctuations

Ocean acidification projections are typically based on open ocean environments that have relatively stable pCO$_2$ through time (Hofmann et al., 2011). The pCO$_2$ at the surface of the open ocean currently fluctuates seasonally by approximately 60-100 µatm (Gallego et al., 2018; McNeil and Sasse, 2016). However, this cycle is predicted to increase to between 200-400 µatm by the end of the century due to the reduced buffering capacity of acidified seawater (Gallego et al., 2018; McNeil and Sasse, 2016). This could result in the surface water of the open ocean reaching pCO$_2$ levels over 900 µatm, on a seasonal basis, far earlier than predicted by a simple extrapolation from atmospheric CO$_2$ concentrations (Gallego et al., 2018; McNeil and Sasse, 2016). Moreover, different marine habitats exhibit different pCO$_2$ ranges and temporal dynamics. Time series data show that pCO$_2$ fluctuations in coastal marine habitats often exceed those in the open ocean by an order of magnitude, and fluctuations can even exceed mean pCO$_2$ projections for the end of the century in some shallow water habitats (Hofmann et al., 2011; Shaw et al., 2013a).

Natural pCO$_2$ fluctuations across different spatial and temporal scales are common in the marine environment. Shallow water coastal habitats, such as coral reefs, are known to experience relatively large diel pCO$_2$ fluctuations when compared to the open ocean. The pCO$_2$ variations in shallow marine habitats, such as coral reefs, macroalgal beds, and seagrass meadows are primarily driven by the processes of photosynthesis and respiration across a day-night cycle. However, pCO$_2$ variations are also being modified by physical properties, such as water flow rates and residence time, which alter contact time between the seawater and the benthic communities (Cyronak et al., 2018; Falter et al., 2013). These cycles also vary seasonally, as they are greater during the warmer months due to decreased solubility of CO$_2$ and increased community production (Albright et al., 2013; Kline et al., 2015). Although

it is understood that benthic organisms play a major role in CO_2 dynamics at the ecosystem scale, relatively little is known about pCO_2 variation at the microhabitat scale that the organisms directly experience. It might be expected, for example, that a sandy microhabitat would experience different CO_2 dynamics compared with an adjacent coral dominated microhabitat, due to their different benthic communities. Yet, there is a lack of sufficient *in situ* measurements of pH and pCO_2 fluctuations across different microhabitats within coral reefs or other coastal habitats (Kapsenberg and Hofmann, 2016; Price et al., 2012). Despite knowing that spatial and temporal pCO_2 fluctuations exist in shallow water environments, the vast majority of experimental research into the effects of ocean acidification on coastal marine organisms has been performed using stable pCO_2 treatments. This problem is exacerbated by the fact that natural fluctuations in pCO_2 may be increased by up to 10-fold in the future due to a declining buffering capacity of seawater at higher CO_2 levels (McNeil and Sasse, 2016). Additional information regarding current-day pH and pCO_2 fluctuations is important to help inform how these conditions will change in the future.

1.3   Effects of CO_2 on marine organisms

To date more than 900 papers have been published on the impacts of ocean acidification on marine organisms (data complitaion - IAEA Ocean Acidification International Coordination Centre (OA-ICC) as described in Yang et al., 2016). A meta-analysis of 228 of these studies on marine organisms ranging from algae to fishes found net negative impacts of stable elevated pCO_2 levels on survival, calcification, growth, development, and abundance but no effect to photosynthesis or metabolism (Kroeker et al., 2013). The majority of studies and resulting negative effects have been observed for calcifying organisms. Research has focused on calcifying organisms due to the concern that ocean acidification will cause a reduction in

carbonate saturation states, which affects these organisms' ability to form skeletons (Fabry et al., 2008; Heuer and Grosell, 2014; Orr et al., 2005). While many ocean acidification experiments have detected negative impacts to marine organisms, the effects on coastal marine organisms are highly variable (Kroeker et al., 2013; Wittmann and Pörtner, 2013). These varied responses may be related to that fact that the majority of ocean acidification studies to date have not taken the naturally fluctuating pCO_2 conditions that the organisms experience into account. Instead, the majority of studies have focused on pCO_2 treatments based on relatively stable open ocean predictions (McElhany and Busch, 2013; Wahl et al., 2015).

It has been suggested that natural fluctuations in pCO_2 in shallow water habitats, such as coral reefs, could mitigate the impact of ocean acidification on marine organisms, by providing a temporary refuge from the stress of constant elevated pCO_2 (Bracken et al., 2018; Hendriks et al., 2014). Indeed, some studies have shown that including CO_2 variability can ameliorate the negative effects of stable ocean acidification treatments (Chan and Eggins, 2017; Comeau et al., 2014; Dufault et al., 2012; Enochs et al., 2018; Frieder et al., 2014; Jarrold and Munday, 2019; Ou et al., 2015). However, other studies have reported no effects (Clark and Gobler, 2016) or negative effects (Mangan et al., 2017) of fluctuating pCO_2. These results indicate that incorporating realistic natural CO_2 variation into experimental treatments will help to more accurately assess the responses of shallow water marine organisms to ocean acidification.

1.4 Effects of ocean acidification on fishes

Teleost fishes were initially expected to be tolerant to stable elevated pCO_2 conditions (Kroeker et al., 2010; Melzner et al., 2009a; Pörtner, 2008). Their skeletons consist primarily

of calcium phosphate, which is much less susceptible to changes in ocean carbon chemistry when compared to calcium carbonate that constitutes the primary building blocks of invertebrate shells (Toppe et al., 2007). Additionally, teleost fishes are known to possess an efficient acid-base regulatory system whereby, during an acidosis, fishes accumulate HCO_3^- ions in exchange for Cl^- ions and excrete H^+ ions to compensate for the acid-base disturbance (Brauner et al., 2019; Esbaugh, 2017; Heuer and Grosell, 2014). Most studies show that, upon exposure to stable elevated pCO_2 conditions, full pH compensation can be achieved within hours to days (Baker et al., 2009; Brauner et al., 2019; Esbaugh et al., 2012; Heuer and Grosell, 2014). However, while efficient, this acid-base compensatory mechanism is also predicted to be energetically costly (Ishimatsu et al., 2008; Melzner et al., 2009a). In fact, any type of regulation has the potential to have an energetic cost. The cost of osmoregulation in marine fishes has been estimated to be 6-15% of their resting oxygen uptake rates (Ishimatsu et al., 2008). Acid-base regulation, on top of this baseline cost of osmoregulation, has the potential to increase energy expenditure. Further, without this efficient acid-base regulation, decreased pH can have large effects on protein charge, which can impact molecular, cellular, organ, and whole organism performance (Brauner et al., 2019).

Some studies have revealed that fishes can experience a range of physiological disturbances upon exposure to stable elevated pCO_2 (Cattano et al., 2018; Heuer and Grosell, 2014; Lefevre, 2019). A meta-analysis by Cattano et al., (2018) found impacts of stable elevated CO_2 treatments on calcification, resting oxygen uptake, and yolk formation. Additionally, a meta-analysis by Heuer and Grosell (2014) found impacts of stable elevated CO_2 conditions on otolith growth, mitochondrial function, and metabolic rates. A meta-analysis by Hannan and Rummer (2018) also found increases in the resting oxygen uptake rates, but no effect to maximum oxygen uptake rates or aerobic scope upon exposure to stable elevated pCO_2. Negative impacts to oxygen uptake metrics were initially predicted to occur due to

acidification of the blood and respiratory organs (Pörtner et al., 2004). However, the physiological disturbances observed upon exposure to stable elevated $p\text{CO}_2$ may dramatically differ from those documented when fish are exposed to fluctuating elevated $p\text{CO}_2$ conditions (Jarrold and Munday, 2019; Jarrold et al., 2017; Laubenstein et al., 2020; Methling et al., 2013; Ou et al., 2015). Some fish species exhibit no effect or even benefit from exposure to fluctuating elevated $p\text{CO}_2$ conditions when compared to stable present-day conditions, and some studies have found that when fish are exposed to fluctuating elevated $p\text{CO}_2$, the negative effects of stable elevated $p\text{CO}_2$ are ameliorated. For example, Jarrold and Munday (2019) found increased survival of juvenile *Amphiprion melanopus* when parents and their offspring were exposed to diel-cycling elevated $p\text{CO}_2$ compared to when the parents and offspring were exposed to stable present-day $p\text{CO}_2$ conditions. Similarly, the wet mass of juvenile *Acanthochromis polyacanthus* increased upon exposure to fluctuating elevated $p\text{CO}_2$ conditions when compared to fish exposed to stable present-day $p\text{CO}_2$ (Jarrold and Munday 2019). Methling et al. (2013) found that ammonia (nitrogen) excretion rates post-feeding returned to pre-feeding levels earlier in freshwater *Anguilla anguilla* that had been exposed to fluctuating elevated $p\text{CO}_2$ conditions when compared to those exposed to stable present-day $p\text{CO}_2$ conditions. Finally, two separate studies found that exposure to stable elevated $p\text{CO}_2$ had negative effects on oxygen uptake metrics, but exposure to fluctuating elevated $p\text{CO}_2$ ameliorated this response (Laubenstein et al., 2020; Ou et al., 2015). Specifically, Ou et al. (2015) found that the negative impacts of stable elevated $p\text{CO}_2$ on maximum oxygen uptake rates in pink salmon were ameliorated when pink salmon were exposed to fluctuating elevated $p\text{CO}_2$, and Laubenstein et al. (2020) found that the negative impacts of stable elevated $p\text{CO}_2$ on minimum oxygen uptake rates and aerobic scope in *A. polyacanthus* were ameliorated upon exposure to fluctuating elevated $p\text{CO}_2$ conditions. Thus, biological and

physiological metrics have the potential to be differentially influenced by fluctuating elevated pCO_2 when compared to stable elevated or even present-day pCO_2.

1.5   Physiological metrics

1.5.1 Swimming

The majority of published studies on the effects of stable elevated pCO_2 on the critical swimming speed (U_{crit}) of fishes have shown no effect on swimming performance (reviewed by Lefevre, 2019). Moreover, when reductions in swimming speeds have been documented, they have generally been relatively modest in magnitude (i.e., 8-17%) (McMahon et al., 2020; Watson et al., 2018). Reduced swimming performance upon exposure to stable elevated pCO_2 might be related to aerobic metabolism, as U_{crit} represents the maximum sustained swimming speed that can be aerobically supported. Thus, a decrease in the capacity for oxygen uptake and/or delivery would result in a lower U_{crit}. However, the U_{crit} of the vast majority of marine fishes tested to date has been unaffected by exposure to stable elevated pCO_2 (90%) (Lefevre, 2019), suggesting that there are mechanisms in place to maintain aerobic performance during exposure to these conditions. A range of mechanisms that fishes may be using to maintain aerobic performance upon exposure to elevated CO_2 include sufficient capacity for acid-base regulation (Melzner et al., 2009a), increased pumping ability of the heart (Gräns et al., 2014), increased respiratory surface area (Rummer et al., 2013a), behavioural alterations leading to increases in swimming during U_{crit} tests (Couturier et al., 2013), and the unique oxygen transport system of teleost fishes (Hannan and Rummer, 2018). Although these mechanisms have been suggested, the majority have not been examined in fishes under ocean acidification relevant CO_2 levels.

1.5.2 Aerobic scope

One trait of aerobic metabolism that can be measured alongside swimming is the maximum

oxygen uptake rate, which can be used in conjunction with resting rates to calculate aerobic

scope. Aerobic scope (the difference between resting and maximal oxygen uptake rates) is

closely tied to metabolism and energetics, with a reduced aerobic scope resulting in less

energy available for oxygen requiring activities that support biological functions, such as

growth and reproduction (Pörtner and Peck, 2010). A reduction in aerobic scope, such as

what Laubenstein et al. (2020) observed in a coral reef fish exposed to stable elevated pCO_2,

can happen if additional energy is allocated to the maintenance of physiological homeostasis,

such as increased energy required for acid-base regulation in high pCO_2. Though reductions

in aerobic scope are predicted under ocean acidification conditions (Pörtner and Farrell,

2008), the majority of studies have found that aerobic scope is unaffected by stable elevated

pCO_2 exposure (reviewed by Hannan and Rummer 2018). Additionally, of the studies on

coral reef fishes, some studies have reported increases in aerobic scope upon exposure to

ocean acidification-relevant conditions (Couturier et al., 2013; Nadler et al., 2016; Rummer et

al., 2013a). Maintained or increased energy availability in fishes exposed to stable elevated

pCO_2 conditions suggests that these conditions are not energetically stressful for these species

and that additional mechanisms may be responsible for maintaining performance under these

conditions. The discrepancy in responses at the level of aerobic scope across fish species

exposed to ocean acidification-relevant conditions reveals knowledge gaps that, once filled,

can improve our understanding as to how coral reef fishes will respond to ocean acidification,

both now and into the future.

1.5.3 Haematological metrics

Haematological parameters have been used to evaluate the physiological responses of

organisms for more than 70 years (Burgos-Aceves et al., 2019). Various studies suggest that

the red blood cell and the heamoglobin play an important role in responding to an acidosis. For instance, adrenergic activation of transporters on the red blood cell can lead to the cells either swelling or shrinking, which would affect the ability of the red blood cell to transport oxygen (Borgese et al., 1987; Forster and Steen, 1969; Guizouarn et al., 1993; Nikinmaa et al., 1990). A measure of red blood cell swelling, mean corpuscular haemoglobin concentration, has traditionally been examined in response to an extreme acidosis (~4.8 pH) (Walker et al., 1988; Witters et al., 1991; Wood and Munger, 1994). These studies observed decreases in mean corpuscular haemoglobin concentrations, which suggested red blood cell swelling, that occurred in conjunction with an increase in haematocrit (Hct) and/or decrease in blood haemoglobin concentrations ([Hb]). The observed decreases in mean corpuscular haemoglobin concentrations (suggesting red blood cell swelling) is thought to protect red blood cell intracellular pH (pH_i) and therefore maintain oxygen uptake once the blood reaches the gills and then subsequent oxygen delivery to the tissues. However, more recent studies using stable ocean acidification relevant pCO_2 levels (i.e. ~1000 µatm) have documented increases in mean corpuscular haemoglobin concentrations, which suggests red blood cell shrinkage (Crespel et al., 2019; Heinrich et al., 2014; Noor et al., 2019). Although, red blood cell swelling is thought to be a result of catecholamine release to maintain oxygen transport, RBC shrinkage has also been suggested to be influenced by catecholamine release (van Dijk et al., 1993), in an effort to aid oxygen carrying capacity (Crespel et al., 2019). The differences in the swelling or shrinking of the red blood cells in teleost fishes may be due to the different levels of acidosis measured. Additionally, the extreme levels of acidosis are often implemented by using mineral acids (e.g., sulfuric acid (H_2SO_4) or hydrochloric acid (HCl)) rather than the CO_2 used in ocean acidification studies. It might be expected that extreme levels of acidosis are more stressful than the levels expected due to ocean acidification and would therefore result in the use of different haematological mechanisms.

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1.6 Mechanistic basis for maintained performance under elevated pCO_2

Improved understanding of the mechanisms underpinning the ability of fishes to maintain and/or increase energy availability and swimming performance can contribute to our understanding as to how fishes will fare at higher CO_2 levels in the future. Studies on model teleost fishes, such as the salmonids, have identified a potential mechanism responsible for enhanced tissue oxygen (O_2) delivery when fish are exposed to stable elevated pCO_2 (Alderman et al., 2016; Harter et al., 2019; Rummer and Brauner, 2011; Rummer et al., 2013b). Most teleost fish species possess extremely pH-sensitive haemoglobins (Hb), where reductions in pH not only reduce Hb-O_2 affinity (Bohr Effect), but also reduce Hb-O_2 carrying capacity (Root Effect). In the presence of stressful conditions, many fish species release catecholamines (e.g., adrenaline, noradrenaline) into circulation to activate sodium/proton (Na^+/H^+) exchange (β-NHE) on the surface of the red blood cell to remove H^+ and elevate intracellular pH (Rummer et al., 2013b). Elevated red blood cell pH increases Hb-O_2 affinity, and therefore O_2 uptake at the gill is safeguarded. To enhance O_2 delivery at select locations (e.g., red muscle, heart, etc.), plasma-accessible carbonic anhydrase (paCA) short-circuits this protective mechanism (Alderman et al., 2016). The result is a re-acidifying of the red blood cell, decreasing Hb-O_2 affinity, and increasing O_2 release to the tissues where paCA is present (Rummer et al., 2013b). In salmonids, this mechanism may also be operating under a mild acidosis, where catecholamines are not released, perhaps driven by a more general 'housekeeping' sodium/proton exchanger (Rummer et al., 2013b). Regardless, the link between enhanced O_2 delivery and enhanced performance (i.e., oxygen uptake metrics), although widely assumed, has not yet been conclusively made. Additionally, it is unclear whether this mechanism for enhanced O_2 delivery via Root Effect Hbs and paCA

exists in more derived teleosts (e.g., coral reef fishes) and whether it can be initiated under the elevated pCO_2 levels used in ocean acidification studies (~850-1,000 µatm), where maintained or even increased aerobic scope has been documented (reviewed in Hannan and Rummer, 2018; Heuer and Grosell, 2014).

Species with different life-history strategies may use different physiological mechanisms that confer benefits to performance under future ocean acidification conditions. For instance, nocturnal versus diurnal activity may influence the mechanisms that fishes utilize while exposed to elevated pCO_2. On coral reefs and other shallow water habitats, pCO_2 is elevated at night due to respiring benthic organisms releasing CO_2, whereas photosynthetic activity draws down CO_2 during the day. Thus, nocturnal fishes are active during times of relatively high pCO_2 when compared to fish species that are active during the day, and thus may have developed mechanisms to maintain performance when pCO_2 is elevated. Environment and life-history strategies can play an important role in the physiological tolerance to changing environmental conditions (Pörtner and Farrell, 2008). Consequently, it is important to consider not only the current environmental and microhabitat pCO_2 conditions to which species are exposed, but also their life-history strategies and physiological mechanisms when predicting how these species will be affected by climate change in the future.

While we have learnt a great deal about the effects of elevated CO_2 on fishes, we are not yet able to reliably predict the effects of ocean acidification on fish populations. For instance, a range of different responses have been reported in fishes with regard to O_2 uptake rates under future climate change scenarios (Heuer and Grosell, 2014; Lefevre, 2016; Lefevre, 2019). It is becoming increasingly evident that incorporating ecologically relevant pCO_2 fluctuations into experimental treatments is important to make robust predictions about the effects of future elevated pCO_2 levels. Especially as, at a local scale, the rate, magnitude, and

variability of future conditions depend on how chemical, biological, and physical processes mediate the uptake of anthropogenic CO_2. Yet, most past studies have used stable elevated pCO_2 treatments based on predictions for the open ocean, rather than shallow water habitats that often have spatially and temporally variable CO_2 dynamics. Moreover, the physiological responses of fish to higher environmental CO_2 may depend on whether experiments capture the natural CO_2 dynamics that the species are adapted to in their habitats. Further, these responses may depend on life-history strategies and physiological mechanisms utilized. This thesis examines natural fluctuations in pCO_2 and how the inclusion of ecologically relevant CO_2 variation affects the physiological responses of coral reef fishes with different life-history strategies.

1.7 Thesis aims

In **chapter 2**, I quantify the pCO_2 dynamics among different microhabitats on coral reefs at Lizard Island on the Great Barrier Reef, Australia. To do this, I placed SeaFET pH sensors at three different microhabitats (sandy, hard coral, or soft coral) at three different reefs over a 9-d period. Additionally, I collected water samples to measure total alkalinity and dissolved inorganic carbon at every part of the tide cycle (high, low, and both mid tides). I then used these data, as well as data sourced from the Bureau of Meteorology to calculate the pCO_2 ranges at different microhabitats at the different reefs. I hypothesized that different microhabitats with different benthic communities will have varying diel ranges of pCO_2. I also predicted that different reefs with different water flow regimes would have different diel ranges in pCO_2 influenced by the hydrodynamics. These data examining pCO_2 at various scales are relevant to organismal performance and will help in better framing how we predict pCO_2 changes into the future.

In **chapter 3**, I investigated how diel pCO_2 fluctuations affect the oxygen uptake rates of resting and swimming fishes. In order to test this, I exposed two species of coral reef damselfishes (*Acanthochromis polyacanthus* and *Amblyglyphidodon curacao*) to one of four different pCO_2 treatments (i.e., ambient, elevated, increasing, or decreasing) for 8 h while the fish were either resting or swimming at their optimum swimming speed. Following the 8 h exposure, the fish were exposed to a critical swimming speed (U_{crit}) test to determine their maximum sustained swimming speed and maximum oxygen uptake rates. I hypothesized that, because these coral reef fishes experience diel fluctuations in their natural environment, fluctuating pCO_2 will be less energetically costly than stable pCO_2 conditions. The results of this chapter are important in demonstrating how diel CO_2 cycles can modulate responses of coral reef fishes to elevated pCO_2.

In **Chapter 4**, I expanded upon chapter 3 by examining the effects of prolonged exposure to projected end-of-century ocean acidification conditions on the oxygen transport of coral fishes with different ecological strategies (i.e., nocturnal versus diurnal). Nocturnal fishes perform during times of elevated pCO_2 compared to diurnal fishes and thus may exhibit different physiological responses to future ocean acidification conditions. In order to examine this, I exposed four species of coral reef fishes (*Lutjanus fulviflamma, Caesio cuning, Abudefduf whitleyi,* and *Cheilodipterus quinquelineatus)* to one of three different pCO_2 treatments (i.e., ambient, fluctuating elevated, and stable elevated) for 9-11 d. Then, I exposed each fish to a U_{crit} trial to examine swimming and oxygen uptake metrics. I hypothesized that all four coral reef fish species would be more negatively impacted by stable elevated pCO_2 conditions than fluctuating elevated pCO_2 conditions, because the latter are closer to what they experience in their environment. I further predicted that nocturnal fishes would be less negatively impacted by elevated pCO_2 when compared to diurnal fishes because they are active at night when pCO_2 on the reefs is high. Data from this chapter can

help inform how longer exposures to fluctuating elevated pCO$_2$ and different life-history strategies can differentially affect coral reef fishes.

In **chapter 5**, I examined a possible mechanism that coral reef fishes use to maintain oxygen uptake rates and swimming performance during exposure to elevated pCO$_2$ conditions. This mechanism, which is related to teleost fishes' unique pH sensitive haemoglobin (i.e., Root effect) and plasma accessible carbonic anhydrase, has been examined in salmonids, but has yet to be tested in coral reef fishes, or under ecologically relevant pCO$_2$ conditions. In this chapter, I exposed *A. polyacanthus* to one of three different pCO$_2$ treatments (i.e., ambient, fluctuating elevated, and stable elevated) for 12 d. Following exposure, I injected each fish with either a carbonic anhydrase enzyme inhibitor or a sham (i.e., control) solution and then measured maximum oxygen rates and haematological metrics. I hypothesized that fish injected with the carbonic anhydrase inhibitor would have lower maximum oxygen uptake rates when compared to fish injected with the sham solution and that this will be exacerbated by exposure to elevated pCO$_2$. The results of this chapter help identify the physiological mechanism that enables many marine fishes to maintain their aerobic performance during exposure to elevated pCO$_2$. Identifying the mechanistic basis for physiological phenotypes is key to identifying which fish species can maintain performance in the future (i.e., winners and losers) and therefore, how ocean acidification will affect fish communities as a whole.

Together these four experimental chapters advance our knowledge regarding the impacts of projected future pCO$_2$ levels on the physiological performance of coral reef fishes, as well as the underlying mechanisms that are potentially responsible for maintaining performance under future ocean acidification conditions. Importantly, examining the linkages between pCO$_2$ dynamics on coral reefs and the mechanisms fish use to maintain their physiological performance under these conditions can help improve predictions about how reef fish

communities will be affected by future ocean acidification conditions. If the mechanisms fish employ to maintain aerobic performance during elevated CO_2 exposures are identified, we may be able to determine which fishes can take advantage of this, which will help inform which species will thrive in the future, and which will not. Further, understanding the physiological mechanisms that fish use to respond to elevated CO_2 may help identify their capacity to adapt to a rapidly changing environment.

Chapter 2: Diel pCO$_2$ variation among coral reefs and microhabitats at Lizard Island, Great Barrier Reef

2.1 Summary

Most laboratory experiments examining the effect of ocean acidification on marine organisms use stable pH/pCO$_2$ treatments based on average projections for the open ocean. However, pH/pCO$_2$ levels vary spatially and temporally in marine environments, and this variation can affect organism responses to pH/pCO$_2$. On coral reefs, diel pH/pCO$_2$ variability at the individual reef scale has been reported in a few studies, but variation among microhabitats within a reef remains poorly understood. This study determined the pH/pCO$_2$ variability of three different reefs, and three contrasting coral reef microhabitats (dominated by hard coral, soft coral, or open substrate) within each reef. Three SeaFET pH loggers were deployed simultaneously at the three microhabitats within a reef over a 9-d period. This was repeated at three different reefs around the Lizard Island lagoon. The loggers recorded pH$_T$ and temperature every 5 min. Water samples were collected from each microhabitat during four points of the tidal cycle (high, low, rising, and falling) and analysed for total alkalinity and dissolved inorganic carbon. The data show a clear diel pCO$_2$ cycle, increasing overnight and decreasing during the day, in association with photosynthesis and respiration cycles. Diel pCO$_2$ differed more between reefs than between microhabitats within reefs. Variation between reefs was most likely influenced by water flow, with the more protected (low flow) reefs experiencing a greater range in pCO$_2$ (Δ 250 µatm) than the exposed (high flow) reefs (Δ 116 µatm). These results add to a growing body of the literature on the diel variation of pCO$_2$ of shallow, nearshore environments and suggest that when projecting future pCO$_2$

levels, it is important to consider reef metabolism as well as physical and hydrodynamic factors.

2.2 Introduction

Anthropogenic activities are emitting steadily-increasing amounts of carbon dioxide (CO_2) into the atmosphere. As a result, the concentration of atmospheric CO_2 has increased by over 45% during the Industrial Age, from approximately 280 ppm before the Industrial Revolution to over 410 ppm in 2019 (Dlugokencky and Tans, 2020). The oceans have absorbed about 30% of the additional CO_2 released into the atmosphere by humans (Doney et al., 2009; Sabine et al., 2004). This additional CO_2 reduces seawater pH and changes the equilibrium of the carbonate system (Zeebe and Wolf-Gladrow, 2001), a process known as ocean acidification (OA) (Doney et al., 2009). If the current CO_2 emissions trajectories are maintained, atmospheric CO_2 concentrations could reach over 900 µatm by the year 2100 (McNeil and Matsumoto, 2019), and the pH of ocean surface water would decline by an average of ~0.32 units compared to pre-industrial values (IPCC, 2019). However, it is important to recognise that these are average projections for the open ocean. Coastal and shallow water environments, in particular, experience substantial natural fluctuations in pH and partial pressure of CO_2 (pCO_2) on a variety of temporal and spatial scales (Duarte et al., 2013; Hofmann et al., 2011; Kayanne et al., 1995; Ohde and van Woesik, 1999; Schmalz and Swanson, 1969; Yates and Halley, 2006); consequently, seawater in these environments may be over- or under-saturated with CO_2 when compared to the atmosphere at any point in time.

On coral reefs, pH and pCO_2 often fluctuate on a diel (day-night) cycle (Albright et al., 2013; Hofmann et al., 2011; Ohde and van Woesik, 1999; Shaw et al., 2012). These fluctuations are

mainly driven by the processes of photosynthesis/respiration and calcification/dissolution across the day-night cycle (Falter et al., 2013; Waldbusser and Salisbury, 2014). During the day, photosynthetic activity consumes CO_2, which increases the pH; however, at night, respiration consumes O_2, and produces CO_2 decreasing the pH (Wootton et al., 2008; Yates et al., 2007). The magnitude of natural diel fluctuations on coral reefs can exceed the mean pCO_2 levels projected to occur in the open ocean by the year 2100 (Shaw et al., 2012; Silverman et al., 2012). Meanwhile, the mean pCO_2 of some coral reefs has been increasing 3.5 times faster than in the open ocean (Cyronak et al., 2014a), and models show that the future pCO_2 cycle may be further amplified several-fold when the reducing buffering capacity of seawater at higher CO_2 levels is taken into account (Gallego et al., 2018; McNeil and Sasse, 2016). Thus, the average and magnitude of pCO_2 fluctuations may increase in the future, exposing marine organisms to a much larger range of pCO_2 than experienced in the current-day.

Fluctuations in pCO_2 are also influenced by a variety of physical and local hydrodynamic factors (Yan et al., 2016). Though it is well known that residence time heavily influences water chemistry, much less research has focused on the influence of these variables on the pH and pCO_2 of coral reefs (Falter et al., 2013). For most shallow coral reef systems, residence time is driven by wave action, tides, and in some cases, wind (Lowe et al., 2009; Taebi et al., 2011; Zhang et al., 2012). These variables can affect the amount of mixing coral reef waters have with offshore waters, which can cause large changes in the pH/pCO_2 of coral reefs. However, most studies focus on the diel influence of reef metabolism when predicting current day and future pH/pCO_2 regimes. Thus, it is important to examine the extent that hydrodynamic variables have on the pH/pCO_2 fluctuations on coral reefs to better ascertain how these reefs will be affected in the future.

The diel range of pCO_2 of coral reefs on the Great Barrier Reef has been reported to vary from 188 – 1697 μatm in an enclosed lagoon (Silverman et al., 2012) to 275 – 542 μatm on a mid-shelf reef (Albright et al., 2013). A recent study has found that depth can be a reliable predictor of diel ranges of pH on coral reefs, but that there was widespread environmental variability within those reefs (Cyronak et al., 2020). However, few studies have examined diel variation in pCO_2 among different microhabitats within a reef. Consequently, there is a lack of knowledge of the variability of the range of pCO_2 among microhabitats within a reef. This is important, because many coral reef organisms exhibit a high degree of microhabitat specificity (Komyakova et al., 2019) and therefore may be exposed to different pH/pCO_2 environments at the scale of the microhabitats they occupy. For example, the microhabitats that coral reef fishes use have previously been classified into four main categories: soft coral, sand/rubble, caves, and hard coral (Depczynski and Bellwood, 2004). If there are differences in the rates of respiration/photosynthesis, calcification/dissolution, or water residence time (flushing rates) in these microhabitats, we may expect to see differences in the daily pH/pCO_2 regime.

While it is known that natural fluctuations in pCO_2 occur in coral reef environments, the majority of experiments investigating the effects of future OA conditions on marine organisms have used stable pH/pCO_2 treatments, even when studying species from locations where these parameters can vary over hours, days or weeks (McElhany and Busch, 2013). Importantly, some recent OA studies that have incorporated fluctuating treatments have found that diel pCO_2 cycles can modify organismal responses compared to stable treatments (Cornwall et al., 2018; Dufault et al., 2012; Jarrold and Munday, 2018a; Jarrold and Munday, 2019; Jarrold et al., 2017; Jiang et al., 2019). Despite the marked increase in OA research over the past decade, coral reef *in situ* monitoring efforts have not increased at the same rate, despite increasing efforts to characterise the carbonate chemistry dynamics of nearshore

environments due to technological advancements (Miller et al., 2018). Therefore, although we know that fluctuating pH/pCO$_2$ can have different effects on marine organisms compared with stable exposures (Baumann, 2019), data are still insufficient to properly parameterize experimental treatments for many nearshore and shallow water environments. Documenting the temporal and spatial variability in pH/pCO$_2$ that organisms must presently cope with is the first step to understanding how organisms may respond to changes in the pH/pCO$_2$ projected to occur in the future.

The objective of this study was to determine (i) how pCO$_2$ (and therefore pH) varies among common coral reef microhabitats (hard coral, soft coral, or sand/rubble) on the same reef, and (ii) how this variation compares with variation in pCO$_2$ among adjacent reefs. The Lizard Island lagoon has been the focus of decades of research into the biology and ecology of coral reef organisms, but no studies have parameterized the microhabitat-scale temporal dynamics of pCO$_2$ on reefs, except for some pH spot measurements by Gagliano et al. (2010), but they did not define any other components of the carbonate system. In this study, we used SeaFET loggers combined with water sampling at four points of the tide cycle (high, low, rising, and falling) to examine the diel variation of pH and pCO$_2$ at three different reefs and three different microhabitats within each reef in the Lizard Island lagoon.

2.3 Methods

Lizard Island (14.40'08'' S, 145.27'34'' E) is a tropical island located in the mid-shelf of the Northern Great Barrier Reef (GBR), approximately 270 km north of Cairns and 30 km off the Australian mainland. The Lizard Island Group consists of four islands (Lizard, Palfrey and South Island, and Bird Islet) connected by a 10 m deep lagoon which is semi-enclosed by

well-established fringing and patch reefs (Figure 2-1). The lagoonal waters surrounding

Lizard Island are influenced by tides and wind/weather patterns, affecting water residence

times (Kiene and Hutchings, 1994). During the trade wind season (March – September)

winds average 15 – 20 knots from the south-east, resulting in a north-north westerly water

flow (Frith et al., 1986). The lagoon has a semi-diurnal tidal cycle with an average daily

range of 0.9 – 2.5 m, with a maximum of 3.2 m and a minimum of 0.1 m during our sampling

period (Table 2-1). Three of the larger reefs within the lagoon, Trawler Reef, Big Vicki's

Reef, and Palfrey Reef were the chosen study sites (Figure 2-1).
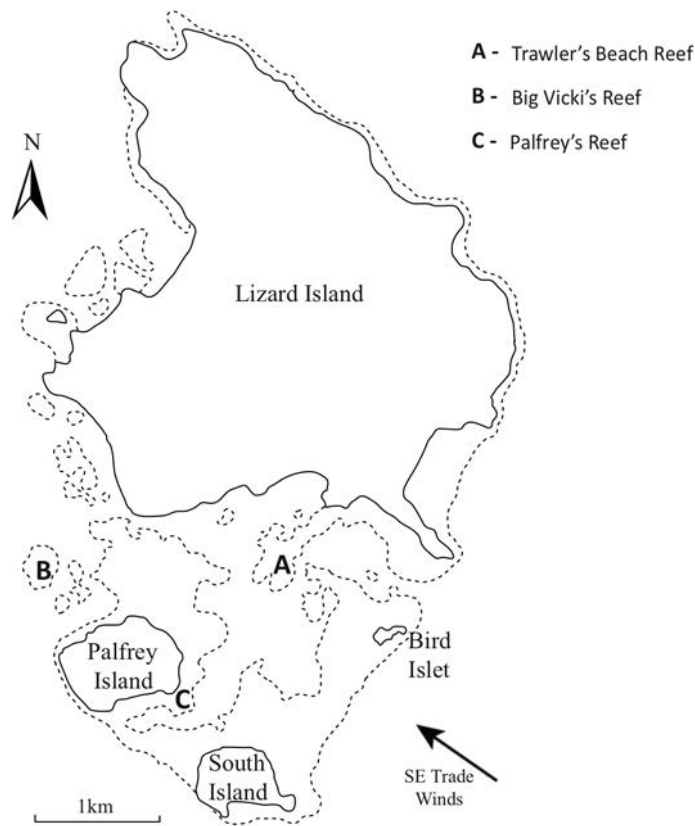
Figure 2-1. Map of the Lizard Island group, depicting the three reefs where the SeaFETs were deployed. The dashed line outlines the reefs surrounding the island group and enclosing the lagoon between the four islands.

Table 2-1. Physical parameters at each habitat within all three reefs. Data are represented as means ± SD, [in brackets: ranges].

Reef	Habitat	pH$_T$ (measured)	Total alkalinity (µmol kg^{-1}) (measured)	DIC (µmol kg^{-1}) (measured)	Salinity	Temperature (°C)	pCO$_2$ (µatm) (calculated)	fCO$_2$ (µatm) (calculated)	Tide (m)	Daylight (hrs) [sunrise, sunset]	Wind speed (km h^{-1})	Total cloud amount (oktas)
Trawler	Open/ sand	7.999 ± 0.044 [7.923, 8.136]	2289.3 ± 4.5 [2282.0, 2295.6]	2005.1 ± 24.3 [1970.5, 2043.8]	35.3 ± 0.0 [35.3, 35.3]	24.1 ± 0.3 [23.5, 25.1]	453.0 ± 53.2 [304.9, 554.5]	451.5 ± 53.1 [303.9, 552.7]	1.6 ± 0.3 [0.7, 2.6]	11.4 ± 0.0 [06:42, 18:03]	17.2 ± 6.6 [0.0, 29.5]	4.4 ± 2.8 [1.0, 8.0]
	Soft coral	8.011 ± 0.020 [7.971, 8.064]	2289.3 ± 4.9 [2282.2, 2296.1]	2003.0 ± 19.7 [1972.6, 2040.3]	35.3 ± 0.0 [35.3, 35.3]	24.1 ± 0.3 [23.5, 25.1]	434.9 ± 24.4 [374.2, 486.2]	433.5 ± 24.3 [372.9, 484.6]	1.6 ± 0.3 [0.7, 2.6]	11.4 ± 0.0 [06:42, 18:03]	17.2 ± 6.6 [0.0, 29.5]	4.4 ± 2.8 [1.0, 8.0]
	Hard coral	8.001 ± 0.035 [7.940, 8.131]	2283.4 ± 6.4 [2274.2, 2398.4]	1989.1 ± 8.0 [1971.1, 2001.5]	35.3 ± 0.0 [35.3, 35.3]	24.1 ± 0.3 [23.5, 25.1]	447.3 ± 41.5 [307.6, 527.4]	445.9 ± 41.4 [306.6, 525.7]	1.6 ± 0.3 [0.7, 2.6]	11.4 ± 0.0 [06:42, 18:03]	17.2 ± 6.6 [0.0, 29.5]	4.4 ± 2.8 [1.0, 8.0]
Big Vicki's	Open/ sand	8.044 ± 0.017 [8.012, 8.085]	2285.0 ± 7.9 [2266.4, 2294.5]	1991.8 ± 18.1 [1969.6, 2035.2]	35.3 ± 0.0 [35.3, 35.4]	24.0 ± 0.2 [23.5, 24.6]	396.4 ± 18.8 [351.3, 432.4]	395.1 ± 18.8 [350.2, 431.0]	1.6 ± 0.6 [0.1, 3.2]	11.4 ± 0.0 [06:38, 18:05]	22.2 ± 4.0 [13.0, 29.5]	6.9 ± 2.2 [1.0, 8.0]
	Soft coral	8.044 ± 0.023 [7.997, 8.092]	2284.0 ± 9.8 [2268.4, 2298.9]	1984.7 ± 14.7 [1967.5, 2005.9]	35.3 ± 0.0 [35.3, 35.4]	24.0 ± 0.2 [23.5, 24.6]	394.6 ± 25.4 [343.1, 449.5]	393.3 ± 25.3 [342.0, 448.1]	1.6 ± 0.6 [0.1, 3.2]	11.4 ± 0.0 [06:38, 18:05]	22.2 ± 4.0 [13.0, 29.5]	6.9 ± 2.2 [1.0, 8.0]
	Hard coral	8.033 ± 0.022 [7.992, 8.075]	2288.7 ± 5.8 [2275.4, 2295.9]	1995.8 ± 18.3 [1967.1, 2032.9]	35.3 ± 0.0 [35.3, 35.4]	24.0 ± 0.2 [23.6, 24.6]	409.0 ± 25.0 [362.4, 458.6]	407.6 ± 24.9 [361.2, 457.1]	1.6 ± 0.6 [0.1, 3.2]	11.4 ± 0.0 [06:38, 18:05]	22.2 ± 4.0 [13.0, 29.5]	6.9 ± 2.2 [1.0, 8.0]
Palfrey	Open/ sand	8.056 ± 0.028 [7.973, 8.126]	2285.3 ± 9.4 [2268.7, 2293.4]	1986.7 ± 22.3 [1964.3, 2031.2]	35.4 ± 0.0 [35.3, 35.4]	24.1 ± 0.3 [23.4, 24.8]	382.9 ± 30.8 [312.5, 481.9]	381.7 ± 30.7 [311.5, 480.3]	1.7 ± 0.4 [0.6, 2.8]	11.6 ± 0.0 [06:34, 18:07]	12.5 ± 7.8 [0.0, 25.9]	6.5 ± 2.8 [1.0, 8.0]
	Soft coral	8.048 ± 0.036 [7.954, 8.159]	2278.4 ± 7.4 [2268.1, 2292.2]	1981.9 ± 22.7 [1960.5, 2039.1]	35.4 ± 0.0 [35.3, 35.4]	24.1 ± 0.3 [23.5, 24.8]	391.7 ± 39.6 [283.6, 506.6]	390.5 ± 39.5 [282.7, 505.0]	1.7 ± 0.4 [0.6, 2.8]	11.6 ± 0.0 [06:34, 18:07]	12.5 ± 7.8 [0.0, 25.9]	6.5 ± 2.8 [1.0, 8.0]
	Hard coral	8.056 ± 0.027 [7.976, 8.109]	2281.7 ± 8.3 [2266.2, 2295.9]	1982.8 ± 23.9 [1965.9, 2035.2]	35.4 ± 0.0 [35.3, 35.4]	24.1 ± 0.3 [23.5, 24.8]	382.5 ± 29.4 [327.2, 477.5]	381.3 ± 29.3 [326.1, 475.9]	1.7 ± 0.4 [0.6, 2.8]	11.6 ± 0.0 [06:34, 18:07]	12.5 ± 7.8 [0.0, 25.9]	6.5 ± 2.8 [1.0, 8.0]

pH logger deployments, seawater collection and calibration

Three SeaFET Ocean pH sensors (Satlantic, Canada) were deployed to record pH on the total hydrogen scale (pH_T) and temperature (°C) every five min. SeaFETs are a modified version of the Honeywell DuraFET and consist of an ion sensitive field effect transistor (ISFET) and an integrated programmable data logger and power supply (Martz et al., 2010).

Data were collected from 27th July to 21st August 2014: Trawler Reef (27 July to 4 August), Big Vicki's Reef (4 August to 12 August), and Palfrey Reef (13 August to 21 August). On each reef, the SeaFET loggers were deployed simultaneously at ~5 m depth on the substratum, putting the sensor ~10 cm above the substrate, in three different microhabitats – hard coral, soft coral, or open substrate, within 5 m of each other. Each microhabitat consisted of, at minimum, a 2 m^2 patch of the same species. The hard coral microhabitat was comprised of thickets of branching coral (*Porites cylindrica*). The soft coral microhabitat was made up of live *Sarcophyton* sp., and the open sandy bottom microhabitat was an area of sandy substrate approximately 1 m adjacent to the reef. The SeaFETs were rotated between the microhabitat types between the different sites, to exclude confounding between microhabitat types and the SeaFET instruments. Water samples were collected to provide calibrations for each SeaFET unit following Hofmann et al., (2011). Two to three water samples for each SeaFET were collected at four time points during the tide cycle at each site: high tide, low tide, rising, and falling tide. Water samples were collected within 5 cm of the pH_T and temperature sensors. The bottles were placed on ice while being transported to shore. Samples were preserved (0.05% saturated solution of $HgCl_2$; Dickson et al., 2007) within 30 min of collection and stored in the dark before being transported to the Australian Institute for Marine Science (AIMS) in Townsville for analyses.

Water samples were analysed at AIMS for total alkalinity (TA) and dissolved inorganic carbon (DIC) using a VINDTA 3C (Versatile INstrument for the Determination of Total inorganic carbon and titration Alkalinity; Mariana, Kiel, Germany). The measurements were corrected for salinity and verified against certified seawater reference material (CRM 126 with 33.46 salinity, A. Dickson, Scripps Institute of Oceanography, Dickson et al., 2007). Tide height and sunrise/sunset data were sourced from the Australian Bureau of Meteorology (BoM). Wind speed and cloud cover data at 3 h intervals was sourced from BoM at the Cape Flannery Station, 30219. Salinity data was sourced from the Seabird buoy (SBE37-IM MicroCat CTD with Pressure) owned by the Integrated Marine Observing System (IMOS) (accessed via the Australian Ocean Data Network: https://portal.aodn.org.au January 2015).

All three SeaFETs were deployed at the same location prior to the start of the experiment (July 25th) for temperature calibration (Appendix A - Table S1). SeaFET pH_T values were calibrated against the calculated pH_T from the water samples at each microhabitat and site to account for sensor drift (Appendix A - Table S1). The pCO_2 was calculated from the corrected SeaFET temperature (during the July 25th control deployment, Appendix A - Table S1), IMOS sourced salinity, measured TA averaged across each microhabitat at each reef (VINDTA 3C), pressure (dbars), and pH_T using the program CO2SYS (Lewis and Wallace, 1998), and the constants K1, K2 from Mehrbach et al. (1973) refit by Dickson and Millero (1987), and Dickson (1990) for KHSO4.

Data analyses

Drivers of pH and pCO_2 were explored via gradient boosted regression tree (BRT) models for all reefs together and individually (Ridgeway, 2007). Boosted regression tree models construct many small regression trees, training individual trees on a random subset of the residuals from the previous tree, and shrinking the contribution of each one (De'ath, 2007;

Elith et al., 2008). Overfitting is counteracted through cross-validation to balance predictive performance and model fit (Hastie et al., 2009). Boosted regression trees are robust to multicollinearity and non-linearity; therefore, BRTs are well suited to exploring relative impacts of a large number of complex predictors, such as microhabitat, hour, reef, wind speed, cloud amount, temperature, and tide.

A total of 20,000 trees were fit to an interaction depth of 5, bag fraction of 0.5, and shrinkage rate of 0.001. The optimal number of boosting iterations was determined by the out-of-bag method (Breiman et al., 1984). The relative importance of each predictor was calculated as the amount of splits involving each variable, weighted by the associated squared improvement in the model over all trees, which was averaged and scaled out of 100 (i.e., larger values denote stronger influence). Uncertainty in partial effects and relative importance estimates were incorporated by bootstrapping (sampling with replacement) each BRT 10 times. For each bootstrap BRT, parameter values associated with maximizing the responses were estimated by optimizing the predicted response across all predictors. Within each bootstrap BRT, for both the full model as well as each of the individual predictors, quasi-R^2 values were calculated using correlation according to the following:

$$\text{quasi } R^2 = \text{cor}(O_i, F_i)$$

where O_i and F_i are the ith observed and fitted response values, respectively. In the case of individual predictor R^2 calculations, F_i values are the partial fitted values (values predicted when all other predictors are held constant at their respective means). All BRT models were fit via the gbm package (Ridgeway, 2007) within the R statistical and graphical environment (R Core Team, 2017). The quasi R^2 of all BRT models was $\geq 92\%$.

Boosted regression tree models that included pH when examining the best predictors of pCO_2, found pH to have at least a 99.8% relative influence on pCO_2. Due to the role of pH in calculating pCO_2, pH was removed from the BRTs and run separately to pCO_2.

The slope of TA-DIC regressions can be used to show the balance between organic carbon cycle (net community production, NCP, i.e., CO_2 fluxes from photosynthesis and respiration) to the inorganic carbon cycle (net community calcification, NCC, i.e., carbonate precipitation and dissolution) (Cyronak et al., 2018). If the slope of the TA-DIC regression at constant temperature and salinity is ~1, then NCP and NCC are at equilibrium; however, if the slope approaches 0, the system is dominated by NCP, and if the slope approaches 2 it is dominated by NCC. For TA-DIC regression analysis, the TA and DIC data measured in duplicate/triplicate were averaged for each time point at each habitat. Type II linear regression analysis was performed on TA and DIC data using package lmodel2 using the ordinary least squares (OLS) method.

2.4   Results

Environmental parameters

During the deployments, seawater temperature averaged 24.0 ± 0.3 °C (mean ± S.D.) across deployments and reefs, and day length averaged 11.45 hrs (Appendix A - Figure S1, Table 2-1). Tidal ranges were Δ1.90 m during the deployment at Trawler Reef, Δ3.10 m at Big Vicki's Reef, and Δ2.20 m at Palfrey (Figure 2-2, Table 2-1). Salinity was 35.4 ± 0.0 PSU at Palfrey Reef, and 35.3 ± 0.0 PSU at Big Vicki's and Trawler Reef. TA was similar across all reefs, (Trawler: 2284.3 ± 5.3 µmol kg^{-1}, Big Vicki's: 2285.9 ± 7.8 µmol kg^{-1}, and Palfrey: 2281.8 ± 8.4 µmol kg^{-1}). Wind speed was highest during Big Vicki's deployment, (22.15 ± 4.02 km h^{-1}), followed by the Trawler deployment (17.16 ± 6.63 km h^{-1}), and lowest during

Palfrey deployment (12.48 ± 7.82 km h^{-1}). Cloud cover was similar across Big Vicki's and Palfrey deployments (6.85 ± 2.24 oktas and 6.49 ± 2.76 oktas, respectively) and lower during Trawlers deployment (4.41 ± 2.82 oktas) (Table 2-1).

pH and pCO$_2$

The pH$_T$ varied between $7.92 - 8.16$ over the experimental period (Figure 2-2). The highest pH value was recorded at Palfrey Reef and the lowest at Trawler Reef (Figure 2-2, Table 2-1). The lagoonal Trawler Reef experienced the largest range of pH (0.213), followed by the fringing Palfrey Reef (0.205), and the outer patch reef Big Vicki's Reef (0.100).
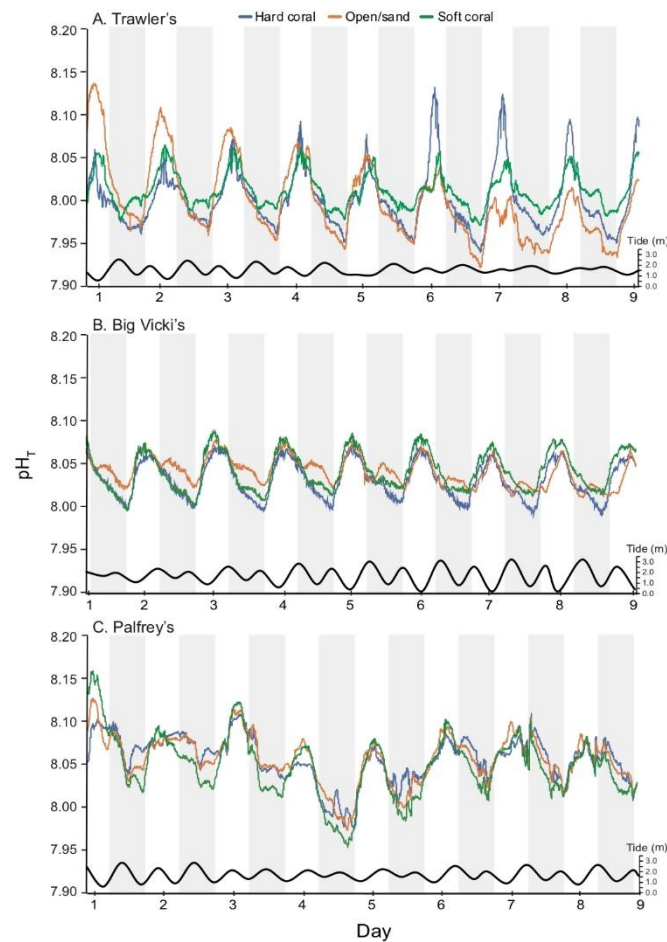
Figure 2-2. The variation of pH$_T$ at three reef sites around the Lizard Island lagoon over the 9-day trial. A) Trawler Reef, B) Big Vicki's Reef, and C) Palfrey Reef. The different microhabitats are represented by different colours (hard coral - blue; soft coral - green; open substrate - orange). White and grey bars represent day and night, respectively. Tide height is represented by the black line at the bottom of each graph.

The pCO$_2$, calculated from pH, salinity, temperature, and TA, ranged from 283.6 – 554.5

µatm over the entire experimental period (Figure 2-3, Table 2-1). The highest pCO$_2$ was seen

at Trawler Reef and the lowest at Palfrey Reef. The range of pCO$_2$ follows the same order as

the range of pH, with the largest range of pCO$_2$ levels across microhabitats at Trawler Reef

(Δ250 µatm), followed by Palfrey Reef (Δ223 µatm), and then Big Vicki's Reef with the

lowest range of pCO$_2$ values (Δ116 µatm).
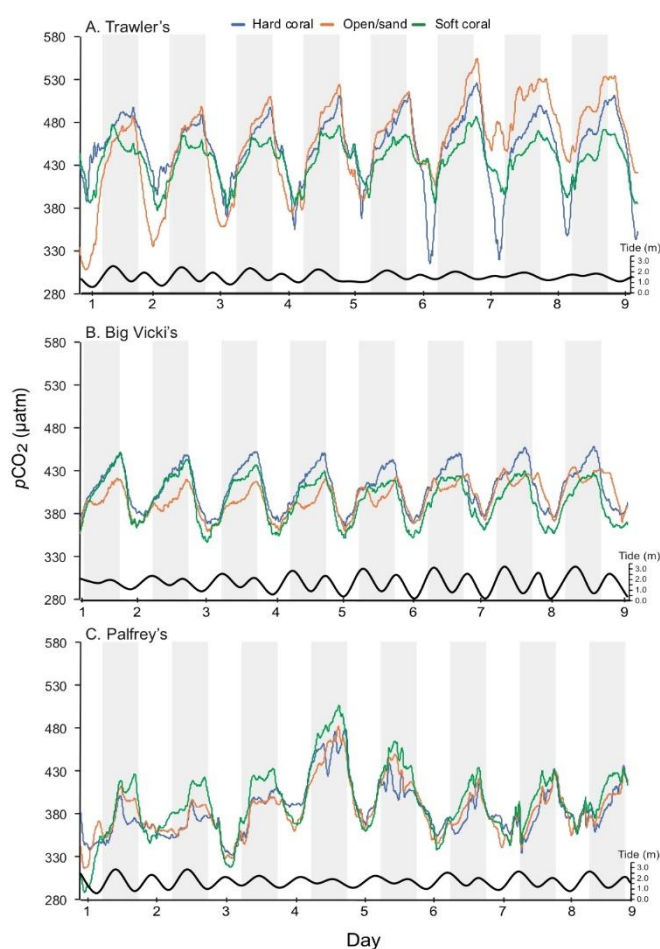
Figure 2-3. The variation of calculated pCO$_2$ over the 9-day trial at three reef sites around the Lizard Island lagoon at A) Trawler Reef, B) Big Vicki's Reef, and C) Palfrey Reef. Colours and symbols as in Figure 2-2.

Predictors of variation in pCO₂

When all reefs were analysed together, the BRT models found the best predictors for pH and pCO_2 to be time of day (averaged across hr) and reef (Figure 2-4). These variables were the only ones with median values of importance above the threshold of expected values given the number of predictors (Figure 2-4). These two variables together accounted for ~63% of variation in pCO_2 (Table 2-2). The pH was lowest and pCO_2 highest between the night and morning times of 02:30 and 06:20, and the reverse was true after noon (between 12:30 and 14:20, Figure 2-4). When all other factors were controlled for, the pH was lowest and pCO_2 highest at the lagoonal Trawler Reef, and the pH was highest and pCO_2 lowest at Palfrey Reef (Figure 2-4). All other variables, including microhabitat type, were of limited importance to pH and pCO_2 (R^2 values <0.03 and had low variability) (Figure 2-4, Table 2-2).
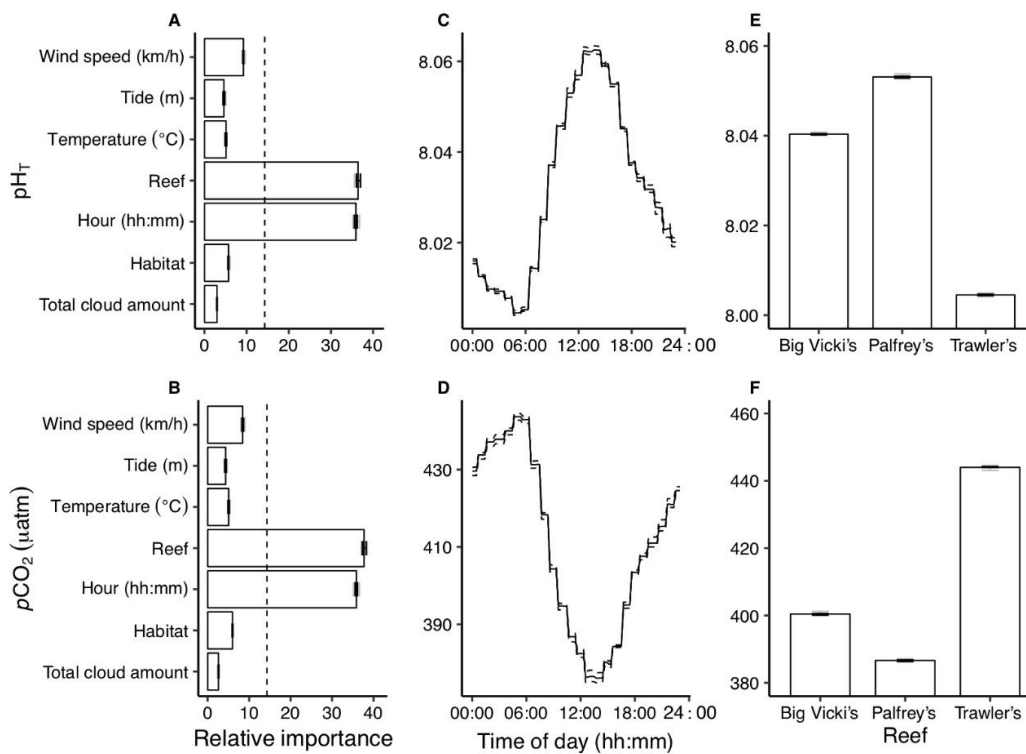
Figure 2-4. Relative importance of the seven predictors for A) pH, and B) pCO_2 (bootstrapped regression trees, all reefs analysed together). The bars in the relative importance plots represent

medians, and black and grey error bars represent 50 and 95% quantiles, respectively. The vertical dashed line represents the threshold (100/7), above which predictors are represented in trees more than would be expected by chance. The partial effects plots the significant predictor variables, time of day and reef are displayed for pH and pCO_2; C) time of day vs pH, D) time of day vs pCO_2,E) reef vs pH, and F) reef vs pCO_2. In plots C) and D), the solid and dashed lines represent median and lower/upper 95% quantiles, respectively. The bars in E) and F) represent medians, and black and grey bars represent 50 and 95% quantiles, respectively.

Table 2-2. Mean Quasi R^2 and their lower and upper 95% confidence intervals over all predictors for both pH and pCO_2. Predictors that performed best (i.e., that were disproportionately represented in trees) are highlighted in **bold**.

	Predictors	Reefs combined			Trawler Reef			Big Vicki's Reef			Palfrey Reef		
		R^2	Lower	Upper	R^2	Lower	Upper	R^2	Lower	Upper	R^2	Lower	Upper
pH_T	Habitat	0.002	0.002	0.002	0.021	0.017	0.024	0.005	0.003	0.011	0.001	0.001	0.002
	Hour (hh:mm)	**0.296**	**0.283**	**0.304**	**0.624**	**0.619**	**0.627**	**0.712**	**0.700**	**0.719**	**0.362**	**0.352**	**0.372**
	Reef	**0.331**	**0.328**	**0.333**	-	-	-	-	-	-	-	-	-
	Temperature (°C)	0.003	0.000	0.014	0.028	0.002	0.065	0.265	0.247	0.285	0.006	0.004	0.008
	Tide (m)	0.019	0.013	0.026	0.117	0.051	0.160	0.014	0.000	0.068	0.002	0.000	0.006
	Total cloud amount	0.012	0.006	0.020	0.010	0.006	0.013	0.027	0.006	0.057	0.001	0.000	0.003
	Wind speed (km/h)	0.005	0.000	0.011	0.036	0.001	0.098	0.104	0.030	0.309	0.381	0.373	0.388
pCO_2	Habitat	0.003	0.003	0.003	0.027	0.023	0.030	0.007	0.003	0.011	0.003	0.002	0.003
	Hour (hh:mm)	**0.286**	**0.276**	**0.303**	**0.632**	**0.627**	**0.636**	**0.695**	**0.682**	**0.706**	**0.358**	**0.350**	**0.365**
	Reef	**0.350**	**0.344**	**0.354**	-	-	-	-	-	-	-	-	-
	Temperature (°C)	0.003	0.000	0.009	0.018	0.000	0.049	0.263	0.249	0.271	0.006	0.004	0.007
	Tide (m)	0.020	0.013	0.030	0.101	0.068	0.121	0.012	0.002	0.030	0.001	0.000	0.002
	Total cloud amount	0.010	0.006	0.017	0.010	0.006	0.013	0.026	0.005	0.040	0.000	0.000	0.001
	Wind speed (km/h)	0.008	0.001	0.014	0.030	0.002	0.071	0.125	0.078	0.191	0.377	0.367	0.385

When Trawler Reef was examined alone, the BRT models found that the best predictor of pH and pCO_2 was time of day (hr) (Figure 2-5A, B), accounting for ~63% of the variation in the pH and pCO_2 (Table 2-2). The pH was lowest and pCO_2 highest between the hours of 02:30 –

05:20 and the reverse at 12:30 – 14:30 (Figure 2-6A, B). All other variables were of limited importance to the pH and pCO$_2$ levels (Figure 2-5A, B).
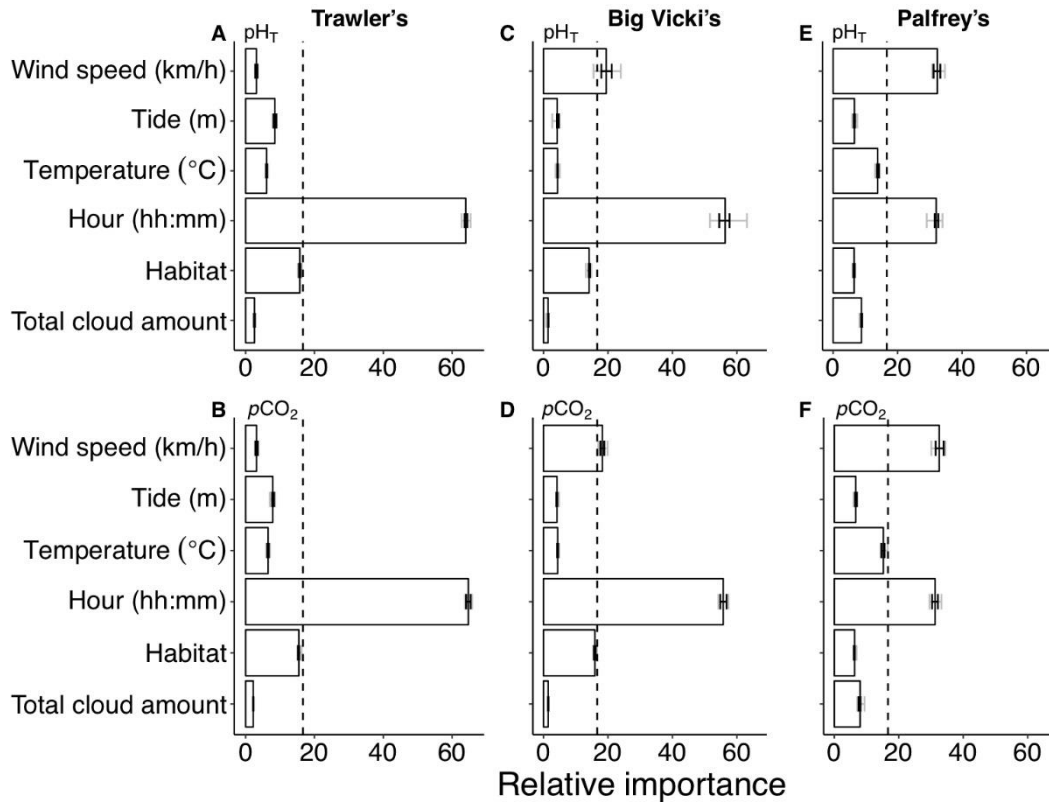
Figure 2-5. Relative importance of the six predictor variables when all reefs were analysed separately. Trawler Reef: A) pH, B) pCO$_2$, Big Vicki's Reef: C) pH, D) pCO$_2$, and Palfrey Reef: E) pH, F) pCO$_2$. The bars in the relative importance plots represent medians, and black and grey bars represent 50 and 95% quantiles, respectively. The vertical dashed line represents the threshold (100/6), above which predictors are represented in trees more than would be expected by chance.

At Big Vicki's Reef, the best predictors of pH and pCO$_2$ were time of day and wind speed (Figure 2-5C, D) together accounting for ~82% of the variation of pH and pCO$_2$. The pH was lowest and pCO$_2$ highest between the hours of 04:40 – 06:20 and the reverse occurred between the hours of 11:40 – 15:20 (Figure 2-6C, D). There was a positive relationship between wind speed and pH where increasing wind speed corresponded with increasing pH (Appendix A - Figure S2) and a negative relationship with pCO$_2$ (Appendix A - Figure S2).
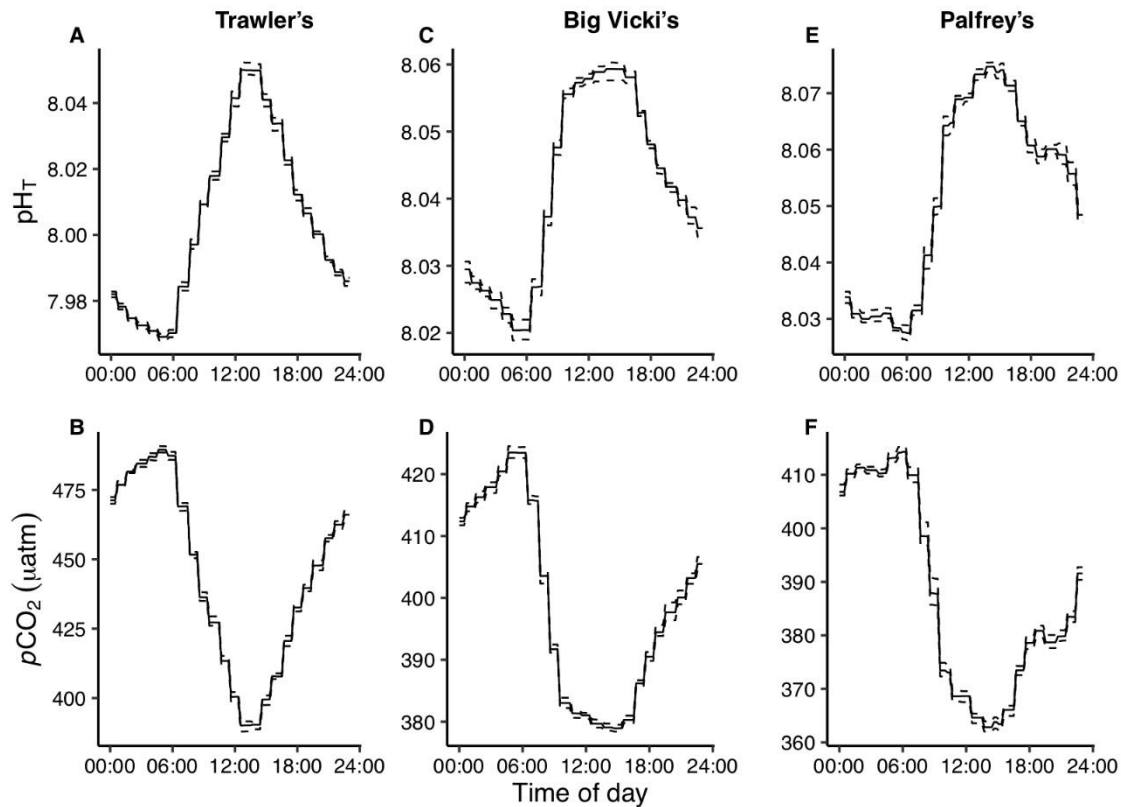
Figure 2-6. Bootstrap regression tree partial effects plots, with reefs analysed separately, of the changes in pH and pCO$_2$ with the significant predictor variable, time of day. Trawler Reef: A) and B), Big Vicki's Reef: C) and D), Palfrey Reef: E) and F). Solid and dashed lines represent median and lower/upper 95% quantiles, respectively.

At Palfrey Reef, the best predictors of pH and pCO$_2$ were time of day and wind speed (Figure 2-5E, F). The pH was lowest and pCO$_2$ highest between 05:40 – 06:20 and the reverse between 13:40 and 14:30 (Figure 2-6E, F). There was a positive relationship between wind speed and pH where increasing wind speed corresponded with increasing pH (Appendix A - Figure S2) and a negative relationship with pCO$_2$ (Appendix A - Figure S2). The two best predictors combined accounted for ~74% of the variation in pH and pCO$_2$ (Table 2-2). All other variables were of limited importance to the pCO$_2$ levels.

TA-DIC regression

The slope and intercept of the TA-DIC regression using the ordinary least squares (OLS) method were 0.17 and 1949.1, respectively (Appendix A - Figure S3).

2.5   Discussion

There is a growing appreciation that coral reefs and other shallow costal environments can experience substantial natural variability in pH and pCO$_2$ (Hofmann et al., 2011; Shaw et al., 2013a; Wahl et al., 2015). Here, we show that pH and pCO$_2$ vary on a regular diel cycle in the Lizard Island lagoon, one of the best researched coral reef locations on the planet. Yet, there is also temporal and spatial variation in the dynamics of the diel cycle. Time of day (hr) was the most influential variable on both pCO$_2$ and pH regardless of reef examined. The highest levels of pCO$_2$ were observed at night over four hours between 02:00 and 06:00, and the lowest levels were observed during the day over a two-hour period between 12:00 and 14:00. This diel trend is consistent with other work on the Great Barrier Reef (Cyronak et al., 2014b; Shaw et al., 2012), and attributable to the drawdown of CO$_2$ during the day from photosynthesis and CO$_2$ release at night from respiration (Wootton et al., 2008; Yates et al., 2007). However, though this diel trend was observed on all reefs, the reefs themselves also influenced pCO$_2$ values. This indicates that other reef-specific factors affect the pCO$_2$ regime on individual reefs. This is consistent with previous studies, which have also found that different reefs have different diel trends in both magnitude and timing of pCO$_2$ fluctuations (Drupp et al., 2013; Frankignoulle et al., 1996; Hofmann et al., 2011; Lantz et al., 2014).

There are many possible reasons why different reefs experience different pCO$_2$ regimes, such as contrasting physical and spatial environmental conditions (e.g., speed and directions of currents, tide cycles, wind events, residence time, light, temperature, depth, geomorphology)

and reef metabolism (respiration/photosynthesis and calcification/dissolution) (Albright et al., 2013; Anthony et al., 2011; Cyronak et al., 2014a; Cyronak et al., 2018; Davis et al., 2019; Falter et al., 2013; Long et al., 2013). Of the three reefs, Trawler Reef is the most lagoonal reef (Figure 2-1), and experienced the lowest tidal fluctuations during the measurements (Table 2-1), suggesting prolonged water residence times. Trawler Reef also experienced the greatest range of pCO_2 (Figure 2-3, Table 2-1). This lends support to the idea that longer residence times lead to higher pCO_2 values and higher offset values compared to the open ocean (Andersson et al., 2013; Suzuki and Kawahata, 2004). Further support for this can be observed on Big Vicki's Reef, which is the most exposed reef (Figure 2-1), and had the highest tidal range, the highest wind speeds (Table 2-1), and hence probably the lowest residence time. Big Vicki's Reef also had the narrowest range of pCO_2 (Figure 2-3, Table 2-1).

When Palfrey Reef was analysed separately from the other two reefs, wind speed had a higher relative influence than would be expected by chance (Figure 2-5). The partial effects plot shows that as wind speed increased, the pCO_2 decreased, which could reflect increases in air-sea flux, thus bringing seawater closer to equilibrium with atmospheric pCO_2, or increased wind-driven currents reducing water residence time (Shaw et al., 2013a). This trend was also reflected in the pCO_2 relationship with wind speed at Big Vicki's Reef. Further, when wind speed was included in the model, time of day and wind speed combined account for as much variation if not more than time of day alone at both Trawler Reef and Big Vicki's Reef. Wind speed can contribute to water residence time, as high wind speeds can move water quickly reducing water residence time, increasing water exchange, causing the pCO_2 to be more similar to surrounding waters; whereas, low wind speeds can increase residence time, which can increase the pCO_2.

Interestingly, microhabitat type had very little influence on the pCO_2 levels observed at each reef. This is likely due to hydrodynamic mixing at the reef microhabitat scale, as the sensors were placed about 10 cm above the bottom of the substrate with the canopy above open to the water column. The microhabitats chosen where within 5 m of each other, and seawater exposed to one microhabitat was likely well mixed with water from nearby microhabitats due to wind- and tidal-driven currents. The currents at Lizard Island are known to be directly influenced by the wind speed, and during the winter, when this study was conducted, the south-easterly trade winds are known to be stronger and more consistent than they are during the summer months (Frith et al., 1986; Johansen, 2014). Therefore, even if different microhabitats do have different pCO_2 dynamics due to different ratios and magnitudes of photosynthesis and respiration, water from these different microhabitats will be well mixed on these spatial scales. These data suggest that it is unlikely that species utilizing these three different microhabitats on the reef would experience significantly different pCO_2 regimes. However, sedentary species living within the reef matrix or within the sediments, where pCO_2 from respiration will usually exceed that from photosynthesis (Anthony et al., 2011; Eyre et al., 2014; Ravaglioli et al., 2019), may experience different pCO_2 regimes compared to more mobile organisms, such as fishes, that utilize a wider range of reef microhabitats. This emphasises the need for future studies that further investigate biogeochemically distinct habitats that are hydrologically separate.

This study is consistent with the hypothesis that coral reefs are a source of CO_2 to the atmosphere. The pCO_2 averaged at each reef was 400.0 ± 24.1 at Big Vicki's, 385.7 ± 33.8 at Palfrey, and 445.0 ± 42.1 at Trawler. Global average atmospheric CO_2 was 395.9 and 394.8 ppm in July and August of 2014, respectively (Dlugokencky and Tans, 2020). This suggests that both Big Vicki's and Trawler Reefs are sources of CO_2 to the atmosphere rather than sinks. Further, if averaged at the island scale, the pCO_2 was 410.5 ± 42.6, which adds further

support that coral reefs, on the whole, are sources of CO_2 to the atmosphere (Cyronak et al., 2014a; Gattuso et al., 1999; Longhini et al., 2015; Suzuki and Kawahata, 2004; Ware et al., 1991). This being said, it is important to keep in mind that three months prior to these measurements, Lizard Island was subjected to Cyclone Ita. This cyclone resulted in a significant decrease in hard coral cover coverage from ~27% in 2011 to ~12% 10 months post-cyclone (Ceccarelli et al., 2016). This damage to the benthic community may mean that the pCO_2 values in this study may be underestimating the contribution of these reefs to atmospheric CO_2 due to disruption of carbonate chemistry and the benthic community. Overall, there is evidence that even post-disturbance in 2014, reefs surrounding Lizard Island were sources of CO_2 to the atmosphere. However, this may have changed in recent times due to repeated disturbances (cyclones and bleaching events) that have reduced the hard coral cover coverage at Lizard Island to 2.6%, which has resulted in a net respiratory system (McMahon et al., 2019).

Both TA and DIC are often used to examine the net organic carbon metabolism of a reef. The slope of the TA-DIC regression in this study was 0.17 (Appendix A - Figure S3). Thus, Lizard Island reefs during this study were heavily dominated by NCP, indicating that the carbon cycle is dominated by photosynthesis and respiration. Although, it is important to note that all of the TA/DIC samples were collected during daylight hours, and dissolution mainly occurs at night. The low slope in this study coincides with data from a meta-analysis of the TA-DIC slopes of 23 coral reef locations that found Indo-Pacific reefs (including the GBR) had relatively lower slopes when compared to Atlantic reefs (Cyronak et al., 2018). Cyronak et al. (2018) also found that studies that included larger spatial scales (>10 km^2) had higher TA-DIC slopes when compared to studies characterizing seawater carbonate chemistry at only one site (Cyronak et al., 2018). Our study was conducted in the Indo-Pacific region at a small spatial scale, and measurements were only taken during the day, which would

collectively contribute to a low TA-DIC slope. However, this low value is in agreement with previous studies on the GBR, which have found slopes ranging from 0.28 – 0.59 and thus an overarching NCP dominance (Albright et al., 2013; Gattuso et al., 1997; Kline et al., 2015; McMahon et al., 2013; Shaw et al., 2012; Shaw et al., 2015; Silverman et al., 2012; Silverman et al., 2014).

The measurements of pCO$_2$ in this study fall in the low to average range of pCO$_2$ observed during winter at a depth greater than 1m on coral reefs in a variety of locations (Figure 2-7, Table 2-3). We observed a maximum total range over the sampling period of 116 – 250 μatm at 5 m on three reefs in the Lizard Island lagoon. Recent studies have shown a wide range of variability in seawater pCO$_2$ at different reef sites, from a range of 130 μatm (Bates et al., 2001) at 6 m in Bermuda during the winter to a range of 1509 μatm on the Great Barrier Reef in the summer (Silverman et al., 2012) (Figure 2-7, Table 2-3). This study was performed during the Austral winter, and the range of water temperatures on Lizard Island in 2014 was approximately 23.5 – 28.5 °C. Thus, during the summer, we would expect to see a larger range in pCO$_2$ driven by increased reef metabolism. As expected, for all studies combined, the lowest reef pCO$_2$ ranges were observed during the winter (306 ± 229 μatm, mean ± S.D.) and the highest during the summer (748 ± 365 μatm) (Figure 2-7, Table 2-3). The diel variation is expected to be smaller in winter because of the reduced rates of metabolism and calcification at lower water temperatures and reduced daylight hours. These studies show that the majority of reefs studied (~52%) have a pCO$_2$ range that is less than 500 μatm (Figure 2-7). In the future, not only will the average pCO$_2$ increase, but the natural pCO$_2$ cycles may also be amplified due to the change in seawater buffering capacity as more CO$_2$ is absorbed by the oceans (Gallego et al., 2018; McNeil and Sasse, 2016).
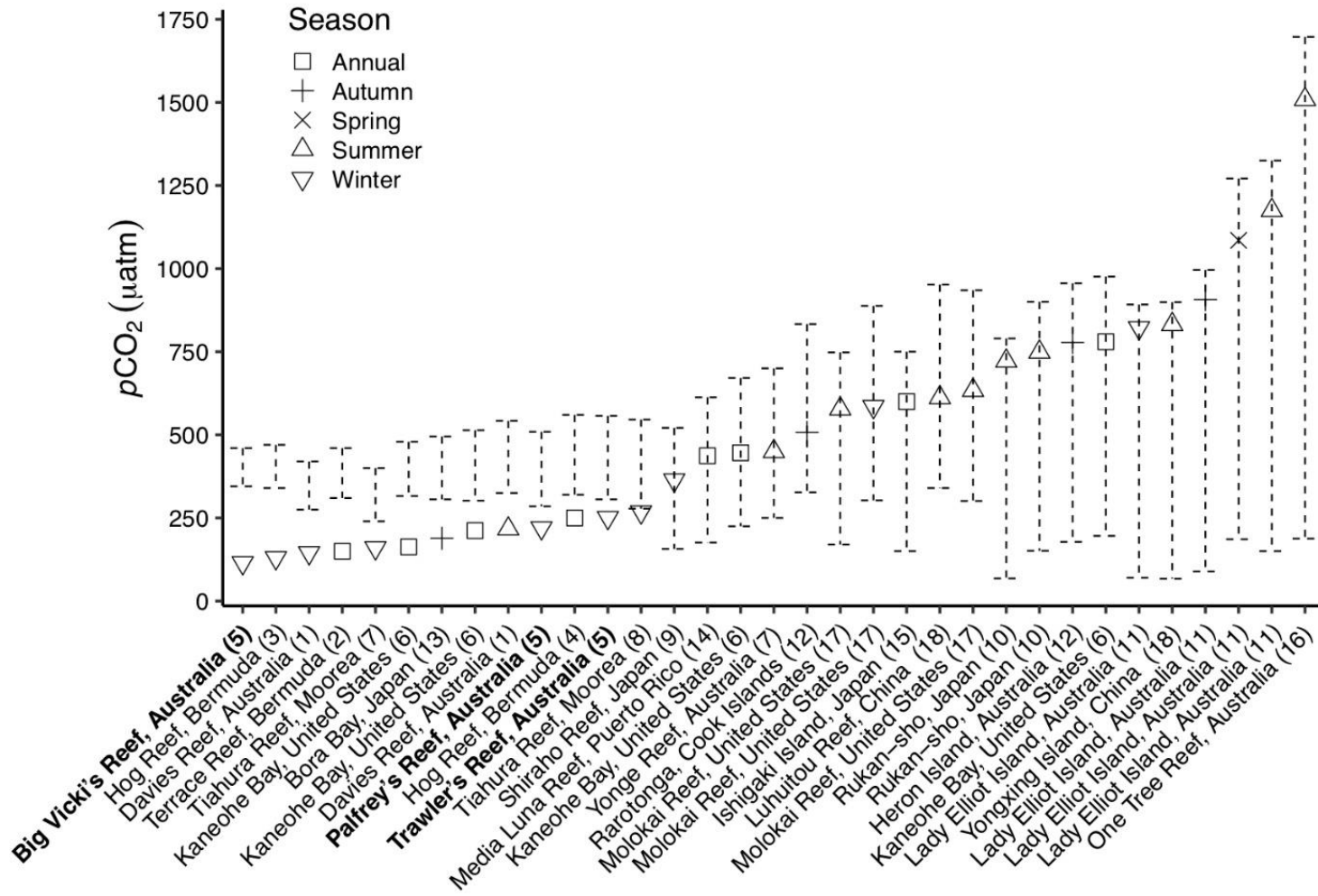
Figure 2-7. Published maximum and minimum pCO_2 values (dashed lines) and range (O, +, x, ∇, Δ, calculated as max – min) of pCO_2 in coral reef environments, separated by study, season and reef sites. The labels on the x-axis correspond to the respective locations, the number in the parentheses correspond to the manuscript found in Table 2-3.

Table 2-3. Studies that report pCO_2 ranges (µatm) at different locations and reef types separated by season. Studies with < 10 measurements of pCO_2 measurements were not included.

Number	Study	Season	Year	Days	Recording intervals (min)	Location	Reef name	Type of Reef	Depth (m)	pCO_2 min, max (µatm)	Δ pCO_2 (µatm)
5	Current study	Winter	2014	9	5	Australia	Big Vicki's Reef	Lagoon	5	345-460	115
3	Bates et al., 2001	Winter	1998	24	60	Bermuda	Hog Reef	Lagoon	6	340-470	130
1	Albright et al., 2013	Winter	2012	9	120	Australia	Davies Reef	Lagoon	1.8	275-420	145
2	Bates 2002	Annual	1994-1998	-	12	Bermuda	Terrace Reef	Terrace	-	310-460	150
7	Frankignoulle et al., 1996	Winter	1992	3	30	Moorea	Tiahura Reef	Back reef	1.3	240-400	160
6	Drupp et al., 2013	Annual	2008-2011	-	180	United States	Kaneohe bay	Reef crest	12	316-479	163
13	Gattuso and Frankignoulle 1998	Autumn	1995	2	-	Japan	Bora Bay	Back reef	2.3	306-495	189
6	Drupp et al., 2013	Annual	2008-2011	-	180	United States	Kaneohe bay	Reef crest	12	302-514	212
1	Albright et al., 2013	Summer	2012	10	120	Australia	Davies Reef	Lagoon	1.8	325-542	217
5	Current study	Winter	2014	9	5	Australia	Palfrey Reef	Lagoon	5	285-509	220
4	Bates et al., 2010	Annual	2002-2003	-	60	Bermuda	Hog Reef	Lagoon	2	320-560	250
5	Current study	Winter	2014	9	5	Australia	Trawler Reef	Lagoon	5	306-557	251
8	Gattuso et al., 1997	Winter	1992	2	180	Moorea	Tiahura Reef	Fringing reef	0.61	278-546	268
9	Kayanne et al., 1995	Winter	1993-1994	3	720	Japan	Shiraho Reef	Fringing reef	2	157-521	364
14	Gray et al., 2012	Annual	2007-2008	16	30	Puerto Rico	Media Luna Reef	Fringing reef	5	176-613	437
6	Drupp et al., 2013	Annual	2005-2008	-	180	United States	Kaneohe bay	Lagoon	0.5	225-671	446
7	Frankignoulle et al., 1996	Summer	1993	3	1	Australia	Yonge Reef	Back reef	1.7	250-700	450
12	Cyronak et al. 2014	Autumn	2012	4	5	Cook Islands	Rarotonga	Fringing reef	0.69	327-833	507
17	Yates and Halley 2006	Summer	2001	9	240	United States	Molokai reef	Fringing reef	1-2	170-748	578
17	Yates and Halley 2006	Winter	2000	9	240	United States	Molokai reef	Fringing reef	1-2	303-888	585
15	Kayanne et al., 2005	Annual	1998-1999	375	1	Japan	Ishigaki Island	Fringing reef	2	150-750	600
18	Yan et al., 2011	Summer	2009	6	60	China	Luhuitou reef	Fringing reef	-	340-952	612
17	Yates and Halley 2006	Summer	2003	4	240	United States	Molokai reef	Fringing reef	1-2	301-935	634
10	Ohde et al., 1999	Summer	1994	2	20	Japan	Rukan-sho	Atoll	1	68-790	722
10	Ohde et al., 1999	Summer	1993	2	20	Japan	Rukan-sho	Atoll	3	151-900	749

12	Cyronak et al. 2014	Autumn	2012	10	5	Australia	Heron Island	Fringing reef	0.95	178-956	778
6	Drupp et al., 2013	Annual	2008-2011	-	180	United States	Kaneohe bay	Lagoon	3	196-976	780
11	Shaw et al., 2012	Winter	2010	4	60	Australia	Lady Elliot Island	Reef flat	0.74	70-892	822
18	Yan et al., 2011	Summer	2008	6	120	China	Yongxing Island	Reef flat	9-1.1	67-899	832
11	Shaw et al., 2012	Autumn	2010	8	60	Australia	Lady Elliot Island	Reef flat	0.74	89-996	907
11	Shaw et al., 2012	Spring	2009	3	60	Australia	Lady Elliot Island	Reef flat	0.74	186-1271	1085
11	Shaw et al., 2012	Summer	2010	6	60	Australia	Lady Elliot Island	Reef flat	0.74	150-1325	1175
16	Silverman et al., 2012	Summer	2009	27	720	Australia	One Tree Reef	Platform reef	-	188-1697	1509

In conclusion, we found that three reefs in the Lizard Island lagoon display a distinct diel fluctuation in pCO$_2$ and that there was far greater variation in pCO$_2$ among nearby reefs than among microhabitats within reefs. However, variation in pH and pCO$_2$ on these reefs overall was relatively low during each 9-day observation period, which is consistent with the majority of previous studies examining the range of pCO$_2$ on coral reefs (Figure 2-7, Table 2-3). Our results highlight that, when projecting future ocean acidification values, physical, hydrological, and biological properties need to be taken into account to best represent how organisms from different ecosystems will respond. Finally, these data emphasise that studies on the physiological effects of ocean acidification on shallow nearshore organisms should try to include fluctuating treatments when drawing conclusions about the future impacts of ocean acidification.

Chapter 3:   The effects of short-term constant and fluctuating elevated pCO$_2$ levels on oxygen uptake rates of damselfishes

3.1   Summary

Ocean acidification, resulting from increasing atmospheric carbon dioxide (CO$_2$) emissions, can affect the physiological performance of some fishes. Most studies investigating ocean acidification have used stable pCO$_2$ treatments based on open ocean predictions. However, nearshore systems can experience substantial spatial and temporal variations in pCO$_2$. Notably, coral reefs are known to experience diel fluctuations in pCO$_2$, which are expected to increase on average and in magnitude in the future. Though we know these variations exist, relatively few studies have included fluctuating treatments when examining the effects of ocean acidification conditions on coral reef species. To address this, we exposed two species of damselfishes, *Amblyglyphidodon curacao* and *Acanthochromis polyacanthus*, to ambient pCO$_2$, a stable elevated pCO$_2$ treatment, and two fluctuating pCO$_2$ treatments (increasing and decreasing) over an 8 h period. Oxygen uptake rates were measured both while fish were swimming and resting at low-speed. These 8 h periods were followed by an exhaustive swimming test (U_{crit}) and blood draw examining swimming metrics and haematological parameters contributing to oxygen transport. When *A. polyacanthus* were exposed to stable pCO$_2$ conditions (ambient or elevated), they required more energy during the 8 h trial regardless of swimming type than fish exposed to either of the fluctuating pCO$_2$ treatments (increasing or decreasing). These results were reflected in the oxygen uptake rates during the U_{crit} tests, where fish exposed to fluctuating pCO$_2$ treatments had a higher factorial aerobic scope than fish exposed to stable pCO$_2$ treatments. By contrast, *A. curacao* showed no effect

of $p\mathrm{CO_2}$ treatment on swimming or oxygen uptake metrics. Our results show that responses to stable versus fluctuating $p\mathrm{CO_2}$ differ between species - what is stressful for one species may not be stressful for another. Such asymmetries may have population- and community-level impacts under higher more variable $p\mathrm{CO_2}$ conditions in the future.

3.2 Introduction

Carbon dioxide ($\mathrm{CO_2}$) concentrations in the atmosphere have been rising at an unprecedented rate since the Industrial Revolution (Dlugokencky and Tans, 2015; Tian et al. 2016). Continuing along this trajectory, atmospheric $\mathrm{CO_2}$ is projected to exceed 900 ppm by 2100 (Collins et al., 2013). As atmospheric $\mathrm{CO_2}$ concentrations increase, the partial pressure of $\mathrm{CO_2}$ ($p\mathrm{CO_2}$) in the oceans increases as well (Doney, 2010). This increase in ocean $p\mathrm{CO_2}$ is accompanied by a decrease in pH, referred to as ocean acidification (OA) (Doney, 2010). Since the Industrial Revolution, ocean pH has declined by 0.1 units and is predicted to decrease an additional 0.3-0.4 units by 2100 (IPCC, 2019).

Future ocean pH and $p\mathrm{CO_2}$ projections are often based on open ocean environments that are relatively stable over time (Doney, 2010), but temporal fluctuations in $p\mathrm{CO_2}$ and pH are common in many nearshore systems (Hofmann et al., 2011). In some coastal habitats, daily $p\mathrm{CO_2}$ fluctuations due to tides and benthic respiration can already approach or, in select cases, exceed the projections for average $p\mathrm{CO_2}$ by the year 2100 (Degrandpre et al., 1995; Melzner et al., 2013; Shaw et al., 2013a). Specifically, coral reef ecosystems experience diel pH and $p\mathrm{CO_2}$ fluctuations due to daily cycles of calcification, respiration, and photosynthesis, which are further modified by water flow and residence time (Duarte et al., 2013; Hofmann et al., 2011; Kayanne et al., 1995; Manzello et al., 2012; Ohde and van Woesik, 1999; Schmalz and Swanson, 1969; Yates and Halley, 2006). Further, seasonal changes in $p\mathrm{CO_2}$ due to

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changes in reef metabolism can vary between nearshore and offshore sites (Manzello et al., 2012). Shaw and colleagues (2013) found that nightly pCO_2 levels in a reef flat ecosystem could already exceed predicted levels for 2100. Other studies have documented diel pCO_2 variations on coral reef systems varying anywhere from ± 50 to 600 µatm around the mean (Albright et al., 2013; Kayanne et al., 1995; Kline et al., 2015). On the Great Barrier Reef, the average daily variation in pCO_2 around the mean is ~ 300 µatm; however, season and depth are influential, resulting in an average variation around the mean of 91 µatm for deeper habitats (>1m) and 411 µatm for shallower habitats (<1m) during the winter, and 167 and 671 µatm for deeper and shallower habitats, respectively, during the summer (Albright et al., 2013; Frankignoulle et al., 1996; Hannan et al., *in press*; Shaw et al., 2012; Silverman et al., 2012). Though we know these fluctuations exist, long-term *in situ* pCO_2 data on coral reef habitats is lacking in most locations (Hofmann et al., 2011), which limits our understanding as to how ecosystems cope with such fluctuations. Further, these natural pCO_2 fluctuations may increase by up to 10-fold in the future due to climate change and the resulting decline in the buffering capacity of the ocean (McNeil and Matsumoto, 2019; McNeil and Sasse, 2016).

The majority of studies assessing the impacts of OA on marine organisms have used stable pCO_2 and pH treatments that reflect open ocean predictions, rather than fluctuating treatments that may be more relevant to what nearshore organisms experience (McElhany and Busch, 2013; Wahl et al., 2015). The few studies that have compared fluctuating and stable pCO_2 treatments on coral reef organisms have found that the effects commonly associated with elevated pCO_2 exposure can be modified or even ameliorated under fluctuating pCO_2 conditions (Chan and Eggins, 2017; Comeau et al., 2014; Dufault et al., 2012; Enochs et al., 2018; Jarrold and Munday, 2018a; Jarrold et al., 2017). One of the studies investigating the effects of fluctuating pCO_2 on marine fishes found that the behavioural, growth, and developmental effects that have been previously observed under stable elevated pCO_2

exposure were ameliorated when fishes were exposed to elevated fluctuating pCO_2 levels (Jarrold et al., 2017). In another study, Laubestein et al. (2020) found that resting oxygen uptake rates increased in fish exposed to stable elevated pCO_2 conditions, but was restored to control levels when a diel pCO_2 cycle was included in the elevated pCO_2 treatment. Thus far, there have only been a few studies examining the effects of fluctuating pCO_2 exposure on physiological performance of fishes (Laubenstein et al., 2020; Methling et al., 2013; Ou et al., 2015).

In order to predict how organisms may respond to anthropogenic and environmental stressors, it is important to measure physiological performance over a range of relevant activities. Because performance is often tightly linked to oxygen utilization, an important metric related to oxygen uptake for fish is the maximum sustained swimming speed (U_{crit}). While U_{crit} values for various fish species have been measured under stable pCO_2 conditions across many studies (reviewed by Heuer and Grosell, 2014), no study to date has examined U_{crit} under fluctuating pCO_2 conditions. Typically, exposure to stable elevated pCO_2 levels has not had an effect on swimming performance in fish (reviewed by Heuer and Grosell, 2014); although, there are some exceptions (e.g., McMahon et al., 2020). However, for nearshore coral reef fishes, exposure to fluctuating pCO_2 may yield more ecologically relevant responses.

Measuring U_{crit} also facilitates the measurement of maximum oxygen uptake rates ($\dot{M}O_{2\ Max}$), which represent the upper bound of a fish's capacity to uptake oxygen and can be used as a proxy for maximum metabolic rates (Roche et al., 2013; Rummer et al., 2016). Oxygen uptake rates during rest ($\dot{M}O_{2\ Min}$) can be calculated by extrapolating the non-linear swimming speed-$\dot{M}O_2$ relationship to a swimming speed of zero (Roche et al., 2013) and can be used as a proxy for standard metabolic rates. The difference between these two metrics

($\dot{M}O_{2\ Max} - \dot{M}O_{2\ Min}$) is the absolute scope of aerobic activity, termed aerobic scope. Aerobic

scope represents the energy available for life-history processes, including but not limited to

growth, reproduction, foraging, and escaping predators (Pörtner and Peck, 2010). The

majority of studies to date have found that OA-relevant, stable elevated pCO_2 levels (700-

2,000 μatm) do not affect the aerobic scope of marine fishes (reviewed by Hannan and

Rummer, 2018; Lefevre, 2016); however, little is known as to the effects of fluctuating pCO_2

on aerobic scope. To date, only three studies have investigated the effects of fluctuating pCO_2

on oxygen uptake metrics of fishes, and only two of these studies used OA-relevant pCO_2

levels (Laubenstein et al., 2020; Ou et al., 2015). These studies found that exposure to stable,

elevated pCO_2 had a negative effect on oxygen uptake metrics, but exposure to fluctuating

pCO_2 ameliorated this response. However, only early-life stages of fishes were investigated,

which may respond differently to environmental stressors when compared to adult fishes

(Pörtner and Farrell, 2008). In addition to whole organism performance metrics that rely on

oxygen uptake, we can also use haematological parameters and metabolites to, for example,

examine the physiological status of a fish prior to and immediately following exercise.

Haematological parameters, such as, haemoglobin [Hb], haematocrit, and mean cell Hb

concentration, as well as metabolites such as glucose and lactate can provide further insight

into how elevated or fluctuating pCO_2 levels influence exercise as well as post-exercise

recovery. Indeed, physiological performance metrics can be important in understanding how

changing environmental conditions affect fishes, both now and into the future.

Ecologically relevant fluctuating pCO_2 levels, when compared to stable pCO_2 levels may

have different effects on the physiological performance of fishes, thus representing a critical

knowledge gap limiting our ability to predict the potential impacts of OA on marine species.

Therefore, the objectives of this study were to determine: (i) how the exercise physiology of

two coral reef fish species (Family Pomacentridae) is affected by exposure to stable versus

fluctuating pCO_2, and (ii) whether the responses to stable versus fluctuating pCO_2 are different depending on activity level (i.e., swimming and resting at low-speed). We measured oxygen uptake rates of both species during an 8 h exposure to one of two stable or two fluctuating pCO_2 treatments while fish were either swimming or resting at low-speed. Additional metabolic, swimming, and haematological parameters were examined following the swimming 8 h trial. We predicted that these coral reef fishes, which experience fluctuating conditions in their natural environment, will respond negatively to stable elevated pCO_2 when compared to fluctuating pCO_2 conditions, as has been suggested in previous studies, but that these differences will be even more evident during swimming trials when more energy is needed to maintain performance.

3.3 Methods

Experimental animals

Amblyglyphidodon curacao (N = 23) were collected from the reef at Orpheus Island on the central Great Barrier Reef (GBR) and transported to James Cook University's Marine and Aquaculture Research Facilities Unit (MARFU). *Acanthochromis polyacanthus* (N = 20) were bred at MARFU from breeding pairs collected in the Cairns region of the GBR by Cairns Marine. The two damselfish species were chosen because they are common in shallow coral reef habitats, including lagoonal areas that can experience substantial diel pCO_2 fluctuations. Prior to commencing experimentation, all animals were maintained in individual (30 L) aquariums under ambient temperature (27.9 ± 0.2 °C), salinity (33.6 ± 0.1), dissolved oxygen (6.61 ± 0.03), pH_T (7.952 ± 0.004), and pCO_2 (468.9 ± 5.6 µatm) conditions with a continuous flow of filtered seawater for >14 days and fed ad libitum twice daily (NRD Aquaculture Nutrition pellets; INVE Aquaculture, Salt Lake City, UT, USA). Prior to

experimentation, all fish were fasted for 48 h to ensure they were in a post-absorptive state (Niimi and Beamish, 1974). All collections and experimentation were carried out under approval from the James Cook University animal ethics committee (permits: A2414 and A2089) and according to the University's animal ethics guidelines.

Experimental set-up

Oxygen uptake rates for both species of damselfishes were measured using a 5 L intermittent-closed Brett-type swimming respirometry chamber (described in Johansen and Jones, 2011; Steffensen et al., 1984). Flow straighteners were used to create laminar flow within the swimming section (7.0 × 36.0 × 7.0 cm; width, length, depth, respectively), and a motor (2600/5400rpm Permanent Magnet Brushless Motor, Jay Electronic Equipment) was used to control the speed. A flush pump flushed the swimming respirometry chamber with clean, fully-oxygenated water every 15 min for 5 min. Oxygen was measured using a fiber-optic probe connected to a Firesting Optical Oxygen Meter every 2 seconds (Pyro Science e. K., Aachen, Germany). Sensors were calibrated daily according to the manufacturer's instructions. Each 'flush-wait-measurement' cycle lasted 15 min (5 min 'flush' to exchange the respirometry chamber water, 1 min 'wait' mixing phase in closed mode, 9 min 'measurement' period in closed mode). Water was continually pumped from a 500 L external buffer tank through a UV-filter into the water bath surrounding the swimming respirometry chamber, and then water was gravity fed back to the main sump (7,000 L). The main sump fed the buffer tank, which was the location of pCO_2 and temperature manipulation.

Elevated pCO_2 was achieved by dosing water in the buffer tank with CO_2 gas to a set pH, following standard techniques (Gattuso and Hansson, 2011). A pH controller (Aqua Medic, Germany) maintained the pH at the desired level. Water quality metrics (temperature,

dissolved oxygen concentration, salinity, and pH$_{NBS}$) were measured daily using a multimeter (WTW Multi meter 3630 IDS SET F 2FD57F, Xylem Analytics, Germany) that was calibrated weekly, according to manufacturing instructions (Table 3-1). Total alkalinity (A$_T$) of seawater was estimated daily by Gran titration (Gran, 1950; 1952) using certified reference material from Dr. A. G. Dickson (Scripps Institution of Oceanography), and pH$_T$ measurements were performed on the same water samples using the indicator dye meta/m-cresol purple (mCP; m-cresol purple sodium salt 99%, non-purified, Acros Organic; Table 3-1). Seawater pCO$_2$ was calculated with CO2SYS (http://cdiac.ornl.gov/oceans/co2rprt.html) from measured A$_T$, temperature, salinity, barometric pressure, and pH$_T$ and using the constants of Mehrbach et al. (1973) refit by Dickson and Millero (1987) (Table 3-1). The pCO$_2$ levels were verified with a non-dispersive infrared (NDIR) gas analyzer before and after the experimental period (Table 3-1; for further description see; Watson et al., 2017).

Table 3-1. As there were no significant differences in the water quality parameters within pCO$_2$ treatments across species or trials, the average, minimum, maximum and range of pH$_T$ and pCO$_2$, as well as the average total alkalinity (TA), temperature (°C), salinity are reported below as means ± SEM.

Water quality parameter	Stable ambient pCO$_2$ (~480 µatm)	Stable elevated pCO$_2$ (~1,100 µatm)	Fluctuating increasing pCO$_2$ (~480-1,100 µatm)	Fluctuating decreasing pCO$_2$ (~1,100-480 µatm)
Average pH$_T$	7.938 ± 0.005	7.612 ± 0.004	7.783 ± 0.025	7.764 ± 0.023
Min. pH$_T$	—	—	7.588	7.582
Max. pH$_T$	—	—	7.969	7.965
pH$_T$ range	—	—	0.381	0.383
Average pCO$_2$ (µatm)	489.7 ± 7.4	1163.8 ± 12.6	806.4 ± 50.5	842.7 ± 49.0
Min. pCO$_2$ (µatm)	—	—	451.1	465.8
Max. pCO$_2$ (µatm)	—	—	1202.3	1264.9
pCO$_2$ range (µatm)	—	—	751.2	799.1
TA (µmol kg^{-1})	2083.1 ± 5.2	2094.2 ± 5.4	2080.4 ± 4.9	2088.8 ± 6.0
Temperature (°C)	28.0 ± 0.15	27.7 ± 0.15	27.3 ± 0.13	27.6 ± 0.16
Salinity	33.2 ± 0.18	33.3 ± 0.18	33.0 ± 0.20	33.3 ± 0.19

8 h resting at low-speed (U_{rest}) trial

Prior to experimentation, each fish was measured in order to determine mass (g), standard length (mm), height (mm), and width (mm) and then transferred into the swimming respirometry chamber. Fish were allowed to habituate to the swimming chamber overnight (>10 h) at the lowest speed (15 cm s^{-1}) that maintained water flow. Following this habituation period, each fish was randomly exposed to one of four pCO$_2$ treatments (stable ambient: ~480 µatm, stable elevated: ~1,100 µatm, fluctuating increasing: ~480-1,100 µatm, or fluctuating decreasing: ~1,100-480 µatm) over an 8 h measurement period (Table 3-1). The stable ambient and stable elevated pCO$_2$ treatments represent typical levels used in OA studies; whereas, the fluctuating increasing and fluctuating decreasing pCO$_2$ treatments represent daily cycles (~17:00-01:00 and 05:00-13:00, respectively) meant to simulate extreme daily pH and pCO$_2$ fluctuations on a coral reef (Shaw et al., 2012). For both fluctuating treatments, pH changed at a rate of ~0.02 pH units every 30 min over the 8 h period, which provided two (15 min) oxygen uptake rate ($\dot{M}O_2$) measurements at each of the 16 pH levels. The swimming chamber was maintained at the lowest speed (15 cm s^{-1}) that maintained water flow, which we term U_{rest}. Following this 8 h U_{rest} trial, the fish were again weighed and returned to their individual aquaria to recover for 8 d prior to further experimentation.

8 h swimming (U_{opt}) trial

Following the 8 d recovery period, each fish was returned to the swimming respirometry chamber and allowed to habituate to the chamber overnight (>10 h) at a speed of 15 cm s^{-1}. Then, the fish was again randomly exposed to one of the aforementioned pCO$_2$ treatments, but not necessarily the same treatment as the U_{rest} trial. This resulted in 1-2 fish in each pCO$_2$ treatment repeating the same treatment from the U_{rest} test. Once the 8 h trial began, the water

velocity in the swimming chamber was increased to a speed of 4.0 body lengths (BL) s^{-1} over a period of 5 min. The maximum diameter of the fish can impede the flow of water in the swimming chamber, and so these solid blocking effects were corrected for following Bell and Terhune (1970) and did not exceed 5% for any individual. This speed of 4.0 BL s^{-1} was determined to be the optimal swimming (U_{opt}) speed from previous *A. polyacanthus* swimming studies (Rummer et al., unpublished data). This speed calculated as $U_{opt} = \{a / [(c-1)b]\}^{1/c}$ (m s^{-1}), where a, b, and c are constants also calculated from previous U_{crit} data (Rummer et al. 2016) from the average nonlinear regression of oxygen uptake measures following y = a + be^{cU}, and U = maximum metabolic rate ($\dot{M}O_{2\ Max}$) (Videler, 1993). Therefore, U_{opt} is the speed that minimizes energy expenditure per unit of travel distance (Videler, 1993). Fish were maintained at U_{opt} throughout the entire 8 h trial.

Critical swimming speed (U_{crit})

The U_{crit} trial commenced during the last two measurements of the 8 h U_{opt} trial. From 4.0 BL s^{-1}, water velocity was increased (increments of 0.75 BL s^{-1}) following standard U_{crit} protocols (Jain et al., 1997) every 30 min. Thus, each fish swam at each speed for two 15 min cycles. The trial was complete when the fish was exhausted (i.e., when it rested at the back grid of the swimming chamber for >10 s). Throughout the U_{crit} test, oxygen uptake was measured using a fiber-optic probe, as stated above.

Haematological and metabolite parameters

Immediately following the U_{crit} test, fishes were cranially concussed, and blood (0.1 mL) was immediately drawn from the caudal artery/vein via a sterile lithium-heparin-rinsed syringe (1 mL syringe, 23-G needle). Blood samples were immediately used to determine haemoglobin concentration ([Hb]), haematocrit (Hct), and whole blood glucose and lactate concentrations. Haemoglobin concentration in the blood was determined using 10 µl of whole blood and the

HemoCue Hb 201 system, Australia Pty Ltd, Tumbi Umb, NSW, Australia. The [Hb] whole

blood is reported as grams per 100 mL using a calibration curve according to Clark et al.

(2008) corrected for tropical reef species by Rummer et al. (2013). The Hct was determined

by centrifuging 60 µl of whole blood in heparinized micro-capillary tubes for 3 min and

calculated as the ratio of packed red blood cells to total blood volume (as a percentage). Both

[Hb] and Hct were used to calculate the mean cell haemoglobin concentration (MCHC) of the

blood. Whole blood glucose and lactate concentrations (millimolar) were determined from

two 15 µl samples using the Accutrend Plus (Roche Diagnostics Australia Pty Ltd Dee Why,

NSW, Australia).

Data analyses

All $\dot{M}O_2$ data were calculated from linear decreases in the swimming respirometry chamber

oxygen concentration using the appropriate constants for oxygen solubility in seawater

(Boutilier et al., 1984) using the following equation:

$$\dot{M}O_2 = SV_{resp}M^{-1},$$

modified from (Bushnell et al., 1994; Schurmann and Steffensen, 1997) where S is the slope

(in milligrams of O_2 per litre per second), V_{resp} is the volume of the respirometer minus the

volume of the fish (in litres), and M is the mass of the fish (in kilograms). We subtracted the

proportional background O_2 uptake rate (measured as O_2 depletion in the empty respirometer

before and after each trial, assumed linear) from each $\dot{M}O_2$ measurement. The critical

swimming speed (U_{crit}) was calculated using the following equation (Brett, 1964):

$$U_{crit} = u_i + [u_{ii}(t_i/t_{ii})],$$

with u_i being the highest swimming velocity maintained for the entire time interval

(BL s^{-1}), t_i the time interval spent at the exhaustion velocity (min), t_{ii} the time interval at each

swimming speed (30 min), and u_{ii} the velocity increment (0.75 BL s^{-1}). The lower of the two

measured $\dot{M}O_2$ values during each swimming step were plotted against swimming speed.

Standard metabolic rate was estimated from $\dot{M}O_{2\,Min}$ and calculated by extrapolating the

$\dot{M}O_2$ vs. swimming velocity curves to zero swimming speed (Reidy et al., 2000; Roche et al.,

2013). Absolute aerobic scope (AS) was calculated by subtracting $\dot{M}O_{2\,Min}$ from the highest

$\dot{M}O_2$ value recorded during the entire experiment ($\dot{M}O_{2\,Max}$). Factorial AS was calculated by

dividing $\dot{M}O_{2\,Max}$ by $\dot{M}O_{2\,Min}$ for each fish.

Statistical analyses

All statistical analyses were performed using R v3.4.3 (R Development Core Team, 2016).

The mass and standard length of individuals within each species were not different across any

of the planned comparisons for the 8 h trials, oxygen uptake metrics, swimming metrics, and

haematological parameters. Due to the differences in rearing conditions (i.e., reef vs.

laboratory) the two damselfish species were analysed separately for all variables. All data are

represented as means ± SEM where appropriate, and differences were considered significant

if p was <0.05.

8 h U_{opt} and U_{rest} tests

The effect of pCO$_2$ treatment (stable ambient pCO$_2$, stable elevated pCO$_2$, fluctuating

increasing pCO$_2$, and fluctuating decreasing pCO$_2$) and swimming type (U_{rest} and U_{opt}) on

$\dot{M}O_2$ averaged over 8 h was analysed separately for each species using a generalized linear

mixed-effects model (GLMM) with a Gaussian distribution (package 'lme4', Bates et al.,

2015). Initially, time and mass were added into the model as covariates; however, they were

removed from the final models because they had no significant effect on model fit. Treatment

and swimming type were treated as fixed variables, and the random variable was specified as

Fish ID nested in swimming test (i.e., U_{rest} or U_{opt}). The overall effects of pCO$_2$ treatment and

swimming were assessed by performing a likelihood test on the full model. The analysis was followed by planned comparison tests to assess significant differences between treatments using the package 'multcomp' (Hothorn et al., 2008) and applying a Tukey correction for multiple testing. Planned comparisons included the following: (1) comparisons between the stable ambient and stable elevated pCO_2 treatments, (2) comparisons between the fluctuating increasing and fluctuating decreasing treatments, (3) comparisons between stable pCO_2 and fluctuating pCO_2, and (4) comparisons between U_{opt} and U_{rest} tests.

Oxygen uptake, U_{crit}, and haematological parameters

The effect of pCO_2 treatment (i.e., stable ambient, stable elevated, fluctuating increasing, and fluctuating decreasing) on the response variables (i.e., $\dot{M}O_{2\ Min}$, $\dot{M}O_{2\ Max}$, absolute AS, factorial AS, U_{crit}, [Hb], Hct, MCHC, glucose, and lactate) were measured separately for each species using a GLMM with a Gaussian distribution from the 'stats' package. Initially, pre-treatment was added into the model as a covariate; however, it was removed from the final models because it had no significant effect on model fit. The pCO_2 treatment was a fixed variable, and Akaike information criterions (AICs) were used to determine if mass should be included in the model. As stated above, the overall effect of pCO_2 treatment was assessed using planned comparisons including the following: (1) comparisons between the stable ambient and stable elevated pCO_2 treatments, (2) comparisons between the fluctuating increasing and fluctuating decreasing treatments, (3) comparisons between stable pCO_2 and fluctuating pCO_2, and (4) comparisons between stable ambient and all other pCO_2 treatments. Due to the relatively small size of some *A. polyacanthus* individuals the amount of blood collected was insufficient to test all haematological parameters in all fish, leading to a small sample size (n < 3) for some haematological parameters in *A. polyacanthus*. Haematological

parameters with n < 3 were unable to be compared via statistics and were removed from the models.

3.4   Results

8 h U_{opt} and U_{rest} tests

When *A. polyacanthus* were exposed to the stable pCO$_2$ treatments over the 8 h period, they exhibited a significant increase in oxygen uptake rates when compared to *A. polyacanthus* that were exposed to the fluctuating pCO$_2$ treatments, regardless of swimming type (planned comparisons; Figure 3-1A, Table 3-2). Increased oxygen uptake rates were also evident in *A. polyacanthus* while swimming (U_{opt}) when compared to resting at a low speed (U_{rest}). Similarly, *A. curacao* increased their oxygen uptake rates over the 8 h period while swimming (U_{opt}) when compared to resting at a low speed (U_{rest}), regardless of pCO$_2$ treatment (Figure 3-1A, Table 3-2).
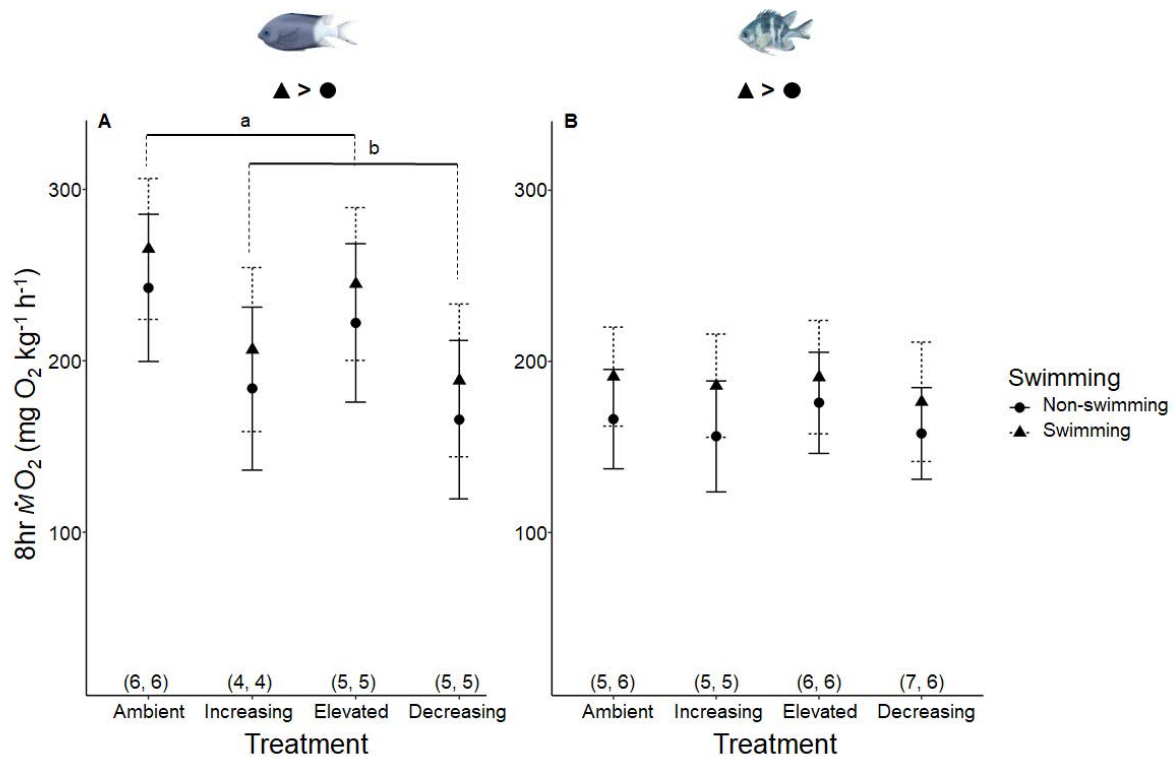
Figure 3-1. Oxygen uptake rates ($\dot{M}O_2$) over the 8 h trial for both A) *Acanthochromis polyacanthus* and B) *Amblyglyphidodon curacao* exposed to one of four different pCO_2 treatments (Ambient ~480 µatm, increasing ~480-1,100 µatm, stable elevated ~ 1,100 µatm, and decreasing ~1,100-480 µatm). The least-squares means of 8 h U_{opt} tests are represented by triangles and dashed error bars. The least-squares means of 8 h U_{rest} tests are represented by circles and solid error bars. Error bars represent 95% confidence intervals. Significant differences resulting from the planned comparisons between pCO_2 treatments are represented by different letters. Significance and direction of differences between swimming treatments are represented by the respective shapes separated by > or < symbols. The numbers in the parentheses represent sample sizes (static, swimming, respectively).

Table 3-2. Results from planned comparison tests examining the effect of the 8 h tests at the four pCO_2 treatments (stable ambient ~480 µatm; stable elevated ~ 1,100 µatm; fluctuating increasing ~480-1,100 µatm; and fluctuating decreasing ~1,100-480 µatm) on the oxygen uptake rates ($\dot{M}O_2$) of two species of damselfishes during both U_{opt} and U_{rest} trials. d.f. – for the entire model was calculated as, nrow(data)-length(coefficients of model).

Species	Main effects	Estimate	Standard Error	z-value	P-value	df
Acanthochromis polyacanthus	Ambient vs Elevated	20.39	25.44	0.80	0.423	1307
	Increase vs Decrease	18.05	27.39	0.66	0.510	
	Fluctuating vs Constant	57.58	18.66	3.09	0.002	
	U_{opt} vs U_{rest}	-61.75	23.70	-2.61	0.009	
Amblyglyphidodon curacao	Ambient vs Elevated	-6.57	14.312	-0.46	0.646	1486
	Increase vs Decrease	3.82	14.45	0.26	0.792	
	Fluctuating vs Constant	12.22	10.36	1.18	0.238	
	U_{opt} vs U_{rest}	-24.60	10.33	-2.38	0.017	

Acanthochromis polyacanthus

When *A. polyacanthus* were exposed to stable pCO_2 levels, $\dot{M}O_{2\,Min}$ was higher than in *A. polyacanthus* exposed to fluctuating pCO_2 levels (Figure 3-2A, Appendix B - Table S1). Though, there was no effect of pCO_2 treatment on $\dot{M}O_{2\,Max}$ or absolute AS (Appendix B - Table S1). The factorial AS of *A. polyacanthus* reflects the results for $\dot{M}O_{2\,Min}$, where fish that were exposed to stable pCO_2 levels (ambient or elevated) had a lower factorial AS than fish from both fluctuating pCO_2 treatments (increasing and decreasing) (Figure 3-3A, Appendix B - Table S1).
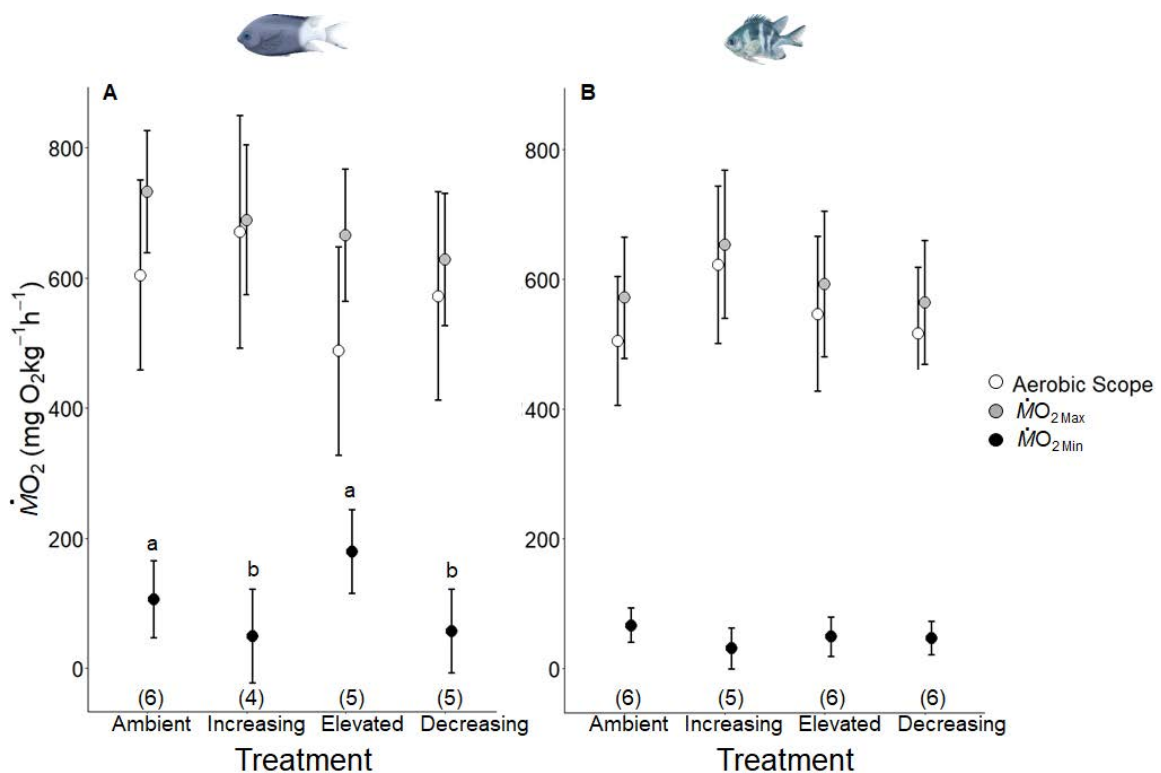
Figure 3-2. Three oxygen uptake metrics ($\dot{M}O_{2\,min}$, $\dot{M}O_{2\,max}$, and aerobic scope) of A) *Acanthochromis polyacanthus* and B) *Amblyglyphidodon curacao* exposed to one of four different pCO_2 treatments (Ambient ~480 μatm, increasing ~480-1,100 μatm, stable elevated ~ 1,100, and decreasing ~1,100-480 μatm). Circles represent least-squares means, and error bars represent 95% confidence intervals. Significant differences of the planned comparisons are represented by differing letters. The numbers in the parentheses represent sample sizes.
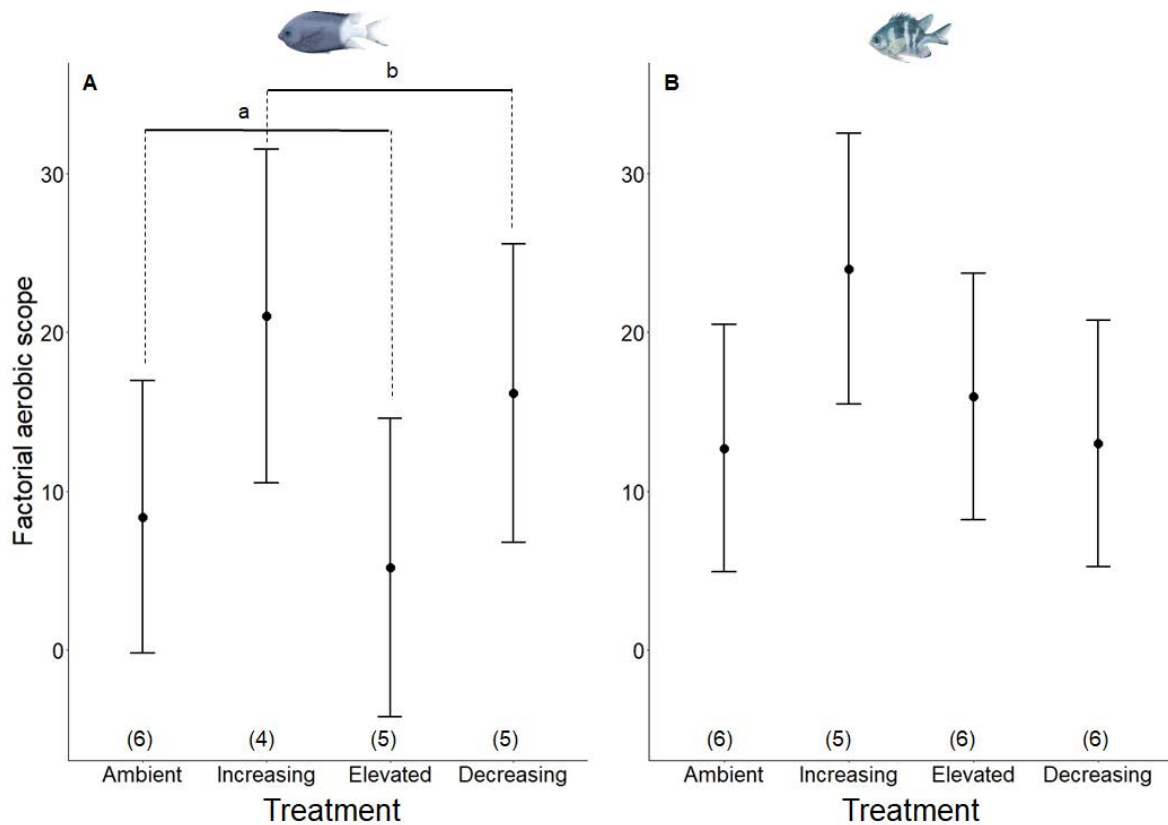
Figure 3-3. Factorial aerobic scope of A) *Acanthochromis polyacanthus* and B) *Amblyglyphidodon curacao* exposed to one of four different $p\text{CO}_2$ treatments (Ambient ~480 µatm, increasing ~480-1,100 µatm, stable elevated ~ 1,100 µatm, and decreasing ~1,100-480 µatm). Circles represent least-squares means, and error bars represent 95% confidence intervals. Significant differences of the planned comparisons are represented by differing letters. The numbers in the parentheses represent sample sizes.

Neither U_{crit}, [Hb], Hct, lactate, nor MCHC (Figure 3-4A) were affected by $p\text{CO}_2$ treatment (Appendix B - Table S1). When *A. polyacanthus* were exposed to stable ambient $p\text{CO}_2$, blood glucose concentrations were higher than in fish exposed to the other $p\text{CO}_2$ treatments (Figure 3-5A, Appendix B - Table S1). Regardless of $p\text{CO}_2$ treatment, however, there was an inverse relationship between AS, $\dot{M}\text{O}_{2 \text{ Max}}$ and U_{crit} with body mass of *A. polyacanthus*.
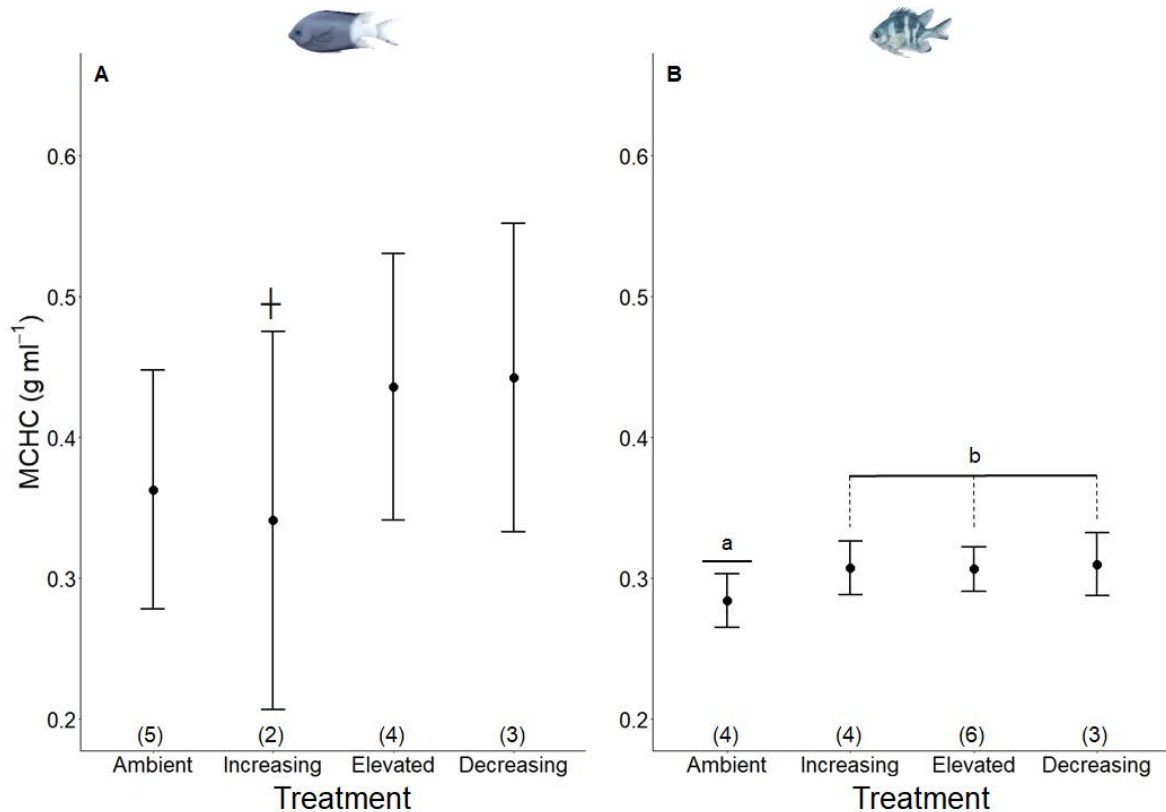
Figure 3-4. Mean cell haemoglobin concentration (MCHC) of A) *Acanthochromis polyacanthus* and B) *Amblyglyphidodon curacao* exposed to one of four different $p\mathrm{CO_2}$ treatments (Ambient ~480 µatm, increasing ~480-1,100 µatm, stable elevated ~ 1,100 µatm, and decreasing ~1,100-480 µatm). Circles represent least-squares means, and error bars represent 95% confidence intervals. Significant differences resulting from the planned comparisons are represented by different letters. The numbers in the parentheses represent sample sizes. The cross (┼) symbol above the error bars represents when the sample size was insufficient for statistical analyses.

Amblyglyphidodon curacao

There was no significant effect of $p\mathrm{CO_2}$ treatment on U_{crit} or oxygen uptake metrics (i.e., aerobic scope, factorial aerobic scope, $\dot{M}\mathrm{O_{2\ Min}}$, $\dot{M}\mathrm{O_{2\ Max}}$) in *A. curacao*. However, some haematological metrics were affected. When *A. curacao* were exposed to fluctuating increasing $p\mathrm{CO_2}$, [Hb] was higher than in fish exposed to fluctuating decreasing $p\mathrm{CO_2}$ (Appendix B - Table S2). Further, MCHC was lower in fish exposed to stable ambient $p\mathrm{CO_2}$ when compared to fish exposed to the other $p\mathrm{CO_2}$ treatments (Figure 3-4B, Appendix B - Table S2). However, none of the other haematological metrics – Hct, lactate concentrations,

or glucose concentrations (Figure 3-5B) – were affected by pCO_2 treatment (Appendix B - Table S2).
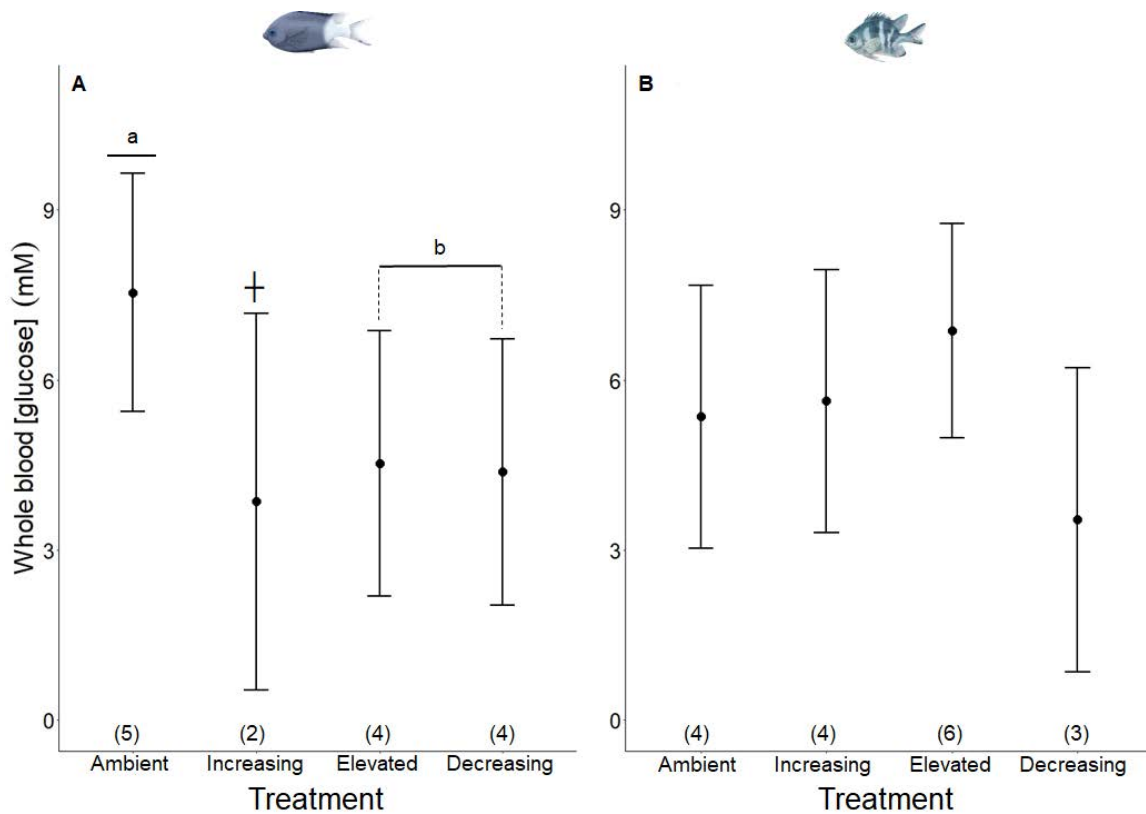
Figure 3-5. Whole blood glucose concentration of A) *Acanthochromis polyacanthus* and B) *Amblyglyphidodon curacao* exposed to one of four different pCO_2 treatments (Ambient ~480 µatm, increasing ~480-1,100 µatm, stable elevated ~ 1,100 µatm, and decreasing ~1,100-480 µatm). Circles represent least-squares means, and error bars represent 95% confidence intervals. Significant differences resulting from the planned comparisons are represented by different letters. The numbers in the parentheses represent sample sizes. The cross (+) symbol above the error bars represents when the sample size was insufficient for statistical analyses.

While there was no significant impact to oxygen uptake metrics, there was a non-significant trend. When *A. curacao* were exposed to stable ambient pCO_2 levels, $\dot{M}O_{2\ Min}$ was higher than in fish exposed to the other three pCO_2 treatments ($p = 0.064$; Figure 3-2B, Appendix B - Table S2). There was also a trend suggesting that when *A. curacao* were exposed to elevated increasing pCO_2, factorial AS was higher than in fish exposed to fluctuating decreasing pCO_2

conditions ($p = 0.061$; Figure 3-3B, Appendix B - Table S2). Similar to *A. polyacanthus*, U_{crit} decreased with increasing fish mass, regardless of pCO_2 treatment; however, in *A. curacao*, blood lactate increased with increasing fish mass, regardless of pCO_2 treatment.

3.5 Discussion

This study shows that fluctuating conditions can have a beneficial impact on oxygen uptake metrics in coral reef fishes when compared to stable ambient pCO_2. However, this was only observed in *A. polyacanthus* and the oxygen uptake rates of *A. curacao* were unaffected by pCO_2 treatment. As predicted, during the 8h trials *A. polyacanthus* required less energy upon exposure to both fluctuating pCO_2 treatments (increasing and decreasing) when compared to both the stable ambient and stable elevated pCO_2 treatments regardless of swimming trial (U_{rest} or U_{opt}). Further, and as expected, both fish species required more oxygen while swimming (U_{opt}) compared to while resting at low-speed (U_{rest}).

Oxygen uptake

A. polyacanthus required less energy to perform basic maintenance functions (i.e., $\dot{M}O_{2\ Min}$) under fluctuating pCO_2 conditions when compared to fish that had been exposed to stable pCO_2 conditions. This matches the results of Laubenstein et al. (2020) for the same species. The differences in $\dot{M}O_{2\ Min}$ contributed to an enhanced factorial aerobic scope under fluctuating pCO_2 conditions, and thus, *A. polyacanthus* exposed to fluctuating conditions would be expected to have more energy available for life-history processes than fish that had been exposed to stable pCO_2 levels. These results suggest that *A. polyacanthus* may possess physiological mechanisms that allow them to survive and maintain metabolic function under

fluctuating pCO_2 conditions that are similar to extreme *in situ* conditions on shallow coral reefs.

Other fish species have also demonstrated benefits of fluctuating pCO_2 exposure when compared to stable ambient exposure (Jarrold and Munday, 2018b; Jarrold and Munday, 2019; Methling et al., 2013). For example, Jarrold and Munday (2019) found increased survival of juvenile *Amphiprion melanopus* when parents and their offspring were exposed to cycling pCO_2 as opposed to when the parents and offspring were exposed to stable ambient pCO_2 conditions. Jarrold and Munday (2019) also found that the wet mass of juvenile *A. polyacanthus* increased during fluctuating pCO_2 treatment regardless of temperature when compared to fish exposed to stable ambient pCO_2. Methling et al. (2013) found that ammonia (nitrogen) excretion rates post-feeding returned to pre-feeding levels earlier in freshwater *Anguilla anguilla* exposed to fluctuating pCO_2 treatments when compared to those exposed to stable ambient pCO_2 treatments. Thus, there is evidence that some physiological and biological metrics for both nearshore coral reef fishes as well as some freshwater fishes may benefit from fluctuating pCO_2 exposure, which contrasts their responses upon exposure to stable ambient pCO_2 treatments. In contrast to *A. polyacanthus* and our predictions, the oxygen uptake rates and absolute and factorial AS of *A. curacao* were unaffected by pCO_2 treatment. This suggests that the amount of energy available for life-history processes of *A. curacao* when exposed to fluctuating pCO_2 was unaffected compared those exposed to stable elevated pCO_2. This maintained energy and oxygen uptake across all treatments, suggest that *A. curacao*, unlike *A. polyacanthus*, may possess physiological mechanisms that allow them to survive and maintain metabolic function under elevated pCO_2 conditions, regardless as to whether conditions are stable or fluctuating.

Species specific differences

The species-specific differences in the oxygen uptake rates in the present study might be associated with differences in the life-history and physiology of the two damselfish species examined. *Acanthochromis polyacanthus* possesses a unique reproductive strategy that does not include a pelagic larval phase (Bay et al., 2006). Whereas, *A. curacao* have a pelagic larval phase that ranges from 15-22 d in the open ocean prior to settlement on a coral reef (Bay et al., 2006). Thus, *A. curacao* experiences a change in pCO$_2$ in their environment from constant (i.e., in the open ocean) to fluctuating as they settle on nearshore reefs (Ferrari et al., 2015). However, *A. polyacanthus* may never experience stable pCO$_2$ conditions in their environment, as they spend their entire lives in coral reef habitats. Additionally, in a study of depth gradients of coral reef fishes, Jankowski et al. (2015) consistently observed *A. polyacanthus* at shallower depths than *A. curacao*. This suggests that *A. polyacanthus* spend the entirety of their lives on shallower reef habitats that are known to have highly variable diel fluctuations. Whereas, *A. curacao* experience the open ocean early in life, a relatively stable habitat in terms of pCO$_2$, before settling onto deeper coral reefs, which may still experience pCO$_2$ fluctuations, but perhaps to a lesser degree than shallow reef habitats (Hannan et al., 2020a; Hofmann et al., 2011; Shaw et al., 2012). Such differences in the pCO$_2$ environments experienced throughout these damselfishes' life-history may have underpinned the responses we observed. However, another difference between the species in this study is that *A. polyacanthus* were laboratory-reared and presumably never experienced diel fluctuations in pCO$_2$; whereas, *A. curacao* were wild-caught and had experienced fluctuating pCO$_2$ in the environment. That being said, the results were relatively similar. When exposed to elevated fluctuating pCO$_2$, *A. polyacanthus* possessed more energy, and although not statistically significant, the trend was evident in *A. curacao* as well upon exposure to fluctuating as well as stable elevated pCO$_2$ treatments.

Possible mechanism of enhanced metabolic traits

The enhanced metabolic traits of *A. polyacanthus* and the trends observed in *A. curacao* during exposure to elevated pCO_2 (stable or fluctuating) may be facilitated by the mechanism described by Rummer and colleagues (Rummer and Brauner, 2015; Rummer et al., 2013b). During a mild acidosis (e.g., exposure to elevated pCO_2), the combination of pH-sensitive Root effect Hbs and plasma-accessible carbonic anhydrase (CA) result in enhanced oxygen release to tissues such as red muscle and heart (reviewed by Hannan and Rummer, 2018). Thus, as the pCO_2 of the water is increasing, the internal pH of the fish decreases, but various ion transporters on the red blood cell (RBC) membranes (e.g., sodium proton exchange; NHE) remove H^+ from the RBCs to protect intracellular pH and therefore oxygen uptake at the gills. This ion transport can result in an osmotic gradient causing water to enter the RBC, which results in swelling. However, at select locations, plasma-accessible CA short-circuits the NHE by catalysing the H^+ and HCO_3^- that are in the plasma to form H_2O and CO_2, the latter of which back-diffuses into the RBC, thus re-acidifying the RBC and resulting in an increased release of oxygen (from Hb) to those sites, which may underpin maintained performance.

Haematological metrics

As mentioned, RBC swelling is hypothesised to be a product of some of the mechanisms that are in place to protect RBC pH and oxygen transport during stress. A decrease in MCHC can indicate RBC swelling. However, in *A. curacao* exposed to fluctuating increasing, fluctuating decreasing, or stable elevated pCO_2 treatments, MCHC increased compared to fish in the stable ambient pCO_2 treatment. The changes observed in MCHC, but not in either Hct or [Hb] in fish exposed to ambient compared to fish exposed to the other pCO_2 treatments, suggest that RBC shrinking, rather than swelling, occurred over the short term to compensate for elevations in pCO_2. Of the previous studies examining the effects of ocean acidification

on MCHC of fishes (Crespel et al., 2019; Green and Jutfelt, 2014; Heinrich et al., 2014; Noor et al., 2019), three demonstrated an increase in MCHC with pCO_2 treatment, similar to the results observed for *A. curacao* in this study (Crespel et al., 2019; Heinrich et al., 2014; Noor et al., 2019). Van Dijk et al. (1993) also found significant shrinkage in fish RBCs during exposure to acidified water and suggested that this was in response to an adrenalin infusion. This shift of electrolytes from the RBC to the extracellular compartment causes water movement in the same direction. Therefore, although RBC swelling (decreases in MCHC) has previously been thought to aid in protecting pH and maintaining O_2 uptake, the majority of studies show that the RBC shrinks with exposure to elevated pCO_2. It is also important to note that due to the size of some individuals, blood volume was insufficient for some analyses and thus, the sample sizes for some haematological metrics were small. The conclusions drawn should therefore be regarded conservatively; however, due to the similarity of our results to the above mentioned studies, we have included their discussion.

Metabolite parameters

Whole blood glucose concentrations were only affected by pCO_2 for *A. polyacanthus* but not for *A. curacao*. In *A. polyacanthus* exposed to elevated pCO_2 (stable elevated and fluctuating decreasing), we observed a decrease in whole blood glucose concentrations when compared to fish from ambient pCO_2. Because glucose is known to contribute to oxidative metabolism (Pagnotta and Milligan, 1991), increases following exercise suggest that *A. polyacanthus* exposed to ambient pCO_2 either performed anaerobically during swimming or started using anaerobic metabolism for recovery following swimming when compared to those exposed to elevated pCO_2 treatments. However, the oxygen uptake rates of *A. polyacanthus* do not reflect the trends observed in blood glucose concentrations. Increased plasma glucose can also occur following stress to satisfy increased energy demands in response to catecholamine

release (Wendelaar Bonga, 1997). Whole blood lactate concentrations, however, were unaffected by pCO$_2$ treatment. This suggests that neither species relied on anaerobic metabolism while swimming under any pCO$_2$ treatment, and the increase in plasma glucose observed in *A. polyacanthus* was more likely to satisfy increased energy demands. Similarly to the haematological metrics, due to the size of some individuals, blood volume was insufficient for some analyses, and the sample sizes for some metabolite parameters were therefore small. The conclusions drawn should therefore be regarded conservatively.

U$_{crit}$

Though we observed effects of elevated pCO$_2$ on both oxygen uptake and haematological metrics, exhaustive swimming was not affected by pCO$_2$ treatment. The way in which both *A. polyacanthus* and *A. curacao* responded is consistent with other studies showing that U_{crit} is generally unaffected by OA-relevant pCO$_2$ levels (reviewed in Heuer and Grosell, 2014). However, recent studies have found that exposure to stable elevated pCO$_2$ negatively affected U_{crit} swimming in juvenile yellowtail kingfish, *Seriola lalandi* (Watson et al., 2018) and juvenile Australasian snapper, *Chrysophrys auratus* (McMahon et al., 2020). These juvenile fishes may be more negatively impacted than the adult fishes measured in this study. Further, both of these species inhabit deep, offshore, rocky reefs that do not experience large, daily fluctuations in pCO$_2$ and may be more affected than the coral reef fishes measured in this study. Here, U_{crit} was heavily influenced by mass – decreasing with increasing body mass – in both species. This result is not surprising; however, as U_{crit} is a relative measure based on the fish's body length and thus would also be influenced by the fish's body mass (Goolish, 1991). Indeed, it even seems that, in this study, U_{crit} is affected more by body mass than by pCO$_2$ treatment in both species. This may be due to other mechanisms contributing to

swimming ability. For example, enhanced O_2 delivery during times of elevated CO_2 (Root Effect) may contribute to maintained swimming performance.

3.6 Conclusions

Our results are a starting point for understanding how coral reef fishes physiologically respond to fluctuating pCO$_2$. *Acanthochromis polyacanthus* appears to be adapted to fluctuating as opposed to stable pCO$_2$ conditions. These data suggest that testing fishes that predominately experience diel pCO$_2$ fluctuations under stable pCO$_2$ conditions (i.e., those meant to simulate open ocean conditions in the future with OA) may over- or under-estimate responses. However, fishes that experience both stable and fluctuating pCO$_2$ exposures across their life-history (e.g., *A. curacao* in this study) may be less affected by fluctuating or stable elevated pCO$_2$. However, we have examined only two damselfish species here. Findings may differ for other species, even within Family Pomacentridae.

The majority of studies measuring the effects of fluctuating pCO$_2$, this study included, have compared fluctuating pCO$_2$ to stable ambient pCO$_2$ conditions, but this assumes that stable conditions are what the species of interest experiences in their environment. Yet, we know that is not the case, because coral reefs and other shallow coastal habitats experience diel pCO$_2$ fluctuations. Thus, we suggest that fluctuating conditions should be treated as 'control' or 'ambient' in future OA experiments, and elevated pCO$_2$ conditions should also be fluctuating, but to a greater degree (i.e., range and magnitude). These results demonstrate that, for some fish, exposure to fluctuating compared to stable pCO$_2$ conditions can alter oxygen uptake rates, where individuals have more energy availability during fluctuating exposures. Our findings add another layer to the growing discussion regarding the mixed responses observed across species in ocean acidification studies. The discussion is further

complicated by the fact that differences here were observed in two species from the same family. It is important to consider that different pCO_2 exposures may have not only species-specific, but also family-specific, or life-history specific effects. In other words, what is stressful for one species many not be stressful for another. The differences in responses to elevated pCO_2 may have population- and/or community-level impacts in the future. Moving forward to understand and predict the effects of ocean acidification on coral reef fishes, it is critical to consider pCO_2 exposure in terms of fluctuations, ranges, relative extremes, and overall ecological relevance.

Chapter 4:   Contrasting effects of long-term constant and fluctuating pCO$_2$ conditions on the exercise physiology of coral reef fishes

4.1   Summary

Ocean acidification (OA) is predicted to affect the physiology of some fishes. To date, most studies have investigated this issue using stable pCO$_2$ levels based on open ocean projections. Yet, most shallow, nearshore systems experience temporal and spatial pCO$_2$ fluctuations. For example, pCO$_2$ on coral reefs is highest at night and lowest during the day, but as OA progresses, both the average pCO$_2$ and magnitude of fluctuations are expected to increase. We exposed four coral reef fishes – *Lutjanus fulviflamma*, *Caesio cuning*, *Abudefduf whitleyi*, and *Cheilodipterus quinquelineatus* – to ambient, stable elevated, or fluctuating elevated pCO$_2$ conditions for 9–11 d. Then, we measured swimming performance, oxygen uptake rates, and haematological parameters during the day and at night. When compared to ambient pCO$_2$ conditions, *L. fulviflamma*, *C. cuning*, and *A. whitleyi* exposed to fluctuating elevated pCO$_2$ increased swimming performance, maximum oxygen uptake rates, and aerobic scope, regardless of time of day; whereas, the only nocturnal species studied, *C. quinquelineatus*, decreased maximum oxygen uptake rates and aerobic scope. Our findings suggest that exposure to fluctuating or stable elevated pCO$_2$ can physiologically benefit some coral reef fishes; however, other species, such as the cardinalfish examined here, may be more sensitive to future OA conditions.

4.2   Introduction

The increased uptake of carbon dioxide (CO_2) by the oceans is causing a reduction in pH and changes to the equilibrium of the carbonate system (Zeebe and Wolf-Gladrow, 2001), a process referred to as ocean acidification (OA) (Doney et al., 2009). Atmospheric CO_2 concentrations are expected to exceed 900 µatm by the year 2100 if current emissions trajectories are maintained (McNeil and Matsumoto, 2019) and the pH of seawater at the surface of the oceans is expected to decline by an average of 0.3-0.4 units compared to pre-industrial values (Caldeira and Wickett, 2003). However, these numbers are based on average projections for the open ocean, not nearshore, coastal regions where much of the ocean's biodiversity resides (Doney et al., 2009). Coastal and shallow water systems are known to experience fluctuations in both pH and the partial pressure of CO_2 (pCO_2) on a variety of spatial and temporal scales (Hofmann et al., 2011; Kayanne et al., 1995; Schmalz and Swanson, 1969). Thus, at a given point in time, seawater in these habitats may be over- or under-saturated in terms of CO_2 when compared to the atmosphere.

Coral reefs are known to experience diel pH and pCO_2 fluctuations (Albright et al., 2013; Hannan et al., 2020a; Hofmann et al., 2011; Ohde and van Woesik, 1999; Shaw et al., 2012) mainly caused by photosynthesis/respiration and calcification/dissolution of benthic organisms across the day-night cycle combined with hydrodynamic factors (Falter et al., 2013; Waldbusser and Salisbury, 2014). These diel fluctuations cause pCO_2 to be elevated at night and low during the day. In some cases, the diel fluctuations that already occur on reefs can exceed the mean pCO_2 levels projected to occur with global climate change by 2100 (Shaw et al., 2012). Additionally, while the average pCO_2 of seawater is projected to increase in the future, these natural pCO_2 fluctuations are also expected to increase in magnitude (Gallego et al., 2018; McNeil and Sasse, 2016). Such increases in the magnitude of pCO_2 fluctuations will expose shallow water inhabitants to a much larger range of pCO_2 than

current day levels. Moreover, pCO_2 fluctuations might differentially affect coral reef organisms that are active at different times of the day. For example, nocturnal fishes are active at night when pCO_2 on coral reefs is the highest; consequently, these species may possess different adaptations for coping with elevated pCO_2 now and into the future.

Though it is known that coral reefs experience diel pCO_2 fluctuations, to date, experimental OA studies have focused on stable open ocean predictions leaving pCO_2 fluctuations largely overlooked, until recently (McElhany and Busch, 2013). This lack of research on environmentally-relevant pCO_2 conditions could lead to over- or under-estimates of the effects of future OA conditions on nearshore organisms. However, some of the studies that have included fluctuating pCO_2 treatments have observed that marine organisms respond differently when exposed to pCO_2 fluctuations when compared to traditionally-used stable pCO_2 treatments (Alenius and Munguia, 2012; Clark and Gobler, 2016; Dufault et al., 2012; Frieder et al., 2014; Hannan et al., 2020b; Jarrold and Munday, 2019; Jarrold et al., 2017; Laubenstein et al., 2020). For fishes, the emphasis to date has been life-history traits, such as growth and survival, or behavioural metrics (Davidson et al., 2016; Jarrold and Munday, 2018a; Jarrold and Munday, 2019; Jarrold et al., 2017; Laubenstein et al., 2020; Lifavi et al., 2017; Ou et al., 2015), with very few studies addressing physiological processes.

Understanding the physiological mechanisms underpinning changes in individual performance under fluctuating pCO_2 conditions will improve our ability to predict the effects of OA on fishes (Heuer and Grosell, 2014; Melzner et al., 2009a; Pörtner and Peck, 2010; Pörtner et al., 2004). Swimming performance is an important and established physiological metric with significant implications for fish health and survival (Fulton, 2007). In addition to swimming performance itself, the amount of energy available for swimming can inform how a fish will function under environmental stressors. Energy availability is often estimated by

measuring oxygen uptake rates as a proxy. For instance, aerobic scope (AS; the difference between maximum and minimum oxygen uptake rates) represents the energy available for oxygen-consuming processes above what is required for maintenance. In other words, AS represents the energy available for processes such as swimming, foraging, growth, and reproduction. In fishes, AS, was initially predicted to decrease with increasing $p\mathrm{CO_2}$ levels (Pörtner and Peck, 2010); however, all possible responses have been observed; from decreases (McMahon et al., 2020; Munday et al., 2009a), to increases (Couturier et al., 2013; Rummer et al., 2013a) in AS (reviewed by Lefevre, 2019). There are likely species-specific responses to elevated $p\mathrm{CO_2}$, but the mechanisms underpinning these varied responses have not been determined. Further, it is not yet understood how these species-specific responses to traditionally-used stable elevated $p\mathrm{CO_2}$ conditions will change under environmentally-relevant fluctuating $p\mathrm{CO_2}$ conditions.

We designed this study to investigate the effects of both stable and diel fluctuating elevated $p\mathrm{CO_2}$ levels on the exercise physiology of four different species of coral reef fishes during both day- and night-time hours. The $p\mathrm{CO_2}$ treatments included ambient (400 µatm), traditionally-used stable elevated $p\mathrm{CO_2}$ (1000 µatm), and fluctuating elevated $p\mathrm{CO_2}$ (1000 ± 300 µatm). The fluctuating elevated $p\mathrm{CO_2}$ was meant to represent future diel cycling on a coral reef (McNeil and Sasse, 2016). Trials were conducted both during the day (08:00) and night (20:00). We used swimming respirometry to calculate maximum sustained swimming speeds (U_{crit}), maximum oxygen uptake rates ($\dot{M}\mathrm{O_{2\ Max}}$), and minimum oxygen uptake rates ($\dot{M}\mathrm{O_{2\ Min}}$), with the latter two used to calculate AS. Following swimming protocols, we also measured haematological parameters that provide information regarding energy use and oxygen transport (i.e., lactate and glucose concentrations, haemoglobin concentration, haematocrit, and mean cell haemoglobin concentration). Initially, *L. fulviflamma* and *C. quinquelineatus* were chosen as nocturnally-active species, and *A. whitleyi* and *C. cuning*

represented diurnally-active species. *Lutjanus fulviflamma* are often thought to be purely nocturnal (Connell, 1998; Dorenbosch et al., 2004; Newman, 1995); however, some studies suggest that they are opportunistic feeders that are active both during the day and at night (Kamukuru and Mgaya, 2004). Therefore, although *L. fulviflamma* may be mostly nocturnal, this species may also possess adaptations that allow it to be active during the day as well; whereas, *C. quinquelineatus* is known to be purely nocturnal (Marnane and Bellwood, 2002). We hypothesized that: (i) compared to ambient pCO_2 exposed fish, exposure to stable elevated pCO_2 will have negative and stronger impacts on coral reef fishes when compared to fluctuating elevated pCO_2 because coral reef fishes are adapted to fluctuating pCO_2 conditions in their natural habitats; and (ii) coral reef fishes that are active at night may possess mechanisms to maintain performance under elevated pCO_2 conditions, which could be advantageous under future elevated pCO_2 conditions; whereas, the opposite may be the case for fishes that are active during the day when pCO_2 levels are low.

4.3   Methods

Experimental animals

This study was conducted at the Lizard Island Research Station on the Great Barrier Reef, Australia (14°40'S; 145°28'E) between May and August (austral winter) of 2019. Adult *L. fulviflamma* (sample size n = 9-12; standard length, 22.0 ± 2.7 cm, wet mas 245 ± 38.7 g; means ± SD)*, C. cuning* (n = 8-9; 22.0 ± 2.7 cm; 163.2 ± 38.7 g)*, A. whitleyi* (n = 8-9; 10.0 ± 1.2 cm; 29.8 ± 10.3 g)*, and C. quinquelineatus* (n = 9-12; 8.3 ± 1.1 cm; 9.2 ± 3.5 g) were collected from the reefs around Lizard Island and immediately brought back to the laboratory where they were maintained in flow-through seawater aquaria (Table 4-1).

Table 4-1. Seawater parameters across the experiment. Values are means ± SD for daily average, minimum, maximum, and range of pH_T, and pCO_2. Mean ± SD for total alkalinity (TA), temperature, and salinity across the experiment are also shown.

Parameter	pCO_2 treatment		
	Ambient ~400 μatm	Stable elevated ~1000 μatm	Fluctuating elevated ~1000 ± 300 μatm
pH	8.02 ±0.03	7.75 ± 0.06	7.70 ± 0.04
Min. pH	-	-	7.61 ± 0.00
Max. pH	-	-	7.82 ±0.01
pH range	-	-	0.22 ± 0.01
pCO_2 (μatm)	410 ± 31	1082 ± 108	979 ± 152
Min. pCO_2	-	-	708 ± 11
Max. pCO_2	-	-	1249 ± 25
pCO_2 range (μatm)	-	-	541 ± 19
TA (μmol kg^{-1})	2307 ± 36	2318 ± 25	2308 ± 25
Temperature (°C)	24.9 ± 0.9	24.9 ± 1.0	24.8 ± 1.1
Salinity	35.3 ± 0.2	35.3 ± 0.1	35.3 ± 0.2

Both *A. whitleyi* and *C. quinquelineatus* were collected using a monofilament barrier net and hand nets, and *C. cuning* and *L. fulviflamma* were caught using hand lines. Fishes were maintained in ambient flow-through conditions for 4-5 d prior to the onset of experiments. *Caesio cuning, L. fulviflamma,* and *C. quinquelineatus* were fed daily to satiation with pieces of raw prawn, while *A. whitleyi* were fed daily to satiation with INVE NRD G12 Aquaculture Nutrition pellets. All animal care and experimental protocols complied with regulations at James Cook University and Lizard Island Research Station and were approved by the James Cook University Ethics Committee (Approval # A2414). Fishes were collected under permit G19/42131.1 from the Great Barrier Reef Marine Park Authority.

CO₂ treatment

Fishes were exposed to either ambient (~400 µatm), stable elevated pCO_2 (~1000 µatm), or fluctuating elevated pCO_2 (~1000 ± 300 µatm) for 9-11 d (Table 1). The elevated CO_2 treatment matched CO_2 projections for year 2100 under RCP8.5 (Meinshausen et al., 2011). The pCO_2 of the reefs around Lizard Island, according to *in situ* measurements, ranges from 283.6 – 554.5 µatm (Hannan et al., 2020a). Thus, the future fluctuating treatment was chosen as 1000 ± 300 µatm to account for the projected several-fold increase in the magnitude of the fluctuation in the future. The pCO_2 of treatment seawater was manipulated by CO_2-dosing to a set pH. Seawater was pumped from the ocean into replicate 300 L sumps (5-6 per pCO_2 treatment) where it was diffused with ambient air (ambient treatment) or CO_2 to achieve the desired pH (pCO_2 treatment). CO_2 dosing was controlled by solenoid valves (M-Ventil Standard, Aqua Medic, Germany) connected to a pH control system (Aqua Medic AT Control System, Aqua Medic, Germany) with laboratory grade pH electrodes (Neptune Systems, USA). When the solenoid valve opened, CO_2 flowed through a fine needle valve and into the impeller of a small pump (Aqua One Maxi 101 Power Head Pump 400 LPH) where it was immediately dissolved and mixed throughout the sump tank. This method ensured a slow steady flow of CO_2 into the sump and rapid mixing to prevent overdosing that leads to unstable CO_2 treatments. The Aqua Medic AT Control System has a curve function which creates fluctuating pCO_2 profiles (see, Jarrold et al., 2017). The pH profiles in the fluctuating pCO_2 treatment were recorded every other day using a pH meter (pH SD Card Datalogger: Model 850060, Sper Scientific) set to take a reading every 20 min. Temperature was recorded daily with the same model of pH meter. Seawater pH on the total hydrogen ion concentration scale (total scale, pH_T) was measured daily at either the middle, top, or bottom of the fluctuations using a spectrophotometer following standard operating procedures (Dickson et al., 2007) using indicator dye (meta/*m*-cresol purple sodium salt 99%, non-

purified; mCP, Acros Organic). Salinity data were obtained from the Lizard Island Observing

System (LIOS) part of the Integrated Marine Observing System (IMOS) ocean monitoring

sensors deployed at Lizard Island (platform: SF4-Research Bay). Water samples were

collected for total alkalinity (TA) three times for each sump throughout the experimental

period. TA was measured using Gran-titrations (Metrohm 888 Titrando Titrator Metrohm,

AG, Switzerland) and referenced with certified material from Dr. A.G. Dickson (Scripps

Institute of Oceanography, La Jolla, CA). Values of pH_T, TA, salinity, and temperature were

entered into CO2SYS (Lewis and Wallace, 1998) using the constants K1 from (Mehrbach et

al., 1973) refit by (Dickson and Millero, 1987) and (Dickson, 1990) to calculate pCO_2. Mean

salinity, temperature, pH, and carbonate system parameters are reported in Table 1. This

standard method of estimating pCO_2 has equivalent accuracy to direct measurements of pCO_2

using high resolution NDIR (Watson et al., 2017).

Swimming respirometry

All fishes were fasted for 18–24 h prior to experimentation to ensure a post-absorptive state

(Niimi and Beamish, 1974). Oxygen uptake rates ($\dot{M}O_2$) were measured in either a 4.8 L (*A.

whitleyi* and *C. quinquelineatus*) or a 90 L (*C. cuning* and *L. fulviflamma*) Steffensen-type

Plexiglas swimming respirometer (Johansen and Jones, 2011; Roche et al., 2013; Steffensen

et al., 1984) both during the day and at night for all fish from each pCO_2 treatment. The

working section of the 4.8 L swimming chamber was 7.0 × 36.0 × 7.0 cm (width × length ×

depth) and 20.0 × 66.0 × 20.0 cm for the 90 L tunnel, and each respirometer was immersed in

a temperature-controlled water bath. Flow straighteners were used to create laminar flow

within the swimming section, and flow was calibrated using a digital vane wheel flow sensor

(Höntzsch GmbH, Waiblingen, Germany; model #ZS30GFE-md20T). Fish size and solid

blocking effects in the swimming section were corrected following (Bell and Terhune, 1970) and did not exceed 5% for any individual.

Prior to each trial, a fish was placed in the swimming section of the chamber and left to habituate for 6–8 h at a swimming speed of 0.5 body lengths (Bl) s^{-1}. Day trials started at 08:00, and night trials commenced at 20:00. Once the trial started, the speed was incrementally increased by 0.5 Bl s^{-1} every 20 min for the duration of the experiment. Fish in the 4.8 L swimming respirometer swam at each speed for two 10 min cycles. Each 10 min cycle consisted of a 6 min measurement period and a 4 min flush period to replenish the chamber with filtered, well-aerated seawater. Fish in the 90 L swimming respirometer swam at each speed for one 20 min cycle. Each 20 min cycle consisted of a 12 min measurement period and an 8 min flush period. Trials were complete once fish reached their critical swimming speed (U_{crit}), which occurred when they could no longer maintain their position in the swim chamber and were forced to rest against the back grid of the chamber for >10 s (Johansen and Jones, 2011). We calculated U_{crit} following (Brett, 1964):

$$U_{crit} = U + U_i \, (t/t_i),$$

where U is the last swimming speed maintained for 20 min before the fish fatigued and stopped swimming, U_i is the swimming speed at which the fish was unable to continue swimming, t is the length of time the fish swam at the final swimming speed where fatigue occurred, and t_i is the amount of time fish were swam at each speed interval (i.e., 20 min). Dissolved oxygen concentrations were measured every 2 s and logged using a Fire-Sting fibre-optic oxygen meter (Pyroscience, Germany) connected to a computer. The $\dot{M}O_2$ (mg O_2 kg^{-1} h^{-1}) was calculated with LabChart v. 6.1.3 (ADInstruments, Dunedin, New Zealand) as the slope of the linear regression of oxygen concentration decline over time for each cycle using the equation (Bushnell et al., 1994; Schurmann and Steffensen, 1997):

$$\dot{M}O_2 = sV_{resp}M^{-1},$$

where s is the slope (in milligrams of O_2 per litre per second), V_{resp} is the volume of the

respirometer minus the volume of the fish, and M is the mass of the fish (kg). One cycle was

executed before and after each trial to measure background rates of microbial respiration in

each swim tunnel, which were subtracted from $\dot{M}O_2$ values upon calculation. The system was

rinsed in freshwater every third day to ensure that background oxygen uptake rates remained

below 15% of the resting oxygen uptake rate of the fish. We calculated $\dot{M}O_2$ at $U = 0.5$ Bl s^{-1}

by averaging the three lowest $\dot{M}O_2$ measurements ($\dot{M}O_{2\ Min}$) before increasing U to start the

U_{crit} test (Schurmann and Steffensen, 1997). Maximum metabolic rate was estimated from the

$\dot{M}O_2$ that was measured at the maximum swimming speed where fish completed at least one

10 min cycle ($\dot{M}O_{2\ Max}$). Absolute aerobic scope (AS $= \dot{M}O_{2\ Max} - \dot{M}O_{2\ Min}$) was calculated for

each fish.

Metabolite and haematological parameters

Immediately following the U_{crit} test, fishes were cranially concussed, and blood was drawn

from the caudal artery/vein via a lithium heparin rinsed syringe (1 mL syringe, 23-G needle)

according to A2414 James Cook University Animal Ethics Committee protocols. Blood

samples were used to determine haemoglobin concentration ([Hb]), hematocrit (Hct), and

whole blood glucose and lactate concentrations. Haemoglobin concentration was determined

using 10 µl of whole blood and the HemoCue Hb 201 system, Australia Pty Ltd, Tumbi Umb,

NSW, Australia. The [Hb] whole blood is reported as grams per 100 mL using a calibration

curve according to (Clark et al., 2008) corrected for tropical reef species by (Rummer et al.,

2013a). The Hct was determined by centrifuging 60 µl of whole blood in heparinized micro-

capillary tubes for 3 min and calculated as the ratio of packed red blood cells to total blood

volume (as a percentage). Both [Hb] and Hct were used to calculate the mean corpuscular

haemoglobin concentration (MCHC) of the blood. Whole blood glucose and lactate

concentrations (millimolar) were determined from two 15 µl samples using the Accutrend

Plus (Roche Diagnostics Australia Pty Ltd Dee Why, NSW, Australia).

Statistical analyses

Bayesian linear mixed models were used to analyse differences in the U_{crit}, absolute aerobic

scope, $\dot{M}O_{2\,Max}$, $\dot{M}O_{2\,Min}$, [Hb], Hct, MCHC, lactate, and glucose of *A. whitleyi, C.*

quinquelineatus, C. cuning, and L. fulviflamma between pCO_2 treatments and time of day.

Analyses were conducted in R version 3.3.2 (R Core Team, 2017), and the models were fit in

STAN with Markov chain Monte Carlo sampling (Carpenter et al., 2017) using the rstanarm

package version 2.13.1 (Goodrich et al., 2018). The broom (version 0.4.4; Robinson, 2017)

and emmeans (version 1.3.2; Lenth, 2016) packages were used to summarise model outputs

using highest posterior density intervals with a probability level of 0.95. Plots were produced

using ggplot2 version 2.2.1 (Wickham and Chang, 2008). To analyse differences in measured

variables the generalized linear mixed model 'stan_glm' was used. The additive models

included pCO_2 treatment (i.e., ambient vs. stable elevated vs. fluctuating elevated) and time

of day (day vs. night) as fixed factors. All models used a Gaussian error distribution with

5000 iterations, a warmup of 500, 3 chains, and a thinning factor of two. For all models,

diagnostic plots were visually examined to ensure there was convergence of chains and no

evidence of heteroscedasticity or autocorrelation. Medians and central intervals from prior

and posterior distributions were compared to ensure that the chosen priors were sufficiently

wide so as to not dictate any trends, without being flat (non-informative).

We used the highest posterior density uncertainty intervals. In this study, strong evidence for

an effect (i.e., statistical 'significance') is defined when the 95% uncertainty interval (UI)

does not intersect with zero. Moderate evidence for an effect is inferred when 85% of the UI lies to one side of zero.

4.4   Results

Critical swimming speeds (U_{crit})

There was strong evidence that the U_{crit} of *L. fulviflamma* exposed to fluctuating and stable elevated pCO_2 conditions was elevated compared to fish under ambient pCO_2 conditions (100 and 96% UI, respectively). *Lutjanus fulviflamma* exhibited a 16% higher U_{crit} under fluctuating elevated pCO_2 conditions and an 11% higher U_{crit} under stable elevated pCO_2 conditions when compared to fish from ambient pCO_2 conditions (Figure 4-1A, Appendix C - Table S1). *Lutjanus fulviflamma* maintained under fluctuating elevated pCO_2 conditions exhibited a U_{crit} that was 6% higher than *L. fulviflamma* from stable elevated pCO_2 conditions (86% UI: Figure 4-1A, Appendix C - Table S1). *Caesio cuning* maintained under fluctuating elevated pCO_2 conditions exhibited a U_{crit} that was 6% higher than fish from ambient pCO_2 and stable elevated pCO_2 conditions (85% UI: Figure 4-1B, Appendix C - Table S1). However, *C. cuning* examined during the day exhibited a U_{crit} that was 5% higher than fish examined at night (86% UI: Figure 4-1B, Appendix C - Table S1). *Abudefduf whitleyi* maintained under fluctuating elevated pCO_2 conditions exhibited a U_{crit} that was 6% higher than fish maintained under ambient pCO_2 conditions (97% UI: Figure 4-1C, Appendix C - Table S1), and those exposed to stable elevated pCO_2 conditions exhibited a 5% higher U_{crit} than fish maintained under ambient pCO_2 conditions (92% UI: Figure 4-1C, Appendix C - Table S1). *Cheilodipterus quinquelineatus* maintained under either of the elevated pCO_2 treatments did not exhibit changes in U_{crit} exposed when compared to the ambient treatment (Figure 4-1D, Appendix C - Table S1). However, *C. quinquelineatus* maintained under

fluctuating elevated pCO_2 conditions, they exhibited a U_{crit} that was 4% higher than that of *C. quinquelineatus* maintained under stable elevated pCO_2 conditions (89% UI: Figure 4-1D, Appendix C - Table S1).
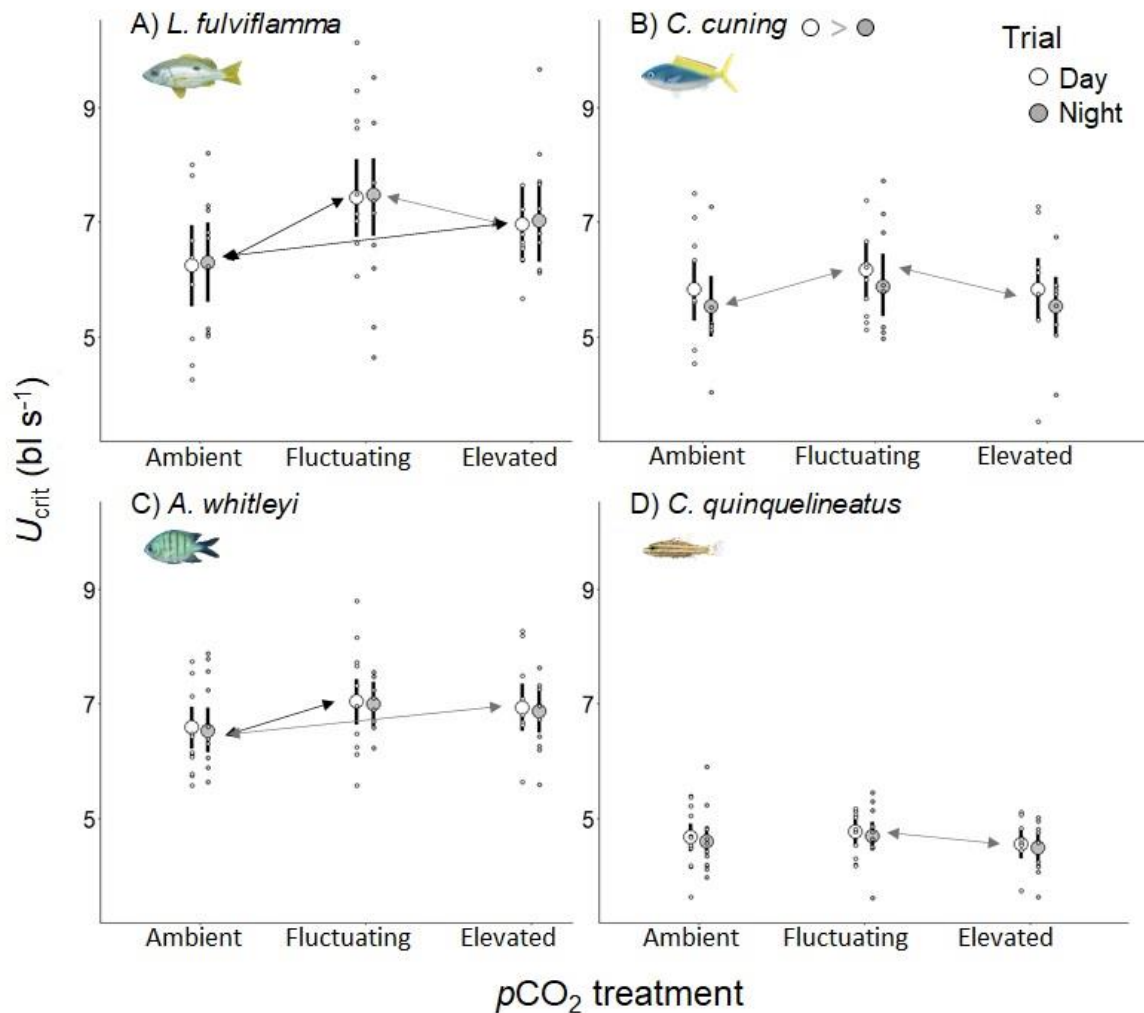
Figure 4-1. The Ucrit of A) *Lutjanus fulviflamma*, B) *Caesio cuning*, C) *Abudefduf whitleyi*, and D) *Cheilodipterus quinquelineatus* exposed to one of three different $pCO2$ treatments (ambient 400 µatm, stable elevated 1,000 µatm, and fluctuating elevated 1,000 ± 300 µatm). Circles represent least-squares means and error bars represent 95% confidence intervals. Differences between pCO_2 treatments are displayed by black arrows (strong evidence for an effect: i.e., > 95% of the UI does not intersected zero) and grey arrows (moderate evidence for an effect: i.e., > 85% of the UI does not intersected zero). Differences between time of day are depicted by greater than or less than symbols (i.e., ○ > ● or ○ < ●).

Maximum O$_2$ uptake rates ($\dot{M}O_{2\ Max}$)

The results for $\dot{M}O_{2\ Max}$ mirrored that of U_{crit} for *L. fulviflamma*. When this species was maintained under fluctuating or stable elevated pCO_2 conditions, $\dot{M}O_{2\ Max}$ was 40% and 22% higher, respectively, when compared to fish maintained under ambient pCO_2 conditions (100 and 88% UI, respectively: Figure 4-2A, Appendix C - Table S1). Further, *L. fulviflamma* maintained under fluctuating elevated pCO_2 conditions exhibited a 24% higher $\dot{M}O_{2\ Max}$ than fish maintained under stable elevated pCO_2 conditions (96% UI: Figure 4-2A, Appendix C - Table S1). When *C. cuning* were maintained under fluctuating elevated pCO_2 conditions, their $\dot{M}O_{2\ Max}$ was 13% higher than that of fish maintained under ambient pCO_2 conditions (88% UI: Figure 4-2B, Appendix C - Table S1). The $\dot{M}O_{2\ Max}$ of *A. whitleyi* also mirrored the U_{crit} results. *A. whitleyi* maintained under fluctuating or stable elevated pCO_2 conditions exhibited $\dot{M}O_{2\ Max}$ values that were 8% higher than fish maintained under ambient pCO_2 conditions (85 and 87% UI, respectively: Figure 4-2C, Appendix C - Table S1). When *C. quinquelineatus* were maintained under fluctuating elevated pCO_2 conditions, they exhibited $\dot{M}O_{2\ Max}$ values that were 12% lower than fish maintained under ambient pCO_2 conditions and 12% lower than fish maintained under stable elevated pCO_2 conditions (96 and 85% UI, respectively; Figure 4-2D, Appendix C - Table S1).
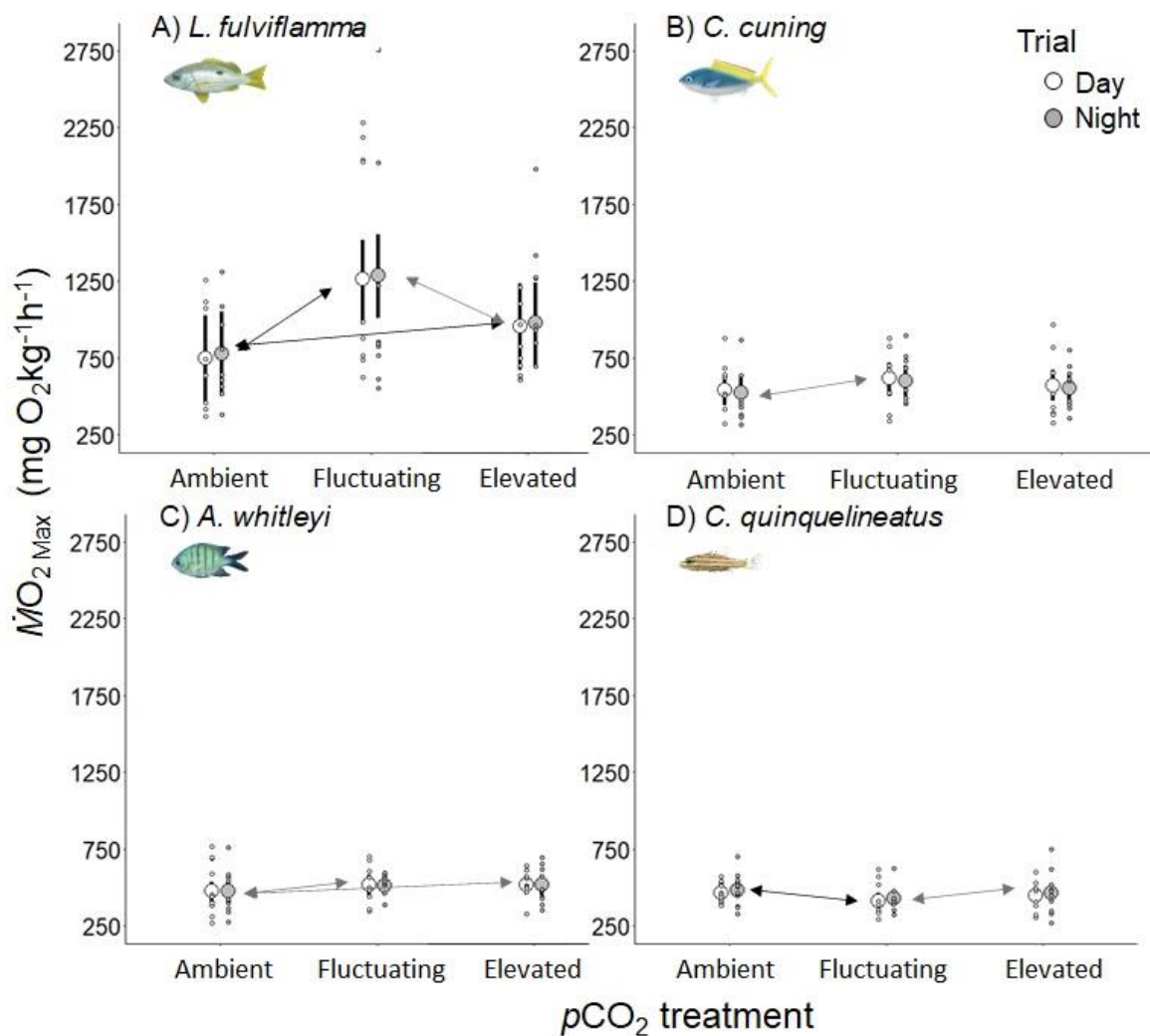
Figure 4-2. The $\dot{M}O_{2\,Max}$ of A) *Lutjanus fulviflamma*, B) *Caesio cuning*, C) *Abudefduf whitleyi*, and D) *Cheilodipterus quinquelineatus* exposed to one of three different pCO_2 treatments (ambient 400 µatm, stable elevated 1,000 µatm, and fluctuating elevated 1,000 ± 300 µatm). The circles represent least-squares means and error bars represent 95% confidence intervals. Differences between pCO_2 treatments are displayed by black arrows (strong evidence for an effect: i.e., > 95% of the UI does not intersected zero) and grey arrows (moderate evidence for an effect: i.e., > 85% of the UI does not intersected zero). Differences between time of day are depicted by greater than or less than symbols (i.e., ○ > ● or ○ < ●).

Minimum O_2 uptake rates ($\dot{M}O_{2\,Min}$)

C. cuning, *A. whitleyi*, and *C. quinquelineatus*, when examined during the night, exhibited $\dot{M}O_{2\,Min}$ values that were, respectively, 11, 8, and 15% higher than the $\dot{M}O_{2\,Min}$ values for each species when examined during the day (95, 93, and 95% UI, respectively: Figure

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4-3B/C/D, Appendix C - Table S1). By contrast, *L. fulviflamma* exhibited 7% higher $\dot{M}O_{2\text{ Min}}$

values during the day than those measured at night (85% UI, Figure 4-3A, Appendix C - Table

S1). Additionally, when *A. whitleyi* were maintained under stable elevated pCO$_2$ conditions,

they exhibited $\dot{M}O_{2\text{ Min}}$ values that were 8% lower than when fish were maintained under

ambient pCO$_2$ conditions (89% UI: Figure 4-3C, Appendix C - Table S1).
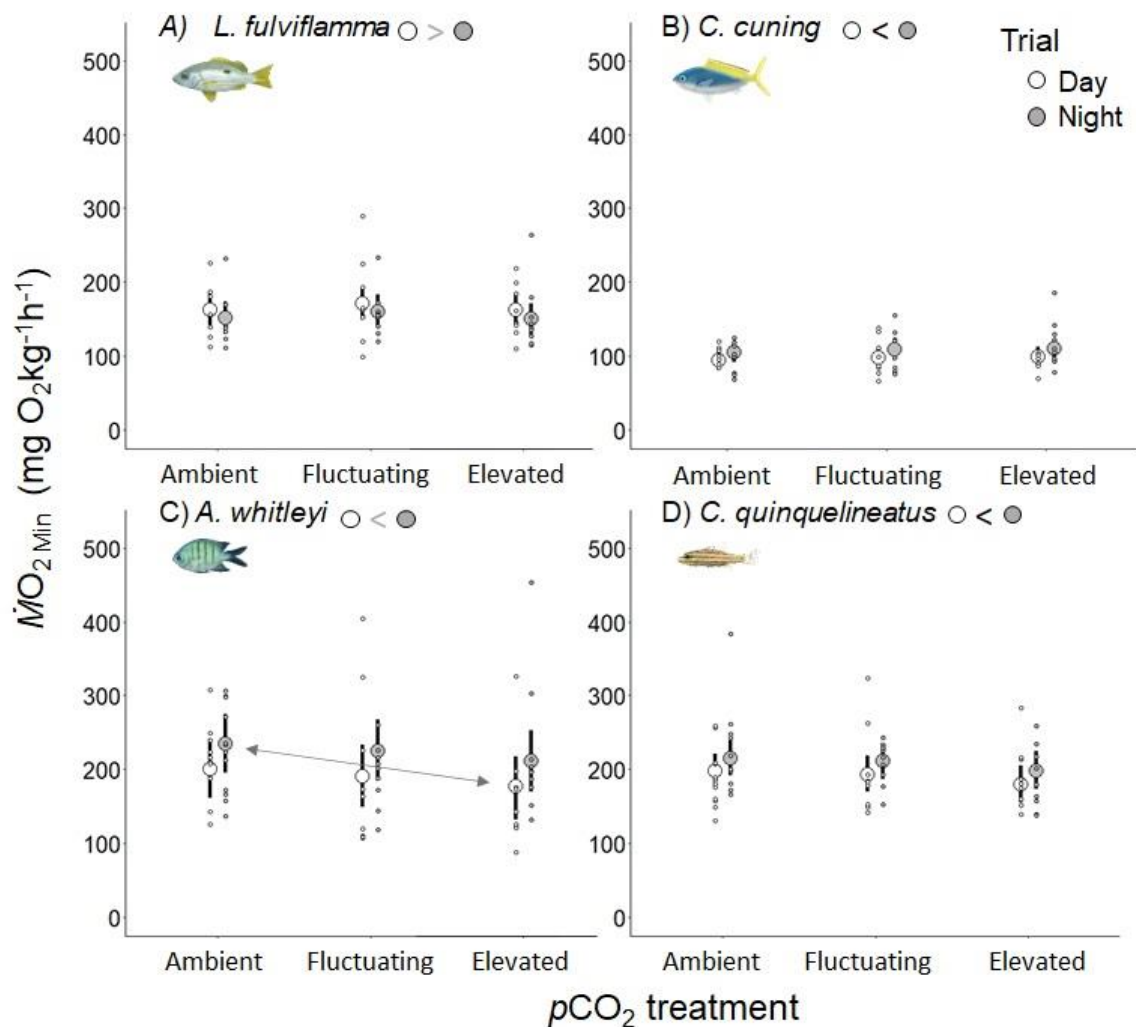
Figure 4-3. The $\dot{M}O_{2\text{ Min}}$ of A) *Lutjanus fulviflamma*, B) *Caesio cuning*, C) *Abudefduf whitleyi*, and D) *Cheilodipterus quinquelineatus* exposed to one of three different pCO$_2$ treatments (ambient 400 µatm, stable elevated 1,000 µatm, and fluctuating elevated 1,000 ± 300 µatm). The circles represent least-squares means and error bars represent 95% confidence intervals. Differences between pCO$_2$ treatments are displayed by black arrows (strong evidence for an effect: i.e., > 95% of the UI does not intersected zero) and grey arrows (moderate evidence for an effect: i.e., > 85% of the UI does not intersected zero). Differences between time of day are depicted by greater than or less than symbols (i.e., ○ > ● or ○ < ●).

Aerobic scope

The aerobic scope (i.e., the difference between $\dot{M}O_{2\,Max}$ and $\dot{M}O_{2\,Min}$) of *L. fulviflamma* exhibited the same trends that were observed for U_{crit} and $\dot{M}O_{2\,Max}$. When *L. fulviflamma* were maintained under fluctuating or stable elevated pCO_2 conditions, their aerobic scope was, respectively, 46% and 26% higher than the AS of fish maintained under ambient pCO_2 conditions (100 and 89% UI; Figure 4-4A, Appendix C - Table S1). Additionally, when *L. fulviflamma* were maintained under fluctuating elevated pCO_2 conditions, their AS was 27% higher than the AS of fish maintained under stable elevated pCO_2 conditions (96% UI: Figure 4-4A, Appendix C - Table S1). *Caesio cuning* exhibited the same trends in AS as those observed for $\dot{M}O_{2\,Max}$. Specifically, when *C. cuning* were maintained under fluctuating elevated pCO_2 conditions, their AS was 15% higher than the AS of fish maintained under ambient pCO_2 conditions (89% UI: Figure 4-4B, Appendix C - Table S1). When *A. whitleyi* were maintained under fluctuating or stable elevated pCO_2 conditions, their AS was, respectively, 18% and 14% higher than the AS of fish maintained under ambient pCO_2 conditions (88 and 95% chance, respectively: Figure 4-4C, Appendix C - Table S1). *Cheilodipterus quinquelineatus* exhibited the same trends in AS as those observed for $\dot{M}O_{2\,Max}$, where fish maintained under fluctuating elevated pCO_2 conditions exhibited an AS that was 17% lower than the AS of fish from ambient pCO_2 conditions and 19% lower than fish from stable elevated pCO_2 conditions (96 and 98% UI, respectively: Figure 4-4D, Appendix C - Table S1).
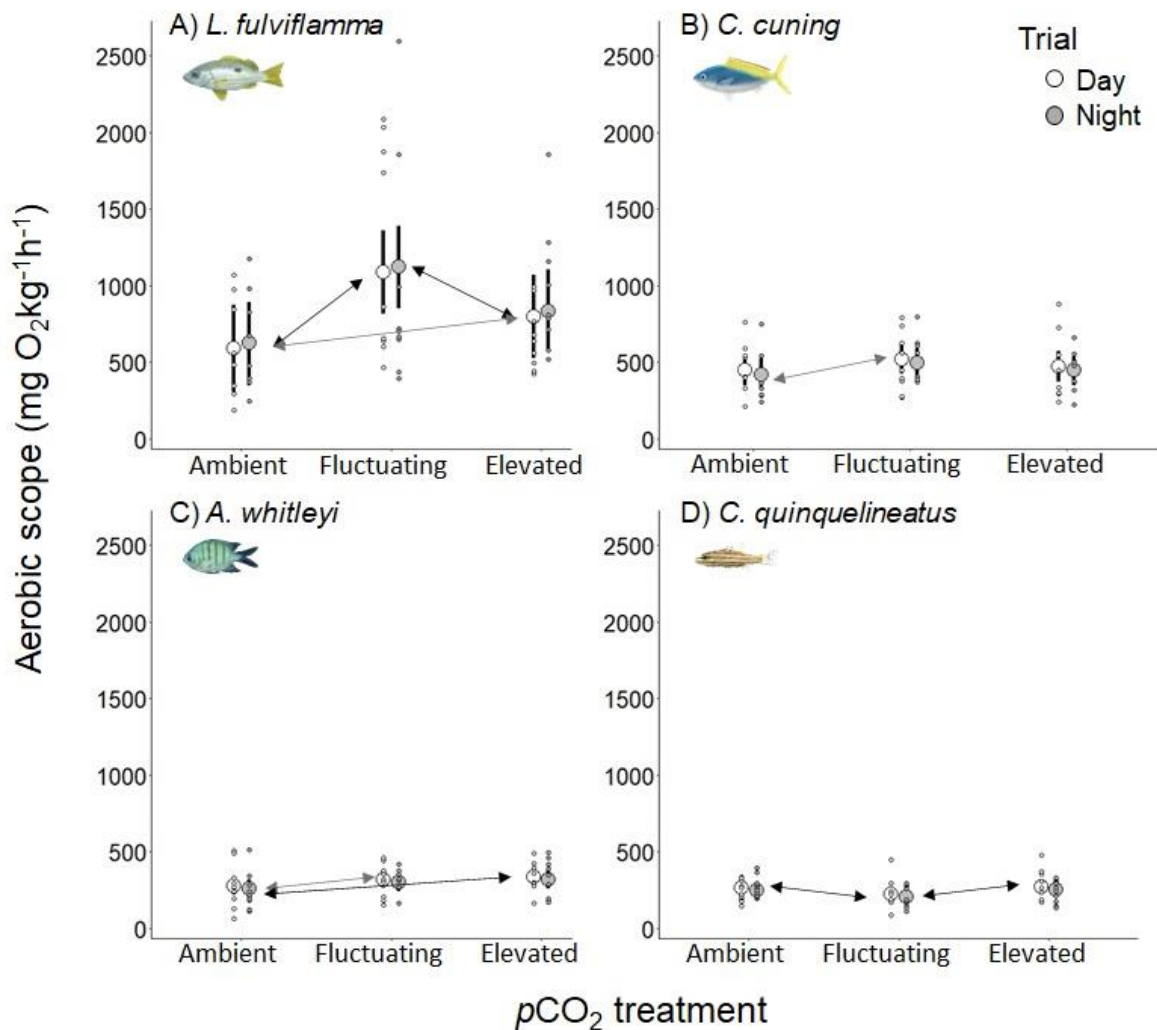
Figure 4-4. The aerobic scope of A) *Lutjanus fulviflamma*, B) *Caesio cuning*, C) *Abudefduf whitleyi*, and D) *Cheilodipterus quinquelineatus* exposed to one of three different pCO_2 treatments (ambient 400 µatm, stable elevated 1,000 µatm, and fluctuating elevated $1,000 \pm 300$ µatm). The circles represent least-squares means and error bars represent 95% confidence intervals. Differences between pCO_2 treatments are displayed by black arrows (strong evidence for an effect: i.e., > 95% of the UI does not intersected zero) and grey arrows (moderate evidence for an effect: i.e., > 85% of the UI does not intersected zero). Differences between time of day are depicted by greater than or less than symbols (i.e., ○ > ● or ○ < ●).

$\dot{M}O_2$ across the U_{crit} trial

Throughout each swimming test, $\dot{M}O_2$ was elevated at night when compared to the day for *L. fulviflamma, C. cuning,* and *C. quinquelineatus* but unaffected by time of day for *A. whitleyi*. Additionally, $\dot{M}O_2$ increased from the beginning of the swimming trial to U_{crit} by

~988%, 633%, 218% and 171% for *L. fulviflamma, C. cuning, A. whitleyi*, and *C. quinquelineatus,* respectively (Appendix C - Figures S1-S4).

Metabolites

There was no evidence (i.e., >85% of the UI intersected zero) of any effects of pCO_2 conditions between day and night time trials on whole blood lactate or glucose concentrations in *L. fulviflamma* or *A. whitleyi* (Appendix C - Figure S5, Table 4-2). When *C. cuning* were maintained under stable elevated pCO_2 conditions, their whole blood lactate concentrations were 36% lower than concentrations measured in fish maintained under ambient pCO_2 conditions and 39% lower than concentrations measured in fish maintained under fluctuating elevated pCO_2 conditions (90 and 93% UI, respectively: Appendix C - Figure S5, Table 4-2). When *C. cuning* were maintained under fluctuating elevated pCO_2 conditions, their whole blood glucose concentrations were 26% higher than concentrations measured in fish maintained under ambient pCO_2 conditions (92% UI: Appendix C - Figure S6, Table 4-2). When *C. quinquelineatus* were maintained under fluctuating elevated pCO_2 conditions, their whole blood lactate concentrations were 38% lower than concentrations measured in fish maintained under ambient pCO_2 conditions and 32% lower than concentrations measured in fish maintained under stable elevated pCO_2 conditions (92 and 87% UI, respectively: Appendix C - Figure S5, Table 4-2). When *C. quinquelineatus* were examined during daytime hours, their whole blood glucose concentrations were 24% higher than concentrations determined for fish during night time hours (95% UI, respectively: Appendix C - Figure S6, Table 4-2).

Table 4-2. Bayesian posterior means, 95% highest posterior density uncertainty intervals (UI), and the amount of UI that intersect 0 (%) of whole blood lactate and glucose. Bolded values indicate that the % UI that does not intersect 0 is more than 85%, i.e., moderate evidence of an effect. Bold values with an asterisk (*) indicate that the % UI that does not intersection with 0 is above than 95%, i.e., strong evidence of an effect.

Variables	Contrasts	*Lutjanus fulviflamma*				*Caesio cuning*				*Abudefduf whitleyi*				*Cheilodipterus quinquelineatus*			
		Mean	Lower UI	Upper UI	UI (%)	Mean	Lower UI	Upper UI	UI (%)	Mean	Lower UI	Upper UI	UI (%)	Mean	Lower UI	Upper UI	UI (%)
Lactate (mM)	Ambient - Fluctuating	-0.71	-3.82	2.65	67	-0.32	-3.58	3.14	58	0.12	-0.229	0.47	75	2.21	-0.83	5.51	92
	Ambient - Stable	-1.28	-4.62	1.95	78	2.24	-1.08	5.86	90	0.10	-0.271	0.44	71	1.84	-1.45	5.18	87
	Fluctuating - Stable	-0.57	-3.59	2.71	65	2.60	-0.82	6.11	93	-0.02	-0.37	0.37	54	-0.38	-3.52	2.84	60
	Day - Night	1.09	-1.43	3.82	80	1.07	-1.83	3.90	76	0.13	-0.161	0.43	80	1.02	-1.65	3.60	78
Glucose (mM)	Ambient - Fluctuating	-0.25	-3.52	2.88	56	-1.18	-2.78	0.49	92	-0.33	-1.31	0.64	75	0.90	-0.92	2.76	83
	Ambient - Stable	-0.35	-3.42	2.80	59	-0.64	-2.22	1.00	78	-0.29	-1.31	0.71	72	0.43	-1.41	2.34	67
	Fluctuating - Stable	-0.12	-3.10	3.02	53	0.54	-1.15	2.10	74	0.03	-1.02	1.08	52	-0.48	-2.18	1.33	71
	Day - Night	-0.90	-3.53	1.63	76	0.08	-1.22	1.49	55	0.28	-0.53	1.11	76	1.24	-0.20	2.81	95*

Haematological parameters

When *L. fulviflamma* were examined during daytime hours, their [Hb] was 4% higher than concentrations determined for fish examined at night (96% UI: Figure 4-5A, Appendix C - Table S2). Similarly, when *L. fulviflamma* were examined at night, Hct was 3% lower than *L. fulviflamma* examined during daytime hours (88% UI: Figure 4-6A, Appendix C - Table S2). When *L. fulviflamma* were maintained under stable elevated pCO_2 conditions exhibited MCHC values that were 4% higher than in fish from ambient pCO_2 conditions (91% UI, Figure 4-7A, Appendix C - Table S2). Neither pCO_2 nor time of day affected the [Hb] of *C. cuning* blood (Appendix C - Table S2). However, when *C. cuning* were maintained under fluctuating elevated pCO_2 conditions, their Hct values were 6% higher than those measured in fish maintained under stable elevated pCO_2 conditions (90% UI: Figure 4-6B, Appendix C - Table S2). There was no evidence that pCO_2 or time of day affected MCHC in *C. cuning* (Figure 4-7B, Appendix C - Table S2). When *A. whitleyi* were maintained under fluctuating elevated pCO_2 conditions, they exhibited [Hb] that were 5% higher than concentrations measured in fish maintained under ambient pCO_2 conditions (89% UI: Figure 4-5C, Appendix C - Table S2). When *A. whitleyi* were maintained under stable elevated pCO_2 conditions, Hct values were 4% lower than Hct values in fish maintained under ambient pCO_2 conditions and 5% lower than Hct values in fish maintained under fluctuating elevated pCO_2 conditions (90 and 95% UI, respectively; Figure 4-6C, Appendix C - Table S2). Further, when *A. whitleyi* were examined during night time hours, their Hct values were 3% higher than fish examined during the day (90% UI: Figure 4-6C, Appendix C - Table S2). When *A. whitleyi* were maintained under stable elevated pCO_2 conditions their MCHC was 5% higher than MCHC values measured in fish maintained under ambient pCO_2 conditions (92% UI). When *A. whitleyi* were examined during night time hours, MCHC was 4% lower than when fish were examined during the day (90% UI: Figure 4-7C, Appendix C - Table S2). When *C.*

*quinquelineatu*s were maintained under fluctuating elevated pCO_2 conditions, both [Hb] and

Hct were each 5% higher than fish maintained stable elevated pCO_2 conditions (93 and 86%

UI, respectively; Figure 4-5D and Figure 4-6D, Appendix C - Table S2). However, there was

no evidence that pCO_2 or time of day affected MCHC in *C. quinquelineatus* (Figure 4-7D,

Appendix C - Table S2).
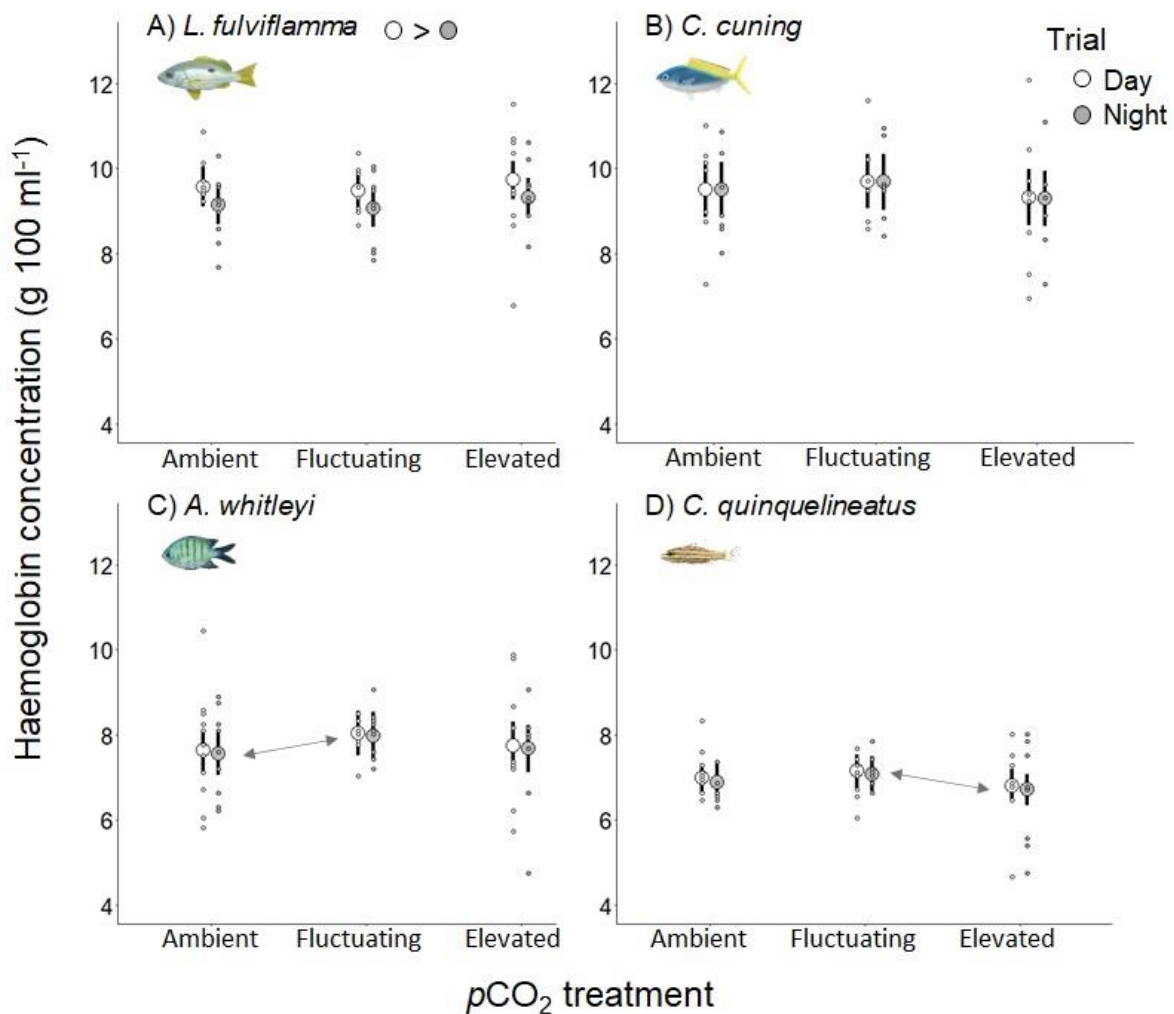
Figure 4-5. The concentration of haemoglobin ([Hb]) of A) *Lutjanus fulviflamma*, B) *Caesio cuning*, C) *Abudefduf whitleyi*, and D) *Cheilodipterus quinquelineatus* exposed to one of three different pCO_2 treatments (ambient 400 µatm, stable elevated 1,000 µatm, and fluctuating elevated $1,000 \pm 300$ µatm). Circles represent least-squares means and error bars represent 95% confidence intervals. Differences between pCO_2 treatments are displayed by black arrows (strong evidence for an effect: i.e., > 95% of the UI does not intersected zero) and grey arrows (moderate evidence for an effect: i.e., > 85% of the UI does not intersected zero). Differences between time of day are depicted by greater than or less than symbols (i.e., ○ > ● or ○ < ●).

Figure 4-6. The haematocrit (expressed as a percent to represent the ratio of packed red blood cells to total blood volume) of A) *Lutjanus fulviflamma*, B) *Caesio cuning*, C) *Abudefduf whitleyi*, and D) *Cheilodipterus quinquelineatus* exposed to one of three different pCO_2 treatments (ambient 400 µatm, stable elevated 1,000 µatm, and fluctuating elevated 1,000 ± 300 µatm). Circles represent least-squares means and error bars represent 95% confidence intervals. Differences between pCO_2 treatments are displayed by black arrows (strong evidence for an effect: i.e., > 95% of the UI does not intersected zero) and grey arrows (moderate evidence for an effect: i.e., > 85% of the UI does not intersected zero). Differences between time of day are depicted by greater than or less than symbols (i.e., ○ > ● or ○ < ●).

Figure 4-7. The mean cell haemoglobin concentration of A) *Lutjanus fulviflamma*, B) *Caesio cuning*, C) *Abudefduf whitleyi*, and D) *Cheilodipterus quinquelineatus* exposed to one of three different pCO$_2$ treatments (ambient 400 µatm, stable elevated 1,000 µatm, and fluctuating elevated 1,000 ± 300 µatm). Circles represent least-squares means and error bars represent 95% confidence intervals. Differences between pCO$_2$ treatments are displayed by black arrows (strong evidence for an effect: i.e., > 95% of the UI does not intersected zero) and grey arrows (moderate evidence for an effect: i.e., > 85% of the UI does not intersected zero). Differences between time of day are depicted by greater than or less than symbols (i.e., ○ > ● or ○ < ●).

4.5   Discussion

Our findings support the hypothesis that when fish are maintained under fluctuating pCO$_2$

conditions, traits associated with exercise physiology respond differently than when fish are

maintained under traditionally-used stable elevated pCO$_2$ conditions. However, contrary to

our predictions, we did not observe negative impacts, but rather some positive impacts, in

fish maintained under stable elevated pCO_2 conditions when compared to fish maintained under ambient pCO_2 conditions. Further, when fish were maintained under fluctuating elevated pCO_2 conditions, swimming and oxygen uptake metrics were positively affected in three of the four species investigated. We also predicted that nocturnal fishes, given that they would be most active during times when pCO_2 on the reef would be naturally high, would possess mechanisms to maintain or enhance physiological performance under elevated pCO_2 conditions; however, our results suggested the contrary. The diurnal species tested in this study benefited upon exposure to fluctuating pCO_2 conditions in terms of swimming performance and energy availability when compared to their counterparts from ambient conditions. However, the one nocturnal species examined showed no such benefits in terms of swimming performance and even exhibited decreased energy availability. Therefore, while most species we tested seem robust to future OA conditions on coral reefs, there are species-specific differences that may suggest greater sensitivity in this nocturnal fish species.

Maximum sustained swimming speed (U_{crit})

When exposed to fluctuating elevated pCO_2 conditions, three of the four species examined, *L. fulviflamma*, *C. cuning*, and *A. whitleyi*, exhibited a higher U_{crit} than their counterparts from ambient conditions. This enhanced U_{crit} pattern was also evident in *L. fulviflamma* and *A. whitleyi* maintained under stable elevated pCO_2 conditions. To our knowledge, an increase in maximum sustained swimming speed in response to elevated pCO_2 has not been previously documented. Most studies have found no effect of stable elevated pCO_2 conditions on U_{crit} (Bignami et al., 2017, 2014, 2013; Cominassi et al., 2019; Hamilton et al., 2017; Kunz et al., 2018; Melzner et al., 2009; Munday et al., 2009b; Silva et al., 2016), and several recent studies have even found decreases in U_{crit} (McMahon et al., 2020; Watson et al., 2018). However, most previous studies have been performed on juvenile or larval life stages. This

suggest that early life-stages may be more sensitive to elevated pCO_2, possibly due to concurrent development of their physiological regulatory mechanisms (Grosell et al., 2019). Indeed, a previous study found two species of adult damselfishes maintained swimming performance upon brief (i.e., 8h) exposure to fluctuating or stable elevated pCO_2 conditions (Hannan et al., 2020b). There is a possibility that the increases in U_{crit} documented in the current study are driven by increases in energy availability (e.g., maximum oxygen uptake rates and aerobic scope).

Minimum oxygen uptake rates ($\dot{M}O_{2\ Min}$)

The amount of energy required by organisms to perform basic maintenance tasks is typically represented by $\dot{M}O_{2\ Min}$. The $\dot{M}O_{2\ Min}$ of this study was determined when the fish was habituating to the swimming respirometer while exposed to minimal water flow and therefore may also include the energy necessary to remain upright and face into the current. Exposure to elevated pCO_2 is hypothesized to increase $\dot{M}O_{2\ Min}$ in fishes due to increased acid-base regulation requirements (Strobel et al., 2013). However, the majority of published studies show that $\dot{M}O_{2\ Min}$ is not affected by elevated pCO_2 (reviewed in Hannan and Rummer, 2018; Lefevre, 2016). Similarly, this study found that fluctuating pCO_2 conditions did not affect $\dot{M}O_{2\ Min}$ of four coral reef fish species. This is also consistent with most previous studies investigating the effects of stable versus fluctuating pCO_2 on $\dot{M}O_{2\ Min}$ that show $\dot{M}O_{2\ Min}$ to be unaffected by fluctuating pCO_2 conditions (Laubenstein et al., 2020; Methling et al., 2013; Ou et al., 2015). Similarly, stable elevated pCO_2 conditions did not affect $\dot{M}O_{2\ Min}$ for three of the four species we examined. The exception was *A. whitleyi* for which $\dot{M}O_{2\ Min}$ decreased in response to exposure to stable elevated pCO_2. Other studies have observed decreases in $\dot{M}O_{2\ Min}$ in response to elevated pCO_2 (Pimentel et al., 2014; Rummer et al., 2013a), which may represent a decrease in the amount of energy required to perform basic maintenance tasks. It

is not yet understood why elevated pCO_2 should result in reduced energetic demands. However, it may be that OA relevant pCO_2 levels are lower than levels in the plasma pCO_2 of resting fish (Esbaugh et al., 2012) and may therefore not be stressful for fishes. Moreover, the influence that changes of $\dot{M}O_{2\ Min}$ in response to elevated pCO_2 has on AS is relatively weak; whereas, changes of $\dot{M}O_{2\ Max}$ in response to elevated pCO_2 typically have a greater influence on AS (Lefevre, 2016). This is consistent with our results, where AS was influenced almost exclusively by $\dot{M}O_{2\ Max}$. The only consistent response in $\dot{M}O_{2\ Min}$ that was observed across three of the four species was that $\dot{M}O_{2\ Min}$ was higher at night than during daytime hours, regardless of pCO_2 treatment. This may suggest that some coral reef fishes perform more maintenance tasks at night and require more energy to perform basic tasks at night than during the day.

Maximum oxygen uptake rates ($\dot{M}O_{2\ Max}$)

The maximum amount of oxygen that is taken up during peak aerobic exercise is represented in this study by $\dot{M}O_{2\ Max}$. As expected, increases in $\dot{M}O_{2\ Max}$ observed in this study corresponded to increases in U_{crit}. On the other hand, the fishes that exhibited no change or a decrease in $\dot{M}O_{2\ Max}$ were unable to increase U_{crit}. When *L. fulviflamma*, *C. cuning*, and *A. whitleyi* were exposed to fluctuating pCO_2 conditions, they exhibited an increase in $\dot{M}O_{2\ Max}$ when compared to fishes from ambient pCO_2 conditions. The opposite occurred for *C. quinquelineatus*, where exposure to fluctuating pCO_2 conditions resulted in a lower $\dot{M}O_{2\ Max}$ compared to fish from ambient pCO_2 conditions. In two of the four previous studies investigating oxygen uptake metrics of fishes exposed to fluctuating elevated pCO_2, the negative effects of stable elevated pCO_2 conditions on $\dot{M}O_{2\ Max}$ were ameliorated under fluctuating pCO_2 conditions (Methling et al., 2013; Ou et al., 2015); whereas, no effects of stable or fluctuating elevated pCO_2 on $\dot{M}O_{2\ Max}$ were observed in the other two studies

(Hannan et al., 2020b; Laubenstein et al., 2020). Interestingly, in most of these previous studies, there was no difference in the $\dot{M}O_2$ $_{Max}$ of fishes exposed to fluctuating and ambient pCO_2 conditions, which was not the case in the current study. We found that fishes exposed to fluctuating elevated pCO_2 conditions either exhibited a higher or lower $\dot{M}O_2$ $_{Max}$ when compared to their ambient pCO_2 counterparts. Further, two fish species in this study, *L. fulviflamma* and *A. whitleyi*, also increased $\dot{M}O_2$ $_{Max}$ upon exposure to stable elevated pCO_2 conditions when compared to ambient exposed fishes. The previous studies have observed increases in $\dot{M}O_2$ $_{Max}$ upon exposure to stable elevated pCO_2 have attributed the response to increases in respiratory surface area (Rummer et al., 2013a), increases in oxygen release to tissues during hypercapnia (Rummer and Brauner, 2015; Rummer et al., 2013b), or removal of the behavioural inhibition that suppresses maximal exercise, leading to increases in U_{crit} (Couturier et al., 2013; Kunz et al., 2018; Rummer et al., 2013a). These proposed mechanisms can result in a range of responses at the level of oxygen uptake metrics ($\dot{M}O_2$ $_{Max}$, $\dot{M}O_2$ $_{Min}$, and aerobic scope).

Aerobic scope (AS)

Aerobic scope – the difference between $\dot{M}O_2$ $_{Max}$ and $\dot{M}O_2$ $_{Min}$ – represents the energy available above what is required for basic maintenance, energy that can be used for life-history processes, such as, but not limited to, swimming, foraging, growth, and reproduction. Three of the four species examined here increased AS upon exposure to one or both elevated pCO_2 treatments. However, for one species, *C. quinquelineatus*, a decrease in AS was observed in response to fluctuating elevated pCO_2 when compared to ambient or stable elevated pCO_2 exposed fish. This suggests that this elevated fluctuating exposure was more energetically costly to *C. quinquelineatus* than the other two treatments. Though elevated pCO_2 was initially predicted to have negative impacts on AS due to increased energy demand

(Pörtner and Farrell, 2008), most of the fishes tested, including those in the current study, have exhibited no impact of elevated pCO_2 on AS (Hannan and Rummer, 2018; Lefevre, 2016) and even increases in AS in response to elevated pCO_2 (Couturier et al., 2013; Gräns et al., 2014; Hannan et al., 2020b; Kunz et al., 2018; Rummer et al., 2013a). Previously observed increases in AS of fishes in response to stable elevated pCO_2 have been attributed to increases in cardiac function (Gräns et al., 2014) and/or increased release of oxygen from the haemoglobin to select tissues during hypercapnia (Rummer and Brauner, 2015; Rummer et al., 2013b). Further, studies examining the effects of stable versus fluctuating pCO_2 on AS found that exposure to stable elevated pCO_2 decreased AS, but exposure to fluctuating elevated pCO_2 ameliorated this response (Laubenstein et al., 2020; Methling et al., 2013). Another study found that the magnitude by which $\dot{M}O_2$ can increase (i.e., from minimum or resting values to maximum; factorial AS) was elevated in fishes exposed to fluctuating pCO_2 conditions when compared to fish exposed to either stable ambient or stable elevated pCO_2 conditions. Overall, the results from previous studies and the current study, excluding *C. quinquelineatus*, demonstrate that when fishes are exposed to fluctuating elevated pCO_2 conditions, many species are able to maintain or increase AS when compared to their ambient pCO_2 counterparts. This may mean that some fishes are able to maintain or increase the amount of energy available for life-history traits upon exposure to OA relevant pCO_2 fluctuations. Nevertheless, there are exceptions, and OA is not occurring in isolation. For example, juvenile snapper decrease their AS upon exposure to elevated pCO_2 by 31-35% under current-day and predicted future summer temperatures (McMahon et al., 2020).

Haematological metrics

Red blood cell (RBC) swelling in teleost fishes – as noted by decreases in MCHC – is an indication of the adrenergic stress response, which, among other functions, is thought to also

aid in protecting RBC pH and therefore oxygen transport. While swelling is indicated by decreases in MCHC, in this study, we observed an increase in MCHC in both *L. fulviflamma* and *A. whitleyi* exposed to stable elevated pCO_2 conditions. For *A. whitleyi*, this was underpinned by a decrease in Hct but no change in total [Hb], thus suggesting RBC shrinkage. In response to stress, RBC shrinkage rather than swelling, has been previously observed under OA relevant pCO_2 levels; whereas, RBC swelling has typically been observed under extreme pCO_2 levels (Crespel et al., 2019; Hannan et al., 2020b; Heinrich et al., 2014; Noor et al., 2019). Interestingly, *A. whitleyi* also decreased MCHC during the day compared to night. However, it is important to note that these haematological metrics were also measured directly following exhaustive swimming, which is also stressful on some levels and causes changes to haematological metrics. This may make it challenging to separate the responses related to the swimming challenge from those related to pCO_2 exposure as well as the interactions between the two stressors, a topic worthy of future investigations.

Metabolite parameters

For the most part, lactate and glucose concentrations in the species investigated here were not affected by elevated pCO_2 conditions. However, *C. cuning* exhibited higher whole blood lactate concentrations under ambient and fluctuating pCO_2 conditions than under stable elevated pCO_2 conditions. This may indicate a heavier reliance on or earlier shift to anaerobic metabolism during the swimming test. This may have also been the case for *C. quinquelineatus* under ambient pCO_2 conditions. When *C. cuning* were maintained under fluctuating elevated pCO_2 conditions, they exhibited an increase in whole blood glucose concentrations when compared to their ambient pCO_2 counterparts. This response may have been to aid the increased energy requirements and/or the stress response associated with

managing a general acidosis, which can occur during swimming (Wendelaar Bonga, 1997). Plasma glucose concentrations were also elevated in *C. quinquelineatus* sampled during the day, which could represent the increased energy demand these nocturnal fish bear when forced to be diurnally active. Again, it is important to keep in mind that these parameters were measured following exercise, and the resulting differences observed between pCO_2 treatments may indicate that pCO_2 treatments alter metabolite production and how fish respond to exercise stress.

Comparing fluctuating and stable pCO₂

Both *C. cuning* and *C. quinquelineatus* exhibited different responses upon exposure to fluctuating elevated pCO_2 conditions when compared to stable elevated pCO_2 conditions. Typically, there were no differences between fish maintained under ambient and stable elevated pCO_2 conditions, but when fish were examined under fluctuating elevated pCO_2 conditions, differences arose. Thus, examining species under stable elevated pCO_2 conditions may potentially mask the effects of future OA conditions, especially for species that experience fluctuating pCO_2 conditions in their natural habitats. We observed this trend in *C. cuning* for U_{crit}, $\dot{M}O_{2\ Max,}$ AS, and glucose and in *C. quinquelineatus* for $\dot{M}O_{2\ Max}$ and AS. The different responses, depending on fluctuating or stable elevated pCO_2 conditions, may account for so many previous studies reporting no physiological effects associated with exposure to elevated pCO_2 conditions (reviewed by Heuer and Grosell, 2014). Our findings further emphasise that studies investigating nearshore species should include fluctuating pCO_2 conditions in order to gain a better understanding as to how fishes will respond to OA into the future.

Species differences

Few trends could be consistently detected when comparing daytime and night time measurements; however, the one nocturnal species examined (i.e., *C. quinquelineatus*) exhibited the opposite trends in terms of oxygen uptake rate metrics when compared to the two diurnal species (i.e., *A. whitleyi* and *C. cuning*) and the opportunistic feeder (i.e., *L. fulviflamma*). These three species, *L. fulviflamma, A. whitleyi*, and *C. cuning,* all exhibited an elevated AS during one or both of the elevated pCO_2 treatments when compared to the ambient pCO_2 treatment. Declines in AS upon exposure to stable elevated pCO_2 conditions have been previously observed in cardinalfishes (e.g., *Ostorhinchus cyanosoma*) (Munday et al., 2009a). These earlier findings, in combination with our results here for *C. quinquelineatus*, could suggest that nocturnal fish species, or perhaps specifically cardinalfishes, are more sensitive to changes in pCO_2 than other coral reef fishes. Further, upon exposure to other stressors (i.e., elevated temperatures) cardinalfishes have also been shown to be more sensitive than other coral reef fish species, such as damselfishes (Gardiner et al., 2010; Nilsson et al., 2009). One possible explanation for this sensitivity is that the circadian rhythms of nocturnal species may be more strongly affected by elevated pCO_2 than in diurnal species. There is evidence that heart rate is governed by circadian rhythms in fishes, likely based on time of activity (Borch et al., 1993). Further, heart metrics have been suggested to be responsible for increased aerobic scope during exposure to elevated pCO_2 (Gräns et al., 2014). A study on the molecular signatures of transgenerational exposure to elevated pCO_2 found that the circadian rhythm genes *nr1d1* and purvalbumin *(pvalb)* were differently expressed at both the transcript and protein levels upon exposure to elevated pCO_2 (Schunter et al., 2016). Taken together with the results of this study, it seems that circadian rhythms could be affected by exposure to elevated pCO_2. Alternatively, cardinalfishes, rather than nocturnal fishes in general, may just be more sensitive to environmental stressors. Regardless, this could potentially have implications for the composition of coral reef fish

communities in the future. More research is needed to tease apart this nocturnal/cardinalfish specific response, and further examine the mechanisms underpinning differences in sensitivity to elevated pCO$_2$ among fishes that occupy the same coral reef habitat.

4.6   Conclusions

Previous studies as well as the current study report differences in oxygen uptake rates of teleost fishes upon exposure to elevated fluctuating pCO$_2$ conditions when compared to ambient and even stable elevated pCO$_2$ conditions. In many of the previous studies, examples are given where the negative responses associated with exposure to stable elevated pCO$_2$ conditions are ameliorated upon exposure to fluctuating elevated pCO$_2$ conditions. In these cases, it was thought that the organisms were products of their environment, in other words, adapted to fluctuating pCO$_2$ conditions, such as those documented on coral reefs. By contrast, we found little evidence that exposure to stable elevated pCO$_2$ conditions negatively affected the swimming performance, oxygen uptake rates, or haematological metrics in any of the four species examined in this study. The only negative response was related to oxygen uptake rates/energy availability in one of the four species upon exposure to fluctuating pCO$_2$ conditions. Moreover, the other three species seemed to benefit, overall, in terms of physiological performance upon exposure to elevated fluctuating pCO$_2$ conditions. Thus, future OA conditions may not negatively impact coral reef fishes, universally, at least at the level of oxygen uptake rates and swimming performance. However, our results, as well as those of previous studies, suggest that cardinalfishes and perhaps other nocturnal species may be more sensitive to future OA conditions than other coral reef fishes. Further investigating the differential responses between species can help inform how fish populations and communities will be affected in the future by ocean acidification. Additionally, it is important

to keep in mind that OA is not occurring in isolation; future research should focus on a combination of fluctuating elevated pCO$_2$ and other environmental factors (e.g., temperature) to reveal additive or synergetic effects. That being said, these findings highlight the importance of considering other aspects that may be influence the tolerance of fishes to future OA conditions, such as time of activity.

Chapter 5: Evidence for a mechanism underpinning enhanced tissue oxygen delivery during elevated $p\text{CO}_2$ exposure in a coral reef fish species

5.1 Summary

Ocean acidification (OA) is expected to be energetically expensive to fishes due to the increased costs of acid-base regulation. However, the majority of studies to date have found that fishes are able to maintain metabolic performance under OA relevant increased partial pressures of carbon dioxide ($p\text{CO}_2$). The mechanisms fish employ to maintain metabolic performance (measured as oxygen uptake rates) have yet to be verified, but could involve the oxygen transport system that is unique to teleost fishes. Most teleost fishes have Root effect haemoglobins (Hb) that are extremely pH sensitive. In salmonids, there is evidence that the presence of plasma-accessible carbonic anhydrase (paCA; an enzyme that catalyses proton production from CO_2) at select tissues can serve to re-acidify the red blood cell, thereby promoting increased oxygen release from Root effect Hbs to the tissues. Moreover, this effect could be magnified during a generalised acidosis. However, these mechanisms have yet to be examined when fish are exposed to ecologically relevant elevated $p\text{CO}_2$ levels. To experimentally test the role of paCA in maintaining oxygen delivery in fish at OA relevant $p\text{CO}_2$ levels, I exposed *Acanthochromis polyacanthus* to one of three different $p\text{CO}_2$ treatments (i.e., ambient: 400 µatm, stable elevated: 1,000 µatm, or fluctuating elevated: 1,000 ± 300 µatm) for 12 d. Fish were then injected with either a paCA inhibitor (C18-injection) or a control (sham-injection) solution. Following the injection, fish were chased to exhaustion and then air exposed to elicit maximum oxygen uptake rates ($\dot{M}\text{O}_{2\,max}$). Blood was drawn at three different sampling intervals: immediately after the exhaustive exercise

protocol, 30 min after injection, and after overnight recovery. Results show that: (i) paCA inhibition decreased $\dot{M}O_{2\,max}$ in stable elevated pCO$_2$ exposed fish when compared to fish exposed to ambient conditions, indicating a role of paCA in promoting oxygen release to the tissues during pCO$_2$ exposure; (ii) upon exposure to stable elevated pCO$_2$, paCA inhibition elevated whole blood lactate and glucose concentrations 30 min post-injection and following overnight recovery. Together these results are some of the first to confirm that coral reef fishes may be employing the same mechanism as salmonids to increase oxygen release to the tissues during a generalised acidosis.

5.2   Introduction

Atmospheric carbon dioxide (CO$_2$) levels have been increasing at unprecedented rates since the Industrial Revolution (Doney et al., 2009). About a fourth of the anthropogenic CO$_2$ released into the atmosphere is being absorbed by the oceans (Fabricius et al., 2020), which is causing an increase in the partial pressure of CO$_2$ (pCO$_2$) and decrease in ocean pH, a process referred to as ocean acidification (OA) (Doney et al., 2009). In extreme emissions scenarios, atmospheric CO$_2$ concentrations are expected to exceed 900 μatm by the year 2100, and the pH of the surface of the oceans is expected to decline by an average of 0.3-0.4 units compared to pre-Industrial values (IPCC, 2019). These changes in ocean chemistry have been predicted to have a range of negative impacts on marine organisms (Doney et al., 2009; Pörtner and Farrell, 2008). However, coastal and shallow water habitats also have variable pH and pCO$_2$ dynamics on a variety of temporal and spatial scales (Hofmann et al., 2011; Kayanne et al., 1995). Coral reefs, for example, experience diel fluctuations in pCO$_2$, where the pCO$_2$ is elevated at night and lower during the day (Albright et al., 2013; Hannan et al., 2020a; Hofmann et al., 2011; Ohde and van Woesik, 1999; Shaw et al., 2012). These cycles

are mainly driven by the photosynthesis/respiration and calcification/dissolution cycles of benthic organisms (Albright et al., 2013; Falter et al., 2013).

A recent meta-analysis examining the response of teleost fishes to OA conditions suggests that the majority of metrics measured, including growth, reproduction, and aerobic scope, are (on average) unaffected by OA-relevant pCO_2 conditions (Cattano et al., 2018). Additionally, most fishes examined have been able to maintain performance – in terms of swimming, energy availability, and oxygen uptake rates – upon exposure to elevated pCO_2 conditions (reviewed by Lefevre, 2019). Swimming, a critical performance metric for teleosts, depends on adequate oxygen transport from the respiratory surfaces to the tissues. The mechanisms by which fish are able to maintain swimming during elevated pCO_2 can be related to the capacity for aerobic metabolism. Critical swimming speed (U_{crit}) is assumed to be the maximum sustained speed that can be supported aerobically; thus, one would assume that a higher capacity for oxygen uptake would confer a higher capacity for sustained swimming. One mechanism that may be responsible for increased release of oxygen to the tissues during exposure to elevated CO_2 is the oxygen transport system that is unique to teleosts (Rummer et al., 2013b).

In the 1900s, oxygen (O_2) transport, from the respiratory surfaces to the tissues, was determined to be a passive process in vertebrates, driven by diffusion alone (Krogh, 1919a; Krogh, 1919b). Albeit passive, this process is pH sensitive, where decreases in the intracellular pH (pH_i) of red blood cells (RBC) reduces the haemoglobin (Hb)-O_2 affinity (Bohr effect) but can also reduce the oxygen carrying capacity of the blood (Root effect) (Harter and Brauner, 2020). In the RBC, Hb acts as the main transporter of O_2, elevating the O_2-carrying capacity of the blood. However, the Hb of teleosts is more pH sensitive (Bohr-Root effect) than other vertebrates (e.g., humans; Rummer and Brauner, 2015). This suggests

that, during a generalised acidosis, the pH-sensitive Hb will have lower O_2 affinity, which will result in a reduced O_2 carrying capacity of the blood that would be detrimental to overall oxygen transport. To combat this, fish have evolved a mechanism to protect RBC pH_i, where β-adrenergic stimulation can activate sodium (Na^+) proton (H^+) exchangers (β-NHE) on the RBC that extrude H^+ in exchange for Na^+, which activates other transporters, all of which ultimately maintain pH_i (Nikinmaa and Salama, 1998). This mechanism protects the Hb-O_2 affinity and binding in the RBC, which is especially important at the gills. However, if this mechanism were to act in isolation, it would inhibit O_2 release to metabolically active tissues during a generalised acidosis. Therefore, there must be an active process associated with O_2 transport that would not be possible by diffusion alone (Rummer et al., 2013b).

Studies in the first few decades of the 21st century show that fish can facilitate the passive process of oxygen transport (Bohr-Root effects), thus making it active, with a plasma accessible isoform of the enzyme carbonic anhydrase (paCA) (reviewed by Harter and Brauner, 2020). The presence of this enzyme at specific tissues can short-circuit β-NHE activity and re-acidify RBCs. This acidification of the RBC reduces the affinity and carrying capacity of Hb for O_2, thus increasing the release of O_2 to metabolically active tissues where paCA is present. The paCA in teleosts, unlike other vertebrates, is not distributed homogeneously throughout the body (Harter and Brauner, 2017). It is absent at the gills, allowing β-NHE to protect RBC pH_i and facilitate O_2 uptake from the water, and present at tissues where O_2 unloading occurs. This increased unloading of O_2 to the tissues increases arterial to venous PO_2 gradient, thus allowing for increased O_2 binding at the gills where the pH is protected. Thus, paCA activity can facilitate oxygen unloading from the highly pH-sensitive teleost Hbs to metabolically active tissues during stress, thus enabling higher tissue O_2 delivery and in turn O_2 uptake therefore maintained or even enhanced performance.

Evidence also suggests that the mechanism involving paCA and Root Effect Hbs increases tissue partial pressure of O_2 (PO_2) and CO_2 hydration rates during acidosis in teleost fishes (Alderman et al., 2016; Rummer et al., 2013b). When rainbow trout are exposed to extreme pCO_2, red muscle PO_2 increases; however, when paCA is inhibited, red muscle PO_2 decreases (Rummer et al., 2013b). This suggests that during this acidic exposure paCA increases the release of O_2 to metabolically active red muscle tissues, thus increasing tissue PO_2. This may be true in cardiac tissue as well. For example, in the heart atrium of coho salmon exposed to CO_2 saturated water, paCA inhibition resulted in a threefold decrease in CO_2 hydration reaction velocities (Alderman et al., 2016). This suggests that paCA is present in the heart of these fish and can serve to increase PO_2 at this location as well. These two studies demonstrate that, during a CO_2-induced blood acidosis, paCA at specific tissues can facilitate O_2 unloading from the highly pH-sensitive teleost Hb. Functional support for the role of paCA in cardiovascular O_2 transport during swimming has also been demonstrated in Atlantic salmon (Harter et al., 2019). Inhibition of paCA resulted in increased cardiac output at lower swimming speeds, and fish were unable to continue swimming upon paCA inhibition at higher swimming speeds. This further suggests that paCA plays an active role in maintaining performance and oxygen transport during exercise. These experiments collectively demonstrate that paCA located in the red muscle and/or heart may serve to increase tissue PO_2 and assist swimming performance, at least in several salmonid species. Indeed, the majority of research on paCA activity has been performed on salmonids; no study to date has investigated the role of this mechanism in enhancing performance in coral reef fishes or upon exposure to OA relevant pCO_2 levels.

To address this knowledge gap, I exposed a coral reef fish, *Acanthochromis polyacanthus*, to OA-relevant pCO_2 conditions for 12 d. After pCO_2 exposure, fish were either injected with C18 (an enzyme inhibitor targeting paCA) or a sham solution, as a control. Following the

injection, fish were chased and subsequently air exposed to elicit maximum oxygen uptake rates ($\dot{M}O_{2\,max}$). Blood was either sampled immediately, or fish were placed into a static respirometer to measure oxygen uptake rates, and then blood was sampled during various points of recovery. Whole blood lactate and glucose concentrations were also measured as an indicator of tissue hypoxia. I hypothesised that the oxygen uptake rates of *A. polyacanthus* would be unaffected by exposure to elevated pCO$_2$ because fish are using the combination of Root effect Hbs and paCA to maintain aerobic performance. I also predicted that, in fish where paCA is inhibited, $\dot{M}O_{2\,max}$ would decrease regardless of pCO$_2$ treatment, but that this burden to $\dot{M}O_{2\,max}$ would be exacerbated in fish exposed to elevated pCO$_2$ conditions. Finally, I predicted that, when paCA is inhibited, fish would shift to alternative mechanisms (e.g., anaerobic metabolism, as reflected by changes in blood lactate and glucose concentrations) to maintain oxygen delivery and swimming performance during and following the exhaustive challenge. Collectively, this information will show if a more derived coral reef fish uses β-NHE short-circuiting with Root Effect Hbs and paCA as a fundamental aspect of O$_2$ transport and whether this is taken advantage of upon exposure to elevated pCO$_2$ conditions.

5.3   Methods

Experimental animals

This study was conducted at the Lizard Island Research Station on the Great Barrier Reef, Australia (14°40'S; 145°28'E) between August - October of 2020. Adult *Acanthochromis polyacanthus* (sample size n = 9-10; standard length, 10.5 ± 1.3 cm, wet mas 24.2 ± 7.2 g; means ± SD) were collected using a monofilament barrier net and hand nets from the reefs around Lizard Island and immediately brought back to the laboratory where they were

maintained in flow-through seawater aquaria for 4-5 d prior to the onset of experiments. Fish were fed daily to satiation with INVE Aquaculture Nutrition pellets. All animal care and experimental protocols complied with regulations at James Cook University and Lizard Island Research Station and were approved by the James Cook University Ethics Committee (Approval # A2414). Fish were collected under permit G19/42131.1 and CSE105 from the Great Barrier Reef Marine Park Authority to James Cook University.

CO₂ treatments

Fish were exposed to either ambient (~400 µatm), stable elevated pCO_2 (~1000 µatm), or fluctuating elevated (~1000 ± 300 µatm) pCO_2 for 12 d (Figure 5-1, Table 5-1). The elevated CO_2 treatment matched CO_2 projections for the year 2100 under RCP8.5 (Meinshausen et al., 2011). *In situ* measurements show that the pCO_2 on reefs around Lizard Island ranges from 283.6 – 554.5 µatm (Hannan et al., 2020a). Thus, the fluctuating elevated pCO_2 treatment was chosen as 1000 ± 300 µatm to account for the projected several-fold increase in the magnitude of fluctuations in the future. The pCO_2 of treatment seawater was manipulated by CO_2-dosing to a set pH. To do this, seawater was pumped from the lagoon into replicate 300 L sumps (3 per pCO_2 treatment) where it was diffused with ambient air (ambient treatment) or CO_2 to achieve the desired pH (elevated CO_2 treatment). The CO_2 dosing was controlled by solenoid valves (M-Ventil Standard, Aqua Medic, Germany) connected to a pH control system (Aqua Medic AT Control System, Aqua Medic, Germany) with laboratory grade pH electrodes (Neptune Systems, USA). When the solenoid valve opened, CO_2 flowed through a fine needle valve and into the impeller of a small pump (Aqua One Maxi 101 Power Head Pump 400 LPH) where it was immediately dissolved and mixed throughout the sump tank. This method ensured a slow steady flow of CO_2 into the sump and rapid mixing to prevent overdosing that leads to unstable CO_2 treatments. The Aqua Medic AT Control System has a

curve function which creates fluctuating pCO_2 profiles (see Jarrold et al., 2017). The pH

profiles in the fluctuating pCO_2 treatment were recorded every other day using a pH meter

(pH SD Card Datalogger: Model 850060, Sper Scientific) set to take a reading every 20 min.

Temperature was recorded daily with the same model of pH meter. Seawater pH on the total

hydrogen ion concentration scale (total scale, pH_T) was measured daily at either the middle,

top, or bottom of the fluctuations using a spectrophotometer following standard operating

procedures (Dickson et al., 2007) and using indicator dye (meta/m-cresol purple sodium salt

99%, non-purified; mCP, Acros Organic). Salinity data were obtained from the Lizard Island

Observing System (LIOS) part of the Integrated Marine Observing System (IMOS) ocean

monitoring sensors deployed at Lizard Island (platform: SF4-Research Bay). Water samples

were collected three times for each sump throughout the experimental period, and total

alkalinity (TA) was measured using Gran-titrations (Metrohm 888 Titrando Titrator

Metrohm, AG, Switzerland) and referenced with certified material from Dr. A.G. Dickson

(Scripps Institute of Oceanography, La Jolla, CA). All pH_T, TA, salinity, and temperature

data were entered into CO2SYS (Lewis and Wallace, 1998) using the constants K1 from

(Mehrbach et al., 1973) refit by (Dickson and Millero, 1987) and (Dickson, 1990) to calculate

pCO_2. Mean salinity, temperature, pH, and carbonate system parameters are reported in Table

5-1.

Table 5-1. Seawater parameters across the duration of the experiment. Values are means ± SD for daily average, minimum, maximum, and range of pH_T, and pCO_2. Mean ± SD for total alkalinity (TA), temperature, and salinity across the experiment are also shown.

Parameter	pCO_2 treatment		
	Ambient ~400 µatm	Stable elevated ~1000 µatm	Fluctuating elevated ~1000 ± 300 µatm
pH	8.02 ± 0.03	7.69 ± 0.03	7.72 ± 0.09
Min. pH	-	-	7.60 ± 0.02
Max. pH	-	-	7.82 ± 0.04
pH range	-	-	0.22 ± 0.02
pCO_2 (µatm)	419 ± 35	1036 ± 90	949 ± 270
Min. pCO_2	-	-	722 ± 66

Max. pCO_2	-	-	1278 ± 50
pCO_2 range (µatm)	-	-	556 ± 16
TA (µmol kg^{-1})	2265 ± 23	2291 ± 17	2283 ± 13
Temperature (°C)	25.3 ± 0.8	25.2 ± 0.6	25.2 ± 0.6
Salinity	35.2 ± 0.3	35.2 ± 0.3	35.1 ± 0.4
Pressure (dbars)	10.13 ± 0.01	10.14 ± 0.01	10.14 ± 0.01
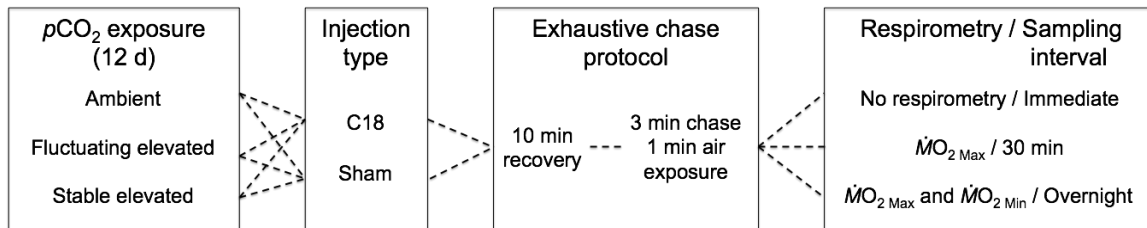
Figure 5-1. The experimental design. 12 d pCO_2 exposure (either ambient, fluctuating elevated, or stable elevated) and an injection (sham or C18) followed by an exhaustive chase protocol and respirometry and/or blood sampling.

Exhaustive exercise

Following exposure to either ambient, stable elevated pCO_2, or fluctuating elevated pCO_2 for 12 d, but prior to the exhaustive exercise challenge, each fish was fasted for 24 h to ensure a post-absorptive state (Niimi and Beamish, 1974). Then, fish were weighed and injected with either a sham solution (0.9% NaCl, 0.1% DMSO), which acted as the control, or a CA inhibitor (C18) solution (0.9% NaCl, 0.1% DMSO, 10 µmol kg^{-1} C18) with limited membrane permeability for up to 45 min (Figure 5-1) (Supuran et al., 2000). Fish were injected in the caudal vasculature (average volume 0.12 ml), and injections took less than a minute. The injection volume was based on the body mass of the fish assuming that the total blood volume is 5% of body mass. Fish were then allowed to recover from the injection and orientate to the circular chase container (60 cm diameter, 58 cm height) for 10 min (Figure 5-1). Fish were individually chased for 3 min and then exposed to air for 1 min; this protocol has been previously determined sufficient to exhaust small coral reef fishes (Figure 5-1) (Laubenstein et al., 2019; Laubenstein et al., 2020). Following the exhaustive exercise

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challenge, fish were either placed into respirometry chambers to measure oxygen uptake rates ($\dot{M}O_2$) or cranially concussed so that blood could be sampled (Figure 5-1).

Haematological metrics

Fish were sampled for blood immediately following the injection and exhaustive exercise protocol, 30 min after the injection and exhaustive exercise protocol, or after overnight recovery from the injection and exhaustive exercise protocol (21.6 ± 0.4 hr) (Figure 5-1). Fish from the latter two groups were placed into respirometry chambers immediately following the exhaustive exercise protocol so that oxygen uptake rates could be measured during recovery and prior to blood sampling (Figure 5-1). Prior to blood sampling, fish were cranially concussed, and then blood was drawn from the caudal artery/vein via a lithium heparin rinsed syringe (1 mL syringe, 23-G needle) according to A2414 James Cook University Animal Ethics Committee protocols. Whole blood glucose and lactate concentrations (millimolar) were immediately determined from two 15 µl samples using the Accutrend Plus (Roche Diagnostics Australia Pty Ltd Dee Why, NSW, Australia).

Respirometry

An intermittent-flow respirometry system, as per standard respirometry methods (Roche et al., 2013), was used to measure the $\dot{M}O_2$ of fish following the exhaustive exercise protocol (Figure 5-1). Immediately following the exhaustive exercise protocol, each fish was placed individually into a darkened glass respirometry chamber (541 mL total volume, including tubing) submerged in a water bath. The water bath received a continuous inflow of treatment water, such that fish in the respirometry chambers experienced the same conditions as they had over their prior 12 d exposure to ambient, stable elevated pCO_2, or fluctuating elevated pCO_2 conditions. A purpose built computer program, AquaResp V3.0, was used to control a flush pump for each respirometry chamber such that each cycle consisted of a 180 s

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measurement period, a 90 s flushing period, and a 30 s wait period. This cycle was repeated over the duration of each trial (i.e., 15 min or overnight). Because each chamber was flushed with clean water from the surrounding water bath for 90s every 5 min, this ensured that the O_2 levels within the chambers did not fall below 80% air saturation during each measurement period. During each measurement period, O_2 levels of the chambers, which would later be used to calculate $\dot{M}O_2$ for each fish, were measured using a Firesting Optical Oxygen Meter (Pyro Science e. K., Aachen, Germany). At the end of each trial, fish were removed from the chambers, and blood was sampled as described above. The $\dot{M}O_2$ was calculated using the following equation (Steffensen et al., 1984):

$$\dot{M}O_2 = s\beta O_2 V_{resp} M^{-1}$$

In this equation, s is the slope of the linear decrease in O_2 while the chamber is closed, βO_2 is the oxygen solubility at a given temperature, salinity, and atmospheric pressure, V_{resp} is the effective respirometer volume (total respirometer volume – fish volume), and M is the mass of the fish. The $\dot{M}O_{2\ Max}$ was determined from the highest oxygen uptake rate (over 5-min intervals) and usually occurred during the first measurement cycle for fish in the 30min and overnight sampling intervals. The $\dot{M}O_{2\ Min}$ was estimated as the average of the lowest 10% of values, provided that the slope R^2 was > 0.98 for fish in the overnight time point. Absolute aerobic scope (AAS) was calculated as $\dot{M}O_{2\ Max}$ - $\dot{M}O_{2\ Min}$, and factorial aerobic scope (FAS) was calculated as $\dot{M}O_{2\ Max}$ / $\dot{M}O_{2\ Min}$ for fish in the overnight time point. Background microbial respiration was subtracted from total chamber respiration to determine the oxygen uptake rates of the fish as per Rummer et al. (2016).

Statistical analyses

Bayesian linear mixed models were used to analyse differences in the AAS, FAS, $\dot{M}O_{2\ Max}$, $\dot{M}O_{2\ Min,}$ lactate, and glucose of *A. polyacanthus* between pCO_2 treatments, injection type, and

time point of the blood draw where appropriate. $\dot{M}O_{2\ Max}$ measurements from fish in the 30min and overnight time points were combined as there were no significant difference in the results. Analyses were conducted in R version 3.3.2 (R Core Team, 2017), and the models were fit in STAN with Markov chain Monte Carlo sampling (Carpenter et al., 2017) using the rstanarm package version 2.13.1 (Goodrich et al., 2018). The broom (version 0.4.4; Robinson, 2017) and emmeans (version 1.3.2; Lenth, 2016) packages were used to summarise model outputs using highest posterior density intervals with a probability level of 0.95. Plots were produced using ggplot2 version 2.2.1 (Wickham and Chang, 2008). To analyse differences in measured variables, the generalised linear mixed model 'stan_glm' was used. The multiplicative models included pCO$_2$ treatment (i.e., ambient vs. stable elevated vs. fluctuating elevated), injection type (sham vs. C18), and blood draw time point (immediate vs. 30min vs. overnight) as fixed factors where applicable. All models used a Gaussian error distribution with 5000 iterations, a warmup of 500, 3 chains, and a thinning factor of two. For all models, diagnostic plots were visually examined to ensure there was convergence of chains and no evidence of heteroscedasticity or autocorrelation. Medians and central intervals from prior and posterior distributions were compared to ensure that the chosen priors were sufficiently wide so as to not dictate any trends, without being flat (non-informative).

The highest posterior density uncertainty intervals were used, and in this study, strong evidence for an effect (i.e., statistical 'significance') was defined when the 95% uncertainty interval (UI) did not intersect with zero.

5.4   Results

Maximum O$_2$ uptake rates ($\dot{M}O_{2\ Max}$)

Among sham-injected fish, there were no differences in $\dot{M}O_{2\,Max}$ between pCO_2 treatments (Figure 5-2, Appendix D - Table S1). However, when paCA was inhibited (C18-injected), $\dot{M}O_{2\,Max}$ was lower in stable elevated pCO_2 exposed fish when compared to ambient pCO_2 exposed fish, regardless of injection type (96 and 100% UI, respectively: Figure 5-2, Appendix D - Table S1). Additionally, C18-injected fish exhibited a higher $\dot{M}O_{2\,Max}$ if they had been exposed to ambient pCO_2 conditions when compared to sham-injected fish from fluctuating elevated or stable elevated pCO_2 conditions (98 and 99% UI, respectively: Figure 5-2, Appendix D - Table S1).
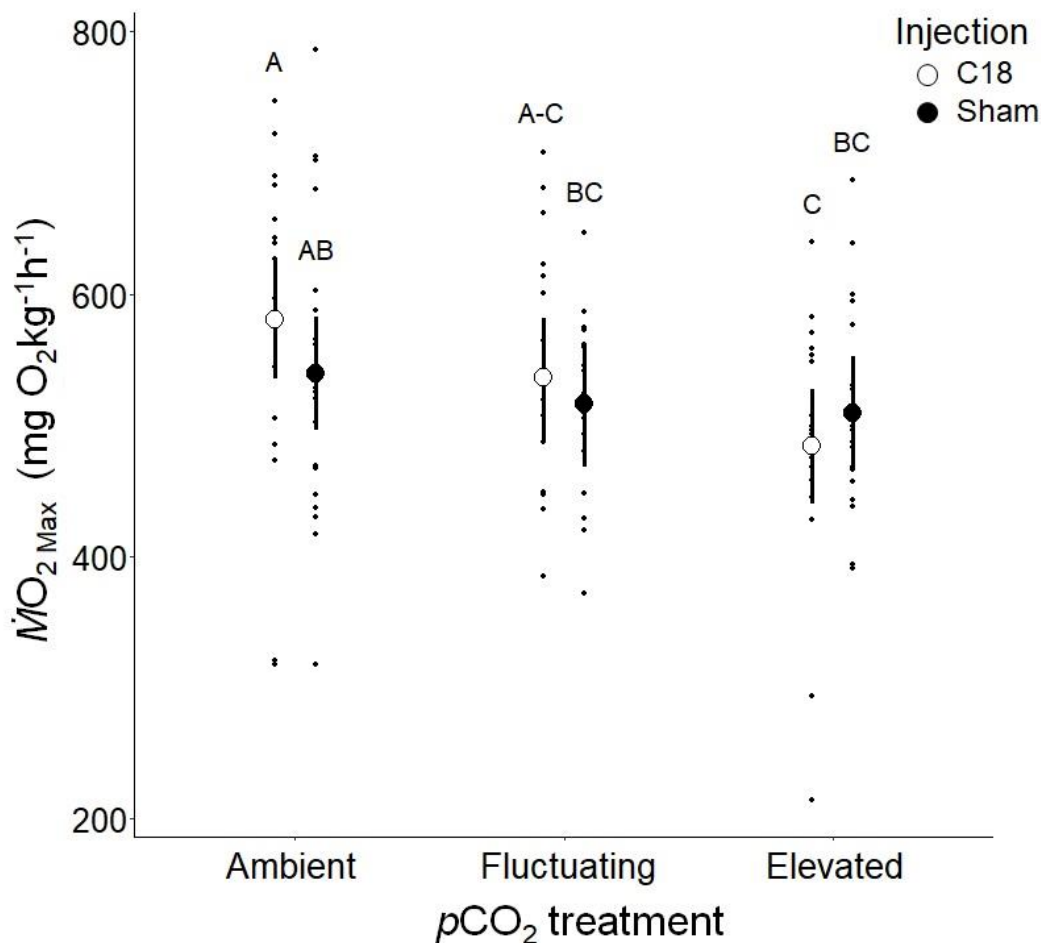
Figure 5-2. The $\dot{M}O_{2\,Max}$ of *Acanthochromis polyacanthus* exposed to one of three different pCO_2 treatments (ambient 400 μatm, stable elevated 1,000 μatm, and fluctuating elevated 1,000 ± 300 μatm). The circles represent least-squares means, and error bars represent 95% confidence intervals. The white circles represent C18-injected fish, and the dark circles represent sham-injected fish. Differences between the interaction of the main effects (pCO_2 treatment*injection) are displayed by dissimilar letters (>95% of the UI does not intersect zero).

Minimum O_2 uptake rates ($\dot{M}O_{2\ Min}$)

Regardless of injection type, $\dot{M}O_{2\ Min}$ was higher in fish exposed to fluctuating elevated $p\text{CO}_2$ conditions when compared to fish exposed to ambient $p\text{CO}_2$ conditions (>95% UI: Figure 5-3, Appendix D - Table S1). In C18-injected fish, $\dot{M}O_{2\ Min}$ was higher in fish that had been exposed to fluctuating elevated $p\text{CO}_2$ conditions compared to stable elevated $p\text{CO}_2$ exposed fish (99 and 100% UI: Figure 5-3, Appendix D - Table S1). Similarly, sham-injected fish exhibited a higher $\dot{M}O_{2\ Min}$ upon exposure to fluctuating elevated $p\text{CO}_2$ conditions when compared to fish exposed to stable elevated $p\text{CO}_2$ conditions (99% UI: Figure 5-3, Appendix D - Table S1).
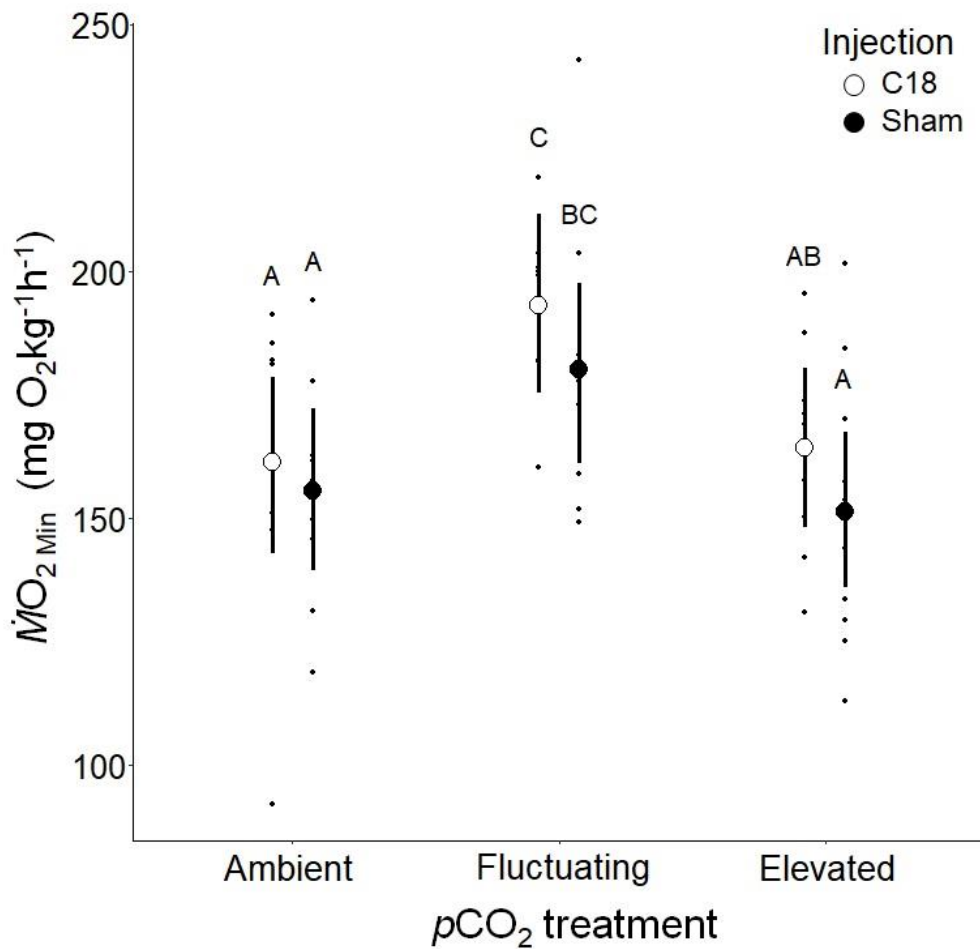
Figure 5-3. The $\dot{M}O_{2\ Min}$ of *Acanthochromis polyacanthus* exposed to one of three different pCO_2 treatments (ambient 400 μatm, stable elevated 1,000 μatm, and fluctuating elevated 1,000 ± 300 μatm). The circles represent least-squares means, and error bars represent 95% confidence intervals. The white circles represent C18-injected fish, and the dark circles represent sham-injected fish. Differences between the interaction of the main effects (pCO_2 treatment*injection) are displayed by dissimilar letters (>95% of the UI does not intersect zero).

Absolute aerobic scope (AAS)

Sham-injected fish were unaffected, in terms of absolute aerobic scope, across all pCO_2 treatments (Figure 5-4, Appendix D - Table S1). However, the absolute aerobic scope of C18-injected fish was higher upon exposure to ambient pCO_2 conditions than exposure to elevated fluctuating pCO_2 conditions (97% UI: Figure 5-4, Appendix D - Table S1).
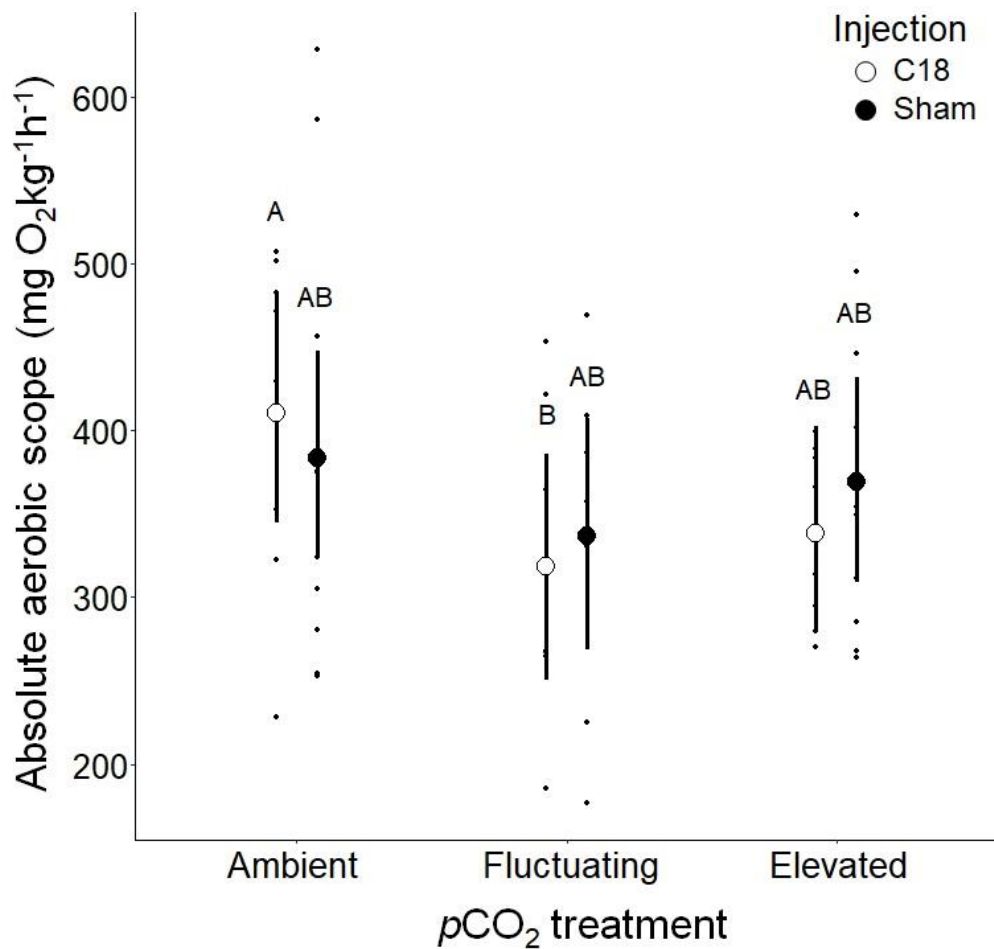
Figure 5-4. The absolute aerobic scope of *Acanthochromis polyacanthus* exposed to one of three different pCO_2 treatments (ambient 400 μatm, stable elevated 1,000 μatm, and fluctuating elevated 1,000 ± 300 μatm). The circles represent least-squares means, and error bars represent 95% confidence intervals. The white circles represent C18-injected fish, and the dark circles represent sham-injected fish. Differences between the interaction of the main effects (pCO_2 treatment*injection) are displayed by dissimilar letters (>95% of the UI does not intersect zero).

Factorial aerobic scope (FAS)

In sham-injected fish, there was no difference in the factorial aerobic scope in fish exposed to ambient conditions compared to both elevated pCO_2 conditions (Figure 5-5, Appendix D - Table S1). However, sham-injected fish exhibited a lower factorial aerobic scope if they had been exposed to fluctuating elevated pCO_2 conditions than if they had been exposed to stable elevated pCO_2 conditions (96% UI: Figure 5-5, Appendix D - Table S1). Additionally, C18-injected fish exhibited a lower factorial aerobic scope under fluctuating elevated pCO_2

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conditions than either C18-injected or sham-injected fish exposed to ambient pCO_2

conditions (99% UI: Figure 5-5, Appendix D - Table S1).
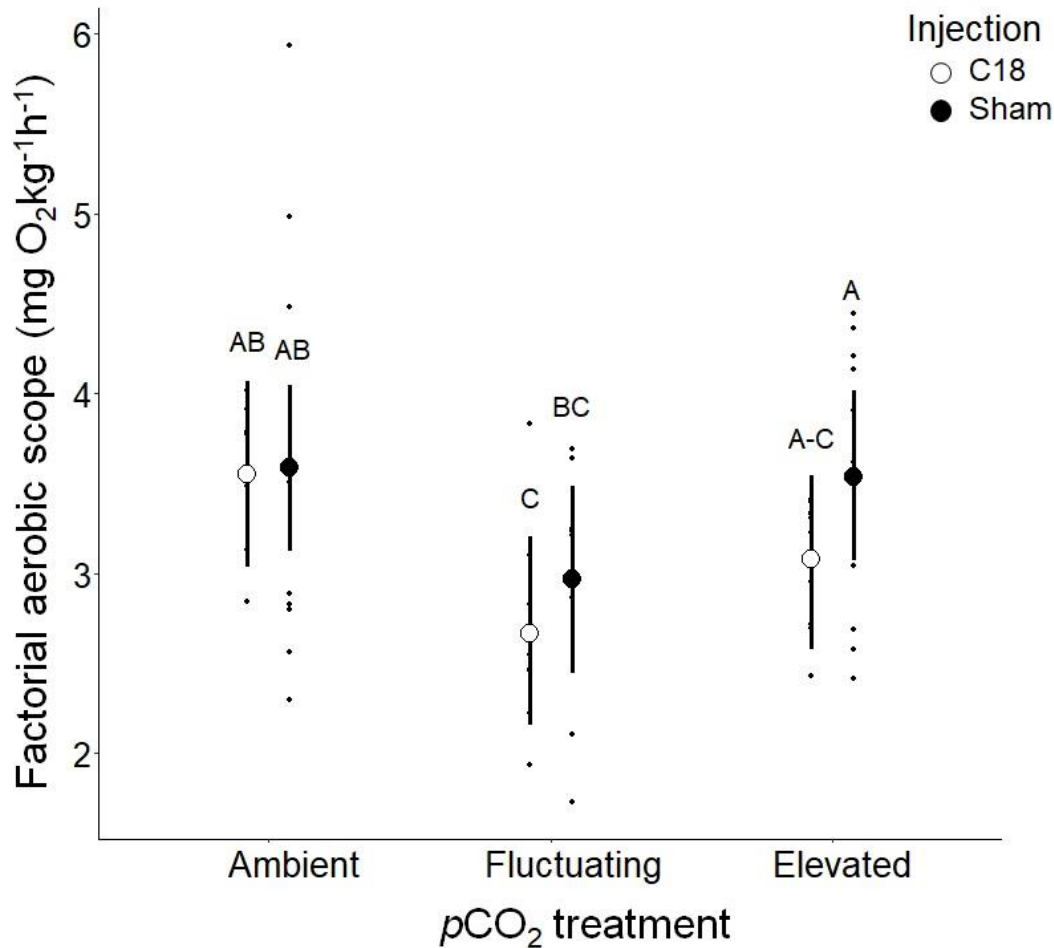
Figure 5-5. The factorial aerobic scope of *Acanthochromis polyacanthus* exposed to one of three different pCO_2 treatments (ambient 400 μatm, stable elevated 1,000 μatm, and fluctuating elevated 1,000 ± 300 μatm). The circles represent least-squares means, and error bars represent 95% confidence intervals. The white circles represent C18-injected fish, and the dark circles represent sham-injected fish. Differences between the interaction of the main effects (pCO_2 treatment*injection) are displayed by dissimilar letters (>95% of the UI does not intersect zero).

Whole blood lactate concentrations

In sham-injected fish, whole blood lactate concentrations did not differ between pCO_2

treatments or between sampling intervals (<95% UI: Figure 5-6, Appendix D - Table S2).

Regardless of injection type, lower lactate concentrations were observed in fish after the

overnight sampling interval when compared to fish from the immediate and 30 min sampling

intervals, regardless of pCO_2 treatment (Figure 5-6, Appendix D - Table S2). The C18-injected (paCA inhibited) fish exhibited decreased whole blood lactate concentrations 30 minutes following the injection when compared to sham-injected fish upon exposure to fluctuating elevated pCO_2 (96% UI: Figure 5-6, Appendix D - Table S2). Within the immediate and 30 min sampling the only C18-injected (paCA inhibited) fish within a pCO_2 treatment occurred upon exposure to stable elevated pCO_2, where fish that were sampled 30 min following the injection had elevated lactate compared to fish immediately sampled (100% UI: Figure 5-6, Appendix D - Table S2).
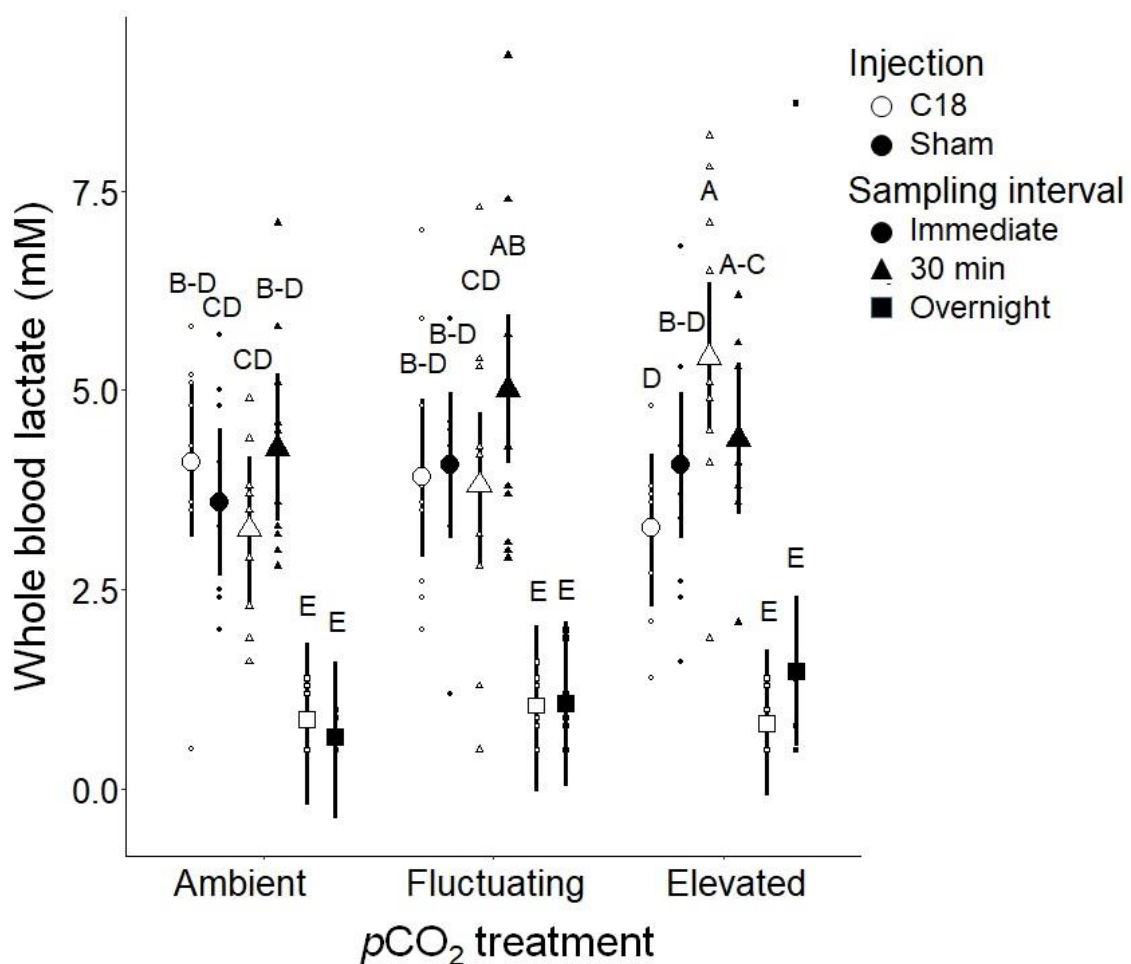
Figure 5-6. The whole blood lactate of *Acanthochromis polyacanthus* exposed to one of three different pCO_2 treatments (ambient 400 µatm, stable elevated 1,000 µatm, and fluctuating elevated 1,000 ± 300 µatm). The circles represent least-squares means, and error bars represent 95% confidence intervals. The white circles represent C18-injected fish, and the dark circles represent sham-injected fish. The different shapes represent the different blood sampling intervals (● - immediate, ▲ - 30 min, and ■ - overnight). Differences between the interaction of the main effects

(pCO$_2$ treatment*injection*sampling interval) are displayed by dissimilar letters (>95% of the UI does not intersect zero).

Whole blood glucose concentrations

Whole blood glucose results were similar to trends observed with whole blood lactate concentrations. Within the sham-injected fish, there were no differences in whole blood glucose concentrations after the immediate and 30 min sampling intervals across all pCO$_2$ treatments (<95% UI: Figure 5-7, Appendix D - Table S2). However, sham-injected fish had lower whole blood glucose concentrations after the overnight sampling interval if they had been exposed to fluctuating pCO$_2$ when compared to those exposed to either ambient or stable elevated pCO$_2$ conditions (Figure 5-7, Appendix D - Table S2). Generally, the sham-injected fish exhibited elevated whole blood glucose concentrations after the overnight sampling interval when compared to immediate and 30 min intervals and across all pCO$_2$ treatments. Additionally, C18-injected fish exhibited higher whole blood glucose concentrations than sham-injected fish after the overnight sampling interval if they had been exposed to stable elevated pCO$_2$ conditions (100% UI: Figure 5-7, Appendix D - Table S2).
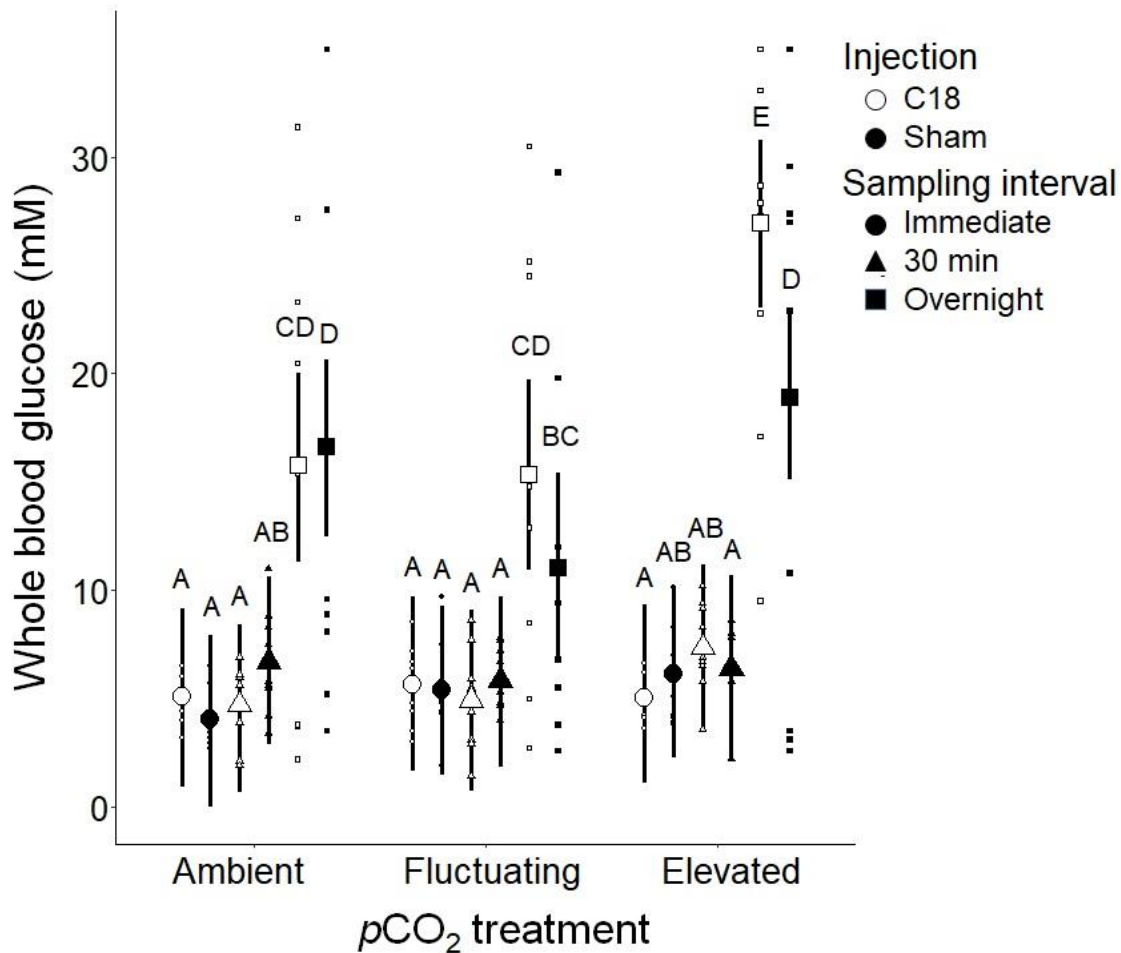
Figure 5-7. The whole blood glucose of *Acanthochromis polyacanthus* exposed to one of three different pCO_2 treatments (ambient 400 µatm, stable elevated 1,000 µatm, and fluctuating elevated 1,000 ± 300 µatm). The circles represent least-squares means, and error bars represent 95% confidence intervals. The white circles represent C18-injected fish, and the dark circles represent sham-injected fish. The different shapes represent the different blood sampling intervals (● - immediate, ▲ - 30 min, and ■ - overnight). Differences between the interaction of the main effects (pCO_2 treatment*injection*sampling interval) are displayed by dissimilar letters (>95% of the UI does not intersect zero).

5.5 Discussion

The overarching hypothesis of this study was that sham-injected fish would be able to maintain oxygen uptake rates upon exposure to elevated pCO_2, and inhibiting paCA (C18-injection) would result in decreased oxygen uptake rates and fish would have to rely on alternative mechanisms to maintain performance. My results generally support this hypothesis in terms of $\dot{M}O_{2\ Max}$, AAS, and FAS, as there is evidence that sham-injected fish

were able to maintain oxygen uptake rates upon exposure to both stable and fluctuating elevated pCO_2. The most significant finding of this study was that inhibiting paCA resulted in a 10% decrease in $\dot{M}O_{2\ Max}$ in fish exposed to stable elevated pCO_2 conditions when compared to sham-injected fish from ambient pCO_2 conditions. This suggests that fish exposed to stable elevated pCO_2 conditions are using paCA to maintain $\dot{M}O_{2\ Max}$. Further, fish exposed to fluctuating elevated pCO_2 that had paCA inhibited (C18-injection) had 17% lower factorial aerobic scope compared to ambient exposed sham-injected fish; whereas, sham-injected CO_2 exposed fish exhibited no difference in factorial aerobic scope. The collective results of this study suggest that paCA activity is important to maintaining oxygen uptake rates in *A. polyacanthus* during exposure to both stable and fluctuating elevated pCO_2 conditions. This is the first time this mechanism has been confirmed in a coral reef fish species, and it may be important for maintaining oxygen uptake rates and swimming performance during future ocean acidification conditions.

Maximum oxygen uptake rates ($\dot{M}O_{2\ Max}$)

It has been predicted that ocean acidification would have negative impacts on the maximum metabolic rates of fishes due to the direct effects of an acidosis on the oxygen carrying capacity of pH sensitive haemoglobins (Pörtner et al., 2005). However, the majority (~70%) of marine fishes that have been studied exhibit maintained $\dot{M}O_{2\ Max}$ upon exposure to elevated pCO_2 conditions (Lefevre, 2019). Similarly, in this study, sham-injected *A. polyacanthus* maintained $\dot{M}O_{2\ Max}$ regardless of pCO_2 treatment. Previous studies on *A. polyacanthus* have also typically observed $\dot{M}O_{2\ Max}$ to be unaffected by elevated pCO_2 conditions (Hannan et al., 2020b; Laubenstein et al., 2019; Laubenstein et al., 2020). However, one study observed an unexpected increase in $\dot{M}O_{2\ Max}$ with a 17 d exposure to elevated stable pCO_2 (~950 μatm) (Rummer et al., 2013a). These collective findings suggest that, during exposure to elevated

$p\text{CO}_2$, *A. polyacanthus* are typically able to maintain or even increase oxygen uptake rates during or following exhaustive exercise events. However, when *A. polyacanthus* in this study had paCA inhibited (C18-injection) during exposure to stable elevated $p\text{CO}_2$, $\dot{M}\text{O}_{2\ \text{Max}}$ was not maintained. This suggests that paCA is short-circuiting β-NHE activity and re-acidifying RBCs in fish exposed to stable elevated $p\text{CO}_2$, thus enabling higher tissue O_2 extraction and maintained $\dot{M}\text{O}_{2\ \text{Max}}$ following exhaustive exercise. This is the first evidence that fish may be using the paCA Root Effect Hb system to maintain oxygen uptake rates and aerobic performance upon exposure to ocean acidification relevant $p\text{CO}_2$ conditions.

Minimum oxygen uptake rates ($\dot{M}\text{O}_{2\ Min}$)

The increasing $p\text{CO}_2$ due to ocean acidification has been predicted to have negative impacts on the minimum metabolic rate of fishes, based on the loading stress hypothesis (Esbaugh, 2017; Heuer and Grosell, 2014). This hypothesis suggests that the additional costs of ion-regulation would increase the basic maintenance costs to fishes. In this study, sham-injected fish exposed to fluctuating elevated $p\text{CO}_2$ exhibited a $\dot{M}\text{O}_{2\ \text{Min}}$ that was 14% higher than fish exposed to ambient $p\text{CO}_2$ conditions. Previous increases in $\dot{M}\text{O}_{2\ \text{Min}}$ that have been attributed to loading stress typically range from 25-60% in marine fishes (Lefevre, 2019). Thus, the 14% increase upon exposure to fluctuating $p\text{CO}_2$ falls on the low end of the typical range and may have more to do with recovery from exhaustive exercise than from loading stress. However, the sham-injected fish exposed to stable elevated $p\text{CO}_2$ were able to maintain $\dot{M}\text{O}_2$ $_\text{Min}$ when compared to ambient exposed fish. This maintenance of $\dot{M}\text{O}_{2\ \text{Min}}$ is similar to what has been demonstrated in the majority (>80%) of marine fishes that have been investigated under elevated $p\text{CO}_2$ conditions to date (Lefevre, 2019). Thus, for the sham-injected *A. polyacanthus* in this study, fluctuating elevated $p\text{CO}_2$ conditions seem to be more energetically stressful than either stable elevated or ambient $p\text{CO}_2$ conditions, either due to

loading stress or recovery from exercise. This finding contrasts my initial hypothesis that exposure to fluctuating elevated pCO$_2$ conditions would be less energetically costly to coral reef fishes, as such fluctuating conditions are more similar coral reef habitats when compared to stable conditions.

Varied responses of $\dot{M}$O$_{2\ Min}$ have also been observed in previous studies on *A. polyacanthus* exposed to different levels of pCO$_2$, examining different life stages, and utilizing different $\dot{M}$O$_{2\ Min}$ methods (Hannan et al., 2020b; Laubenstein et al., 2019; Laubenstein et al., 2020; Rummer et al., 2013a). Collectively, these findings suggest that time of exposure, pCO$_2$ treatment, fish life stage, and method of determining $\dot{M}$O$_{2\ Min}$ should be carefully considered when designing experiments and drawing conclusions from the results obtained. For instance, this is the first study to show that fluctuating elevated pCO$_2$ results in increases in $\dot{M}$O$_{2\ Min}$ in *A. polyacanthus*, but a range of responses to stable elevated pCO$_2$ have been observed from increases to decreases (Hannan et al., 2020b; Laubenstein et al., 2019; Laubenstein et al., 2020; Rummer et al., 2013a).

It is also important to note that the C18 would no longer have been exclusively inhibiting the targeted paCA during the $\dot{M}$O$_{2\ Min}$ measurements in this study. Indeed, after about 45 minutes, C18 crosses RBC membranes, which would be prior to $\dot{M}$O$_{2\ Min}$ measurements. Thus, when considering how C18-injected fish responded in terms of the $\dot{M}$O$_{2\ Min}$, the direct effects of paCA inhibition are no longer being measured at this point. Therefore, the response might be more likely related to recovery from exhaustive exercise. That being said, when paCA was inhibited (C18-injection), all fish, regardless of pCO$_2$ treatment, generally exhibited an increase in $\dot{M}$O$_{2\ Min}$ when compared to fish with functional paCA (sham-injected). Although this trend was not statistically significant, it does suggest that functional paCA may

contribute to recovery following exhaustive exercise, a topic worthy of further investigation in future studies.

Aerobic scope (AAS and FAS)

It has been generally accepted since the early 21st century that fish exposed to elevated pCO$_2$ would exhibit a decrease in aerobic scope largely due to the effects of reduced O$_2$ carrying capacity of haemoglobin on $\dot{M}$O$_{2\ Max}$ and the effects of increased ion-regulation on $\dot{M}$O$_{2\ Min}$ (Pörtner and Farrell, 2008). However, in most (~63%) of the marine fishes that have been investigated to date, aerobic scope, much like the other oxygen uptake metrics, is unaffected by elevated pCO$_2$ (Lefevre, 2019). Similarly, in this study neither absolute (AAS) nor factorial (FAS) aerobic scope of sham-injected *A. polyacanthus* were affected by elevated pCO$_2$. Absolute aerobic scope (AAS) is understood to represent the capacity for oxygen consuming life-history processes above basic maintenance requirements (Killen et al., 2016). However, factorial aerobic scope (FAS) represents the capacity for energy turnover based on resting energy expenditures ($\dot{M}$O$_{2\ Min}$). Clark et al., (2013) suggests that AAS is more informative and robust than FAS because specific activities require specific amounts of oxygen rather than proportions above basic maintenance. Additionally, FAS can vary greatly with small changes in $\dot{M}$O$_{2\ Min}$, which is important to consider for the current study, as the C18-injection would no longer be exclusively inhibiting the targeted paCA. However, the fact that neither AAS nor FAS were affected by elevated pCO$_2$ suggests that *A. polyacanthus* are able to maintain energy availability during this exhaustive exercise challenge.

Although inhibiting paCA resulted in decreased $\dot{M}$O$_{2\ Max}$ during exposure to stable elevated pCO$_2$, this did not translate to a similar decrease in AAS. This suggests that, although paCA is important for maintaining $\dot{M}$O$_{2\ Max}$ during stable pCO$_2$ exposure, paCA inhibition may not affect energy availability for life-history functions. Alternatively, paCA inhibition resulted in

decreased FAS for fish in the fluctuating elevated CO_2 treatment compared to ambient exposed fish, suggesting that paCA is playing a role in the maintained FAS of fluctuating elevated CO_2 exposed fish. Thus, in terms of absolute energy availability, paCA activity does not appear to be playing a large role during exposure to elevated pCO_2 (AAS), but there is evidence of beneficial effects of paCA activity on the ratio of energy availability compared to basic maintenance requirements (FAS). Altogether, in this study there is evidence that paCA is used to maintain $\dot{M}O_{2\ Max}$ during stable elevated pCO_2 exposure but overall energy availability is not affected by the inhibition of this enzyme (AAS). Thus, *A. polyacanthus*, and potentially some other coral reef fishes, may be able to maintain or even increase energy available for life-history functions in the face of future ocean acidification conditions.

Whole blood lactate

Blood lactate has typically been used to indicate anaerobic metabolism, which fuels burst-type exhaustive exercise (Milligan and Girard, 1993). During recovery following exercise, lactate that is released from the muscle can fuel oxidative metabolism, and additional lactate remaining in the blood can be taken up by the muscles in order to replenish glycogen (Milligan and Girard, 1993). Thus, during recovery from exercise, the majority of lactate is taken up into the muscles, potentially for glycogenesis (Milligan and Girard, 1993). This study supports the glycogenesis hypothesis, as the majority of sham-injected *A. polyacanthus* that had recovered from exhaustive exercise overnight had reduced whole blood lactate concentrations when compared to fish that were sampled immediately or 30 min following exhaustive exercise. Immediately following exhaustive exercise under stable elevated pCO_2 conditions fish that had paCA inhibited (C18-injection) exhibited lower whole blood lactate concentrations than fish sampled 30 min following the injection. This suggests that fish without functional paCA had to rely more on anaerobic metabolism to maintain performance

under stable elevated pCO_2 conditions. This evidence supports the notion that the paCA Root Effect Hb system plays a role in maintaining performance immediately following exhaustive exercise.

Whole blood glucose

Hyperglycaemia is a known part of the secondary stress response in fishes, where glucose is released from the liver to fuel metabolic demands following a stressful event, such as exhaustive exercise (Wendelaar Bonga, 1997). In the present study, the overnight sampling interval generally revealed elevated whole blood glucose concentrations in fish when compared to fish sampled immediately and after 30 min. This overnight increase in glucose concentrations corresponded with decreased whole blood lactate concentrations, which supports the notion that the role of glucose aligns more with the secondary stress response than that of glycogen recovery. Inhibiting paCA resulted in higher glucose concentrations in the blood compared to sham-injected fish during the overnight sampling interval upon exposure to stable elevated pCO_2. This suggests that exposure to stable elevated pCO_2 is more stressful for fish than either fluctuating elevated or ambient pCO_2, and more glucose is released from the liver when paCA is inhibited following exhaustive exercise. These metabolite results (whole blood glucose and lactate concentrations) suggest that when *A. polyacanthus* are exposed to stable elevated pCO_2 and have paCA inhibited there is a need for alternative mechanisms to maintain performance during exercise and to facilitate recovery.

5.6 Conclusions

Most of the findings from the current study were observed when paCA was inhibited in *A. polyacanthus* that were exposed to stable elevated pCO_2 conditions. This suggests that stable elevated pCO_2 conditions may be more stressful for coral reef fish species, such as the *A. polyacanthus* examined here, when compared to either fluctuating or ambient pCO_2 conditions. These stable elevated pCO_2 conditions may be more stressful, perhaps because coral reef fishes are accustomed and adapted to fluctuating pCO_2 in their natural habitats or because fluctuating pCO_2 provides a temporary refuge from the stress of constant elevated pCO_2 (Bracken et al., 2018; Jarrold et al., 2017). If this is the case, these stable elevated pCO_2 conditions may initiate the adrenergic stress response, creating conditions when the β-NHE on the RBC membrane could be short-circuited with paCA. This would facilitate enhanced O_2 release to the tissues, thus maintaining or enhancing performance during these times of heightened stress. In contrast, exposure to more ecologically relevant future conditions, such as fluctuating elevated pCO_2, may not be enough to initiate an adrenergic stress response of this magnitude in coral reef fishes, such as the one examined here. Although this study found evidence that inhibiting paCA effects oxygen delivery/transport, there have been no studies confirming that coral reef fishes possess the requirements for enhanced O_2 delivery, i.e., Root effect Hbs, the ability to regulate pH_i with β-NHE, or paCA presence in the tissues. Future research should focus on determining the mechanisms responsible for maintained performance in fishes. Species variation in any of the required conditions for enhanced O_2 delivery could be responsible for the lack of consensus in the literature surrounding exercise performance in fishes exposed to OA-relevant conditions. Once the mechanisms are determined, the presence can be examined in different fishes to determine which fish species will succeed in the face of climate change.

Chapter 6:   General discussion

Predicting the responses of shallow nearshore marine organisms to ocean acidification has been a challenge for marine scientists. Shallow nearshore environments are known to have more variable pH and CO_2 cycles when compared to the open ocean; however, future ocean acidification predictions are typically based on relatively stable open ocean conditions. As a result, the majority of previous studies examining the impacts of ocean acidification have used stable elevated CO_2 treatments that may not be ecologically relevant for coastal species. In order to accurately predict how an organism will respond to future ocean acidification, the pCO_2 environment the animal experiences in its habitat must be considered. This is complicated by the lack of information on natural CO_2 variation among different ecosystems, and different microhabitats within ecosystems. This thesis investigated the current pCO_2 fluctuations on coral reefs and experimentally tested how coral reef fishes respond physiologically to these fluctuations and to predicted future pCO_2 conditions. In general, I found that the majority of reef fish species are robust to projected future ocean acidification conditions and that future fluctuating CO_2 cycles can improve the exercise physiology of coral reef fishes under ocean acidification. Furthermore, I found a potential mechanism fishes are employing to maintain performance during exposure to elevated CO_2.

CO_2 cycles on coral reefs

It is known that coral reefs experience diel cycles in pH and CO_2 that are mainly driven by respiration/photosynthesis and calcification/dissolution cycles of benthic organisms. In Chapter 2 I found the expected diel cycles of pH and CO_2 across all of the microhabitats measured (i.e., sand, hard coral, and soft coral) at all three reefs that I examined.

Unexpectedly, microhabitat did not have a large effect on the CO_2 fluctuations at each reef. The observed variations in CO_2 at a reef level were influenced both by the wind speed as well as hydrodynamic factors (i.e., water residence time). Reefs with longer water residence time (i.e., lagoonal reefs) had larger fluctuations in CO_2; whereas, the most exposed reef had a much smaller and more regular diel variation in CO_2. Thus, it is important to include hydrodynamic, biotic, and abiotic factors when considering the current CO_2 variations at coral reefs and nearshore systems in order to more accurately predict how these systems will change in the future. This is especially important for less mobile organisms or species that complete their life cycle within one reef, such as brooding fishes, because their offspring will experience the same ecosystem as the parents thus providing more potential for adaptation to specific conditions. Seasonal fluctuations in $p$$CO_2$ have the potential to increase up to 10-fold in the future (McNeil and Sasse, 2016), but predictive models that take smaller scale fluctuations (i.e., diel and reefs) into account are lacking. This is important, as I observed that the daily fluctuations around Lizard Island varied in magnitude by reef.

The Lizard Island Research Station is one of the best researched coral reef locations on the planet, more specifically it has been home to >25 studies on the effects of ocean acidification on fishes. Most of these previous studies have used stable CO_2 levels to determine the impacts of ocean acidification on coral reef fishes. My findings in Chapter 2 show that the coral reef fishes inhabiting reefs around Lizard Island experience fluctuating CO_2 conditions in their natural environment. Further, fluctuations in CO_2 have been hypothesized to affect the responses of nearshore marine organisms to elevated CO_2 differently than stable CO_2 levels that have been traditionally used (Shaw et al., 2013b). If fluctuating CO_2 exposures result in mitigation of responses to ocean acidification, the stable CO_2 exposures that have been used traditionally could be over estimating the effects of elevated CO_2 on fishes (Jarrold et al., 2017). Alternatively, larger CO_2 fluctuations could exacerbate the responses to elevated

CO_2, thus causing stable CO_2 exposures to underestimate the responses of fishes to ocean acidification. However, the majority of previous studies have found that many negative impacts of elevated stable pCO_2 are ameliorated by exposure to elevated fluctuating pCO_2. Further, this thesis shows that most of the fishes studied benefited more during exposure to fluctuating elevated pCO_2 compared to stable elevated pCO_2, thus I believe that many of the previous studies have been overestimating the negative impacts of future elevated pCO_2. It is important to consider the environment that fishes are naturally exposed to when designing studies and treatments to more accurately determine how fish will be affected by future conditions.

Effects of ocean acidification and diel CO_2 cycles on oxygen uptake

Ocean acidification has the potential to become a significant stressor for coral reef fishes due to the predicted increased energetic costs of acid-base regulation (Baker and Brauner, 2012). However, I observed that the maintenance metabolism (i.e., minimum oxygen uptake rates, $\dot{M}O_{2\ Min}$) of the majority of fish species studied is unaffected by stable and diel-fluctuating elevated CO_2 (Chapter 3, 4, and 5). The only negative impact I observed was when *A. polyacanthus* were exposed to fluctuating elevated CO_2 conditions for 12 d (Chapter 5). However, the maintenance metabolism of *A. polyacanthus* benefited under fluctuating elevated CO_2 conditions when compared to ambient CO_2 exposed fish during a short-term (8 hr) exposure (Chapter 3). This could suggest that longer exposures (12 d) to fluctuating elevated CO_2 incur costs to maintenance metabolism from long-term acid-base regulation for *A. polyacanthus* (Chapter 5). However, this is still unexpected, as longer-term exposures are typically thought to allow time for fish to offset the cost of maintaining acid-base balance, despite elevated CO_2 (acidosis) in the blood (Lefevre, 2019). Another factor that may be

influencing these data is differing methodology. The benefit to maintenance metabolism was observed when $\dot{M}O_{2\,Min}$ was calculated from extrapolating the relationship of swimming speed and $\dot{M}O_2$ during a U_{crit} test to 0 BL s^{-1} (Brett, 1964; Bushnell et al., 1994; Schurmann and Steffensen, 1997); whereas, the detriment to $\dot{M}O_{2\,Min}$ was observed when fish were allowed to recover from an exhaustive chase in static respirometers overnight after which, the lowest 10% of $\dot{M}O_2$ values were averaged to determine $\dot{M}O_{2\,Min}$. These are both accepted ways to determine $\dot{M}O_{2\,Min}$; however, a previous study demonstrated that, depending on the function used to describe the relationship of oxygen uptake rates over the various swimming speeds, extrapolation can result in lower estimates of $\dot{M}O_{2\,Min}$ when compared to static respirometry (Roche et al., 2013). Thus, it is difficult to determine if the discrepancies are due to different CO_2 exposures (both concentration and length of exposure) or differences in methodology. Although there were conflicting results across experiments for one species, the majority of species showed maintained $\dot{M}O_{2\,Min}$, suggesting that these fishes are not experiencing increased costs of acid-base regulation upon exposure to elevated CO_2. This may mean that exposure to elevated CO_2 conditions is not energetically costly. Alternatively, small increases may be missed by whole animal measurements, as ion-regulation costs can be tissue specific (Heuer and Grosell, 2016).

The exercise performance of coral reef fishes, collectively quantified as maximum metabolic rate, aerobic scope, and maximum sustained swimming speed was generally unaffected by stable and diel-fluctuating elevated CO_2 conditions over both short-term (8 hr: Chapter 3) and longer (9-12 d: Chapter 4 and 5) durations. Further, four species benefited upon exposure to diel-fluctuating elevated CO_2 conditions (Chapter 3 and 4). The only species that was negatively impacted, in terms of exercise performance metrics, was *C. quinquelineatus*; this species exhibited a decreased $\dot{M}O_{2\,Max}$ and aerobic scope upon exposure to diel-fluctuating elevated CO_2 conditions (Chapter 4). The decreases observed in *C. quinquelineatus* for $\dot{M}O_2$

$_{Max}$ may be due to either decreased cardio-respiratory capacity or decreased mitochondrial efficiency of the tissues, but it is not possible to determine which mechanism is responsible without direct measurements. However, the majority of species were able to maintain or increase their exercise performance during exposure to elevated CO_2 conditions. Absolute aerobic scope, a proxy for energy availability for aerobically mediated life-history traits, was maintained or increased during exposure to both stable and diel-fluctuating elevated CO_2 conditions in six of the seven species studied (Chapter 3, 4, and 5). This suggests that the aerobic performance of most coral reef fishes is robust to future elevated CO_2 conditions, which is consistent with previous observations (Lefevre, 2016).

Interestingly, one such aerobically mediated trait, maximum sustained swimming speed, was either maintained or increased for all fish species studied upon exposure to stable and diel-fluctuating elevated CO_2 conditions. I found that increases in $\dot{M}O_2$ $_{Max}$ and aerobic scope lead to increases in U_{crit} for *L. fulvaflamma, C. cuning,* and *A. whitleyi* (Chapter 4). This is possibly due to increased ATP output, thus making more energy available and therefore allowing a higher U_{crit} (Hamilton et al., 2017). Interestingly, this is the first time exposure to elevated CO_2 has resulted in increased U_{crit}, as all previous studies have found that U_{crit} is either maintained or decreased (reviewed by Lefevre, 2019). Collectively, exercise performance was initially expected to decline upon exposure to elevated CO_2 as a result of decreased blood pH affecting the oxygen affinity and carrying capacity of haemoglobin the combined Bohr and Root Effects (Pörtner et al., 2004). However, my results suggest that the exercise performance of the coral reef fishes studied here was maintained or increased upon exposure to elevated pCO$_2$.

The one species that was tested multiple times, *A. polyacanthus*, was able to maintain $\dot{M}O_2$ $_{Max}$ upon exposure to both stable and diel-fluctuating elevated CO_2 conditions across different

time periods (8 h and 12 d; Chapters 3 and 5). However, when *A. polyacanthus* were exposed to ambient CO_2, $\dot{M}O_{2\,Max}$ derived from the U_{crit} method was 24% higher (Chapter 3) than $\dot{M}O_{2\,Max}$ values derived in sham injected chase/air exposed *A. polyacanthus* (Chapter 5). U_{crit} methods resulting in higher within-species $\dot{M}O_{2\,Max}$ measurements when compared to chase/air exposure methods, as has been demonstrated for five other coral reef fish species (Roche et al., 2013; Rummer et al., 2016). However, the opposite trend has been found for Atlantic cod (Reidy et al., 1995). This suggests that the type of swimming test should be carefully considered depending on what question is being posed and which species are being examined. Overall, my data suggest that, for the majority coral reef fishes that I studied, *A. polyacanthus*, *A. curacao, L. fulvaflamma, C. cuning,* and *A. whitleyi*, the ability to perform important exercise performance behaviours, such as escaping predators, should not be compromised by ocean acidification.

Mechanisms used to maintain oxygen uptake

The physiological mechanisms that have been hypothesized to underpin maintained oxygen uptake rates upon exposure to elevated CO_2 include the following: sufficient capacity for acid-base regulation (Melzner et al., 2009a), increased pumping ability of the heart (Gräns et al., 2014), increased respiratory surface area (Rummer et al., 2013a), behavioural alterations leading to increases in swimming during U_{crit} tests (Couturier et al., 2013), and the unique oxygen transport system of teleost fishes (Root effect Hbs and paCA) (Hannan and Rummer, 2018). However, most of these proposed mechanisms have not been directly measured in fishes. I found the first evidence for the role of paCA in maintaining maximum oxygen delivery to the tissues and, as a biproduct of that, oxygen uptake rates during exposure to ocean acidification relevant elevated CO_2 conditions (Chapter 5). There was additional

evidence that when paCA was inhibited fish were forced to use alternative mechanisms, such as switching to anaerobic metabolism sooner. This mechanism had only previously been confirmed in salmonids and had never previously been examined under ocean acidification relevant CO_2 conditions. Further, although this thesis shows impacts of the inhibition of paCA, suggesting that *A. polyacanthus* possess one or more of the mechanisms that allow for enhanced O_2 delivery, there have been no direct studies confirming this in coral reef fishes. Further research should determine if coral reef fishes possess Root effect Hbs, regulate pH_i using β-NHE, and possess paCA in their muscles, as variations in these mechanisms could help explain the lack of consensus on fish performance upon exposure to OA-relevant conditions. Additionally, more research is needed to consider any additional mechanisms beyond enhanced O_2 delivery that teleost fishes may be using to maintain oxygen uptake, as there is the potential for several mechanisms to be acting together.

Effects of ocean acidification and diel CO_2 cycles on hematological metrics and metabolites

Examining how haematological metrics, such as haemoglobin, haematocrit, and mean corpuscular haemoglobin, change with exposure to ocean acidification relevant CO_2 conditions can help inform how oxygen transport will be affected in the future; yet, few studies have examined these metrics. Mean corpuscular haemoglobin concentrations, calculated from whole blood haemoglobin concentrations and haematocrit, were expected to decrease upon exposure to elevated CO_2. This was hypothesized to be a product of cell swelling thought to protect the pH_i of the red blood cell and maintain haemoglobin oxygen affinity (Caldwell et al., 2006). However, I found that the majority of haematological metrics that were measured after an exhaustive chase test were unaffected by exposure to elevated CO_2 (Chapter 3 and 4). I also found no evidence of the expected decreases in mean

corpuscular haemoglobin concentrations, and in fact, three of the six species studied had increased mean corpuscular haemoglobin concentrations upon exposure to elevated CO_2 when compared to ambient CO_2 exposed fish. This has previously been observed in an *Epinephelus fuscoguttatus x lanceolatus* hybrid, *Dicentrarchus labras*, *Salmo salar,* and *Hemiscyllium ocellatum* upon exposure to ocean acidification relevant CO_2 conditions (Crespel et al., 2019; Fivelstad et al., 2007; Heinrich et al., 2014; Noor et al., 2019). The observed increase in mean corpuscular haemoglobin concentrations could be due to either red blood cell shrinkage or the adrenergically mediated release of immature red blood cells from the spleen into circulation. Red blood cell shrinkage is the most plausible mechanism here, as it can take up to 8 months to renew red blood cells (Witeska, 2013). The probable red blood cell shrinkage was expected to negatively impact oxygen transport; however, Brauner et al., (2002) found limited impact of red blood cell shrinkage on blood oxygen transport properties. Similarly, I observed that increases in mean corpuscular haemoglobin concentration (red blood cell shrinking) did not result in decreases to $\dot{M}O_2$, which is consistent with recent suggestions that red blood cell shrinkage upon exposure to elevated CO_2 might aid oxygen carrying capacity (Crespel et al., 2019).

Similar to the above haematological metrics, I found that, immediately following exhaustive exercise, the metabolites including whole blood lactate and glucose in the majority of fish species that I studied were unaffected by exposure to elevated CO_2 conditions. Changes in whole blood lactate concentrations can provide information on the shift from aerobic to anaerobic metabolism during the exhaustive swimming test. I found decreased lactate concentrations in two of the seven fishes that I studied following 9-11 d exposure to both stable and diel-fluctuating elevated CO_2. This may mean that for those two species (i.e., *C. cuning* and *C. quinquelineatus*) exposure to one or both elevated CO_2 treatments resulted in heavier reliance on aerobic metabolism when compared to ambient CO_2 exposed fish. Yet, it

should be noted that in most fish species I examined, whole blood lactate concentrations were unaffected by elevated CO_2. Glucose is a known part of the secondary stress response and often used in conjunction with cortisol to measure the stress response in fishes (Barton and Iwama, 1991; Barton et al., 1988). As glucose is often measured in conjunction with other metrics, caution should be taken when relying on glucose alone to draw conclusions about the stress response in fish. However, because glucose is part of the secondary stress response, we might expect that glucose would start increasing after the acute stress of swimming. So, to measure the different stress responses after swimming, we would need to measure recovery (Chapter 5). When examining the response to an acute stress (i.e., exhaustive swimming) under different CO_2 conditions, I found that exposure to diel-fluctuating elevated CO_2 conditions is less stressful to fish than ambient or stable elevated CO_2 conditions (Chapter 5). The shorter exposure (8 hr) resulted in decreased whole blood glucose concentrations for one species (Chapter 3); whereas, a longer exposure 9-11 d resulted in increased whole blood glucose concentrations for another species (Chapter 4). However, this finding was far from universal, and exposure to elevated CO_2 did not affect whole blood glucose concentrations in the majority of species that I examined (5 of 7). Thus, in terms of metabolites that can be easily measured in small fishes, no obvious trend with exposure to elevated CO_2 could be detected; however, future studies should focus on the secondary stress response associated with exhaustive swimming upon exposure to elevated CO_2 conditions.

Limitations and future directions

This thesis is perhaps limited in scope, i.e., the methodology used is repeated throughout multiple chapters, and is limited to oxygen uptake rates, swimming speeds, and haematological metrics. However, this allows for comparison across chapters and leaves

openings for future research. Future studies should examine the mechanisms that facilitate maintained exercise performance in teleost fishes upon exposure to ecologically relevant CO_2 conditions. Additional research on the blood oxygen transport system that is unique to teleosts (Randall et al., 2014; Rummer and Brauner, 2011), the role of the cardiorespiratory system (Gräns et al., 2014), as well as genomics (Brennan et al., 2018; Nielsen et al., 2009) and transcriptomics (Connon et al., 2018; Schunter et al., 2018) would help to determine mechanisms and the patterns of genes and gene expression underpinning maintained performance. A broader understanding can help us, as a scientific community, identify which types of fishes (i.e., by species, life-history, size, etc.) will be able to adapt to and perform under elevated CO_2 levels and therefore future ocean acidification conditions. Additionally, it is important to note that ocean acidification is not occurring in isolation. Not only are the means and magnitudes of CO_2 fluctuations going to increase, but also a variety of other environmental parameters are expected to increase as well. For instance, the average temperatures of shallow water environments, such as coral reefs, are expected to increase by up to 4°C by the end of the century (Collins et al., 2013). The results derived here from examining the effects of one stressor (i.e., elevated CO_2) could be very different than the effects of multiple stressors that could be additive or synergistic (Laubenstein et al., 2018) and may more closely resemble future ocean conditions. Varying responses to multiple stressors have been observed when compared to individual stressors, for instance when examining elevated temperatures and CO_2 in combination (reviewed by Lefevre, 2016). Examining the responses of multiple stressors will provide more accurate predictions as to how fishes will respond to future climate change conditions. Together, examining the mechanistic basis for altered or maintained performance upon exposure to multiple stressors can help create a more holistic understanding as to how coral reef fishes will respond to and cope with future climate change conditions

Concluding remarks

The results of this thesis can be used to assist in creating future ocean acidification models that incorporate, not only seasonal, but also diel CO_2 fluctuations. Additionally, these data can inform more accurate experimental CO_2 treatments when investigating potential responses of coral reef organisms to ocean acidification. These results add to the growing consensus that it is critical to incorporate natural CO_2 variability in ocean acidification experiments to paint a more ecologically relevant picture of how ocean acidification will affect nearshore marine organisms (Frieder et al., 2014; Jarrold and Munday, 2019; Jarrold et al., 2017; Laubenstein et al., 2020; Ou et al., 2015; Pilditch et al., 2013). However, as a whole, the majority of coral reef fishes studied in this thesis appear robust to future CO_2 conditions, at least in terms of swimming performance and energy availability. This is encouraging for managers, but it does also suggest that impacts will be species-specific, and more research and focus should be placed on sensitive species, such as the nocturnal cardinalfish studied here. The detrimental effects to energy availability of this fish species could potentially have community level impacts. Further, I found evidence that the novel physiological mechanism that has been identified in salmonids may also be enabling coral reef fishes to maintain aerobic performance when exposed to elevated CO_2 conditions. It would behoove managers to determine whether this mechanism is universally utilized by coral reef fishes, as this finding help determine which species will be winners and losers in the face of future ocean acidification conditions.

This thesis is a good example of a conservation physiology approach to an environmental stressor, spanning environmental measurements to examining the mechanisms underpinning whole organism performance. Conservation physiology is an emerging field defined as the

study of physiological responses of organisms to human alteration of the environment that might cause or contribute to population declines (Wikelski and Cooke, 2006). Many of the threats to the persistence of a population of sensitive species have underlying physiological mechanisms (Tracy et al., 2006). Indeed, reproductive success and individual fitness are largely determined by physiology. Thus, environmental change has the potential to affect fitness due to its effect on physiology (Seebacher and Franklin, 2012). Further, significant progress is starting to be made in understanding the mechanistic underpinnings of some aspects of physiological variation among individuals or species (Carey, 2005). The study of such variations will become even more critical than it has been in the past due to the ever increasing threat of a rapidly changing environment (Carey, 2005). Thus, there is an increasingly urgent need to improve the combination of ecological and physiological studies with clear conservation implications to create an accurate picture of how marine organisms will be affected in the future.

References

Albright, R., Langdon, C. and Anthony, K. R. N. (2013). Dynamics of seawater carbonate chemistry, production, and calcification of a coral reef flat, central Great Barrier Reef. *Biogeosciences* **10**, 6747–6758.

Alderman, S. L., Harter, T. S., Wilson, J. M., Supuran, C. T., Farrell, A. P. and Brauner, C. J. (2016). Evidence for a plasma-accessible carbonic anhydrase in the lumen of salmon heart that may enhance oxygen delivery to the myocardium. *J. Exp. Biol.* **219**, 719–24.

Alenius, B. and Munguia, P. (2012). Effects of pH variability on the intertidal isopod, *Paradella dianae. Mar. Freshw. Behav. Physiol.* **45**, 245–259.

Andersson, A. J., Yeakel, K. L., Bates, N. R. and De Putron, S. J. (2013). Partial offsets in ocean acidification from changing coral reef biogeochemistry. *Nat. Clim. Chang.* **4**, 56–61.

Anthony, K. R. N., Kleypas, J. A. and Gattuso, J.-P. (2011). Coral reefs modify their seawater carbon chemistry - implications for impacts of ocean acidification. *Glob. Chang. Biol.* **17**, 3655–3666.

Baker, D. W. and Brauner, C. J. (2012). Metabolic changes associated with acid–base regulation during hypercarbia in the CO_2-tolerant chondrostean, white sturgeon (*Acipenser transmontanus*). *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* **161**, 61–68.

Baker, D. W., Matey, V., Huynh, K. T., Wilson, J. M., Morgan, J. D. and Brauner, C. J. (2009). Complete intracellular pH protection during extracellular pH depression is associated with hypercarbia tolerance in white sturgeon, *Acipenser transmontanus*. *AJP Regul. Integr. Comp. Physiol.* **296**, R1868–R1880.

Barton, B. A. and Iwama, G. K. (1991). Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Annu. Rev. Fish Dis.* **1**, 3–26.

Barton, B. A., Schreck, C. B. and Fowler, L. G. (1988). Fasting and diet content affect stress-induced changes in plasma glucose and cortisol in juvenile chinook salmon. *Progress. Fish-Culturist* **50**, 16–22.

Bates, N. R., Samuels, L. and Merlivat, L. (2001). Biogeochemical and physical factors influencing seawater $f CO_2$ and air-sea CO_2 exchange on the Bermuda coral reef. *Limnol. Oceanogr.* **46**, 833–846.

Bates, D., Mächler, M., Bolker, B. M. and Walker, S. C. (2015). Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* **67**, 1–48.

Baumann, H. (2019). Experimental assessments of marine species sensitivities to ocean acidification and co-stressors: how far have we come? *Can. J. Zool.* **97**, 399–408.

Bay, L. K., Crozier, R. H. and Caley, M. J. (2006). The relationship between population genetic structure and pelagic larval duration in coral reef fishes on the Great Barrier Reef. *Mar. Biol.* **149**, 1247–1256.

Bell, W. and Terhune, L. (1970). Water tunnel design for fisheries research. *J. Fish. Res. Board Canada* **195**, 1–69.

Bignami, S., Sponaugle, S. and Cowen, R. K. (2013). Response to ocean acidification in larvae of a large tropical marine fish, *Rachycentron canadum*. *Glob. Chang. Biol.* **19**, 996–1006.

Bignami, S., Sponaugle, S. and Cowen, R. K. (2014). Effects of ocean acidification on the larvae of a high-value pelagic fisheries species, Mahi-mahi *Coryphaena hippurus*. *Aquat. Biol.* **21**, 249–260.

Bignami, S., Sponaugle, S., Hauff, M. and Cowen, R. K. (2017). Combined effects of elevated pCO$_2$, temperature, and starvation stress on larvae of a large tropical marine fish. *ICES J. Mar. Sci.* **74**, 1220–1229.

Borch, K., Jensen, F. B. and Andersen, B. B. (1993). Cardiac activity, ventilation rate and acid-base regulation in rainbow trout exposed to hypoxia and combined hypoxia and hypercapnia. *Fish Physiol. Biochem.* **12**, 101–110.

Borgese, F., Garcia-Romeu, F. and Motais, R. (1987). Control of cell volume and ion transport by beta-adrenergic catecholamines in erythrocytes of rainbow trout, *Salmo gairdneri*. *J. Physiol.* **382**, 123–144.

Boutilier, R. G., Heming, T. A. and Iwama, G. K. (1984). Physicochemical parameters for use in fish respiratory physiology. In *Fish Physiology* (ed. Hoar, W. S.) and Randall, D. J.), pp. 403–426. New York: Academic Press Inc.

Bracken, M. E. S., Silbiger, N. J., Bernatchez, G. and Sorte, C. J. B. (2018). Primary producers may ameliorate impacts of daytime CO$_2$ addition in a coastal marine ecosystem. *PeerJ* **6**, e4739.

Brauner, C. J., Wang, T. and Jensen, F. B. (2002). Influence of hyperosmotic shrinkage and β-adrenergic stimulation on red blood cell volume regulation and oxygen binding properties in rainbow trout and carp. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **172**, 251–262.

Brauner, C. J., Shartau, R. B., Damsgaard, C., Esbaugh, A. J., Wilson, R. W. and Grosell, M. (2019). *Acid-base physiology and CO2 homeostasis: Regulation and compensation in response to elevated environmental CO2*. 1st ed. Elsevier Inc.

Breiman, L., Friedman, J. H., Olshen, R. A. and Stone, C. J. (1984). *Classification and regression trees*. Boca Raton: Chapman & Hall/CRC.

Brennan, R. S., Healy, T. M., Bryant, H. J., La, M. Van, Schulte, P. M. and Whitehead, A. (2018). Integrative population and physiological genomics reveals mechanisms of adaptation in killifish. *Mol. Biol. Evol.* **35**, 2639–2653.

Brett, J. (1964). The respiratory metabolism and swimming performance of young sockeye salmon. *J. Fish. Res. Board Canada* **21**, 1183–1226.

Burgos-Aceves, M. A., Lionetti, L. and Faggio, C. (2019). Multidisciplinary haematology as prognostic device in environmental and xenobiotic stress-induced response in fish. *Sci. Total Environ.* **670**, 1170–1183.

Bushnell, P. G., Steffensen, J. F., Schurmann, H. and Jones, D. R. (1994). Exercise metabolism in two species of cod in arctic waters. *Polar Biol.* **14**, 43–48.

Caldeira, K. and Wickett, M. E. (2003). Anthropogenic carbon and ocean pH. *Nature* **425**, 365.

Caldwell, S., Rummer, J. L. and Brauner, C. J. (2006). Blood sampling techniques and storage duration: effects on the presence and magnitude of the red blood cell β - adrenergic response in rainbow trout (*Oncorhynchus mykiss*). *Comp. Biochem. Physiol. - A Mol. Integr. Physiol.* **144**, 188–195.

Carey, C. (2005). How physiological methods and concepts can be useful in conservation biology. *Integr. Comp. Biol.* **45**, 4–11.

Carpenter, B., Gelman, A., Hoffman, M. and Lee, D. (2017). Stan: a probabilistic programming language. *J. Stat. Softw.* **76**, 1–29.

Cattano, C., Claudet, J., Domenici, P. and Milazzo, M. (2018). Living in a high CO_2 world: a global meta-analysis shows multiple trait-mediated fish responses to ocean acidification. *Ecol. Monogr.* **88**, 320–335.

Ceccarelli, D. M., Emslie, M. J. and Richards, Z. T. (2016). Post-disturbance stability of fish assemblages measured at coarse taxonomic resolution masks change at finer scales. *PLoS One* **11**, e0156232.

Chan, W. Y. and Eggins, S. M. (2017). Calcification responses to diurnal variation in seawater carbonate chemistry by the coral *Acropora formosa*. *Coral Reefs* **36**, 763–772.

Clark, H. R. and Gobler, C. J. (2016). Diurnal fluctuations in CO_2 and dissolved oxygen concentrations do not provide a refuge from hypoxia and acidification for early-life-stage bivalves. *Mar. Ecol. Prog. Ser.* **558**, 1–14.

Clark, T. D., Eliason, E. J., Sandblom, E., Hinch, S. G. and Farrell, A. P. (2008). Calibration of a hand-held haemoglobin analyser for use on fish blood. *J. Fish Biol.* **73**, 2587–2595.

Clark, T. D., Sandblom, E. and Jutfelt, F. (2013). Aerobic scope measurements of fishes in an era of climate change: respirometry, relevance and recommendations. *J. Exp. Biol.* **216**, 2771–2782.

Collins, M., Knutti, R., Arblaster, J., Dufresne, J.-L., Fichefet, T., Friedlingstein, P., Gao, X., Gutowski, W. J., Johns, T., Krinner, G., et al. (2013). Long-term Climate Change: Projections, Commitments and Irreversibility. In *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* (ed. Stocker, T. F.), Qin, D.), Plattner, G.-K.), Tignor, M.), Allen, S. K.), Boschung, J.), Nauels, A.), Xia, Y.), Bex, B.), and Midgley, P. M.), pp. 1029–1136. Cambridge, United Kingdom and New York, NY, USA: Cambridge University Press.

Comeau, S., Edmunds, P., Spindel, N. and Carpenter, R. (2014). Diel pCO$_2$ oscillations modulate the response of the coral *Acropora hyacinthus* to ocean acidification. *Mar. Ecol. Prog. Ser.* **501**, 99–111.

Cominassi, L., Moyano, M., Claireaux, G., Howald, S., Mark, F. C., Zambonino-Infante, J. L., Le Bayon, N. and Peck, M. A. (2019). Combined effects of ocean acidification and temperature on larval and juvenile growth, development and swimming performance of European sea bass (*Dicentrarchus labrax*). *PLoS One* **14**, 1–22.

Connell, S. D. (1998). Patterns of pisciviory by resident predatory reef fish at One Tree Reef,

Great Barrier Reef. *Mar. Freshw. Res.* **49**, 25–30.

Connon, R. E., Jeffries, K. M., Komoroske, L. M., Todgham, A. E. and Fangue, N. A. (2018). The utility of transcriptomics in fish conservation. *J. Exp. Biol.* **221**, jeb148833.

Cornwall, C. E., Comeau, S., DeCarlo, T. M., Moore, B., D'Alexis, Q. and McCulloch, M. T. (2018). Resistance of corals and coralline algae to ocean acidification: physiological control of calcification under natural pH variability. *Proc. R. Soc. B Biol. Sci.* **285**, 20181168.

Couturier, C. S., Stecyk, J. A. W., Rummer, J. L., Munday, P. L. and Nilsson, G. E. (2013). Species-specific effects of near-future CO_2 on the respiratory performance of two tropical prey fish and their predator. *Comp. Biochem. Physiol. Part A* **166**, 482–489.

Crespel, A., Anttila, K., Lelièvre, P., Quazuguel, P., Le Bayon, N., Zambonino-Infante, J.-L., Chabot, D. and Claireaux, G. (2019). Long-term effects of ocean acidification upon energetics and oxygen transport in the European sea bass (*Dicentrarchus labrax*, Linnaeus). *Mar. Biol.* **166**, 116.

Cyronak, T., Schulz, K. G., Santos, I. R. and Eyre, B. D. (2014a). Enhanced acidification of global coral reefs driven by regional biogeochemical feedbacks. *Geophys. Res. Lett.* **41**, 5538–5546.

Cyronak, T., Santos, I. R., Erler, D. V., Maher, D. T. and Eyre, B. D. (2014b). Drivers of pCO$_2$ variability in two contrasting coral reef lagoons: the influence of submarine groundwater discharge. *Global Biogeochem. Cycles* **28**, 398–414.

Cyronak, T., Andersson, A. J., Langdon, C., Albright, R., Bates, N. R., Caldeira, K., Carlton, R., Corredor, J. E., Dunbar, R. B., Enochs, I., et al. (2018). Taking the metabolic pulse of the world's coral reefs. *PLoS One* **13**, e0190872.

Cyronak, T., Takeshita, Y., Courtney, T. A., DeCarlo, E. H., Eyre, B. D., Kline, D. I., Martz, T., Page, H., Price, N. N., Smith, J., et al. (2020). Diel temperature and pH variability scale with depth across diverse coral reef habitats. *Limnol. Oceanogr. Lett.* **5**, 193–203.

Davidson, M. I., Targett, T. E. and Grecay, P. A. (2016). Evaluating the effects of diel-cycling hypoxia and pH on growth and survival of juvenile summer flounder *Paralichthys dentatus*. *Mar. Ecol. Prog. Ser.* **556**, 223–235.

Davis, K. L., McMahon, A., Kelaher, B., Shaw, E. and Santos, I. R. (2019). Fifty years of sporadic coral reef calcification estimates at One Tree Island, Great Barrier Reef: Is it enough to imply long term trends? *Front. Mar. Sci.* **6**, 282.

De'ath, G. (2007). Boosted trees for ecological modeling and prediction. *Ecology* **88**, 243–251.

Degrandpre, M. D., Hammar, T. R., Smith, S. P. and Sayles, F. L. (1995). In situ measurements of seawater pCO$_2$. *Limnol. Oceanogr.* **40**, 969–975.

Depczynski, M. and Bellwood, D. R. (2004). Microhabitat utilisation patterns in cryptobenthic coral reef fish communities. *Mar. Biol.* **145**, 455–463.

Dickson, A. G. (1990). Standard potential of the reaction: AgCl (s) + 12H$_2$ (g) = Ag (s) + HCl (aq), and the standard acidity constant of the ion HSO$_4^-$ in synthetic sea water from 273.15 to 318.15. *J. Chem. Thermodyn.* **22**, 113–127.

Dickson, A. G. and Millero, F. J. (1987). A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. *Deep Sea Res. Part A, Oceanogr. Res. Pap.* **34**, 1733–1743.

Dickson, A. G., Sabine, C. L. and Christian, J. R. (2007). Guide to best practices for ocean CO_2 measurements. *PICES Spec. Publ.* **3**, 1–191.

Dlugokencky, E. and Tans, P. (2015). Trends in atmospheric carbon dioxide. *Natl. Ocean. Atmos. Adm. Earth Syst. Res. Lab.*

Dlugokencky, E. and Tans, P. (2020). Trends in Atmospheric Carbon Dioxide. *Natl. Ocean. Atmos. Adm. Earth Syst. Res. Lab.*

Doney, S. C. (2010). The growing human footprint on coastal and open-ocean biogeochemistry. *Science (80-.).* **328**, 1512–1516.

Doney, S. C. and Schimel, D. S. (2007). Carbon and climate system coupling on timescales from the precambrian to the anthropocene. *Annu. Rev. Environ. Resour.* **32**, 31–66.

Doney, S. C., Fabry, V. J., Feely, R. A. and Kleypas, J. A. (2009). Ocean acidification: the other CO_2 problem. *Ann. Rev. Mar. Sci.* **1**, 169–192.

Dorenbosch, M., Verweij, M. C., Nagelkerken, I., Jiddawi, N. and Van Der Velde, G. (2004). Homing and daytime tidal movements of juvenile snappers (Lutjanidae) between shallow-water nursery habitats in Zanzibar, western Indian Ocean. *Environ. Biol. Fishes* **70**, 203–209.

Drupp, P. S., De Carlo, E. H., Mackenzie, F. T., Sabine, C. L., Feely, R. A. and Shamberger, K. E. (2013). Comparison of CO_2 dynamics and air-sea gas exchange in differing tropical reef environments. *Aquat. Geochemistry* **19**, 371–397.

Duarte, C. M., Hendriks, I. E., Moore, T. S., Olsen, Y. S., Steckbauer, A., Ramajo, L., Carstensen, J., Trotter, J. A. and McCulloch, M. (2013). Is ocean acidification an open-ocean syndrome? Understanding anthropogenic impacts on seawater pH. *Estuaries and Coasts* **36**, 221–236.

Dufault, A. M., Cumbo, V. R., Fan, T.-Y. and Edmunds, P. J. (2012). Effects of diurnally oscillating *p*CO_2 on the calcification and survival of coral recruits. *Proc. R. Soc. B Biol. Sci.* **279**, 2951–2958.

Elith, J., Leathwick, J. R. and Hastie, T. (2008). A working guide to boosted regression trees. *J. Anim. Ecol.* **77**, 802–813.

Enochs, I. C., Manzello, D. P., Jones, P. J., Aguilar, C., Cohen, K., Valentino, L., Schopmeyer, S., Kolodziej, G., Jankulak, M. and Lirman, D. (2018). The influence of diel carbonate chemistry fluctuations on the calcification rate of *Acropora cervicornis* under present day and future acidification conditions. *J. Exp. Mar. Bio. Ecol.* **506**, 135–143.

Esbaugh, A. J. (2017). Physiological implications of ocean acidification for marine fish: emerging patterns and new insights. *J. Comp. Physiol. B* **188**, 1–13.

Esbaugh, A. J., Heuer, R. and Grosell, M. (2012). Impacts of ocean acidification on respiratory gas exchange and acid-base balance in a marine teleost, *Opsanus beta*. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **182**, 921–934.

Eyre, B. D., Andersson, A. J. and Cyronak, T. (2014). Benthic coral reef calcium carbonate dissolution in an acidifying ocean. *Nat. Clim. Chang.* **4**, 969–976.

Fabricius, K. E., Neill, C., Van Ooijen, E., Smith, J. N. and Tilbrook, B. (2020). Progressive seawater acidification on the Great Barrier Reef continental shelf. *Sci. Rep.* **10**, 1–15.

Fabry, V., Seibel, B., Feely, R. and Orr, J. (2008). Impacts of ocean acidification on marine fauna and ecosystem processes. *ICES J. Mar. Sci.* **65**, 414–432.

Falter, J. L., Lowe, R. J., Zhang, Z. and McCulloch, M. (2013). Physical and biological controls on the carbonate chemistry of coral reef waters: effects of metabolism, wave forcing, sea level, and geomorphology. *PLoS One* **8**, e53303.

Ferrari, M. C. O., Munday, P. L., Rummer, J. L., Mccormick, M. I., Corkill, K., Watson, S.-A., Allan, B. J. M., Meekan, M. G. and Chivers, D. P. (2015). Interactive effects of ocean acidification and rising sea temperatures alter predation rate and predator selectivity in reef fish communities. *Glob. Chang. Biol.* **21**, 1848–1855.

Fivelstad, S., Waagbø, R., Stefansson, S. and Olsen, A. B. (2007). Impacts of elevated water carbon dioxide partial pressure at two temperatures on Atlantic salmon (*Salmo salar* L.) parr growth and haematology. *Aquaculture* **269**, 241–249.

Forster, B. R. E. and Steen, J. B. (1969). The rate of the "Root Shift" in eel red cells and eel haemoglobin solutions. *Physiology* **204**, 259–282.

Frankignoulle, M., Gattuso, J.-P., Biondo, R., Bourge, I., Copin-Montégut, G. and Pichon, M. (1996). Carbon fluxes in coral reefs. II. Eulerian study of inorganic carbon dynamics and measurement of air-sea CO_2 exchanges. *Mar. Ecol. Prog. Ser.* **145**, 123–132.

Frieder, C. A., Gonzalez, J. P., Bockmon, E. E., Navarro, M. O. and Levin, L. A. (2014). Can variable pH and low oxygen moderate ocean acidification outcomes for mussel larvae? *Glob. Chang. Biol.* **20**, 754–764.

Frith, C. A., Leis, J. M. and Goldman, B. (1986). Currents in the Lizard Island region of the Great Barrier Reef Lagoon and their relevance to potential movements of larvae. *Coral Reefs* **5**, 81–92.

Fulton, C. J. (2007). Swimming speed performance in coral reef fishes: field validations reveal distinct functional groups. *Coral Reefs* **26**, 217–228.

Gagliano, M., McCormick, M. I., Moore, J. A. and Depczynski, M. (2010). The basics of acidification: baseline variability of pH on Australian coral reefs. *Mar. Biol.* **157**, 1849–1856.

Gallego, M. A., Timmermann, A., Friedrich, T. and Zeebe, R. E. (2018). Drivers of future seasonal cycle changes of oceanic $p\mathrm{CO_2}$. *Biogeosciences* **15**, 5315–5327.

Gardiner, N. M., Munday, P. L. and Nilsson, G. E. (2010). Counter-gradient variation in respiratory performance of coral reef fishes at elevated temperatures. *PLoS One* **5**,.

Gattuso, J.-P. and Hansson, L. (2011). *Ocean Acidification*. Oxford University Press.

Gattuso, J., Payri, C. E., Pichon, M., Delesalle, B. and Frankignoulle, M. (1997). Primary production, calcification, and air-sea CO_2 fluxes of a macroalgal-dominated coral reef

community (Moorea, French Polynesia). *J. Phycol.* **33**, 729–738.

Gattuso, J., Frankignoulle, M. and Smith, S. V (1999). Measurement of community metabolism and significance in the coral reef CO_2 source-sink debate. *PNAS* **96**, 13017–13022.

Goodrich, B., Gabry, J., Ali, I. and Brilleman, S. (2018). Rstanarm: Bayesian applied regression modeling via Stan. http://mc-stan.org/.

Goolish, E. M. (1991). Aerobic and anaerobic scaling in fish. *Biol. Rev.* **66**, 33–56.

Gräns, A., Jutfelt, F., Sandblom, E., Jönsson, E., Wiklander, K., Seth, H., Olsson, C., Dupont, S., Ortega-Martinez, O., Einarsdottir, I., et al. (2014). Aerobic scope fails to explain the detrimental effects on growth resulting from warming and elevated CO_2 in Atlantic halibut. *J. Exp. Biol.* **217**, 711–717.

Green, L. and Jutfelt, F. (2014). Elevated carbon dioxide alters the plasma composition and behaviour of a shark. *Biol. Lett.* **10**, 20140538.

Grosell, M., Munday, P. L., Farrell, A. P. and Brauner, C. J. (2019). *Carbon Dioxide*. (ed. Farrell, A. P.), Brauner, C. J.), Hoar, W. S.), and Randall, D. J.) Cambridge, MA: Academic Press.

Guizouarn, B. Y. H., Harvey, B. J., Borgese, F., Gabillat, N., Garcia-romeu, F. and Motais, R. (1993). Volume-avtivated Cl^--independent and Cl^--dependent K^+ pathways in trout red blood cells. *Physiology* **462**, 609–626.

Hamilton, S. L., Logan, C. A., Fennie, H. W., Sogard, S. M., Barry, J. P., Makukhov, A. D., Tobosa, L. R., Boyer, K., Lovera, C. F. and Bernardi, G. (2017). Species-specific responses of juvenile rockfish to elevated $p$$CO_2$: from behavior to genomics. *PLoS One* **12**, e0169670.

Hannan, K. D. and Rummer, J. L. (2018). Aquatic acidification: a mechanism underpinning maintained oxygen transport and performance in fish experiencing elevated carbon dioxide conditions. *J. Exp. Biol.* **221**, jeb154559.

Hannan, K. D., Miller, G. M., Watson, S.-A., Rummer, J. L., Fabricius, K. and Munday, P. L. (2020a). Diel $p$$CO_2$ variation among coral reefs and microhabitats at Lizard Island, Great Barrier Reef. *Coral Reefs* **39**, 1391–1406.

Hannan, K. D., Munday, P. L. and Rummer, J. L. (2020b). The effects of constant and fluctuating elevated $p$$CO_2$ levels on oxygen uptake rates of coral reef fishes. *Sci. Total Environ.* **741**, 140334.

Harter, T. S. and Brauner, C. J. (2017). *The O_2 and CO_2 transport system in teleosts and the specialized mechanisms that enhance Hb–O_2 unloading to tissues*. 1st ed. (ed. Gamperl, A. K.), Gillis, T. E.), Farrell, A. P.), and Brauner, C. J.) Elsevier Inc.

Harter, T. S. and Brauner, C. J. (2020). Teleost red blood cells actively enhance the passive diffusion of oxygen that was discovered by August Krogh. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* **253**, 110855.

Harter, T. S., Zanuzzo, F. S., Supuran, C. T., Gamperl, A. K. and Brauner, C. J. (2019). Functional support for a novel mechanism that enhances tissue oxygen extraction in a teleost fish. *Proc. R. Soc. B Biol. Sci.* **286**, 1–9.

Hastie, T., Tibshirani, R. and Friedman, J. (2009). *The Elements of Statistical Learning Data Mining, Inference, and Prediction*. 2nd ed. Stanford, California: Springer.

Heinrich, D. D. U., Rummer, J. L., Morash, A. J., Watson, S.-A., Simpfendorfer, C. A., Heupel, M. R. and Munday, P. L. (2014). A product of its environment: the epaulette shark (*Hemiscyllium ocellatum*) exhibits physiological tolerance to elevated environmental CO_2. *Conserv. Physiol.* **2**, 1–12.

Hendriks, I. E., Olsen, Y. S., Ramajo, L., Basso, L., Steckbauer, A., Moore, T. S., Howard, J. and Duarte, C. M. (2014). Photosynthetic activity buffers ocean acidification in seagrass meadows. *Biogeosciences* **11**, 333–346.

Heuer, R. M. and Grosell, M. (2014). Physiological impacts of elevated carbon dioxide and ocean acidification on fish. *AJP Regul. Integr. Comp. Physiol.* **307**, R1061–R1084.

Heuer, R. M. and Grosell, M. (2016). Elevated CO_2 increases energetic cost and ion movement in the marine fish intestine. *Sci. Rep.* **6**, 34480.

Hofmann, G. E., Smith, J. E., Johnson, K. S., Send, U., Levin, L. A., Micheli, F., Paytan, A., Price, N. N., Peterson, B., Takeshita, Y., et al. (2011). High-frequency dynamics of ocean pH: a multi-ecosystem comparison. *PLoS One* **6**, e28983.

Hothorn, T., Bretz, F. and Westfall, P. (2008). Simultaneous inference in general parametric models. *Biometrical J.* **50**, 346–363.

IPCC (2019). Summary for Policymakers. In *IPCC Special Report on the Ocean and Cryosphere in a Changing Climate* (ed. Pörtner, H.-O.), Roberts, D. C.), Masson-Delmotte, V.), Zhai, P.), Tignor, M.), Poloczanska, E.), Mintenbeck, K.), Nicolai, M.), Okem, A.), Petzold, J.), et al.), p. 42. In press.

Ishimatsu, A., Hayashi, M. and Kikkawa, T. (2008). Fishes in high-CO_2, acidified oceans. *Mar. Ecol. Prog. Ser.* **373**, 295–302.

Jain, K., Hamilton, J. and Farrell, A. (1997). Use of a ramp velocity test to measure critical swimming speed in rainbow trout (*Onchorhynchus mykiss*). *Comp. Biochem. Physiol. - A Mol. Integr. Physiol.* **117**, 441–444.

Jankowski, M. W., Graham, N. A. J. and Jones, G. P. (2015). Depth gradients in diversity, distribution and habitat specialisation in coral reef fishes: implications for the depth-refuge hypothesis. *Mar. Ecol. Prog. Ser.* **540**, 203–215.

Jarrold, M. D. and Munday, P. L. (2018a). Diel CO_2 cycles do not modify juvenile growth, survival and otolith development in two coral reef fish under ocean acidification. *Mar. Biol.* **165**, 49.

Jarrold, M. D. and Munday, P. L. (2018b). Elevated temperature does not substantially modify the interactive effects between elevated CO_2 and diel CO_2 cycles on the survival, growth and behavior of a coral reef fish. *Front. Mar. Sci.* **5**, 458.

Jarrold, M. D. and Munday, P. L. (2019). Diel CO_2 cycles and parental effects have similar benefits to growth of a coral reef fish under ocean acidification. *Biol. Lett.* **15**, 20180724.

Jarrold, M. D., Humphrey, C., McCormick, M. I. and Munday, P. L. (2017). Diel CO_2 cycles reduce severity of behavioural abnormalities in coral reef fish under ocean acidification. *Sci. Rep.* **7**, 10153.

Jiang, L., Guo, Y. J., Zhang, F., Zhang, Y. Y., McCook, L. J., Yuan, X. C., Lei, X. M., Zhou, G. W., Guo, M. L., Cai, L., et al. (2019). Diurnally fluctuating pCO$_2$ modifies the physiological responses of coral recruits under ocean acidification. *Front. Physiol.* **9**, 1952.

Johansen, J. L. (2014). Quantifying water flow within aquatic ecosystems using load cell sensors: a profile of currents experienced by coral reef organisms around Lizard Island, Great Barrier Reef, Australia. *PLoS One* **9**, e83240.

Johansen, J. L. and Jones, G. P. (2011). Increasing ocean temperature reduces the metabolic performance and swimming ability of coral reef damselfishes. *Glob. Chang. Biol.* **17**, 2971–2979.

Kamukuru, A. T. and Mgaya, Y. D. (2004). The food and feeding habits of blackspot snapper, *Lutjanus fulviflamma* (Pisces: Lutjanidae) in shallow waters of Mafia Island, Tanzania. *Afr. J. Ecol.* **42**, 49–58.

Kapsenberg, L. and Hofmann, G. E. (2016). Ocean pH time-series and drivers of variability along the northern Channel Islands, California, USA. *Limnol. Oceanogr.* **61**, 953–968.

Kayanne, H., Suzuki, A. and Saito, H. (1995). Diurnal changes in the partial pressure of carbon dioxide in coral reef water. *Science (80-.).* **269**, 214–216.

Kiene, W. E. and Hutchings, P. A. (1994). Bioerosion experiments at Lizard Island, Great Barrier Reef. *Coral Reefs* **13**, 91–98.

Killen, S. S., Glazier, D. S., Rezende, E. L., Clark, T. D., Atkinson, D., Willener, A. S. T. and Halsey, L. G. (2016). Ecological Influences and Morphological Correlates of Resting and Maximal Metabolic Rates across Teleost Fish Species. *Am. Nat.* **187**, 592–606.

Kline, D. I., Teneva, L., Hauri, C., Schneider, K., Miard, T., Chai, A., Marker, M., Dunbar, R., Caldeira, K., Lazar, B., et al. (2015). Six month *in situ* high-resolution carbonate chemistry and temperature study on a coral reef flat reveals asynchronous pH and temperature anomalies. *PLoS One* **10**, e0127648.

Komyakova, V., Munday, P. L. and Jones, G. P. (2019). Comparative analysis of habitat use and ontogenetic habitat-shifts among coral reef damselfishes. *Environ. Biol. Fishes* **102**, 1201–1218.

Kroeker, K. J., Kordas, R. L., Crim, R. N. and Singh, G. G. (2010). Meta-analysis reveals negative yet variable effects of ocean acidification on marine organisms. *Ecol. Lett.* **13**, 1419–1434.

Kroeker, K. J., Kordas, R. L., Crim, R., Hendriks, I. E., Ramajo, L., Singh, G. S., Duarte, C. M. and Gattuso, J. P. (2013). Impacts of ocean acidification on marine organisms: Quantifying sensitivities and interaction with warming. *Glob. Chang. Biol.* **19**, 1884–1896.

Krogh, A. (1919a). The supply of oxygen to the tissues and the regulation of the capillary circulation. *J. Physiol.* **52**, 457–474.

Krogh, A. (1919b). The number and distribution of capillaries in muscles with calculations of the oxygen pressure head necessary for supplying the tissue. *J. Physiol.* **52**, 409–415.

Kunz, K. L., Claireaux, G., Pörtner, H.-O., Knust, R. and Mark, F. C. (2018). Aerobic capacities and swimming performance of polar cod (*Boreogadus saida*) under ocean acidification and warming conditions. *J. Exp. Biol.* **221**, jeb184473.

Lantz, C. A., Atkinson, M. J., Winn, C. W. and Kahng, S. E. (2014). Dissolved inorganic carbon and total alkalinity of a Hawaiian fringing reef: chemical techniques for monitoring the effects of ocean acidification on coral reefs. *Coral Reefs* **33**, 105–115.

Laubenstein, T., Rummer, J., Nicol, S., Parsons, D., Pether, S., Pope, S., Smith, N. and Munday, P. (2018). Correlated effects of ocean acidification and warming on behavioral and metabolic traits of a large pelagic fish. *Diversity* **10**, 35.

Laubenstein, T. D., Rummer, J. L., McCormick, M. I. and Munday, P. L. (2019). A negative correlation between behavioural and physiological performance under ocean acidification and warming. *Sci. Rep.* **9**, 1–10.

Laubenstein, T. D., Jarrold, M. D., Rummer, J. L. and Munday, P. L. (2020). Beneficial effects of diel CO_2 cycles on reef fish metabolic performance are diminished under elevated temperature. *Sci. Total Environ.* **735**, 139084.

Lefevre, S. (2016). Are global warming and ocean acidification conspiring against marine ectotherms? A meta-analysis of the respiratory effects of elevated temperature, high CO_2 and their interaction. *Conserv. Physiol.* **4**, 1–31.

Lefevre, S. (2019). *Effects of high CO_2 on oxygen consumption rates, aerobic scope and swimming performance.* 1st ed. (ed. Grosell, M.), Munday, P. L.), Farrell, A. P.), and Brauner, C. J.) Elsevier Inc.

Lenth, R. (2016). Least-squares means: the R Package lsmeans. *J. Stat. Softw.* **69**, 1–33.

Lewis, E. and Wallace, D. (1998). *Program developed for CO_2 system calculations, in: Carbon Dioxide Information Analysis Center*. Oak Ridge, TN.

Lifavi, D. M., Targett, T. E. and Grecay, P. A. (2017). Effects of diel-cycling hypoxia and acidification on juvenile weakfish *Cynoscion regalis* growth, survival, and activity. *Mar. Ecol. Prog. Ser.* **564**, 163–171.

Long, M. H., Berg, P., de Beer, D. and Zieman, J. C. (2013). In situ coral reef oxygen metabolism: an eddy correlation study. *PLoS One* **8**, e58581.

Longhini, C. M., Souza, M. F. L. and Silva, A. M. (2015). Net ecosystem production, calcification and CO_2 fluxes on a reef flat in Northeastern Brazil. *Estuar. Coast. Shelf Sci.* **166**, 13–23.

Lowe, R. J., Falter, J. L., Monismith, S. G. and Atkinson, M. J. (2009). A numerical study of circulation in a coastal reef-lagoon system. *J. Geophys. Res. Ocean.* **114**, C06022.

Mangan, S., Urbina, M. A., Findlay, H. S., Wilson, R. W. and Lewis, C. (2017). Fluctuating seawater pH/$p$$CO_2$ regimes are more energetically expensive than static pH/$p$$CO_2$ levels in the mussel *Mytilus edulis*. *Proc. R. Soc. B Biol. Sci.* **284**, 20171642.

Manzello, D. P., Enochs, I. C., Melo, N., Gledhill, D. K. and Johns, E. M. (2012). Ocean acidification refugia of the florida reef tract. *PLoS One* **7**, e41715.

Marnane, M. J. and Bellwood, D. R. (2002). Diet and nocturnal foraging in cardinalfishes (Apogonidae) at One Tree Reef, Great Barrier Reef, Australia. *Mar. Ecol. Prog. Ser.*

231, 261–268.

Martz, T. R., Connery, J. G. and Johnson, K. S. (2010). Testing the Honeywell Durafet® for seawater pH applications. *Limnol. Oceanogr. Methods* **8**, 172–184.

McElhany, P. and Busch, D. S. (2013). Appropriate pCO_2 treatments in ocean acidification experiments. *Mar. Biol.* **160**, 1807–1812.

McMahon, A., Santos, I. R., Cyronak, T. and Eyre, B. D. (2013). Hysteresis between coral reef calcification and the seawater aragonite saturation state. *Geophys. Res. Lett.* **40**, 4675–4679.

McMahon, A., Santos, I. R., Schulz, K. G., Scott, A., Silverman, J., Davis, K. L. and Maher, D. T. (2019). Coral reef calcification and production after the 2016 bleaching event at Lizard Island, Great Barrier Reef. *J. Geophys. Res. Ocean.* **124**, 4003–4016.

McMahon, S. J., Parsons, D. M., Donelson, J. M., Pether, S. M. J. and Munday, P. L. (2020). Elevated CO_2 and heatwave conditions affect the aerobic and swimming performance of juvenile Australasian snapper. *Mar. Biol.* **167**, 6.

McNeil, B. I. and Matsumoto, K. (2019). *The changing ocean and freshwater CO_2 system*. 1st ed. Elsevier Inc.

McNeil, B. I. and Sasse, T. P. (2016). Future ocean hypercapnia driven by anthropogenic amplification of the natural CO_2 cycle. *Nature* **529**, 383–386.

Mehrbach, C., Culberson, C. H., Hawley, J. E. and Pytkowicx, R. M. (1973). Measurement of the apparent dissociation constants of carbonic acid in seawater at atmospheric pressure. *Limnol. Oceanogr.* **18**, 897–907.

Meinshausen, M., Smith, S., Calvin, K., Daniel, J., Kainuma, M., Lamarque, J.-F., Matsumoto, K., Montzka, S., Raper, S., Riahi, K., et al. (2011). The RCP greenhouse gas concentrations and their extensions from 1765 to 2300. *Clim. Change* **109**, 213–241.

Melzner, F., Gutowska, M. A., Langenbuch, M., Dupont, S., Lucassen, M., Thorndyke, M. C., Bleich, M. and Pörtner, H.-O. (2009a). Physiological basis for high CO_2 tolerance in marine ectothermic animals: pre-adaptation through lifestyle and ontogeny? *Biogeosciences* **6**, 2313–2331.

Melzner, F., Göbel, S., Langenbuch, M., Gutowska, M. A., Pörtner, H. O. and Lucassen, M. (2009b). Swimming performance in Atlantic Cod (*Gadus morhua*) following long-term (4-12 months) acclimation to elevated seawater PCO_2. *Aquat. Toxicol.* **92**, 30–37.

Melzner, F., Thomsen, J., Koeve, W., Oschlies, A., Gutowska, M. A., Bange, H. W., Hansen, H. P. and Körtzinger, A. (2013). Future ocean acidification will be amplified by hypoxia in coastal habitats. *Mar. Biol.* **160**, 1875–1888.

Methling, C., Pedersen, P. B., Steffensen, J. F. and Skov, P. V. (2013). Hypercapnia adversely affects postprandial metabolism in the European eel (*Anguilla anguilla*). *Aquaculture* **416–417**, 166–172.

Miller, C. A., Pocock, K., Evans, W. and Kelley, A. L. (2018). An evaluation of the performance of Sea-Bird Scientific's SeaFET™ autonomous pH sensor: considerations for the broader oceanographic community. *Ocean Sci.* **14**, 751–768.

Milligan, C. L. and Girard, S. S. (1993). Lactate Metabolism in Rainbow Trout. *J. Exp.*

Biol. **180**, 175–193.

Munday, P. L., Crawley, N. E. and Nilsson, G. E. (2009a). Interacting effects of elevated temperature and ocean acidification on the aerobic performance of coral reef fishes. *Mar. Ecol. Prog. Ser.* **388**, 235–242.

Munday, P. L., Donelson, J. M., Dixson, D. L. and Endo, G. G. K. (2009b). Effects of ocean acidification on the early life history of a tropical marine fish. *Proc. R. Soc. B Biol. Sci.* **276**, 3275–3283.

Nadler, L. E., Killen, S. S., Mccormick, M. I., Watson, S. and Munday, P. L. (2016). Effect of elevated carbon dioxide on shoal familiarity and metabolism in a coral reef fish. *Conserv. Physiol.* **4**, 1–13.

Newman, S. J. (1995). Spatial variability in the distribution, abundance, growth, mortality and age structures of tropical snappers (Pisces: Lutjanidae) in the Central Great Barrier Reef, Australia. *Thesis*.

Nielsen, E. E., Hemmer-Hansen, J., Larsen, P. F. and Bekkevold, D. (2009). Population genomics of marine fishes: identifying adaptive variation in space and time. *Mol. Ecol.* **18**, 3128–3150.

Niimi, A. J. and Beamish, F. W. H. (1974). Bioenergetics and growth of largemouth bass (*Micropterus salmoides*) in relation to body weight and temperature. *Can. J. Zool.* **52**, 447–456.

Nikinmaa, M. and Salama, A. (1998). Oxygen Transport in Fish. In *Fish Physiology*, pp. 141–184.

Nikinmaa, M., Tiihonen, K. and Paajaste, M. (1990). Adrenergic control of red cell pH in salmonid fish: roles of the sodium/proton exchange, Jacobs-Stewart cycle and membrane potential. *J. Exp. Biol.* **154**, 257–271.

Nilsson, G. E., Crawley, N., Lunde, I. G. and Munday, P. L. (2009). Elevated temperature reduces the respiratory scope of coral reef fishes. *Glob. Chang. Biol.* **15**, 1405–1412.

Noor, N. M., De, M., Iskandar, A., Keng, W. L., Cob, Z. C., Ghaffar, M. A. and Das, S. K. (2019). Effects of elevated carbon dioxide on the growth and welfare of Juvenile tiger grouper (*Epinephelus fuscoguttatus*) × giant grouper (*E. lanceolatus*) hybrid. *Aquaculture* **513**, 734448.

Ohde, S. and van Woesik, R. (1999). Carbon dioxide flux and metabolic processes of a coral reef, Okinawa. *Bull. Mar. Sci.* **65**, 559–576.

Orr, J. C., Fabry, V. J., Aumont, O., Bopp, L., Doney, S. C., Feely, R. A., Gnanadesikan, A., Gruber, N., Ishida, A., Joos, F., et al. (2005). Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. *Nature* **437**, 681–686.

Ou, M., Hamilton, T. J., Eom, J., Lyall, E. M., Gallup, J., Jiang, A., Lee, J., Close, D. A., Yun, S.-S. and Brauner, C. J. (2015). Responses of pink salmon to CO_2-induced aquatic acidification. *Nat. Clim. Chang.* **5**, 1–24.

Pagnotta, A. and Milligan, C. L. (1991). The role of blood glucose in the restoration of muscle glycogen during recovery from exhaustive exercise in rainbow trout (*Oncorhynchus mykiss*) and winter flounder (*Pseudopleuronectes americanus*). *J. Exp.*

Biol. **161**, 489–508.

Pilditch, C. A., Cornwall, C. E., Hepburn, C. D., McGraw, C. M., Hurd, C. L., Hunter, K. A., Currie, K. I. and Boyd, P. W. (2013). Diurnal fluctuations in seawater pH influence the response of a calcifying macroalga to ocean acidification. *Proc. R. Soc. B Biol. Sci.* **280**, 20132201–20132201.

Pimentel, M., Pegado, M., Repolho, T. and Rosa, R. (2014). Impact of ocean acidification in the metabolism and swimming behavior of the dolphinfish (*Coryphaena hippurus*) early larvae. *Mar. Biol.* **161**, 725–729.

Pörtner, H. O. (2008). Ecosystem effects of ocean acidification in times of ocean warming: a physiologist's view. *Mar. Ecol. Prog. Ser.* **373**, 203–217.

Pörtner, H. O. and Farrell, A. P. (2008). Physiology and climate change. *Science (80-.).* **322**, 690–692.

Pörtner, H. O. and Peck, M. A. (2010). Climate change effects on fishes and fisheries: towards a cause-and-effect understanding. *J. Fish Biol.* **77**, 1745–1779.

Pörtner, H. O., Langenbuch, M. and Reipschläger, A. (2004). Biological impact of elevated ocean CO_2 concentrations: lessons from animal physiology and earth history. *J. Oceanogr.* **60**, 705–718.

Pörtner, H. O., Langenbuch, M. and Michaelidis, B. (2005). Synergistic effects of temperature extremes, hypoxia, and increases in CO2 on marine animals: From Earth history to global change. *J. Geophys. Res. C Ocean.* **110**, 1–15.

Pörtner, H.-O., Karl, D. M. and Boyd, P. W. (2014). *Climate Change 2014: Impacts, Adaptation, and Vulnerability. Part A: Global and Sectoral Aspects*. Contributi. (ed. Field, C. B.), Barros, V. R.), and Dokken, D. J.) Cambridge: Cambridge University Press.

Price, N. N., Martz, T. R., Brainard, R. E. and Smith, J. E. (2012). Diel Variability in Seawater pH Relates to Calcification and Benthic Community Structure on Coral Reefs. *PLoS One* **7**, 1–9.

R Core Team (2017). R: a language and environment for statistical computing. http://www.r-project.org/.

R Development Core Team (2016). R: a language and environmental for statistical computing.

Randall, D. J., Rummer, J. L., Wilson, J. M., Wang, S. and Brauner, C. J. (2014). A unique mode of tissue oxygenation and the adaptive radiation of teleost fishes. *J. Exp. Biol.* **217**, 1205–14.

Ravaglioli, C., Bulleri, F., Rühl, S., Mccoy, S. J., Findlay, H. S., Widdicombe, S. and Queirós, A. M. (2019). Ocean acidification and hypoxia alter organic carbon fluxes in marine soft sediments. *Glob. Chang.* **00**, 1–14.

Reidy, S. P., Nelson, J. A., Tang, Y. and Kerr, S. R. (1995). Post-exercise metabolic rate in Atlantic cod and its dependence upon the method of exhaustion. *J. Fish Biol.* **47**, 377–386.

Reidy, S. P., Kerr, S. R. and Nelson, J. A. (2000). Aerobic and anaerobic swimming

performance of individual Atlantic cod. *J. Exp. Biol.* **203**, 347–357.

Ridgeway, G. (2007). Generalized boosted models: a guide to the gbm package. *Http://Cran.R-Project.Org/Web/Packages/Gbm/Gmb/Pdf* **1**, 1–12.

Robinson, D. (2017). Broom: convert statistical analysis objects into tidy data frames. https://CRAN.R-project.org/package=broom.

Roche, D. G., Binning, S. A., Bosiger, Y., Johansen, J. L. and Rummer, J. L. (2013). Finding the best estimates of metabolic rates in a coral reef fish. *J. Exp. Biol.* **216**, 2103–2110.

Rummer, J. L. and Brauner, C. J. (2011). Plasma-accessible carbonic anhydrase at the tissue of a teleost fish may greatly enhance oxygen delivery: in vitro evidence in rainbow trout, *Oncorhynchus mykiss*. *J. Exp. Biol.* **214**, 2319–28.

Rummer, J. L. and Brauner, C. J. (2015). Root effect haemoglobins in fish may greatly enhance general oxygen delivery relative to other vertebrates. *PLoS One* **10**, e0139477.

Rummer, J. L., Stecyk, J. A. W., Couturier, C. S., Watson, S.-A. A., Nilsson, G. E. and Munday, P. L. (2013a). Elevated CO_2 enhances aerobic scope of a coral reef fish. *Conserv. Physiol.* **1**, 1–7.

Rummer, J. L., Mckenzie, D. J., Innocenti, A., Supuran, C. T. and Brauner, C. J. (2013b). Root effect hemoglobin may have evolved to enhance general tissue oxygen delivery. *Science (80-.).* **340**, 1327–1329.

Rummer, J. L., Stecyk, J. A. W., Couturier, C. S., Watson, S. A., Nilsson, G. E. and Munday, P. L. (2013c). Elevated CO2 enhances aerobic scope of a coral reef fish. *Conserv. Physiol.* **1**, 1–7.

Rummer, J. L., Binning, S. A., Roche, D. G. and Johansen, J. L. (2016). Methods matter: considering locomotory mode and respirometry technique when estimating metabolic rates of fishes. *Conserv. Physiol.* **4**, 1–13.

Sabine, C. L., Feely, R. A., Gruber, N., Key, R., Lee, K., Bullister, J., Wanninkhof, R., Wong, C., Wallace, D., Tillbrook, B., et al. (2004). The oceanic sink for anthropogenic CO_2. *Science (80-.).* **305**, 367–371.

Schmalz, R. F. and Swanson, F. J. (1969). Diurnal variations in the carbonate saturation of seawater. *J. Sediment. Petrol.* **39**, 255–267.

Schunter, C., Welch, M. J., Ryu, T., Zhang, H., Berumen, M. L., Nilsson, G. E., Munday, P. L. and Ravasi, T. (2016). Molecular signatures of transgenerational response to ocean acidification in a species of reef fish. *Nat. Clim. Chang.* **6**, 1014–1018.

Schunter, C., Welch, M. J., Nilsson, G. E., Rummer, J. L., Munday, P. L. and Ravasi, T. (2018). An interplay between plasticity and parental phenotype determines impacts of ocean acidificaiton on a reef fish. *Nat. Ecol. Evol.* **2**, 334–342.

Schurmann, H. and Steffensen, J. F. (1997). Effects of temperature, hypoxia and activity on the metabolism of juvenile Atlantic cod. *J. Exp. Mar. Bio. Ecol.* **50**, 1166–1180.

Seebacher, F. and Franklin, C. E. (2012). Determining environmental causes of biological effects: the need for a mechanistic physiological dimension in conservation biology.

Philos. Trans. R. Soc. B Biol. Sci. **367**, 1607–1614.

Shaw, E. C., McNeil, B. I. and Tilbrook, B. (2012). Impacts of ocean acidification in naturally variable coral reef flat ecosystems. *J. Geophys. Res.* **117**, C03038.

Shaw, E. C., Mcneil, B. I., Tilbrook, B., Matear, R. and Bates, M. L. (2013a). Anthropogenic changes to seawater buffer capacity combined with natural reef metabolism induce extreme future coral reef CO_2 conditions. *Glob. Chang. Biol.* **19**, 1632–1641.

Shaw, E. C., Munday, P. L. and Mcneil, B. I. (2013b). The role of CO_2 variability and exposure time for biological impacts of ocean acidification. **40**, 4685–4688.

Shaw, E. C., Phinn, S. R., Tilbrook, B. and Steven, A. (2015). Natural *in situ* relationships suggest coral reef calcium carbonate production will decline with ocean acidification. *Limnol. Oceanogr.* **60**, 777–788.

Silva, C. S. E., Novais, S. C., Lemos, M. F. L., Mendes, S., Oliveira, A. P., Gonçalves, E. J. and Faria, A. M. (2016). Effects of ocean acidification on the swimming ability, development and biochemical responses of sand smelt larvae. *Sci. Total Environ.* **563– 564**, 89–98.

Silverman, J., Kline, D. I., Johnson, L., Rivlin, T., Schneider, K., Erez, J., Lazar, B. and Caldeira, K. (2012). Carbon turnover rates in the One Tree Island reef: a 40-year perspective. *J. Geophys. Res.* **117**, G03023.

Silverman, J., Schneider, K., Kline, D. I., Rivlin, T., Rivlin, A., Hamylton, S., Lazar, B., Erez, J. and Caldeira, K. (2014). Community calcification in Lizard Island, Great Barrier Reef: a 33 year perspective. *Geochim. Cosmochim. Acta* **144**, 72–81.

Steffensen, J., Johansen, K. and Bushnell, P. (1984). An automated swimming respirometer. *Comp. Biochem. Physiol. Part A Physiol.* **79**, 437–440.

Strobel, A., Leo, E., Pörtner, H. O. and Mark, F. C. (2013). Elevated temperature and PCO_2 shift metabolic pathways in differentially oxidative tissues of *Notothenia rossii*. *Comp. Biochem. Physiol. - B Biochem. Mol. Biol.* **166**, 48–57.

Supuran, C. T., Scozzafava, A., Ilies, M. A. and Briganti, F. (2000). Carbonic anhydrase inhibitors: synthesis of sulfonamides incorporating 2,4,6-trisubstituted-pyridinium-ethylcarboxamido moieties possessing membrane-impermeability and in vivo selectivity for the membrane-bound (CA IV) versus the cytosolic (CA I and CA II. *J. Enzyme Inhib.* **15**, 381–401.

Suzuki, A. and Kawahata, H. (2004). Reef water CO_2 system and carbon production of coral reefs: topographic control of system-level performance. *Glob. Environ. Chang. Ocean L.* 229–248.

Taebi, S., Lowe, R. J., Pattiaratchi, C. B., Ivey, G. N., Symonds, G. and Brinkman, R. (2011). Nearshore circulation in a tropical fringing reef system. *J. Geophys. Res. Ocean.* **116**, C02016.

Tian, H., Lu, C., Ciais, P., Michalak, A. M., Canadell, J. G., Saikawa, E., Huntzinger, D. N., Gurney, K. R., Sitch, S., Zhang, B., et al. (2016). The terrestrial biosphere as a net source of greenhouse gases to the atmosphere. *Nature* **531**, 225–228.

Toppe, J., Albrektsen, S., Hope, B. and Aksnes, A. (2007). Chemical composition, mineral

content and amino acid and lipid profiles in bones from various fish species. *Comp. Biochem. Physiol. - B Biochem. Mol. Biol.* **146**, 395–401.

Tracy, C. R., Nussear, K. E., Esque, T. C., Dean-Bradley, K., Tracy, C. R., DeFalco, L. A., Castle, K. T., Zimmerman, L. C., Espinoza, R. E. and Barber, A. M. (2006). The importance of physiological ecology in conservation biology. *Integr. Comp. Biol.* **46**, 1191–1205.

van Dijk, P. L. M., Van den Thillart, G. E. E. J. M. and Wendelaar Bonga, S. E. (1993). Is there a synergistic effect between steady-state exercise and water acidification in carp? *J. Fish Biol.* **42**, 673–681.

Videler, J. (1993). *Fish swimming*. New York: Chapman and Hall.

Wahl, M., Saderne, V. and Sawall, Y. (2015). How good are we at assessing the impact of ocean acidification in coastal systems? Limitations, omissions and strengths of commonly used experimental approaches with special emphasis on the neglected role of fluctuations. *Mar. Freshw. Res.* **67**, 25–36.

Waldbusser, G. G. and Salisbury, J. E. (2014). Ocean acidification in the coastal zone from an organism's perspective: multiple system parameters, frequency domains, and habitats. *Ann. Rev. Mar. Sci.* **6**, 221–247.

Walker, R., Wood, C. and Bergman, H. L. (1988). Effects of low pH and aluminum on ventilation in the brook trout (*Salvelinus fontinalis*). *Can. J. Fish. Aquat. Sci.* **45**, 1614–1622.

Ware, J. R., Smith, S. V and Reaka-kudla, M. L. (1991). Coral reefs: sources or sinks of atmospheric CO_2. *Coral Reefs* **11**, 127–130.

Watson, S.-A., Fabricius, K. E. and Munday, P. L. (2017). Quantifying pCO_2 in biological ocean acidification experiments: a comparison of four methods. *PLoS One* **12**, e0185469.

Watson, S.-A., Allan, B. J. M., McQueen, D. E., Nicol, S., Parsons, D. M., Pether, S. M. J., Pope, S., Setiawan, A. N., Smith, N., Wilson, C., et al. (2018). Ocean warming has a greater effect than acidification on the early life history development and swimming performance of a large circumglobal pelagic fish. *Glob. Chang. Biol.* **24**, 4368–4385.

Wendelaar Bonga, S. E. (1997). The stress response in fish. *Physiol. Rev.* **77**, 591–625.

Wickham, H. and Chang, W. (2008). Ggplot2: an implementation of the grammar of graphics.

Wikelski, M. and Cooke, S. J. (2006). Conservation physiology. *Trends Ecol. Evol.* **21**, 38–46.

Witeska, M. (2013). Erythrocytes in teleost fishes: a review. *Zool. Ecol.* **23**, 275–281.

Witters, H. E., Van Puymbroeck, S. and Vanderborght, O. L. J. (1991). Adrenergic response to physiological disturbances in Rainbow Trout , *Oncorhynchus mykiss*, exposed to aluminum at acid pH. *Can. J. Fish. Aquat. Sci.* **48**, 414–420.

Wittmann, A. C. and Pörtner, H. O. (2013). Sensitivities of extant animal taxa to ocean acidification. *Nat. Clim. Chang.* **3**, 995–1001.

Wood, C. and Munger, R. (1994). Carbonic anhydrase injection provides evidence for the

role of blood acid-base status in stimulating ventilation after exhaustive exercise in rainbow trout. *J. Exp. Biol.* **194**, 225–53.

Wootton, J. T., Pfister, C. A. and Forester, J. D. (2008). Dynamic patterns and ecological impacts of declining ocean pH in a high-resolution multi-year dataset. *PNAS* **105**, 18848–18853.

Yan, H., Yu, K., Shi, Q., Tan, Y., Liu, G., Zhao, M., Li, S., Chen, T. and Wang, Y. (2016). Seasonal variations of seawater pCO_2 and sea-air CO_2 fluxes in a fringing coral reef, northern South China Sea. *J. Geophys. Res. Ocean.* **121**, 998–1008.

Yang, Y., Hansson, L. and Gattuso, J. P. (2016). Data compilation on the biological response to ocean acidification: an update. *Earth Syst. Sci. Data* **8**, 79–87.

Yates, K. and Halley, R. (2006). CO_3^{2-} concentration and pCO_2 thresholds for calcification and dissolution on the Molokai reef flat, Hawaii. *Biogeosciences* **3**, 357–369.

Yates, K. K., Dufore, C., Smiley, N., Jackson, C. and Halley, R. B. (2007). Diurnal variation of oxygen and carbonate system parameters in Tampa Bay and Florida Bay. *Mar. Chem.* **104**, 110–124.

Zeebe, R. E. and Wolf-Gladrow, D. (2001). *CO_2 in seawater: equilibrium, kinetics, isotopes*. First. (ed. Halpern, D.) Aamsterdam, The Netherlands: Elsevier Science B. V.

Zhang, Z., Falter, J., Lowe, R. and Ivey, G. (2012). The combined influence of hydrodynamic forcing and calcification on the spatial distribution of alkalinity in a coral reef system. *J. Geophys. Res. Ocean.* **117**, C04034.

Appendix A -   Supplementary material for Chapter 2

Diel pCO_2 variation among coral reefs and microhabitats at Lizard Island, Great Barrier Reef

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Table S1. The SeaFET offset by location and habitat calculated from the difference between measured pH_T and pH_T calculated from alkalinity and DIC of the replicate water samples and reported by each SeaFET. The temperature offset was calculated based on the control side-by-side deployment of all SeaFETs at the same location.

Logger	Trawler pH_T	Big Vicki's pH_T	Palfrey pH_T	Temperature (°C)
SeaFET 35	Soft coral -0.0289	Open sand +0.0131	Hard coral +0.0451	+0.0181
SeaFET 36	Hard coral -0.0325	Soft coral -0.0036	Open sand +0.0425	+0.0000
SeaFET 119	Open sand -0.1067	Hard coral -0.0630	Soft coral -0.0281	+0.1339
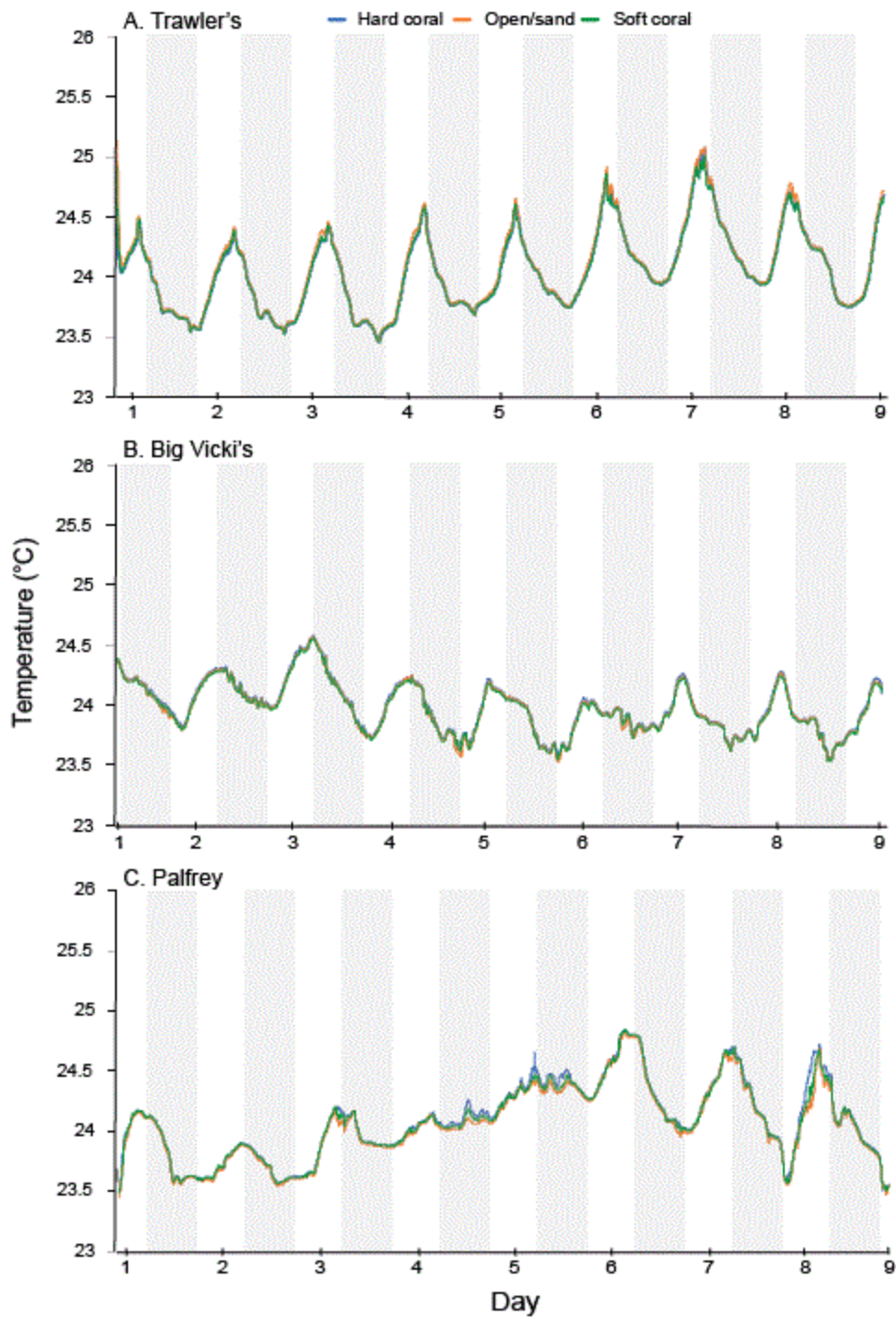
Figure S1. The variation of calculated temperature (°C) over the 9-day trial at three reef sites around the Lizard Island lagoon at A) Trawler Reef, B) Big Vicki's Reef, and C) Palfrey Reef. The different microhabitats are represented by different colours (hard coral - blue; soft coral - green; open substrate - orange). White and grey bars represent day and night, respectively.
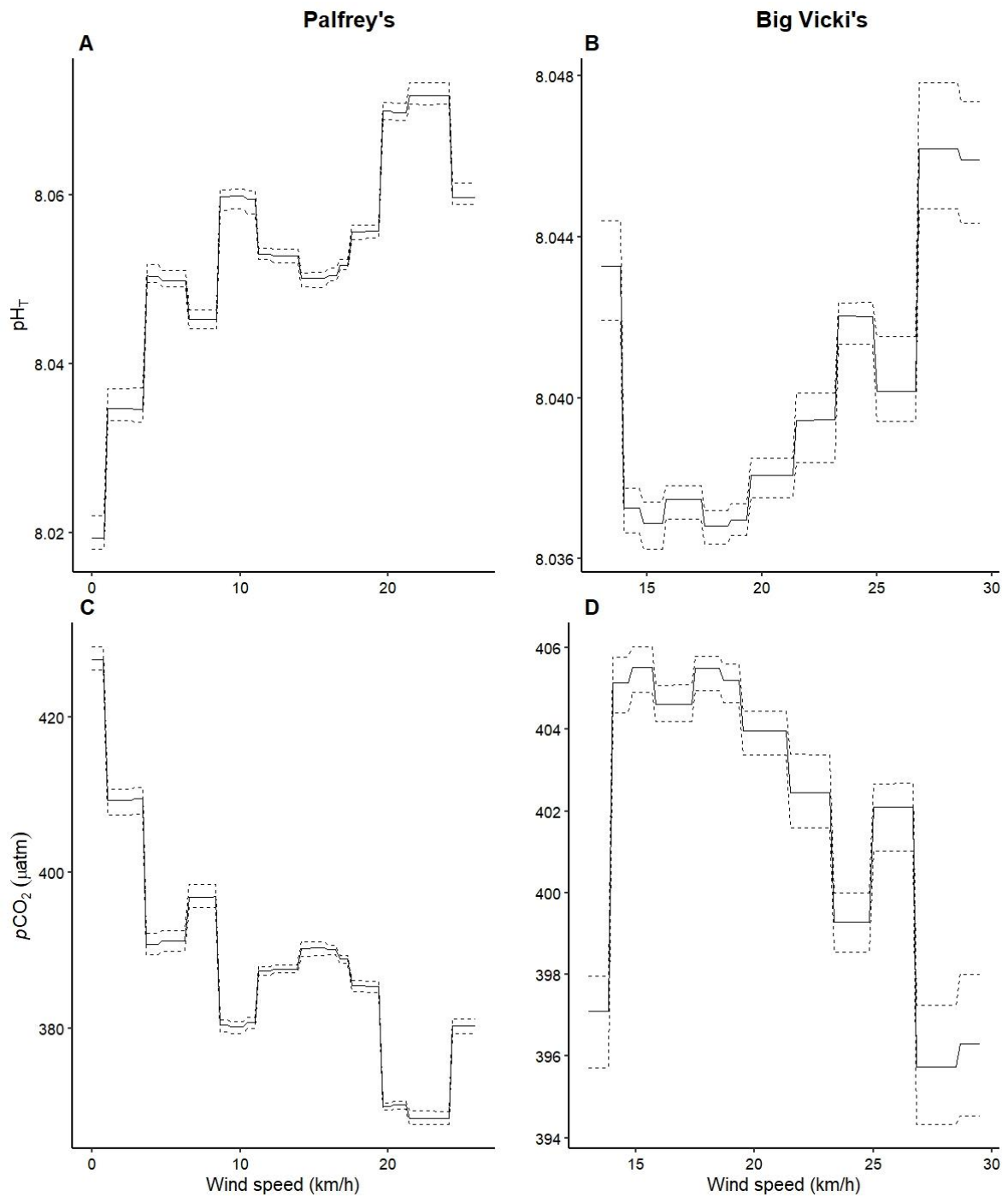
Figure S2. The partial effects plots of the significant predictor variable, wind analysed in relation to A-B) pH and C-D) pCO$_2$ for Palfrey and Big Vicki's Reefs. Solid and dashed lines represent median and lower/upper 95% quantiles, respectively.
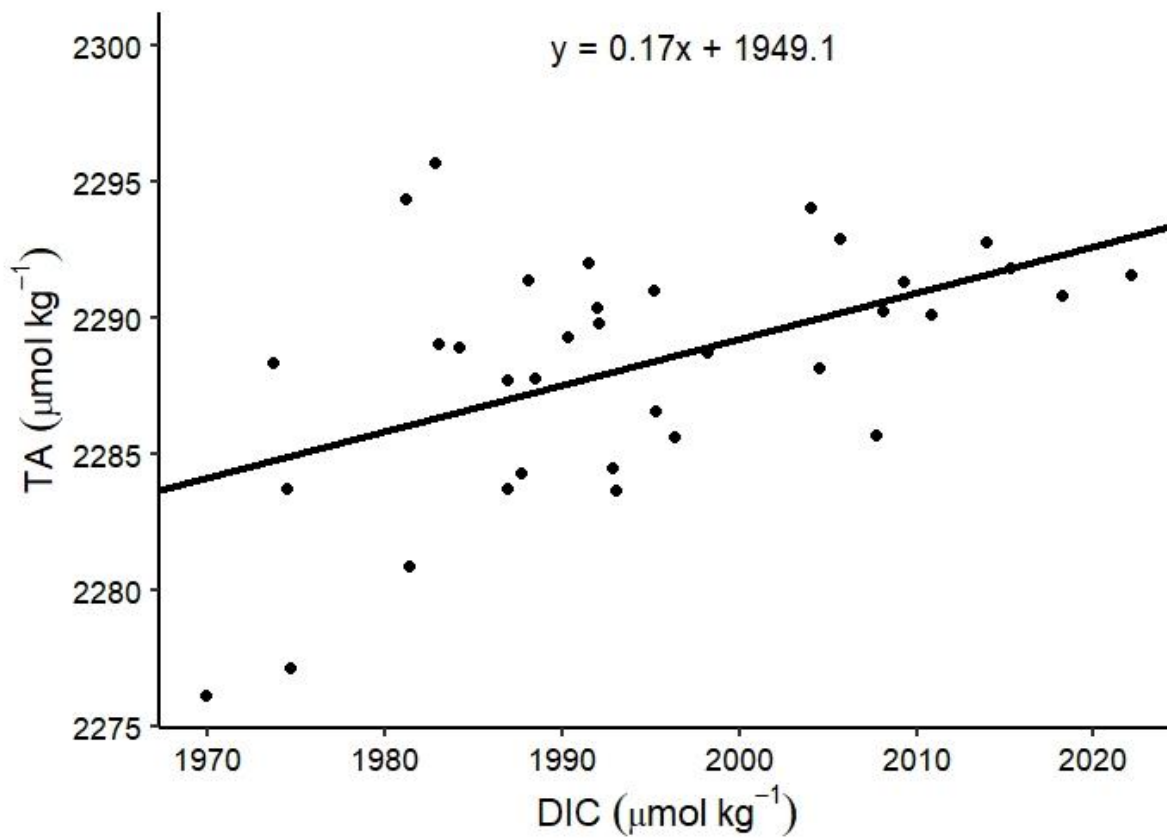
Figure S3. The regression analysis of total alkalinity (TA) and dissolved inorganic carbon (DIC) using ordinary least squares (OLS) method.

Appendix B -   Supplementary material for Chapter 3

The effects of constant and fluctuating elevated pCO$_2$ levels on oxygen uptake rates of coral reef fishes

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Table S1. Results from the planned comparison tests examining the impact of the four pCO_2 treatments (stable ambient ~480 µatm; stable elevated ~ 1,100 µatm; fluctuating increasing ~480-1,100 µatm; and fluctuating decreasing ~1,100-480 µatm) on *Acanthochromis polyacanthus* following an 8 h swimming trial. d.f. – for the entire model was calculated as, nrow(data)-length (coefficients of model).

Measured variable	Main effects	Estimate	Std. Error	z-value	P-value	df
$\dot{M}O_{2\ Min}$	Ambient vs Elevated	-73.84	41.24	-1.79	0.092	17
	Increasing vs Decreasing	7.99	45.69	0.18	0.863	
	Constant vs Fluctuating	89.25	30.77	2.90	0.010	
	Ambient vs pCO_2	-10.28	33.29	-0.31	0.762	
$\dot{M}O_{2\ Max}$*	Ambient vs Elevated	67.19	65.02	1.03	0.318	17
	Increasing vs Decreasing	-60.94	72.10	-0.85	0.411	
	Constant vs Fluctuating	40.24	48.79	0.83	0.422	
	Ambient vs pCO_2	-71.61	52.99	-1.35	0.197	
Aerobic scope*	Ambient vs Elevated	145.13	76.90	1.89	0.079	17
	Increasing vs Decreasing	-63.95	85.27	-0.75	0.465	
	Constant vs Fluctuating	-44.58	57.70	-0.77	0.452	
	Ambient vs pCO_2	-67.03	62.68	-1.07	0.302	
Factorial aerobic scope	Ambient vs Elevated	3.16	6.01	0.53	0.606	17
	Increasing vs Decreasing	-4.85	6.66	-0.73	0.477	
	Constant vs Fluctuating	-11.84	4.49	-2.64	0.018	
	Ambient vs pCO_2	5.79	4.85	1.19	0.251	
Ucrit*	Ambient vs Elevated	0.03	0.24	0.12	0.907	17
	Increasing vs Decreasing	0.48	0.27	1.76	0.099	
	Constant vs Fluctuating	0.01	0.18	0.08	0.937	
	Ambient vs pCO_2	-0.03	0.20	-0.15	0.886	
Haemoglobin concentration	Ambient vs Elevated	0.83	0.59	1.40	0.184	17
	Increasing vs Decreasing	-1.08	0.69	-1.58	0.138	
	Constant vs Fluctuating	0.48	0.45	1.05	0.310	
	Ambient vs pCO_2	-0.87	0.50	-1.75	0.102	
Lactate	Ambient vs Elevated	0.64	0.65	0.99	0.349	11
	Increasing vs Decreasing	-	-	-	-	
	Constant vs Fluctuating	-0.35	0.30	-1.18	0.269	
	Ambient vs pCO_2	-0.15	0.55	-0.27	0.792	
Glucose	Ambient vs Elevated	3.24	1.51	2.14	0.061	11
	Increasing vs Decreasing	-	-	-	-	
	Constant vs Fluctuating	-1.29	0.70	-1.83	0.100	
	Ambient vs pCO_2	-3.30	1.29	-2.56	0.031	
Haematocrit (%)	Ambient vs Elevated	-1.94	5.25	-0.37	0.721	10
	Increasing vs Decreasing	-	-	-	-	
	Constant vs Fluctuating	3.84	5.17	0.74	0.478	
	Ambient vs pCO_2	-0.46	4.56	-0.10	0.921	
Mean cell haemoglobin concentration	Ambient vs Elevated	-0.05	0.05	-1.13	0.290	10
	Increasing vs Decreasing	-	-	-	-	
	Constant vs Fluctuating	-0.05	0.05	-1.11	0.300	
	Ambient vs pCO_2	0.07	0.04	1.60	0.147	

* Mass had a significant effect and was kept in the model

Table S2. Results from the planned comparison tests examining the impact of the four pCO_2 treatments (stable ambient ~480 µatm; stable elevated ~ 1,100 µatm; fluctuating increasing ~480-1,100 µatm; and fluctuating decreasing ~1,100-480 µatm) on *Amblyglyphidodon curacao* following an 8 h swimming trial.

Measured variable	Main effects	Estimate	Std. Error	z-value	*P*-value	df
$\dot{M}O_{2\,Min}$	Ambient vs Elevated	20.77	15.12	1.37	0.186	19
	Increasing vs Decreasing	17.03	15.86	1.07	0.296	
	Constant vs Fluctuating	16.04	10.96	1.46	0.160	
	Ambient vs pCO_2	-24.54	12.45	-1.97	0.064	
$\dot{M}O_{2\,Max}$	Ambient vs Elevated	-21.04	56.35	-0.37	0.713	19
	Increasing vs Decreasing	-89.37	59.10	-1.51	0.147	
	Constant vs Fluctuating	-27.31	40.83	-0.67	0.512	
	Ambient vs pCO_2	32.23	46.39	0.70	0.496	
Aerobic scope	Ambient vs Elevated	-41.80	60.07	-0.70	0.495	19
	Increasing vs Decreasing	-106.41	63.01	-1.69	0.108	
	Constant vs Fluctuating	-43.35	43.53	-1.00	0.332	
	Ambient vs pCO_2	56.77	49.46	1.15	0.265	
Factorial aerobic scope	Ambient vs Elevated	-3.27	5.27	-0.62	0.542	19
	Increasing vs Decreasing	-11.01	5.52	-1.99	0.061	
	Constant vs Fluctuating	-4.17	3.82	-1.09	0.288	
	Ambient vs pCO_2	4.96	4.34	1.14	0.267	
U_{crit}*	Ambient vs Elevated	0.05	0.34	0.16	0.875	19
	Increasing vs Decreasing	-0.02	0.33	-0.06	0.950	
	Constant vs Fluctuating	-0.11	0.22	-0.51	0.620	
	Ambient vs pCO_2	0.04	0.27	0.15	0.886	
Haemoglobin concentration	Ambient vs Elevated	0.14	0.47	0.30	0.768	19
	Increasing vs Decreasing	-1.14	0.52	-2.20	0.045	
	Constant vs Fluctuating	0.19	0.35	0.55	0.590	
	Ambient vs pCO_2	-0.22	0.42	-0.54	0.600	
Whole blood lactate*	Ambient vs Elevated	1.20	0.71	1.68	0.116	19
	Increasing vs Decreasing	0.01	0.76	0.01	0.989	
	Constant vs Fluctuating	0.56	0.50	1.12	0.281	
	Ambient vs pCO_2	-1.17	0.62	-1.88	0.083	
Whole blood glucose	Ambient vs Elevated	-1.52	1.39	-1.09	0.294	19
	Increasing vs Decreasing	-2.09	1.64	-1.27	0.225	
	Constant vs Fluctuating	1.53	1.07	1.42	0.178	
	Ambient vs pCO_2	-0.01	1.24	-0.01	0.995	
Haematocrit (%)	Ambient vs Elevated	-2.06	2.82	-0.73	0.479	19
	Increasing vs Decreasing	-4.49	3.34	-1.34	0.202	
	Constant vs Fluctuating	-0.92	2.19	-0.42	0.680	
	Ambient vs pCO_2	1.99	2.53	0.79	0.445	
Mean cell haemoglobin concentration	Ambient vs Elevated	-0.02	0.01	-1.94	0.074	19
	Increasing vs Decreasing	0.00	0.01	0.19	0.851	
	Constant vs Fluctuating	-0.01	0.01	-1.50	0.157	
	Ambient vs pCO_2	0.02	0.01	2.32	0.038	

Table S3. The output of the emmeans test of the original model A.pol.lmer2<-lmer(MO2~Treatment+Swimming+(Swimming|Fish), data=apol) without the planned comparisons examining the effect of the 8 h tests at the four pCO_2 treatments (stable ambient ~480 µatm; stable elevated ~ 1,100 µatm; fluctuating increasing ~480-1,100 µatm; and fluctuating decreasing ~1,100-480 µatm) on the oxygen uptake rates ($\dot{M}O_2$) of *A. poluacanthus* during both U_{opt} and U_{rest} trials.

Treatment	Swimming test	emmean	SE	df	lower.CI	upper.CI
Ambient	U_{rest}	242.5	20.9	26.1	199.6	285.4
Increasing	U_{rest}	183.8	23.2	27.0	136.3	231.3
Elevated	U_{rest}	222.1	22.5	26.5	175.9	268.3
Decreasing	U_{rest}	165.7	22.5	26.4	119.5	211.9
Ambient	U_{opt}	265.3	19.9	22.8	224.1	306.4
Increasing	U_{opt}	206.5	23.3	26.7	158.7	254.3
Elevated	U_{opt}	244.9	21.6	23.2	200.3	289.5
Decreasing	U_{opt}	188.5	21.6	23.3	143.9	233.0

Table S4. The output of the emmeans test of the original model A.cur.lmer2<-lmer(MO2~Treatment+Swimming+(Swimming|Fish), data=acur) without the planned comparisons examining the effect of the 8 h tests at the four pCO_2 treatments (stable ambient ~480 µatm; stable elevated ~ 1,100 µatm; fluctuating increasing ~480-1,100 µatm; and fluctuating decreasing ~1,100-480 µatm) on the oxygen uptake rates ($\dot{M}O_2$) of *A. curacao* during both U_{opt} and U_{rest} trials.

Treatment	Swimming test	emmean	SE	df	lower.CI	upper.CI
Ambient	U_{rest}	167.1	11.7	40.0	143.4	190.9
Increasing	U_{rest}	160.1	13.2	44.0	133.5	186.7
Elevated	U_{rest}	173.7	12.9	36.0	147.4	199.9
Decreasing	U_{rest}	156.3	11.7	34.1	132.6	180.0
Ambient	U_{opt}	188.9	12.2	39.8	164.3	213.6
Increasing	U_{opt}	181.9	13.4	41.2	154.9	208.9
Elevated	U_{opt}	195.5	13.7	37.2	167.7	223.2
Decreasing	U_{opt}	178.1	13.2	38.7	151.4	204.7

Appendix C -   Supplementary material for Chapter 4

Contrasting effects of constant and fluctuating pCO$_2$ conditions on the exercise physiology of coral reef fishes

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Table S1. Bayesian posterior means, 95% highest posterior density uncertainty intervals (UI), and the amount of UI that intersect 0 (%) of U_{crit}, $\dot{M}O_{2\,Max}$, $\dot{M}O_{2\,Min}$, and Aerobic scope. Bold values indicate that the % UI that does not intersect 0 is more than 85%, i.e., moderate evidence of an effect. Bold values with an asterisk (*) indicate that the % UI that does not intersect with 0 is above than 95%, i.e., strong evidence of an effect.

Variables	Contrasts	*Lutjanus fulviflamma*				*Caesio cuning*				*Abudefduf whitleyi*				*Cheilodipterus quinquelineatus*			
		Mean	Lower UI	Upper UI	UI (%)	Mean	Lower UI	Upper UI	UI (%)	Mean	Lower UI	Upper UI	UI (%)	Mean	Lower UI	Upper UI	UI (%)
U_{crit}	Ambient - Fluctuating	-1.19	-1.99	-0.30	100*	-0.35	-1.02	0.31	85	-0.46	-0.946	0.03	97*	-0.09	-0.38	0.20	73
	Ambient - Stable	-0.73	-1.58	0.09	96*	0.00	-0.66	0.63	50	-0.34	-0.839	0.15	92	0.12	-0.17	0.42	78
	Fluctuating - Stable	0.47	-0.36	1.29	86	0.34	-0.31	0.99	85	0.11	-0.408	0.60	67	0.21	-0.10	0.52	91
	Day - Night	-0.05	-0.75	0.62	56	0.29	-0.21	0.88	86	0.06	-0.381	0.42	61	0.07	-0.17	0.33	72
$\dot{M}O_{2\,Max}$	Ambient - Fluctuating	-508.00	-863.00	-203.00	100*	-75.50	-197.70	46.50	88	-40	-120.40	32.00	85	52.60	-9.86	113.60	96*
	Ambient - Stable	-203.00	-543.00	134.00	88	-28.50	-150.00	91.20	69	-43	-117.50	37.70	87	18.60	-40.01	83.40	73
	Fluctuating - Stable	304.00	-35.00	636.00	96*	46.10	-73.40	171.40	77	-3	-87.10	76.10	53	-33.50	-95.88	30.80	85
	Day - Night	-24.70	-292.00	257.00	57	16.70	-82.20	119.00	62	0.665	-65.3	64.6	51	-14.60	-64.50	36.40	72
$\dot{M}O_{2\,Min}$	Ambient - Fluctuating	-8.44	-34.30	19.60	73	-3.56	-20.00	13.30	66	4.66	-25.2	33.40	62	10.40	-38.70	57.70	66
	Ambient - Stable	1.44	-24.70	29.70	54	-5.24	-21.70	11.10	73	18.13	-12.1	46.90	89	24.80	-25.50	73.70	83
	Fluctuating - Stable	9.78	-18.70	36.50	77	-1.86	-18.70	14.70	59	13.33	-17.3	44.40	81	14.10	-35.50	66.20	71
	Day - Night	12.00	-10.50	33.90	85	-11.10	-24.70	2.25	95*	-17.70	-43.3	5.88	93	-33.50	-73.10	8.19	95*
Aerobic scope	Ambient - Fluctuating	-495.00	-836.20	-164.00	100*	-72.30	-193.50	45.20	89	-42.20	-109.1	30.43	88	43.94	-5.74	87.87	96*
	Ambient - Stable	-204.00	-555.90	110.00	89	-23.60	-147.70	91.20	65	-60.30	-132.2	9.48	95*	-5.25	-54.64	40.64	59
	Fluctuating - Stable	291.00	-52.30	622.00	96*	49.40	-70.20	166.00	79	-17.40	-93.1	53.79	69	-49.36	-99.21	-1.74	98*
	Day - Night	-34.10	-308.00	231.00	60	27.20	-70.80	124.00	71	19.20	-40	75.40	74	19.90	-18.20	59.10	84

Table S2. Bayesian posterior means, 95% highest posterior density uncertainty intervals (UI), and the amount of UI that intersect 0 (%) of haemoglobin concentration [Hb], haematocrit (Hct), and mean cell haemoglobin concentration (MCHC). Bolded values indicate that the % UI that does not intersect 0 is more than 85%, i.e., moderate evidence of an effect. Bold values with an asterisk (*) indicate that the % UI that does not intersect with 0 is above than 95%, i.e., strong evidence of an effect.

Variables	Contrasts	*Lutjanus fulviflamma*				*Caesio cuning*				*Abudefduf whitleyi*				*Cheilodipterus quinquelineatus*			
		Mean	Lower UI	Upper UI	UI (%)	Mean	Lower UI	Upper UI	UI (%)	Mean	Lower UI	Upper UI	UI (%)	Mean	Lower UI	Upper UI	UI (%)
[Hb] (g 100ml^{-1})	Ambient - Fluctuating	0.09	-0.47	0.66	63	-0.19	-1.00	0.59	69	-0.40	-1.036	0.24	89	-0.18	-0.66	0.30	78
	Ambient - Stable	-0.17	-0.72	0.42	72	0.21	-0.56	1.05	70	-0.12	-0.764	0.54	64	0.17	-0.30	0.64	77
	Fluctuating - Stable	-0.26	-0.79	0.33	82	0.40	-0.38	1.22	83	0.28	-0.39	0.96	80	0.36	-0.15	0.82	93
	Day - Night	0.42	-0.02	0.90	96*	0.01	-0.67	0.66	51	0.07	-0.461	0.62	60	0.09	-0.30	0.48	67
Hct (%)	Ambient - Fluctuating	1.11	-1.62	3.89	79	-1.19	-4.13	2.03	78	-0.55	-2.871	1.63	69	-0.94	-4.00	2.14	73
	Ambient - Stable	1.14	-1.64	3.82	79	0.85	-2.33	3.88	71	1.43	-0.795	3.71	90	0.80	-2.34	4.01	70
	Fluctuating - Stable	0.00	-2.80	2.69	50	2.03	-0.98	5.17	90	1.97	-0.326	4.42	95*	1.72	-1.41	4.95	86
	Day - Night	1.36	-0.90	3.57	88	-0.69	-3.17	1.84	70	-1.20	-3.15	0.60	90	0.16	-2.45	2.69	55
MCHC (g ml^{-1})	Ambient - Fluctuating	-0.06	-0.30	0.16	70	0.07	-0.18	0.34	70	-0.11	-0.353	0.11	82	-0.06	-0.38	0.30	63
	Ambient - Stable	-0.16	-0.39	0.07	91	-0.01	-0.26	0.26	53	-0.16	-0.399	0.07	92	-0.16	-0.52	0.17	83
	Fluctuating - Stable	-0.10	-0.33	0.12	81	-0.08	-0.33	0.19	72	-0.05	-0.308	0.20	66	-0.11	-0.45	0.24	73
	Day - Night	0.04	-0.15	0.23	66	0.10	-0.12	0.31	83	0.14	-0.0777	0.32	90	0.10	-0.19	0.38	76
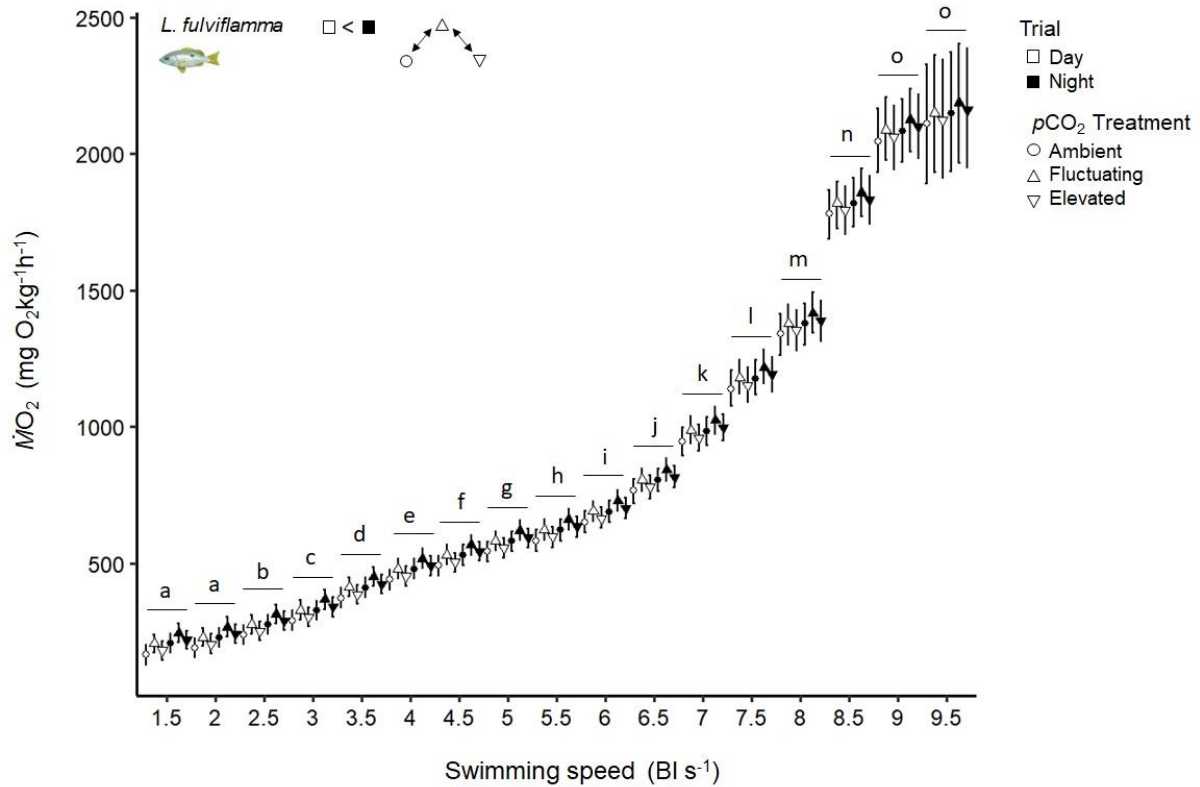
Figure S1. The $\dot{M}O_2$ of *Lutjanus fulviflamma* at different swimming speeds exposed to one of three different pCO_2 treatments (● - Ambient 400 µatm, ▼- stable elevated 1,000 µatm, and ▲ - fluctuating elevated 1,000 ± 300 µatm). The symbols represent least-squares means and error bars represent 95% confidence intervals. Black symbols represent values at night and the white symbols represent daytime values. Black arrows and greater than or less than symbols represent strong evidence for an effect (i.e., > 95% of the UI does not intersected zero), grey arrows and greater than or less than symbols represent moderate evidence for an effect (i.e., > 85% of the UI intersected zero).
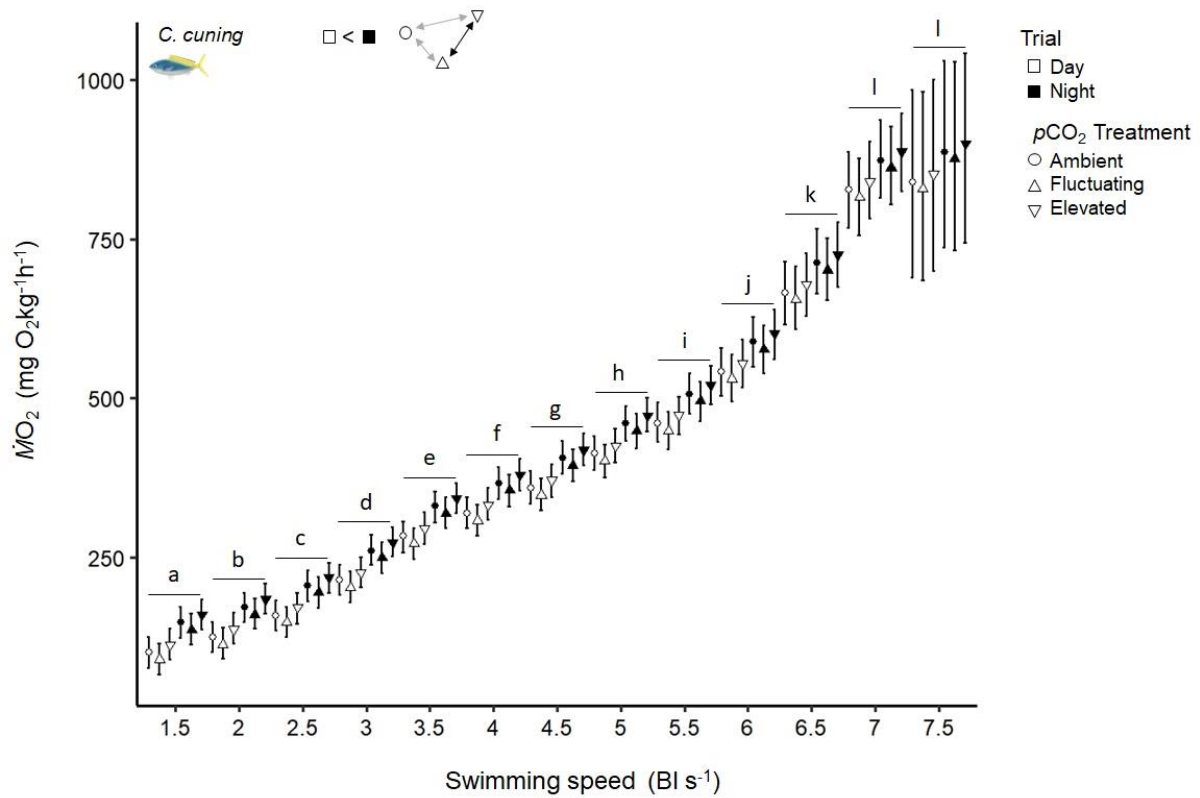
Figure S2. The $\dot{M}O_2$ of *Caesio cuning* at different swimming speeds exposed to one of three different pCO_2 treatments (● - Ambient 400 μatm, ▼- stable elevated 1,000 μatm, and ▲ - fluctuating elevated 1,000 ± 300 μatm). The symbols represent least-squares means and error bars represent 95% confidence intervals. Black symbols represent values at night and the white symbols represent daytime values. Black arrows and greater than or less than symbols represent strong evidence for an effect (i.e., > 95% of the UI does not intersected zero), grey arrows and greater than or less than symbols represent moderate evidence for an effect (i.e., > 85% of the UI intersected zero).
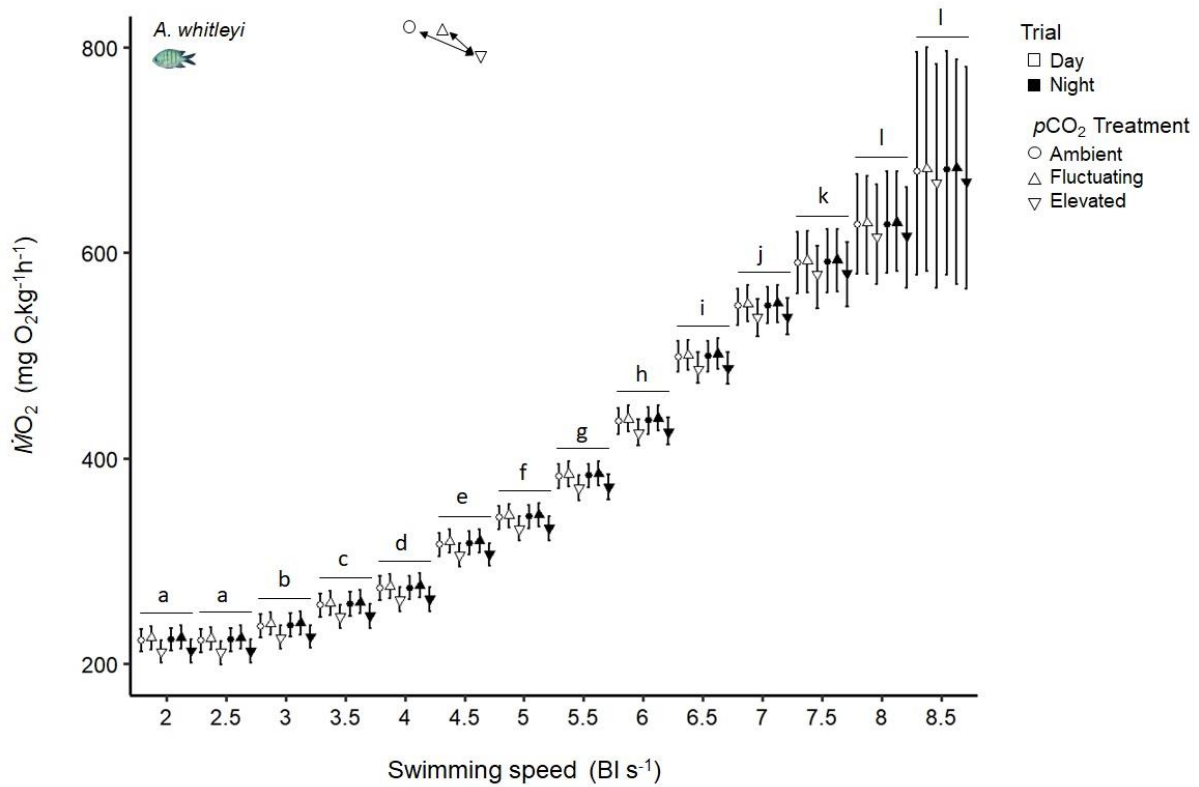
Figure S3. The $\dot{M}O_2$ of *Abudefduf whitleyi* at different swimming speeds exposed to one of three different pCO_2 treatments (● - Ambient 400 µatm, ▼- stable elevated 1,000 µatm, and ▲ - fluctuating elevated 1,000 ± 300 µatm). The symbols represent least-squares means and error bars represent 95% confidence intervals. Black symbols represent values at night and the white symbols represent daytime values. Black arrows and greater than or less than symbols represent strong evidence for an effect (i.e., > 95% of the UI does not intersected zero), grey arrows and greater than or less than symbols represent moderate evidence for an effect (i.e., > 85% of the UI intersected zero).

Figure S4. The $\dot{M}O_2$ of *Cheilodipterus quinquelineatus* at different swimming speeds exposed to one of three different pCO_2 treatments (● - Ambient 400 µatm, ▼ - stable elevated 1,000 µatm, and ▲ - fluctuating elevated 1,000 ± 300 µatm). The symbols represent least-squares means and error bars represent 95% confidence intervals. Black symbols represent values at night and the white symbols represent daytime values. Black arrows and greater than or less than symbols represent strong evidence for an effect (i.e., > 95% of the UI does not intersected zero), grey arrows and greater than or less than symbols represent moderate evidence for an effect (i.e., > 85% of the UI intersected zero).
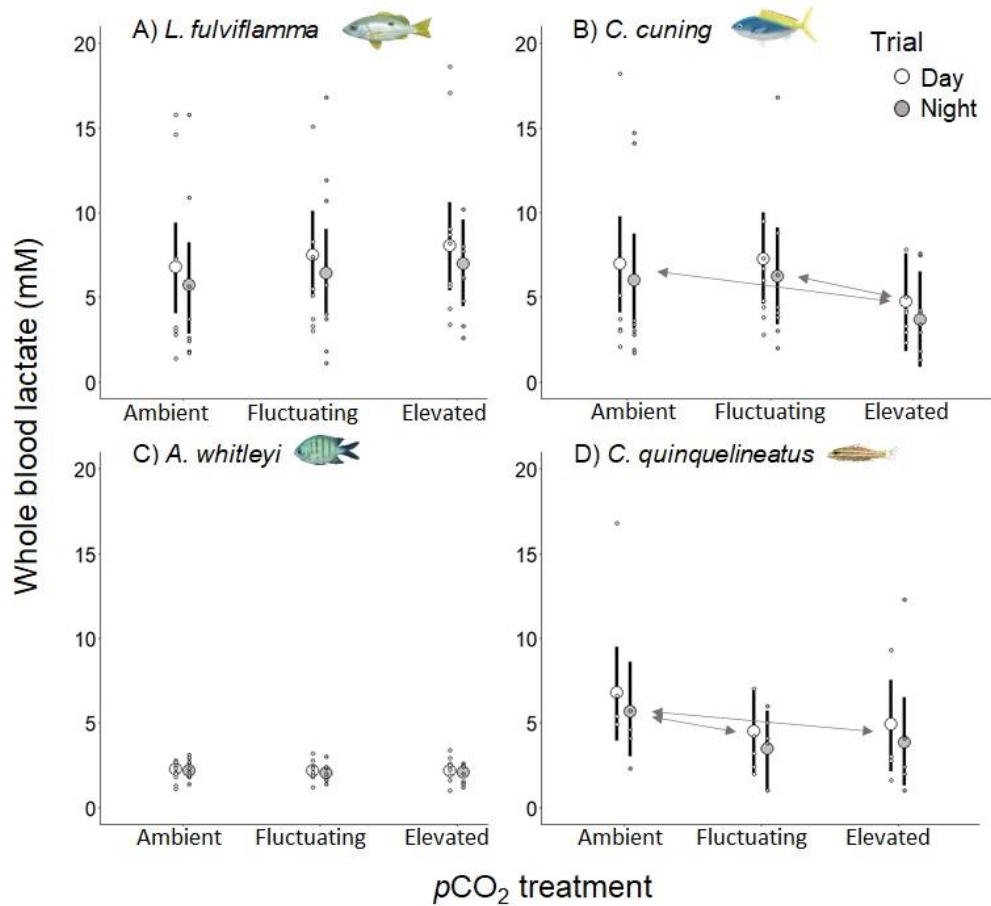
Figure S5. The whole blood lactate of A) *Lutjanus fulviflamma*, B) *Caesio cuning*, C) *Abudefduf whitleyi*, and D) *Cheilodipterus quinquelineatus* exposed to one of three different pCO_2 treatments (Ambient 400 µatm, stable elevated 1,000 µatm, and fluctuating elevated 1,000 ± 300 µatm). Circles represent least-squares means and error bars represent 95% confidence intervals. Differences between pCO_2 treatments are displayed by black arrows (strong evidence for an effect: i.e., > 95% of the UI does not intersected zero) and grey arrows (moderate evidence for an effect: i.e., > 85% of the UI does not intersected zero). Differences between time of day are depicted by greater than or less than symbols (i.e., ○ > ● or ○ < ●).

Figure S6. The whole blood glucose of A) *Lutjanus fulviflamma*, B) *Caesio cuning*, C) *Abudefduf whitleyi*, and D) *Cheilodipterus quinquelineatus* exposed to one of three different pCO_2 treatments (Ambient 400 µatm, stable elevated 1,000 µatm, and fluctuating elevated 1,000 ± 300 µatm). Circles represent least-squares means and error bars represent 95% confidence intervals. Differences between pCO_2 treatments are displayed by black arrows (strong evidence for an effect: i.e., > 95% of the UI does not intersected zero) and grey arrows (moderate evidence for an effect: i.e., > 85% of the UI does not intersected zero). Differences between time of day are depicted by greater than or less than symbols (i.e., ○ > ● or ○ < ●).

Appendix D - Supplementary material for Chapter 5

This chapter has not yet been unpublished

Table S1. Bayesian posterior means, 95% highest posterior density uncertainty intervals (UI), and the amount of UI that intersect 0 (%) of $\dot{M}O_{2\,Max}$, $\dot{M}O_{2\,Min}$, absolute aerobic scope, and factorial aerobic scope. Bold values indicate that the % UI that does not intersect 0 is more than 95%.

Variables	Contrasts	Mean	Lower UI	Upper UI	UI (%)
$\dot{M}O_{2\,Max}$	Ambient C18 - Ambient Sham	38.07	25.26	97.01	88
	Ambient C18 - Fluctuating C18	41.38	21.62	103.97	90
	Ambient C18 - Fluctuating Sham	**62.38**	**1.73**	**128.25**	**98**
	Ambient C18 - Elevated C18	**93.18**	**31.3**	**156.62**	**100**
	Ambient C18 - Elevated Sham	**69.69**	**7.75**	**130.51**	**99**
	Ambient Sham - Fluctuating Sham	24.78	39.44	86.06	79
	Ambient Sham - Elevated Sham	30.9	28.16	95.57	85
	Fluctuating C18 - Ambient Sham	3.79	67.29	60.43	55
	Fluctuating C18 - Fluctuating Sham	20.99	44.73	84.26	74
	Fluctuating C18 - Elevated C18	51.21	13.13	114.31	94
	Fluctuating C18 - Elevated Sham	27.62	31.66	97.09	81
	Fluctuating Sham - Elevated Sham	6.64	61.38	68.35	58
	Elevated C18 - Ambient Sham	**54.73**	**115.4**	**6.38**	**96**
	Elevated C18 - Fluctuating Sham	30.21	92.22	34.25	83
	Elevated C18 - Elevated Sham	23.47	86.97	36.01	77
$\dot{M}O_{2\,Min}$	Ambient C18 - Ambient Sham	6.67	-15.99	30.26	74
	Ambient C18 - Fluctuating C18	**-30.41**	**-55.13**	**-5.70**	**99**
	Ambient C18 - Fluctuating Sham	-18.08	-41.72	8.42	89
	Ambient C18 - Elevated C18	-1.76	-24.67	21.02	58
	Ambient C18 - Elevated Sham	11.12	-13.21	34.14	89
	Ambient Sham - Fluctuating Sham	**-24.65**	**-48.87**	**-0.86**	**97**
	Ambient Sham - Elevated Sham	4.38	-19.27	25.11	74
	Fluctuating C18 - Ambient Sham	**37.07**	**11.74**	**59.66**	**100**
	Fluctuating C18 - Fluctuating Sham	12.29	-11.17	38.53	85
	Fluctuating C18 - Elevated C18	**28.52**	**5.25**	**52.23**	**99**
	Fluctuating C18 - Elevated Sham	**41.28**	**16.68**	**65.07**	**100**
	Fluctuating Sham - Elevated Sham	**28.81**	**4.75**	**51.92**	**99**
	Elevated C18 - Ambient Sham	8.56	-14.65	29.87	67
	Elevated C18 - Fluctuating Sham	-16.22	-40.32	6.62	93
	Elevated C18 - Elevated Sham	12.87	-10.59	34.85	87
Absolute aerobic scope	Ambient C18 - Ambient Sham	20.87	65	104	69
	Ambient C18 - Fluctuating C18	**84.61**	**4.74**	**174.3**	**97**
	Ambient C18 - Fluctuating Sham	68.02	27.55	161.9	92
	Ambient C18 - Elevated C18	64.23	27.24	148.3	93

		Mean	Lower UI	Upper UI	UI (%)
	Ambient C18 - Elevated Sham	33.12	55.56	124.7	76
	Ambient Sham - Fluctuating Sham	47.55	42.13	134.1	85
	Ambient Sham - Elevated Sham	12.22	71.93	99.9	62
	Fluctuating C18 - Ambient Sham	63.5	156.44	25.1	92
	Fluctuating C18 - Fluctuating Sham	15.85	111.14	78.8	64
	Fluctuating C18 - Elevated C18	20.47	108.6	71.7	67
	Fluctuating C18 - Elevated Sham	51.06	141.08	35.5	87
	Fluctuating Sham - Elevated Sham	35.44	125.07	56.2	78
	Elevated C18 - Ambient Sham	42.31	123.25	49.2	84
	Elevated C18 - Fluctuating Sham	3.54	86.82	97.7	53
	Elevated C18 - Elevated Sham	31.31	114.17	57.1	76
Factorial aerobic scope	Ambient C18 - Ambient Sham	0.0476	0.6915	0.5319	56
	Ambient C18 - Fluctuating C18	**0.7448**	**0.0805**	**1.3779**	**99**
	Ambient C18 - Fluctuating Sham	0.4816	0.1776	1.1515	92
	Ambient C18 - Elevated C18	0.3321	0.2589	0.9852	85
	Ambient C18 - Elevated Sham	0.089	0.7344	0.5305	61
	Ambient Sham - Fluctuating Sham	0.5258	0.1181	1.1548	94
	Ambient Sham - Elevated Sham	0.044	0.6334	0.5528	56
	Fluctuating C18 - Ambient Sham	**0.789**	**1.4294**	**0.1491**	**99**
	Fluctuating C18 - Fluctuating Sham	0.2687	0.9537	0.3873	79
	Fluctuating C18 - Elevated C18	0.4118	1.0769	0.2154	90
	Fluctuating C18 - Elevated Sham	**0.8344**	**1.4321**	**0.1594**	**99**
	Fluctuating Sham - Elevated Sham	**0.5604**	**1.2227**	**0.0606**	**96**
	Elevated C18 - Ambient Sham	0.3758	0.9977	0.2266	89
	Elevated C18 - Fluctuating Sham	0.1449	0.5196	0.7627	67
	Elevated C18 - Elevated Sham	0.4252	1.0155	0.1965	91

Table S2. Bayesian posterior means, 95% highest posterior density uncertainty intervals (UI), and the amount of UI that intersect 0 (%) of lactate and glucose scope. Bold values indicate that the % UI that does not intersect 0 is more than 95%.

Variables	Contrasts	Mean	Lower UI	Upper UI	UI (%)
Whole blood lactate	Ambient 30min C18 - Ambient 30min Sham	-1.01	-2.41	0.29	94
	Ambient 30min C18 - Ambient Immediate C18	-0.86	-2.19	0.46	89
	Ambient 30min C18 - Ambient Immediate Sham	-0.34	-1.59	1.03	69
	Ambient 30min C18 - Ambient Overnight C18	**2.41**	**1.05**	**3.77**	**100**
	Ambient 30min C18 - Ambient Overnight Sham	**2.62**	**1.20**	**3.88**	**100**
	Ambient 30min C18 - Fluctuating 30min C18	-0.55	-1.92	0.75	79
	Ambient 30min C18 - Fluctuating 30min Sham	**-1.75**	**-3.07**	**-0.47**	**99**
	Ambient 30min C18 - Fluctuating Immediate C18	-0.65	-2.00	0.77	83
	Ambient 30min C18 - Fluctuating Immediate Sham	-0.81	-2.06	0.56	88
	Ambient 30min C18 - Fluctuating Overnight C18	**2.21**	**0.89**	**3.65**	**100**
	Ambient 30min C18 - Fluctuating Overnight Sham	**2.17**	**0.73**	**3.55**	**100**
	Ambient 30min C18 - Elevated 30min C18	**-2.16**	**-3.37**	**-0.78**	**100**
	Ambient 30min C18 - Elevated 30min Sham	**-1.12**	**-2.45**	**0.22**	**95**
	Ambient 30min C18 - Elevated Immediate C18	-0.02	-1.34	1.30	51
	Ambient 30min C18 - Elevated Immediate Sham	-0.80	-2.11	0.47	89

Ambient 30min C18 - Elevated Overnight C18	**2.43**	**1.10**	**3.76**	**100**
Ambient 30min C18 - Elevated Overnight Sham	**1.78**	**0.43**	**3.08**	**100**
Ambient 30min Sham - Ambient Immediate Sham	0.69	-0.63	1.95	85
Ambient 30min Sham - Ambient Overnight Sham	**3.61**	**2.32**	**5.01**	**100**
Ambient 30min Sham - Fluctuating 30min Sham	-0.73	-2.08	0.56	86
Ambient 30min Sham - Fluctuating Immediate Sham	0.21	-1.11	1.52	62
Ambient 30min Sham - Fluctuating Overnight Sham	**3.20**	**1.84**	**4.59**	**100**
Ambient 30min Sham - Elevated 30min Sham	-0.11	-1.48	1.16	56
Ambient 30min Sham - Elevated Immediate Sham	0.21	-1.10	1.50	63
Ambient 30min Sham - Elevated Overnight Sham	**2.79**	**1.53**	**4.14**	**100**
Ambient Immediate C18 - Ambient 30min Sham	-0.16	-1.50	1.18	60
Ambient Immediate C18 - Ambient Immediate Sham	0.52	-0.87	1.80	78
Ambient Immediate C18 - Ambient Overnight C18	**3.25**	**1.87**	**4.61**	**100**
Ambient Immediate C18 - Ambient Overnight Sham	**3.44**	**2.21**	**4.87**	**100**
Ambient Immediate C18 - Fluctuating 30min Sham	-0.89	-2.20	0.45	91
Ambient Immediate C18 - Fluctuating Immediate C18	0.20	-1.16	1.61	62
Ambient Immediate C18 - Fluctuating Immediate Sham	0.04	-1.36	1.39	53
Ambient Immediate C18 - Fluctuating Overnight C18	**3.07**	**1.69**	**4.51**	**100**
Ambient Immediate C18 - Fluctuating Overnight Sham	**3.02**	**1.68**	**4.40**	**100**
Ambient Immediate C18 - Elevated 30min Sham	-0.27	-1.70	1.04	65
Ambient Immediate C18 - Elevated Immediate C18	0.84	-0.57	2.16	88
Ambient Immediate C18 - Elevated Immediate Sham	0.04	-1.28	1.40	52
Ambient Immediate C18 - Elevated Overnight C18	**3.30**	**1.90**	**4.59**	**100**
Ambient Immediate C18 - Elevated Overnight Sham	**2.64**	**1.26**	**3.99**	**100**
Ambient Immediate Sham - Ambient Overnight Sham	**2.92**	**1.50**	**4.22**	**100**
Ambient Immediate Sham - Fluctuating Immediate Sham	-0.48	-1.77	0.83	76
Ambient Immediate Sham - Fluctuating Overnight Sham	**2.50**	**1.18**	**3.94**	**100**
Ambient Immediate Sham - Elevated Immediate Sham	-0.48	-1.72	0.88	77
Ambient Immediate Sham - Elevated Overnight Sham	**2.11**	**0.75**	**3.37**	**100**
Ambient Overnight C18 - Ambient 30min Sham	**-3.41**	**-4.81**	**-2.06**	**100**
Ambient Overnight C18 - Ambient Immediate Sham	**-2.72**	**-4.14**	**-1.36**	**100**
Ambient Overnight C18 - Ambient Overnight Sham	0.19	-1.19	1.64	61
Ambient Overnight C18 - Fluctuating 30min Sham	**-4.13**	**-5.56**	**-2.76**	**100**
Ambient Overnight C18 - Fluctuating Immediate Sham	**-3.19**	**-4.62**	**-1.89**	**100**
Ambient Overnight C18 - Fluctuating Overnight C18	-0.19	-1.64	1.22	60
Ambient Overnight C18 - Fluctuating Overnight Sham	-0.21	-1.69	1.20	61
Ambient Overnight C18 - Elevated 30min Sham	**-3.52**	**-4.87**	**-2.13**	**100**
Ambient Overnight C18 - Elevated Immediate Sham	**-3.21**	**-4.50**	**-1.84**	**100**
Ambient Overnight C18 - Elevated Overnight C18	0.04	-1.26	1.40	52
Ambient Overnight C18 - Elevated Overnight Sham	-0.62	-2.00	0.77	81
Ambient Overnight Sham - Fluctuating Overnight Sham	-0.43	-1.84	0.97	73
Ambient Overnight Sham - Elevated Overnight Sham	-0.82	-2.20	0.51	88
Fluctuating 30min C18 - Ambient 30min Sham	-0.46	-1.78	0.91	75
Fluctuating 30min C18 - Ambient Immediate C18	-0.30	-1.63	1.04	67
Fluctuating 30min C18 - Ambient Immediate Sham	0.22	-1.13	1.48	63
Fluctuating 30min C18 - Ambient Overnight C18	**2.96**	**1.45**	**4.26**	**100**
Fluctuating 30min C18 - Ambient Overnight Sham	**3.16**	**1.78**	**4.49**	**100**
Fluctuating 30min C18 - Fluctuating 30min Sham	**-1.19**	**-2.51**	**0.11**	**96**
Fluctuating 30min C18 - Fluctuating Immediate C18	-0.09	-1.48	1.28	56

Fluctuating 30min C18 - Fluctuating Immediate Sham	-0.25	-1.61	1.07	65
Fluctuating 30min C18 - Fluctuating Overnight C18	**2.75**	**1.43**	**4.26**	**100**
Fluctuating 30min C18 - Fluctuating Overnight Sham	**2.73**	**1.30**	**4.10**	**100**
Fluctuating 30min C18 - Elevated 30min C18	**-1.60**	**-2.85**	**-0.28**	**99**
Fluctuating 30min C18 - Elevated 30min Sham	-0.57	-1.94	0.77	80
Fluctuating 30min C18 - Elevated Immediate C18	0.53	-0.87	1.86	78
Fluctuating 30min C18 - Elevated Immediate Sham	-0.25	-1.58	1.07	64
Fluctuating 30min C18 - Elevated Overnight C18	**2.98**	**1.70**	**4.27**	**100**
Fluctuating 30min C18 - Elevated Overnight Sham	**2.33**	**0.96**	**3.67**	**100**
Fluctuating 30min Sham - Ambient Immediate Sham	**1.42**	**0.10**	**2.75**	**98**
Fluctuating 30min Sham - Ambient Overnight Sham	**4.34**	**2.93**	**5.65**	**100**
Fluctuating 30min Sham - Fluctuating Immediate Sham	0.95	-0.42	2.22	91
Fluctuating 30min Sham - Fluctuating Overnight Sham	**3.92**	**2.61**	**5.39**	**100**
Fluctuating 30min Sham - Elevated 30min Sham	0.62	-0.73	1.97	81
Fluctuating 30min Sham - Elevated Immediate Sham	0.94	-0.38	2.25	92
Fluctuating 30min Sham - Elevated Overnight Sham	**3.52**	**2.21**	**4.87**	**100**
Fluctuating Immediate C18 - Ambient 30min Sham	-0.36	-1.70	0.97	71
Fluctuating Immediate C18 - Ambient Immediate Sham	0.31	-1.00	1.70	68
Fluctuating Immediate C18 - Ambient Overnight C18	**3.04**	**1.64**	**4.51**	**100**
Fluctuating Immediate C18 - Ambient Overnight Sham	**3.24**	**1.86**	**4.60**	**100**
Fluctuating Immediate C18 - Fluctuating 30min Sham	-1.11	-2.43	0.29	94
Fluctuating Immediate C18 - Fluctuating Immediate Sham	-0.15	-1.48	1.18	59
Fluctuating Immediate C18 - Fluctuating Overnight C18	**2.85**	**1.48**	**4.32**	**100**
Fluctuating Immediate C18 - Fluctuating Overnight Sham	**2.83**	**1.37**	**4.25**	**100**
Fluctuating Immediate C18 - Elevated 30min Sham	-0.47	-1.90	0.84	75
Fluctuating Immediate C18 - Elevated Immediate C18	0.63	-0.68	2.09	80
Fluctuating Immediate C18 - Elevated Immediate Sham	-0.16	-1.46	1.23	59
Fluctuating Immediate C18 - Elevated Overnight C18	**3.09**	**1.70**	**4.34**	**100**
Fluctuating Immediate C18 - Elevated Overnight Sham	**2.42**	**1.08**	**3.79**	**100**
Fluctuating Immediate Sham - Ambient Overnight Sham	**3.40**	**2.05**	**4.72**	**100**
Fluctuating Immediate Sham - Fluctuating Overnight Sham	**2.97**	**1.59**	**4.35**	**100**
Fluctuating Immediate Sham - Elevated Immediate Sham	0.01	-1.37	1.26	50
Fluctuating Immediate Sham - Elevated Overnight Sham	**2.59**	**1.25**	**3.92**	**100**
Fluctuating Overnight C18 - Ambient 30min Sham	**-3.22**	**-4.56**	**-1.79**	**100**
Fluctuating Overnight C18 - Ambient Immediate Sham	**-2.54**	**-4.04**	**-1.27**	**100**
Fluctuating Overnight C18 - Ambient Overnight Sham	0.39	-1.04	1.83	71
Fluctuating Overnight C18 - Fluctuating 30min Sham	**-3.95**	**-5.28**	**-2.48**	**100**
Fluctuating Overnight C18 - Fluctuating Immediate Sham	**-3.01**	**-4.45**	**-1.69**	**100**
Fluctuating Overnight C18 - Fluctuating Overnight Sham	-0.03	-1.49	1.39	52
Fluctuating Overnight C18 - Elevated 30min Sham	**-3.33**	**-4.78**	**-1.93**	**100**
Fluctuating Overnight C18 - Elevated Immediate Sham	**-3.01**	**-4.37**	**-1.64**	**100**
Fluctuating Overnight C18 - Elevated Overnight C18	0.24	-1.17	1.61	63
Fluctuating Overnight C18 - Elevated Overnight Sham	-0.42	-1.84	1.00	73
Fluctuating Overnight Sham - Elevated Overnight Sham	-0.39	-1.78	1.02	71
Elevated 30min C18 - Ambient 30min Sham	**1.16**	**-0.19**	**2.45**	**95**
Elevated 30min C18 - Ambient Immediate C18	**1.30**	**0.04**	**2.66**	**97**
Elevated 30min C18 - Ambient Immediate Sham	**1.84**	**0.55**	**3.17**	**100**
Elevated 30min C18 - Ambient Overnight C18	**4.57**	**3.14**	**5.93**	**100**
Elevated 30min C18 - Ambient Overnight Sham	**4.76**	**3.47**	**6.16**	**100**

	Elevated 30min C18 - Fluctuating 30min Sham	0.42	-0.91	1.69	73
	Elevated 30min C18 - Fluctuating Immediate C18	**1.52**	**0.17**	**2.84**	**99**
	Elevated 30min C18 - Fluctuating Immediate Sham	**1.36**	**0.03**	**2.59**	**98**
	Elevated 30min C18 - Fluctuating Overnight C18	**4.37**	**2.99**	**5.73**	**100**
	Elevated 30min C18 - Fluctuating Overnight Sham	**4.34**	**2.98**	**5.78**	**100**
	Elevated 30min C18 - Elevated 30min Sham	1.04	-0.29	2.36	94
	Elevated 30min C18 - Elevated Immediate C18	**2.15**	**0.82**	**3.47**	**100**
	Elevated 30min C18 - Elevated Immediate Sham	**1.37**	**0.08**	**2.66**	**98**
	Elevated 30min C18 - Elevated Overnight C18	**4.61**	**3.27**	**5.98**	**100**
	Elevated 30min C18 - Elevated Overnight Sham	**3.94**	**2.65**	**5.29**	**100**
	Elevated 30min Sham - Ambient Immediate Sham	0.81	-0.57	2.11	88
	Elevated 30min Sham - Ambient Overnight Sham	**3.72**	**2.32**	**5.06**	**100**
	Elevated 30min Sham - Fluctuating Immediate Sham	0.32	-1.05	1.65	68
	Elevated 30min Sham - Fluctuating Overnight Sham	**3.29**	**1.85**	**4.69**	**100**
	Elevated 30min Sham - Elevated Immediate Sham	0.32	-0.99	1.65	68
	Elevated 30min Sham - Elevated Overnight Sham	**2.90**	**1.60**	**4.30**	**100**
	Elevated Immediate C18 - Ambient 30min Sham	-0.98	-2.32	0.33	93
	Elevated Immediate C18 - Ambient Immediate Sham	-0.32	-1.63	1.00	67
	Elevated Immediate C18 - Ambient Overnight C18	**2.43**	**1.09**	**3.87**	**100**
	Elevated Immediate C18 - Ambient Overnight Sham	**2.61**	**1.24**	**3.98**	**100**
	Elevated Immediate C18 - Fluctuating 30min Sham	**-1.73**	**-3.08**	**-0.37**	**99**
	Elevated Immediate C18 - Fluctuating Immediate Sham	-0.78	-2.11	0.60	87
	Elevated Immediate C18 - Fluctuating Overnight C18	**2.23**	**0.80**	**3.57**	**100**
	Elevated Immediate C18 - Fluctuating Overnight Sham	**2.20**	**0.87**	**3.69**	**100**
	Elevated Immediate C18 - Elevated 30min Sham	**-1.10**	**-2.48**	**0.25**	**95**
	Elevated Immediate C18 - Elevated Immediate Sham	-0.79	-2.09	0.62	87
	Elevated Immediate C18 - Elevated Overnight C18	**2.47**	**1.09**	**3.77**	**100**
	Elevated Immediate C18 - Elevated Overnight Sham	**1.80**	**0.48**	**3.19**	**100**
	Elevated Immediate Sham - Ambient Overnight Sham	**3.41**	**2.08**	**4.77**	**100**
	Elevated Immediate Sham - Fluctuating Overnight Sham	**3.00**	**1.61**	**4.37**	**100**
	Elevated Immediate Sham - Elevated Overnight Sham	**2.58**	**1.29**	**3.93**	**100**
	Elevated Overnight C18 - Ambient 30min Sham	**-3.46**	**-4.77**	**-2.12**	**100**
	Elevated Overnight C18 - Ambient Immediate Sham	**-2.77**	**-4.04**	**-1.48**	**100**
	Elevated Overnight C18 - Ambient Overnight Sham	0.15	-1.24	1.48	60
	Elevated Overnight C18 - Fluctuating 30min Sham	**-4.19**	**-5.52**	**-2.89**	**100**
	Elevated Overnight C18 - Fluctuating Immediate Sham	**-3.25**	**-4.51**	**-1.90**	**100**
	Elevated Overnight C18 - Fluctuating Overnight Sham	-0.27	-1.57	1.16	64
	Elevated Overnight C18 - Elevated 30min Sham	**-3.56**	**-4.95**	**-2.26**	**100**
	Elevated Overnight C18 - Elevated Immediate Sham	**-3.24**	**-4.58**	**-1.99**	**100**
	Elevated Overnight C18 - Elevated Overnight Sham	-0.66	-1.94	0.68	84
Whole blood glucose	Ambient 30min C18 - Ambient 30min Sham	-1.93	-7.25	3.65	77
	Ambient 30min C18 - Ambient Immediate C18	-0.40	-5.96	5.13	55
	Ambient 30min C18 - Ambient Immediate Sham	0.64	-4.66	6.14	59
	Ambient 30min C18 - Ambient Overnight C18	**-11.00**	**-16.70**	**-5.14**	**100**
	Ambient 30min C18 - Ambient Overnight Sham	**-11.90**	**-17.77**	**-6.49**	**100**
	Ambient 30min C18 - Fluctuating 30min C18	-0.30	-5.93	5.20	54
	Ambient 30min C18 - Fluctuating 30min Sham	-1.24	-6.70	4.03	67
	Ambient 30min C18 - Fluctuating Immediate C18	-0.95	-6.60	4.42	63
	Ambient 30min C18 - Fluctuating Immediate Sham	-0.79	-6.13	4.77	61

Ambient 30min C18 - Fluctuating Overnight C18	**-10.50**	**-16.22**	**-4.80**	**100**
Ambient 30min C18 - Fluctuating Overnight Sham	**-6.28**	**-12.27**	**-0.75**	**98**
Ambient 30min C18 - Elevated 30min C18	-2.67	-8.05	2.71	83
Ambient 30min C18 - Elevated 30min Sham	-1.71	-7.34	3.77	73
Ambient 30min C18 - Elevated Immediate C18	-0.32	-5.98	5.14	55
Ambient 30min C18 - Elevated Immediate Sham	-1.59	-7.19	3.76	72
Ambient 30min C18 - Elevated Overnight C18	**-22.20**	**-27.80**	**-16.90**	**100**
Ambient 30min C18 - Elevated Overnight Sham	**-14.20**	**-19.50**	**-8.42**	**100**
Ambient 30min Sham - Ambient Immediate Sham	2.66	-2.83	8.25	82
Ambient 30min Sham - Ambient Overnight Sham	**-9.96**	**-15.53**	**-4.18**	**100**
Ambient 30min Sham - Fluctuating 30min Sham	0.73	-4.60	6.32	61
Ambient 30min Sham - Fluctuating Immediate Sham	1.19	-4.31	6.81	67
Ambient 30min Sham - Fluctuating Overnight Sham	-4.28	-10.51	1.17	93
Ambient 30min Sham - Elevated 30min Sham	0.21	-5.21	6.32	53
Ambient 30min Sham - Elevated Immediate Sham	0.44	-5.37	5.84	56
Ambient 30min Sham - Elevated Overnight Sham	**-12.20**	**-17.84**	**-6.84**	**100**
Ambient Immediate C18 - Ambient 30min Sham	-1.64	-7.22	4.13	70
Ambient Immediate C18 - Ambient Immediate Sham	1.09	-4.53	7.09	65
Ambient Immediate C18 - Ambient Overnight C18	**-10.60**	**-16.68**	**-4.68**	**100**
Ambient Immediate C18 - Ambient Overnight Sham	**-11.50**	**-17.41**	**-5.74**	**100**
Ambient Immediate C18 - Fluctuating 30min Sham	-0.81	-6.75	4.52	61
Ambient Immediate C18 - Fluctuating Immediate C18	-0.58	-6.23	5.15	58
Ambient Immediate C18 - Fluctuating Immediate Sham	-0.37	-5.84	5.36	55
Ambient Immediate C18 - Fluctuating Overnight C18	**-10.20**	**-16.10**	**-4.19**	**100**
Ambient Immediate C18 - Fluctuating Overnight Sham	**-5.88**	**-11.94**	**-0.19**	**98**
Ambient Immediate C18 - Elevated 30min Sham	-1.32	-7.27	4.22	68
Ambient Immediate C18 - Elevated Immediate C18	0.09	-5.67	5.78	51
Ambient Immediate C18 - Elevated Immediate Sham	-1.21	-6.56	4.72	66
Ambient Immediate C18 - Elevated Overnight C18	**-21.80**	**-27.44**	**-16.00**	**100**
Ambient Immediate C18 - Elevated Overnight Sham	**-13.80**	**-19.50**	**-8.09**	**100**
Ambient Immediate Sham - Ambient Overnight Sham	**-12.60**	**-18.50**	**-6.75**	**100**
Ambient Immediate Sham - Fluctuating Immediate Sham	-1.42	-7.04	4.18	69
Ambient Immediate Sham - Fluctuating Overnight Sham	**-6.99**	**-12.81**	**-1.10**	**99**
Ambient Immediate Sham - Elevated Immediate Sham	-2.32	-7.92	3.29	79
Ambient Immediate Sham - Elevated Overnight Sham	**-14.90**	**-20.64**	**-9.27**	**100**
Ambient Overnight C18 - Ambient 30min Sham	**9.01**	**3.16**	**14.90**	**100**
Ambient Overnight C18 - Ambient Immediate Sham	**11.70**	**5.84**	**17.70**	**100**
Ambient Overnight C18 - Ambient Overnight Sham	-1.00	-7.39	4.71	62
Ambient Overnight C18 - Fluctuating 30min Sham	**9.76**	**3.99**	**15.60**	**100**
Ambient Overnight C18 - Fluctuating Immediate Sham	**10.20**	**4.21**	**16.00**	**100**
Ambient Overnight C18 - Fluctuating Overnight C18	0.44	-5.59	6.60	55
Ambient Overnight C18 - Fluctuating Overnight Sham	4.70	-1.73	10.60	93
Ambient Overnight C18 - Elevated 30min Sham	**9.28**	**3.32**	**15.20**	**100**
Ambient Overnight C18 - Elevated Immediate Sham	**9.41**	**3.23**	**15.40**	**100**
Ambient Overnight C18 - Elevated Overnight C18	**-11.30**	**-17.27**	**-5.55**	**100**
Ambient Overnight C18 - Elevated Overnight Sham	-3.22	-9.09	2.91	85
Ambient Overnight Sham - Fluctuating Overnight Sham	**5.62**	**-0.58**	**11.50**	**97**
Ambient Overnight Sham - Elevated Overnight Sham	-2.28	-8.07	3.47	78
Fluctuating 30min C18 - Ambient 30min Sham	-1.68	-7.31	4.15	72

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Fluctuating 30min C18 - Ambient Immediate C18	-0.03	-5.83	5.90	50
Fluctuating 30min C18 - Ambient Immediate Sham	1.02	-4.85	6.65	64
Fluctuating 30min C18 - Ambient Overnight C18	**-10.70**	**-16.75**	**-4.82**	**100**
Fluctuating 30min C18 - Ambient Overnight Sham	**-11.60**	**-17.59**	**-5.95**	**100**
Fluctuating 30min C18 - Fluctuating 30min Sham	-0.92	-6.56	4.91	63
Fluctuating 30min C18 - Fluctuating Immediate C18	-0.61	-6.51	5.10	58
Fluctuating 30min C18 - Fluctuating Immediate Sham	-0.43	-6.28	5.05	56
Fluctuating 30min C18 - Fluctuating Overnight C18	**-10.20**	**-16.06**	**-3.72**	**100**
Fluctuating 30min C18 - Fluctuating Overnight Sham	**-5.99**	**-12.05**	**0.00**	**98**
Fluctuating 30min C18 - Elevated 30min C18	-2.30	-7.87	3.20	79
Fluctuating 30min C18 - Elevated 30min Sham	-1.38	-7.16	4.44	68
Fluctuating 30min C18 - Elevated Immediate C18	0.00	-5.85	5.87	50
Fluctuating 30min C18 - Elevated Immediate Sham	-1.24	-7.05	4.36	67
Fluctuating 30min C18 - Elevated Overnight C18	**-22.00**	**-27.72**	**-16.20**	**100**
Fluctuating 30min C18 - Elevated Overnight Sham	**-13.90**	**-19.78**	**-8.22**	**100**
Fluctuating 30min Sham - Ambient Immediate Sham	1.88	-3.32	7.74	75
Fluctuating 30min Sham - Ambient Overnight Sham	**-10.70**	**-16.58**	**-5.22**	**100**
Fluctuating 30min Sham - Fluctuating Immediate Sham	0.46	-5.38	5.81	57
Fluctuating 30min Sham - Fluctuating Overnight Sham	**-5.14**	**-10.93**	**0.84**	**96**
Fluctuating 30min Sham - Elevated 30min Sham	-0.48	-6.07	5.19	57
Fluctuating 30min Sham - Elevated Immediate Sham	-0.37	-6.22	5.21	55
Fluctuating 30min Sham - Elevated Overnight Sham	**-12.90**	**-18.51**	**-7.42**	**100**
Fluctuating Immediate C18 - Ambient 30min Sham	-1.01	-6.43	4.77	64
Fluctuating Immediate C18 - Ambient Immediate Sham	1.63	-3.96	7.40	72
Fluctuating Immediate C18 - Ambient Overnight C18	**-10.00**	**-16.23**	**-4.20**	**100**
Fluctuating Immediate C18 - Ambient Overnight Sham	**-11.00**	**-16.66**	**-5.31**	**100**
Fluctuating Immediate C18 - Fluctuating 30min Sham	-0.25	-6.13	5.19	53
Fluctuating Immediate C18 - Fluctuating Immediate Sham	0.21	-5.33	5.83	53
Fluctuating Immediate C18 - Fluctuating Overnight C18	**-9.59**	**-15.29**	**-3.45**	**100**
Fluctuating Immediate C18 - Fluctuating Overnight Sham	**-5.33**	**-11.28**	**0.55**	**97**
Fluctuating Immediate C18 - Elevated 30min Sham	-0.74	-6.61	4.71	60
Fluctuating Immediate C18 - Elevated Immediate C18	0.64	-4.98	6.41	59
Fluctuating Immediate C18 - Elevated Immediate Sham	-0.66	-6.52	5.03	59
Fluctuating Immediate C18 - Elevated Overnight C18	**-21.30**	**-27.13**	**-15.90**	**100**
Fluctuating Immediate C18 - Elevated Overnight Sham	**-13.30**	**-18.82**	**-7.48**	**100**
Fluctuating Immediate Sham - Ambient Overnight Sham	**-11.10**	**-16.82**	**-5.55**	**100**
Fluctuating Immediate Sham - Fluctuating Overnight Sham	**-5.51**	**-11.63**	**0.15**	**97**
Fluctuating Immediate Sham - Elevated Immediate Sham	-0.85	-6.28	4.65	62
Fluctuating Immediate Sham - Elevated Overnight Sham	**-13.40**	**-19.09**	**-7.94**	**100**
Fluctuating Overnight C18 - Ambient 30min Sham	**8.61**	**2.79**	**14.50**	**100**
Fluctuating Overnight C18 - Ambient Immediate Sham	**11.30**	**5.33**	**17.00**	**100**
Fluctuating Overnight C18 - Ambient Overnight Sham	-1.36	-7.33	4.74	67
Fluctuating Overnight C18 - Fluctuating 30min Sham	**9.32**	**3.43**	**15.10**	**100**
Fluctuating Overnight C18 - Fluctuating Immediate Sham	**9.86**	**3.63**	**15.50**	**100**
Fluctuating Overnight C18 - Fluctuating Overnight Sham	4.28	-1.88	10.20	91
Fluctuating Overnight C18 - Elevated 30min Sham	**8.83**	**3.00**	**14.90**	**100**
Fluctuating Overnight C18 - Elevated Immediate Sham	**8.97**	**3.16**	**15.20**	**100**
Fluctuating Overnight C18 - Elevated Overnight C18	**-11.70**	**-17.68**	**-5.84**	**100**
Fluctuating Overnight C18 - Elevated Overnight Sham	-3.65	-9.86	1.96	88

Fluctuating Overnight Sham - Elevated Overnight Sham	**-7.87**	**-13.91**	**-1.87**	**99**
Elevated 30min C18 - Ambient 30min Sham	0.65	-4.83	6.05	59
Elevated 30min C18 - Ambient Immediate C18	2.23	-3.21	8.16	79
Elevated 30min C18 - Ambient Immediate Sham	3.27	-2.16	8.79	89
Elevated 30min C18 - Ambient Overnight C18	**-8.36**	**-14.36**	**-2.65**	**100**
Elevated 30min C18 - Ambient Overnight Sham	**-9.23**	**-14.93**	**-3.70**	**100**
Elevated 30min C18 - Fluctuating 30min Sham	1.44	-4.17	6.85	70
Elevated 30min C18 - Fluctuating Immediate C18	1.65	-3.80	7.37	73
Elevated 30min C18 - Fluctuating Immediate Sham	1.86	-3.70	7.22	75
Elevated 30min C18 - Fluctuating Overnight C18	**-7.93**	**-13.52**	**-1.77**	**100**
Elevated 30min C18 - Fluctuating Overnight Sham	-3.62	-10.06	1.57	90
Elevated 30min C18 - Elevated 30min Sham	0.92	-4.57	6.65	63
Elevated 30min C18 - Elevated Immediate C18	2.38	-3.20	7.99	79
Elevated 30min C18 - Elevated Immediate Sham	1.05	-4.67	6.40	64
Elevated 30min C18 - Elevated Overnight C18	**-19.60**	**-25.20**	**-14.20**	**100**
Elevated 30min C18 - Elevated Overnight Sham	**-11.50**	**-17.22**	**-6.15**	**100**
Elevated 30min Sham - Ambient Immediate Sham	2.40	-3.51	8.05	80
Elevated 30min Sham - Ambient Overnight Sham	**-10.20**	**-15.98**	**-4.53**	**100**
Elevated 30min Sham - Fluctuating Immediate Sham	1.00	-4.75	6.44	64
Elevated 30min Sham - Fluctuating Overnight Sham	-4.54	-10.68	1.36	93
Elevated 30min Sham - Elevated Immediate Sham	0.10	-5.52	5.69	51
Elevated 30min Sham - Elevated Overnight Sham	**-12.50**	**-17.91**	**-6.62**	**100**
Elevated Immediate C18 - Ambient 30min Sham	-1.65	-7.34	3.80	72
Elevated Immediate C18 - Ambient Immediate Sham	1.01	-4.70	6.53	64
Elevated Immediate C18 - Ambient Overnight C18	**-10.70**	**-16.82**	**-4.74**	**100**
Elevated Immediate C18 - Ambient Overnight Sham	**-11.60**	**-17.30**	**-5.65**	**100**
Elevated Immediate C18 - Fluctuating 30min Sham	-0.87	-6.88	4.46	62
Elevated Immediate C18 - Fluctuating Immediate Sham	-0.47	-6.10	5.24	56
Elevated Immediate C18 - Fluctuating Overnight C18	**-10.30**	**-16.01**	**-4.28**	**100**
Elevated Immediate C18 - Fluctuating Overnight Sham	**-5.96**	**-12.01**	**0.08**	**97**
Elevated Immediate C18 - Elevated 30min Sham	-1.42	-7.00	4.32	69
Elevated Immediate C18 - Elevated Immediate Sham	-1.34	-7.03	4.53	68
Elevated Immediate C18 - Elevated Overnight C18	**-21.90**	**-27.67**	**-16.20**	**100**
Elevated Immediate C18 - Elevated Overnight Sham	**-13.90**	**-19.74**	**-8.39**	**100**
Elevated Immediate Sham - Ambient Overnight Sham	**-10.30**	**-15.65**	**-4.26**	**100**
Elevated Immediate Sham - Fluctuating Overnight Sham	-4.71	-10.51	1.64	94
Elevated Immediate Sham - Elevated Overnight Sham	**-12.60**	**-18.13**	**-6.97**	**100**
Elevated Overnight C18 - Ambient 30min Sham	**20.30**	**14.77**	**25.90**	**100**
Elevated Overnight C18 - Ambient Immediate Sham	**22.90**	**17.56**	**28.50**	**100**
Elevated Overnight C18 - Ambient Overnight Sham	**10.30**	**4.48**	**15.80**	**100**
Elevated Overnight C18 - Fluctuating 30min Sham	**21.00**	**15.57**	**26.60**	**100**
Elevated Overnight C18 - Fluctuating Immediate Sham	**21.50**	**15.98**	**27.00**	**100**
Elevated Overnight C18 - Fluctuating Overnight Sham	**15.90**	**10.21**	**21.90**	**100**
Elevated Overnight C18 - Elevated 30min Sham	**20.50**	**14.88**	**26.40**	**100**
Elevated Overnight C18 - Elevated Immediate Sham	**20.60**	**14.91**	**26.30**	**100**
Elevated Overnight C18 - Elevated Overnight Sham	**8.02**	**2.35**	**13.40**	**100**