CLIMATE CHANGE IN A STABLE THERMAL ENVIRONMENT: EFFECTS ON THE PERFORMANCE AND LIFE HISTORY OF CORAL REEF FISH

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Declaration of Ethics

The research presented in this thesis was conducted in accordance with the National Health and Medical Research Council (NHMRC) Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 8th Edition (2013) and the Queensland Animal Care and Protection Act (2001). The research received and was conducted under the animal ethics approval from the James Cook University Animal Ethics Committee, approval numbers #A1737, #A2000 and #A2055.

Statement of the Contribution of Others

This thesis includes collaborative work conducted with my thesis supervisors Prof. Mark McCormick, Prof. Philip Munday, Dr. Jennifer Donelson and Dr. Jodie Rummer, as well as A/Prof. Linda Johnson, Dr. Timothy Clark and Mr. Pasang Tenzing. During these collaborations, I was responsible for research concept and design, data collection, analysis, interpretation of results and manuscript construction. My co-authors provided intellectual guidance, editorial assistance, financial support and technical assistance.

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General Abstract

The effects of climate change induced ocean warming are expected to be felt most strongly by species living at low-latitudes due to the thermally stable environments that characterise these locations. This thesis examined how near-equatorial populations of coral reef fishes, particularly *A. polyacanthus*, are likely to respond to increases in ocean temperatures projected to occur by the end of this century. The underlying physiological measures linked to declines in organism fitness at higher temperatures were considered, and the capacity for acclimation of these traits assessed. Whether certain populations of coral reef fishes are more or less vulnerable to projected climate change scenarios was also investigated by comparing results with those obtained in previous studies conducted at higher latitudes.

Initially I sought to understand how chronic exposure to temperatures expected to occur with climate change are likely to impact on the survival and metabolic performance of a low-latitude population of marine damselfish (*Acanthochromis polyacanthus*), and subsequently estimate their potential for long-term reversible acclimation to higher temperatures (**Chapter 2**). To do this, I assigned fish to one of three treatments: (1) current average ocean temperatures for the collection locations, seasonally cycling, (2) 1.5 °C higher than current average temperatures or (3) 3 °C higher than current average temperatures. Fish were kept in these treatments for approximately 10 months. Routine and maximum oxygen consumption were then directly measured and subsequently used to estimate net aerobic scope for each fish during both the summer and winter. Critical thermal maximum was also estimated during summer and fish survival was monitored both before and after metabolic testing. For these low-latitude populations of coral reef fish performance in terms of aerobic scope was maintained up to 31.5 ˚C, the maximum ocean temperature routinely experienced by this population. Once temperatures exceeded 31.5 ˚C, aerobic scope dropped significantly, as did fish survival. Survival was further decreased at much lower temperatures after the introduction of a secondary stressor (metabolic testing). As predicted, potential for reversible acclimation was limited, even after extended periods at elevated temperatures.

The first data chapter of this thesis confirmed that increased temperatures are likely to significantly affect the physiological performance of low-latitude coral reef fish. Studies which consider only a single measure of fitness such as aerobic scope when estimating the impacts of thermal stress on organismal health may however not obtain the best estimate of how an organism will cope under future conditions. In **chapter 3** I aimed to expand the range of physiological estimators of fitness used to determine the effect of elevated temperatures on low-latitude reef fish. This chapter compared the impacts of increased ocean temperatures on aerobic scope, haematological parameters and tissue health for Torres Strait *A. polyacanthus*. Haematological parameters and tissue health were analysed during the summer months and compared with the measures of aerobic scope taken in the previous chapter. Haematological traits, similarly to aerobic scope, suggested an impact on oxygen transport in fish at +3 °C, however a negative impact of temperature on tissue health was observed in fish from both +1.5 and +3 °C treatments. Aneurismal dilations in the gill tissue were larger and more numerous in fish from the warmer temperatures. Findings from **chapter 3** suggest multiple thermal optima, depending on the physiological trait concerned. Of the measures considered in this chapter, gill histopathology provided the best indicator of thermal tolerance, as it was the first measure to show of a decline in organism health and corresponded with mortality observations from **chapter 2**.

Previous research shows that increased temperatures may also effect *A. polyacanthus* in the future by producing populations of offspring with a strongly male biased sex ratio. This could have a significant negative impact by reducing the functional reproductive population. For a shift in sex ratio to occur, juveniles must be exposed to increased temperatures during a specific time during development, known as the thermosensitive period (TSP). In **chapter 4**, I aimed to determine the TSP for *A. polyacanthus* and subsequently predict when projected temperature increases are likely to have the greatest effect on sex ratio for this species. For this chapter only we used fish collected from a higher latitude, and so temperature treatments were adjusted accordingly. To determine the thermosensitive period of sex determination under climate change relevant temperatures for this species, a single-shift design was used to expose juvenile fish to elevated water temperatures (+1.5 and +3 ˚C) at various stages of development.

Increasing grow-out temperature to 1.5 ˚C had no effect on the sex ratio of offspring, however an increase to 3 ˚C above average produced a strong male bias (average ~90%). The thermosensitive period was up to 60 days post hatching, with the bias in sex ratio greater for fish that were exposed to higher temperatures earlier in life. Average summer temperatures will need to increase by ~3˚C before an effect of temperature on sex ratio is seen for this population. Temperatures high enough to drive a bias in sex ratio are likely to be first seen during January and February and would have the greatest effect on clutches produced late in the breeding season.

Based on previous chapters it was determined that the ability to acclimate to higher temperatures would be critical for the persistence of low-latitude populations in a warming world. In **chapter 5** I test the capacity for three low-latitude coral reef damselfishes (*Acanthochromis polyacanthus*, *Pomacentrus moluccensis* and *Pomacentrus wardi*), to developmentally acclimate to ocean temperatures expected to occur by the end of this century, again using metabolic fitness as an estimate of organism health. Newly settled juveniles were collected from reef locations in Torres Strait and reared for 3 months in three different temperature treatments, this time consisting of the current-day summer average (30 ˚C) and 1 and 2 ˚C above the average (31 and 32 ˚C). As with **chapter 2**, aerobic scope was estimated, this time for each fish at both their developmental temperature and at the two remaining treatments after acute exposure. Acclimation capacity differed among species and occurred at similar absolute temperatures but lower relative temperatures than for the same species at higher latitudes. There was some scope to deal with climate change relevant temperature increases in these populations, however low-latitude populations are still more vulnerable to temperature increases than their more southern counterparts. Life history and habitat choice of each species appeared to have a strong influence on their capacity for developmental acclimation, leading to the conclusion that scientists should consider the ecological niche of their study species and take care when making generalisations about the effects of climate change, even on closely related species.

Overall, results from this thesis showed that despite substantial physiological effects of climate change, some near equatorial populations of coral reef fish do have a capacity to acclimate to higher temperatures when exposed to warmer conditions during early

development. In the future, multi-generational research that investigates the capacity for transgenerational plasticity may reveal a further ability to acclimate in these populations, beyond what has been shown in this thesis for developmental acclimation. The importance of understanding the life history of model species was also highlighted, as it was shown that a species' capacity for acclimation is likely to be strongly effected by their ecological niche. This thesis represents the most comprehensive investigation into the effects of projected climate change on low-latitude coral reef fish to date and is the first to examine the acclimation capacity of fish at these latitudes. Although acclimation was observed in low-latitude populations, near-equatorial fish were still more vulnerable to increased temperatures when compared with higher latitude populations. Based on the findings of this thesis, low-latitude populations should be considered a high priority for research and management into the future.

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Chapter 1: General Introduction

Anthropogenic warming of the earth's climate caused by the increased release of carbon dioxide into the atmosphere has been unequivocally demonstrated through rigorous scientific testing (Stocker et al. 2013). Temperatures at the Earth's surface have been progressively warmer for each of the last three decades, and all three have been warmer than any preceding decade since 1850 (Stocker et al. 2013). Global climate change will have many serious implications and is now one of the biggest threats to marine and terrestrial biodiversity, with substantial socioeconomic and political repercussions predicted (Thuiller 2007). Approximately 90% of the total energy accumulated in our climate system due to global warming is stored within our oceans, 60% of this within the upper 700 m (Stocker et al. 2013). In Australia, the rate of sea surface warming is comparable to the current global average, which is at present tracking with models for the worst case (RCP 8.5) scenario, under which emissions continue to increase (Lough 2009; Stocker et al. 2013). If greenhouse gas emissions continue at their current rate, sea surface temperatures (SSTs) in Australia are projected to increase by an average of 4°C by the year 2100 (Stocker et al. 2013).

Tropical oceans are warming at approximately 70% of the global average rate (Lough 2012), and coral reef ecosystems, particularly at lower latitudes, may be significantly impacted by the large changes in SSTs that are occurring with climate change. This is because low-latitude coral reefs naturally experience a narrow range of temperatures, which most often spans <4°C over all seasons (Janzen 1967; Deutsch et al. 2008; Tewksbury et al. 2008; Lough 2012). In contrast, high latitude habitats may experience temperature variations spanning at least 7°C (Crabbe 2008; Lough 2012). In addition, the trajectory of change in tropical regions is extremely high when compared with other regions (Burrows et al. 2011). Elevated temperatures on top of an already narrow seasonal temperature range for tropical waters in the northern regions of Australia may be detrimental for some coral reef organisms and could result in extinction or geographic redistribution of species to more forgiving thermal environments.

Ectotherms and temperature

Ectotherms in particular may be strongly effected by a changing climate as they have very little capacity for somatic temperature regulation and rely primarily on behavioural adjustments for controlling their internal temperature (Cowles & Bogert 1944). An ectotherm's response to temperature can be represented graphically in a performance curve, also known as a thermal reaction norm, which appears as a left skewed normal curve (Tewksbury et al. 2008). An organism's optimal temperature (in terms of fitness) lies at the peak of this curve, and the width of the curve indicates the species thermal range. Studies have suggested that many tropical organisms may be already operating at or above their thermal optimum under current day conditions (Tewksbury et al. 2008; Nilsson et al. 2009).

Regardless of their lack of capacity for internal temperature regulation, many ectotherms occupy large geographic ranges and consequently also experience wide thermal ranges. Ectotherms can evolve various performance curves which could allow them to exist over this wide temperature profile. Populations throughout the range may be made up of locally adapted thermal specialists, with relatively narrow performance curves that have developed to perform best only within a limited range of temperatures specific to their location (Angilletta 2009; Kellermann et al. 2012). Alternatively the species may be a thermal generalist, with a wide thermal performance curve allowing individuals throughout the range to function over a wide array of temperatures (Huey & Hertz 1984; Angilletta 2009; Kellermann et al. 2012). Evolutionary costs and benefits exist for each of these strategies, and this will influence which strategy is selected for (Angilletta et al. 2003).

The thermal reaction norm of species inhabiting tropical environments is typically narrower than that of temperate species, reflecting the narrow temperature range normally experienced in these regions and suggesting local adaptation (Deutsch et al. 2008; Tewksbury et al. 2008; Sunday et al. 2011). If a species is locally adapted, a warming climate could mean that these species will be unable to function normally in the future, and could result in changed seasonal behaviours, disruption of ecological

interactions, changes in abundance, geographic redistribution, or local extinction (Walther et al. 2002; Parmesan & Yohe 2003; Chen et al. 2011).

Climate change and coral reef fish

Changes in SST associated with climate change will have serious implications for coral reef fishes (Munday et al. 2008a). The destruction of important complex coral habitats and food sources through processes such as coral bleaching is likely to have a devastating effect on coral reef ecosystems through declines in fish abundance and physiological condition (Jones et al. 2004; Munday et al. 2004; Pratchett et al. 2004; Pratchett et al. 2006; Pratchett et al. 2008). In addition to this, increased ocean temperatures will also have a more direct affect; many marine organisms are ectothermic, including coral reef fish, and are consequently at a high risk of being significantly affected by increased temperatures associated with climate change, as explained above (Pankhurst & Munday 2011; Munday et al. 2012; Nilsson et al. 2009; Nilsson et al. 2010; Johansen & Jones 2011). Previous literature shows that elevated sea temperatures may influence the reproductive output, sex ratio, growth rates and physiological performance of coral reef fishes (Munday et al. 2008b; Donelson et al. 2010; Johansen & Jones 2011; Donelson et al. 2012; Donelson & Munday 2015). Growth rates at all life stages may decline with increasing temperature (Munday et al. 2008b). Other physiological processes such as aerobic scope, hypoxia tolerance and swimming speed have all been shown to be significantly reduced at higher temperatures for a range of species (Nilsson et al. 2009; Nilsson et al. 2010; Johansen & Jones 2011).

Reproductive output is fundamentally linked to persistence and in coral reef fish has been shown to decline at higher temperatures, ceasing altogether if experienced in combination with further stressors such as a reduction in food availability (Donelson et al. 2008). When fish do reproduce at higher temperatures, a strong gender bias, often towards the production of males, has been observed (Janzen 1994; Hawkes et al. 2007; Laloë et al. 2014; Donelson & Munday 2015). Environmental sex determination driven by increased temperatures associated with climate change could create a gender bias away from a population's optimal sex ratio, resulting in a reduction in effective breeding populations (Milner-Gulland et al. 2003; Wright et al. 2012).

Importance of low-latitude populations

Despite the well described effects of increased ocean temperatures on higher latitude tropical reef species, we have only a limited understanding of how low-latitude, or near equatorial populations may tolerate the higher water temperatures projected to occur over the next 100 years. The majority of studies investigating the effects of increased temperatures on coral reef fish have been undertaken on fishes from the middle and southern sections of Australia's Great Barrier Reef (GBR; ~18˚ latitude and greater). A potential issue with this is that, as previously mentioned, many ectotherms including some fish species span large geographical ranges (Roberts et al. 2002; Munday et al. 2008a) and would therefore naturally experience different local environmental conditions, specifically differences in average temperature, daily variation and the annual range of temperatures (i.e. temperature variation). As an example, southern sections of the GBR experience lower average temperatures and greater temperature variation in comparison to the northern GBR (JCU/AIMS weather station). These regional differences are important to scientists because populations may be adapted to their local conditions (Sanford & Kelly 2011) and this may limit generalized predictions related to the impacts of climate change on marine organisms.

Populations existing close to the equator may be more vulnerable to the elevated temperatures associated with climate change than populations from higher tropical latitudes (Nguyen et al. 2011; Bowden et al. 2014; Rummer et al. 2014a). Only two studies have been conducted to date which examine the thermal sensitivity of low-latitude coral reef fish (Bowden et al. 2014; Rummer et al. 2014a). Rummer et al. (2014a) examined the metabolic capacity of several species of coral reef damselfish from reefs in Papua New Guinea and confirmed that many of the species were already living close to or above their thermal optima, with significant reductions in aerobic health for all species at increased temperatures. Bowden et al. (2014) examined the capacity for fish in the same population to make physiological adjustments to the gills in order to increase their capacity for oxygen uptake, however the study found no capacity for gill remodelling in fish exposed to higher temperatures. Although both of these studies suggest significant consequences of increased temperature on the performance of coral reef fish, the duration of exposure in each case was only 14 days.

No studies have tested the long-term effects of projected future temperatures on low-latitude populations. This is critical as when exposed to temperatures outside of their thermal range for a prolonged period of time, some organisms have the capacity to adjust to the changed environment through phenotypic plasticity (Angilletta 2009; Munday et al. 2013; Seebacher et al. 2015). For this reason it is essential that longer-term studies are conducted in order to obtain a more reliable prediction of the impact of increased temperatures on low-latitude species.

Coping in a warming world

If low-latitude species are in fact as vulnerable to increased temperatures as is predicted by theory and the current literature, it will be critical that these populations can utilise coping mechanisms for dealing with the warmer ocean temperatures associated with projected climate change. Aside from geographic re-distribution, the two primary mechanisms which may allow organisms to persist are adaptation and acclimation (Angilletta 2009; Munday et al. 2012; Sunday et al. 2014). Acclimation and adaptation occur when a phenotype is modified such that the organism can prosper in the altered environment. This can occur through the selective loss of unfavourable genotypes over multiple generations (genetic adaptation), or through non-genetic modifications (acclimation). Non-genetic modifications involve changes to the expression of phenotypic plasticity on physiological, behavioural or morphological traits (Sunday et al. 2014) and may be expressed in a variety of ways. For example, changes could occur within a generation (reversible and developmental acclimation; Angilletta 2009), or between generations (trans-generational acclimation), also known as non-genetic heritability (Bonduriansky & Day 2009). Reversible acclimation involves short-term, regulated responses to environmental variation, often within a life stage, and is commonly associated with a response to diel or seasonal change by species that live in heterogeneous environments (Angilletta 2009; Sunday et al. 2014). Developmental acclimation involves an irreversible response to a stimulus (for example temperature) experienced during ontogeny (Angilletta 2009). In this type of acclimation, an organism responds to a cue during one stage of its life cycle in order to enhance its performance in another (Donelson et al. 2011a; Scott & Johnston 2012). When parent organisms

influence the phenotype of their offspring by non-genetic means, it is known as trans-generational acclimation (Donelson et al. 2012; Miller et al. 2012; Sunday et al. 2014).

The capacity for acclimation of model organisms has become a topic of particular interest in climate change research, as this type of coping mechanism does not rely on the passing on of genes from one generation to the next and so changes can occur over time scales relevant to climate change (Bonduriansky et al. 2011). Traditionally, the majority of literature in this field has focussed on plants and insects (Bonduriansky & Day 2009); however there have been some more recent studies that have investigated coral reef fishes (Donelson et al. 2011a, b; Donelson et al. 2012). These have provided evidence for both developmental and trans-generational acclimation to climate change in central and southern GBR populations of the coral reef damselfish *Acanthochromis polyacanthus* (*Pomacentridae*). Despite this observed acclimation, fish reared at above average temperatures still suffered reduced reproductive fitness and decreases in body condition, indicating that although the fish appeared to have some ability to cope with changes in temperature, the consequences of global warming for these species could still be detrimental and that the ability to acclimate may come at a cost (Donelson et al. 2011a). Acclimation to rapid environmental change can act variably on genetic adaptation, potentially influencing the direction of genetic change or the speed of its progress (Ghalambor et al. 2007; Chevin et al. 2010; Day & Bonduriansky 2011). Work is yet to be carried out to investigate acclimation ability at lower latitudes.

Measuring organism fitness

In order to assess organism health in response to a changing environment, one or more measures of fitness must be analysed. One of the most common methods used in the recent climate change literature is to examine aerobic scope over a range of temperatures (Farrell et al. 2008; Nilsson et al. 2009; Eliason et al. 2011). Total aerobic scope provides an estimate of the energy, after accounting for basic maintenance of the organism, which is available for life-history and ecologically-relevant activities such as finding food, reproduction and predator evasion, activities that are also crucial to the overall fitness of an organism (Pörtner & Knust 2007). Studies show that at both low and high temperatures, restriction of whole-animal aerobic scope may be the first

symptom of problems that develop due to an insufficient uptake, transport, and delivery of oxygen (Pörtner 2001; Pörtner & Knust 2007). The efficiency of this system declines at temperatures above an organism's thermal optimum, whilst the solubility of oxygen in water also decreases at elevated temperatures (Emsley 2001). This hypothesis is known as oxygen- and capacity-limited thermal tolerance (OCLTT), and according to this hypothesis, other measures that determine the fitness of an organism are causally linked to aerobic capacity (Pörtner 2001; Pörtner & Knust 2007).

Over the past several decades, the Pacific salmon have become a popular model fish family for studying aerobic scope. Aerobic scope is tightly linked to the range of temperatures to which each salmon population is adapted (Farrell et al. 2008; Eliason et al. 2011). When higher temperatures constrain aerobic scope, in some cases, this may be enough to prevent successful migration, subsequently compromising future generations of the species (Farrell et al. 2008). Thus a strong connection exists between the aerobic scope and lifetime fitness of these fish. Indeed, temperate species have been well studied in terms of aerobic scope, but there has been a recent increase in the number of tropical species investigated as well (Nilsson et al. 2009; Donelson et al. 2011a). For example, some damselfish and cardinalfish species have also been found to exhibit a strong effect of temperature on aerobic scope (Nilsson et al. 2009; Gardiner et al. 2010; Donelson et al. 2011a; Rummer et al. 2014). This research on tropical species is important because as mentioned above, elevated ocean temperatures may be particularly problematic for these lower latitude tropical populations due to their narrow thermal tolerance range.

Despite its wide application, there are some issues in using aerobic scope as a predictor of energy availability. Whole-organism oxygen consumption is the practical measure traditionally used as a proxy for metabolic rate (Brown et al. 2004). Unfortunately, this indirect method of estimating adenosine triphosphate (ATP) production is likely to provide only a partial representation of energy metabolism (Brand 2005; Salin et al. 2015; Schulte 2015). Oxygen consumption can provide a good indicator of flux through the mitochondrial electron transport chain, however it is a less reliable indicator of the amount of ATP produced per unit of oxygen consumed, as this conversion can vary significantly dependant on a range of individual and environmental factors (Schulte

2015). Variable temperature conditions can have a strong influence on the production of ATP as elevated temperatures can disrupt the stability (proton leak rate) of mitochondrial membranes, reducing the amount of ATP produced per unit of oxygen consumed (Salin et al. 2015; Schulte 2015). Temperature can also control the cells demand for energy and organisms must balance the efficiency of ATP production with the potential for oxidative stress and the production of reactive oxygen species, which is higher at more efficient levels of oxygen conversion (Salin et al. 2015). The way in which an organism navigates such trade-offs may differ over time as its demand for energy changes with food availability or life history stage (Salin et al. 2015).

Variation in the conversion of oxygen to ATP may require careful consideration when trying to determine exactly what a change to whole-organism oxygen consumption under different environmental conditions means ecologically. Recent studies have begun to investigate this issue from a whole organism perspective and this has led to popular hypotheses such as the OCLTT being called into question (Clark et al. 2011; Clark et al. 2013; Donelson et al. 2014; Gräns et al. 2014; Norin et al. 2014). The pink salmon (*Oncorhynchus gorbuscha*) is an example where life history characteristics are at odds with the OCLTT theory, as this species has a thermal optimum of aerobic scope at 21°C, but cannot reproduce at this temperature and most frequently occupies waters at temperatures of approximately 11°C (Clark et al. 2011). In their 2014 study, Gräns et al. use growth rate of Atlantic halibut (*Hippoglossus hippoglossus*) as a measure of organism fitness to test the OCLTT theory against increasing temperature and CO_2-acidified water. The study found that whilst aerobic scope increased with increasing temperature, and further with CO_2-acidified water, growth did not follow the same trend.

Of course aerobic scope is not the only measure of fitness that can be used to assess thermal sensitivity. Alternative theories suggest that multiple measures of fitness should be taken into consideration when investigating tolerance to a stressor, and that any physiological driver has the potential be a limiting factor on organism health (Hadfield 1966; Bustard 1967; Du et al. 2000; Clark et al. 2013). The best indicator of physical fitness may vary between species, life stage, or thermal challenge (Clark et al. 2013). Alternative measures of organism fitness may range from molecular to whole

organism in scale and could include measures such as those mentioned above (reproductive success, growth rate, hypoxia tolerance and swimming speed).

Calculating oxygen consumption

In this thesis, calculations of aerobic scope have been made by subtracting routine oxygen consumption ($\dot{M}O_{2Routine}$; calculated using static respirometry) from maximum oxygen consumption ($\dot{M}O_{2Max}$; circular swim chamber). $\dot{M}O_{2\ Routine}$ is calculated over a short time period (from a single fall in oxygen within a respirometry chamber), unlike standard metabolic rate (SMR) or minimum metabolic rate ($\dot{M}O_{2\ Min}$), which are typically calculated from multiple measurements of oxygen consumption recorded over 24 hours. This use of $\dot{M}O_{2Routine}$, along with the static respirometry, are methods which deviate from what is usually considered standard practice (Steffensen 1989; Clark et al. 2013). The reason for using unconventional methods in this study is the large number of sample fish tested in **chapter 5**. When respirometry techniques are applied in ecological research, logistical challenges may arise in association with the desire to carry out experiments in field locations or, as was the case in this thesis, to carry out experiments which require a large number of sample sizes or treatments. The number of treatments and species tested in **chapter 5** could not logistically have been carried out using standard protocol. In fact, this has also been the case in much of the previous long-term climate change research carried out on this and similar species (Donelson et al. 2011b; Donelson & Munday 2012; Miller et al. 2012). To maintain consistency throughout the thesis and to obtain comparable data with previous studies, the current methodology was chosen.

To ensure that the results obtained using this methodology were meaningful, extensive pilot tests were carried out prior to experimentation. **Appendix 1** shows that for the primary species considered in this thesis, *Acanthochromis polyacanthus,* static respirometry can provide consistent results in which trends in the data are not distorted by the absence of a mixing device. This is because damselfish are sufficiently active to provide mixing of the respiration chamber. A pilot study which examined the oxygen consumption of fish subsequent to being introduced to the respirometry chambers also showed that oxygen consumption was not significantly different at one hour after

introduction to the chambers than from all successive measurements taken up to seven hours. **Appendix 1** also shows that this species does not exhibit diurnal trends in oxygen consumption. This consistency in measurements of oxygen consumption in both the pilot study and in **Appendix 1** indicate that measurements of $MO_{2\ Routine}$ in this thesis are unlikely to significantly differ from SMR, however because a 24 hour trial was not conducted correct terminology has been used throughout. Methods used in this thesis were consistent across treatments and species, so any effect of the protocol used would not affect trends in the data. The methodology used does limit direct comparison of absolute values across studies which retain standard practices in calculating SMR or $MO_{2\ Min.}$

Study species

The spiny chromis damselfish (*Acanthochromis polyacanthus*) was chosen as a model species for this thesis. The species was chosen as it is common across many reef locations on the GBR, is easily maintained in captivity and has been used in a number of previous studies at central and southern GBR locations against which results from this thesis could be compared.

A. polyacanthus is a brooding tropical damselfish, with complete larval development occurring during embryogenesis (Doherty et al. 1994). This restricts distribution, causing the juvenile and parental environment to be the same, and resulting in genetically distinct sub-populations (Planes et al. 2001). As a result of this it may be expected that *A. polyacanthus* is a good candidate for exhibiting local adaptation. Further to this, the *A. polyacanthus* species is widely distributed throughout the Indo Pacific (15°N–26°S and 116°E–169°E), as far north as the Philippines and Indonesia, through to north-eastern Australia and Melanesia (Randall et al. 1997). This restricted dispersal potential in combination with wide geographical distribution makes *A. polyacanthus* an excellent species for latitudinal comparisons of thermal sensitivity.

Across its range *A. polyacanthus* populations experience a total temperature span (inclusive of season) of approximately 20–31°C. This range is comparable with a large number of other coral reef fish species (Munday et al. 2008a). In the final chapter of this thesis two further common damselfish species are also considered for comparison;

these are the lemon damselfish (*Pomacentrus moluccensis*) and Ward's damselfish (*Pomacentrus wardi*). These species have largely overlapping distributions with *A. polyacanthus*.

Thesis aims and objectives

This thesis examines how near-equatorial populations of coral reef fish, particularly *A. polyacanthus*, may respond to the increases in ocean temperatures projected to occur with climate change. Furthermore, this study explores whether or not certain populations of coral reef fish are more/less vulnerable to projected climate change scenarios by providing data that is comparable to previous studies conducted at higher latitudes. This thesis not only examines the effects of elevated temperature in an ecological sense, but also considers underlying physiological performance. Conservation physiology is emerging as an important aspect of environmental research as it provides a mechanistic link between changes seen in the environment and the ecological patterns or behaviours of organisms (Hofmann & Todgham 2010; Seebacher & Franklin 2012). Lastly, the capacity for low-latitude populations to acclimate to projected future warming is also examined.

In **Chapter 2**, *A. polyacanthus* collected from Torres Strait (142°20' to 142°35' and 10°31' to 10°46') were used to test the long-term response of a low-latitude population of a coral reef fish to projected end-of-century increases in water temperature. I subsequently tested the hypothesis that this low-latitude population should have a low capacity for reversible acclimation by comparing the performance of fish exposed to higher temperatures for long time periods to those only exposed to higher temperatures for one week. The effects of elevated temperature on oxygen consumption were considered, as a surrogate for predicting the ability of fish to perform ecologically relevant aerobic activities. The aerobic capacity of fish across three temperature treatments was compared, consisting of the current-day average (seasonally cycling) and up to 3 ˚C above the average, as well as between summer and winter seasons. Survival of fish at each of the temperature treatments both before and after testing was also considered. Both long-term and acute exposure to temperature

changes were applied to fish in order to determine the capacity for reversible acclimation to higher temperatures.

After establishing the long-term thermal sensitivity of *A. polyacanthus* from these near-equatorial locations, a closer examination of the physiological predictors of organism fitness was undertaken in **chapters 3 and 4**. In **chapter 3** I compare the effect of the same three temperature treatments that were used in **chapter 2** on a range of physiological measures, in order to test the hypothesis that there would be some variation in response to increased temperatures among physiological traits. Whole animal metabolic performance, haematological parameters, and tissue health were considered. By comparing thermal performance for each trait, I was able to determine if a single physiological measure could provide sufficient information to indicate the thermal threshold of the whole organism or if a multiple trait approach is required.

The aim of **chapter 4** was to determine the period during early development when environmental temperature may influence sex determination of *A. polyacanthus*. From here I was able to make predictions on how projected temperature increases may affect breeding populations of this species into the future. Only for this chapter did we use fish collected from the central GBR, and so temperature treatments were adjusted accordingly.

Finally, in **chapter 5** the potential for developmental acclimation of three low-latitude coral reef fish species (*Acanthochromis polyacanthus*, *Pomacentrus moluccensis* and *Pomacentrus wardi*) to temperatures projected to occur by the end of this century was tested, at the current-day summer average (30 ˚C) and up to 2 ˚C above the average. Analysis of aerobic scope in three common damselfish species collected from Torres Strait was used to determine fitness after three months of developmental exposure to higher temperatures. Aerobic performance was then compared with fish only acutely exposed to higher temperatures at the end of the experimental period (i.e. those given no opportunity for acclimation). Metabolic performance was compared between developmental groups and between species. It was hypothesised that metabolic performance would be significantly improved in fish that developed at higher

temperatures, compared with the acutely exposed groups, due developmental acclimation in the groups that were raised at higher temperatures.

Research within this thesis greatly expands our knowledge of the long-term physiological effects of climate change on low-latitude populations of coral reef fishes. In addition, it begins to provide us with an understanding of the acclimation potential of these populations and how this potential may differ between species. This type of information is critical for the realistic management of reef resources in a warming world.

Chapter 2: Ocean warming affects aerobic performance and survival of a low-latitude coral reef fish.

This chapter was prepared for submission to *Oikos*

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2.1 Summary

Tropical species are predicted to be particularly vulnerable to the impacts of climate change given the relatively narrow thermal range they naturally experience. Within the tropics the average temperature and thermal variation experienced can differ among populations and consequently low-latitude populations may respond differently to increased temperatures than higher latitude tropical populations. In this study, we investigate the long term effects of climate change relevant temperature increases on the survival and metabolic performance of a low-latitude population of marine damselfish (*Acanthochromis polyacanthus*). We also consider their potential for reversible acclimation by comparing the metabolic performance of fish that were only acutely exposed to higher temperatures with those that were allowed up to 10 months to acclimate. Adult fish were randomly assigned to one of three treatments: (1) current average ocean temperatures for the collection locations, (2) 1.5°C higher than current average temperatures or (3) 3°C higher than current average temperatures. Treatments were maintained for approximately 10 months. Aerobic scope was measured during both summer and winter, and critical thermal maximum was estimated during summer. Fish survival was recorded before and after metabolic testing. Low-latitude *A. polyacanthus* maintained aerobic performance up to 31.5˚C, which is the maximum ocean temperature that fish at this location routinely experience. Above this temperature aerobic scope dropped significantly, as did fish survival. When a secondary stressor (maximal exercise during metabolic testing) was introduced survival was significantly diminished at much lower temperatures. Potential for reversible acclimation was limited, even after 10 months at elevated temperatures. Our results

are consistent with the hypothesis that low-latitude species are already living close to their thermal maximum and appear to have limited acclimation capacity within a generation.

2.2 Introduction

Tropical species are expected to be especially sensitive to projected future environmental warming, because they have evolved in a relatively stable thermal environment which now faces a very high trajectory for temperature change when compared with other regions (Janzen 1967; Deutsch et al. 2008; Tewksbury et al. 2008, Burrows et al. 2011). An organism's performance is often plotted as a function of temperature in what is commonly known as a thermal reaction norm (Angilletta 2009). An individual's optimal temperature lies within this curve and the width of the curve indicates the organism's thermal range. The thermal reaction norm of species from tropical environments is generally observed to be narrower than for temperate species (Deutsch et al. 2008; Tewksbury et al. 2008; Sunday et al. 2011). Furthermore, some tropical organisms appear to live at the edge of their thermal tolerance, even at present day temperatures (Stillman 2003; Kellermann et al. 2012; Rummer et al. 2014a), and may be unable to cope with even small increases in temperature. It is for these reasons that tropical species are predicted to be particularly vulnerable to the effects of climate change (Deutsch et al. 2008). However, a range of thermal environments exist within the tropics, and consequently, the sensitivity of populations is likely to vary throughout tropical regions. Lower latitude and equatorial populations could be more sensitive to elevated temperatures associated with climate change than populations from higher tropical latitudes (Nguyen et al. 2011; Bowden et al. 2014; Rummer et al. 2014a). Despite these predictions, the ability of equatorial and low-latitude populations of marine species to respond to ocean warming is largely unknown. The few studies conducted to date suggest serious consequences for the survival, physiology, and performance of low-latitude organisms; however none have tested the potential for acclimation to long-term exposure to environmental warming, with most studies lasting less than 14 days. When exposed to a change in temperature over a prolonged period of time, some organisms have the capacity to adjust to the changed environment through phenotypic plasticity (Angilletta 2009; Munday et al. 2013; Seebacher et al.

2015). Consequently, longer-term studies are required to predict the impact of increased temperatures on tropical species.

Ectotherms rely primarily on behavioural adjustments for temperature regulation, as their capacity for endogenous temperature regulation is extremely limited (Cowles & Bogert 1944). Despite this lack of ability to regulate their internal temperatures, many ectotherms have large geographic ranges that also span wide temperature ranges. Ectotherms may engage one of two strategies to function over a wide thermal gradient. Populations throughout the range may be locally adapted and composed of thermal specialists who perform best within a narrow range of temperatures. Alternatively the species may be a thermal generalist, with individuals throughout the range adapted to function over a wide range of temperatures (Huey & Hertz 1984; Angilletta 2009; Kellermann et al. 2012). Evolutionary costs and benefits exist for each of these two strategies, and these will influence which strategy is favoured. In populations where there is a strong genotype x environment interaction, in combination with limited gene flow between populations, local adaptation is likely to occur (Kawecki & Ebert 2004). It can be challenging in longer lived organisms to differentiate between local adaptation and phenotypic plasticity because of the time required to conduct the appropriate experiments (Merilä & Hendry 2014). Nevertheless, recent studies provide evidence for local adaptation in species with complex life history strategies, whilst accounting for the effects of plasticity (Kuo & Sanford 2009; Phillimore et al. 2010; Crozier and Hutchings 2014; Muir et al. 2014; Fitzpatrick et al. 2015). If a species is locally adapted and therefore has a thermal range which is particularly specialised, a warming climate may mean that it will be unable to function normally in the future, resulting in altered seasonal behaviours, disruption of ecological interactions, changes in abundance, geographic redistribution, or even extinction (Walther et al. 2002; Parmesan & Yohe 2003; Chen et al. 2011).

Species living in habitats with a narrow thermal range are predicted to have less capacity for thermal acclimation than species that live in habitats with a large thermal range (Stillman 2003; Tewksbury et al. 2008). Consistent with this hypothesis, some polar fishes that experience a narrow temperature range appear to have limited capacity for thermal acclimation (Hop & Graham 1995; Van Dijk et al. 1999; Pörtner

2001; Steffensen 2002). However, other polar fishes have demonstrated more capacity for thermal acclimation than might be predicted (Seebacher et al. 2005; Podrabsky & Somero 2006; Franklin et al. 2007; Seebacher et al. 2015). The few studies to date that have examined thermal acclimation in tropical marine fishes have observed limited capacity for acclimation (Nilsson et al. 2009; Nilsson et al. 2010; Gardiner et al. 2010; Rummer et al. 2014a). A limitation, however, is that these studies were relatively short-term, conducted over a maximum of 22 days. Longer-term studies are missing, especially for low-latitude populations. While physiological traits may change dramatically when animals are first exposed to thermal stress, they may return to normal levels over the longer term. Consequently, short term studies of thermal stress could produce a strong physiological response that may not be indicative of the long-term consequences of climate change.

Aerobic scope is a physiological measure that provides an estimate of the potential for energetic expenditure of non-maintenance processes such as foraging, reproduction and predator evasion (Pörtner & Knust 2007). For aquatic ectotherms, restriction of whole-animal aerobic scope, due to an insufficient uptake, transport, and delivery of O_2, is one of the first symptoms of a negative effect on organism health associated with temperatures outside the optimum thermal range (Pörtner 2001; Pörtner & Farrell 2008). This hypothesis is known as oxygen- and capacity-limited thermal tolerance (OCLTT), and according to this hypothesis, other measures that determine the fitness of an organism are causally linked to aerobic capacity. The value of this theory is that scientists are able to use a single measure to infer the effects of a stressor on overall organism health. The theory could then be used to make predictions about population level responses to climate change (Pörtner & Knust 2007; Eliason et al. 2011). Alternatively, there may not be a single optimal temperature for organism function, but rather different physiological processes could have different thermal optima (Hadfield 1966; Bustard 1967; Du et al. 2000; Clark et al. 2013). In the multiple optima hypothesis, any physiological driver can be a limiting factor on organism health and functions that best indicate physical fitness may vary between species, life stage, or thermal challenge (Clark et al. 2013). The multiple optima hypothesis has received less attention in recent

literature, perhaps because it is more difficult to investigate than the OCLTT hypothesis given that there are no set testing parameters and multiple traits must be investigated.

Research so far on the thermal sensitivity and acclimation capacity of coral reef fish has focused largely on populations at latitudes greater than ~ 18˚. These studies show that elevated sea temperatures can markedly affect reproductive output, growth rates and physiological performance (Pankhurst & Munday 2011; Munday et al. 2012). Increased temperature has a significant effect on physiological performance measures, including aerobic scope, hypoxia tolerance and swimming speed (Nilsson et al. 2009; Nilsson et al. 2010; Johansen & Jones 2011). Fewer fish breed in elevated summer temperatures, and when increased temperature is combined with other potential effects of climate change such as reduced food availability, reproduction ceases completely (Donelson et al. 2008). Growth in both adult and juvenile fish is also restricted at higher temperatures (Munday et al. 2008b). Despite the well described effects of increased ocean temperatures on higher latitude tropical reef species, we have only a limited understanding of how low-latitude populations may tolerate the higher water temperatures projected to occur over the next 100 years. In the only two studies conducted to date, Rummer et al. (2014a) showed that low-latitude fish populations are living at or above their thermal optimum, whilst Bowden et al. (2014) found no capacity for gill remodelling in fish exposed to higher temperatures. Low-latitude populations exhibited a decreased aerobic scope at higher temperatures and significant mortality was observed for one species at 34˚C. Although the study by Rummer et al. (2014a) shows significant effects of temperature on the performance of coral reef fish, the duration of exposure was only 14 days. No studies have tested the long-term effects of projected future temperatures on low-latitude populations, therefore taking into account capacity for reversible acclimation.

Spiny Chromis damselfish (*Acanthochromis polyacanthus*) from Torres Strait (far northern Great Barrier Reef; GBR) were collected as adults and held in three temperature treatments for 10 months (current average ocean temperatures, +1.5°C and +3°C) to test the long-term response of a low-latitude population of a coral reef fish to projected future increases in water temperature. Both the aerobic capacity and survival of fish maintained at 1.5 and 3°C above current sea surface temperature (SST)

averages were examined. We compared the aerobic capacity of fish across temperature treatments, during both summer and winter. Long-term (chronic) and acute (7 day) temperature changes were applied to fish in order to determine the capacity for reversible acclimation. The additional testing after acute exposure to treatments was designed to establish differences in oxygen consumption due to acute temperature exposure from changes due to possible reversible acclimation. In addition to testing aerobic capacity, CT_{max} was calculated, along with the long term survival of fish both before and after metabolic testing. Comparisons between aerobic capacity and survival provided multiple metrics for determining fish health.

2.3 Methodology

Tropical SSTs are projected to increase up to 3°C by year 2100 if the current CO_2 emissions trajectory is maintained (Stocker et al. 2013). In the current study we tested the long-term (10 month) response of a low-latitude population of a coral reef fish to future increases in water temperature under moderate (+1.5°C) and high (+3°C) warming scenarios. The spiny chromis damselfish (*Acanthochromis polyacanthus*) was chosen as a test species because it is a brooding tropical damselfish, with complete larval development occurring during embryogenesis (Doherty et al. 1994). This restricts distribution, causing the juvenile and parental environment to be the same, and resulting in genetically distinct sub-populations (Planes et al. 2001). As a result of this it may be expected that *A. polyacanthus* is a good candidate for exhibiting local adaptation. *A. polyacanthus* is widely distributed throughout the Indo Pacific (15°N–26°S and 116°E–169°E), as far north as the Philippines and Indonesia, through to north-eastern Australia and Melanesia (Randall et al. 1997). This distribution makes *A. polyacanthus* an excellent species for latitudinal comparisons of thermal sensitivity. Across its range populations experience a total temperature span (inclusive of seasons) of approximately 20–31°C.

The three temperature treatments used in this study included: 1) current average ocean temperatures for the collection locations (+0°C; 25.0°C – 30.0°C, seasonally cycling), 2) 1.5°C higher than current average ocean temperatures (+1.5°C), and 3) 3°C higher than current average ocean temperatures (+3°C), each also seasonally cycling. The maximum

temperature experienced by the +1.5°C treatment (31.5°C) is close to the maximum temperature experienced by wild populations of *A. polyacanthus* at the study location. The highest recorded average daily temperature at the study location over all of the years used to calculate temperatures for this study (May 1998 – March 2010) was 31.34°C (Australian Institute of Marine Science (AIMS) SST database; http://data.aims.gov.au/aimsrtds/datatool.xhtml?site=921&param=water%20temperature). The +1.5°C treatment (31.5°C) is also directly comparable with similar studies conducted with more southern populations of *A. polyacanthus* where the +3°C treatment has a maximum summer temperature of 31.5°C (Donelson et al. 2010; 2011a; 2012; Donelson & Munday 2012). The +3°C treatment temperature has a summer maximum of 33°C, which is greater than the population naturally experiences, but would be the average summer temperature by 2100 under RCP 8.5 where emissions continue at their current rate.

Fish collection and treatment allocation

Adult breeding pairs of *Acanthochromis polyacanthus* were collected from three reefs in the Southern Torres Strait, far Northern GBR during December 2011: Dugong Reef, Twin Cays and Kagar Reef (142°20' to 142°35' and 10°31' to 10°46'). The mean (±SE) mass of *A. polyacanthus* was 24.36 ± 0.45 g, with a maximum size up to 45.33 g. The pairs were transported to James Cook in Townsville, Australia where they were maintained in 60 L tanks in a recirculating system. Each tank contained a shelter site (half of a terracotta pot) and pairs were fed *ad libitum*, one to two times per day using commercial fish pellets (INVE NRD G12). Pairs were maintained at the average water temperature for the collection location (30°C) as determined from the AIMS SST database.

In June 2012, pairs were randomly assigned to one of three temperature treatments (10-15 pairs per treatment), with temperatures adjusted over a 7-day period. Fish were maintained in the treatment temperatures for 10 months to test the chronic effects of increased water temperature. Throughout the experimental period all fish deaths were recorded, along with cause of death, fish size and weight.

Metabolic response to temperature

To determine the effects of temperature on aerobic performance of *A. polyacanthus*, routine oxygen consumption ($\dot{M}O_{2Routine}$), maximum oxygen consumption ($\dot{M}O_{2Max}$) and net (absolute) aerobic scope were determined. Routine and maximum oxygen consumption were measured directly, whilst net aerobic scope was calculated by subtracting $\dot{M}O_{2Routine}$ from $\dot{M}O_{2Max}$ for each fish. $\dot{M}O_{2Routine}$ and $\dot{M}O_{2Max}$ were measured for all fish at their mean winter (September/October 2012) and summer (February/March 2013) temperatures. Treatment temperatures were: +0 = 30°C, +1.5 = 31.5°C and +3 = 33°C (summer) and +0 = 25°C, +1.5 = 26.5°C and +3 = 28°C (winter). In addition, fish kept at present-day temperatures (+0°C) were also tested at the two higher temperatures after an acclimation period of one week. This additional testing establishes differences in oxygen consumption due to acute temperature change from changes due to long term (reversible) acclimation.

Respirometry measurements ($\dot{M}O_{2Routine}$) were carried out using closed chamber respirometry (Sinclair et al. 2006; Seebacher et al. 2014). Previous work has shown that for *A. polyacanthus* this type of respirometry can provide reliable results in which trends in the data are not distorted by the absence of a mixing device (Rodgers et al. 2016 **Appendix 1**). This is because damselfish are sufficiently active to provide mixing of the respiration chamber and have not been shown to exhibit diurnal trends in oxygen consumption. Food was withheld for 24 hours prior to testing, so that specific dynamic action increases in O_2 consumption associated with digestion would not affect the results. Each fish was placed inside a 3.33 L respirometer, which was submerged for 1h in a water bath in order to allow the fish to habituate to the chamber. A pilot study which examined the oxygen consumption of fish subsequent to being introduced to the respirometry chambers showed that oxygen consumption was not significantly different at 1h after introduction to the respiration chambers from all successive measurements taken up to seven hours (Repeated measures ANOVA; $F_{5,15} = 11.47$, $P < 0.001$; Post hoc: $P > 0.05$ for all subsequent measures compared with 1h). Therefore it was determined that 1h was sufficient time to recover from any handling stress that may have elevated $\dot{M}O_2$ in this study. Blank (empty) chambers were also measured during this pilot study and showed that microbial (background) respiration was not

significant until approximately 7 hours after commencement of measurements. For this reason no microbial oxygen consumption was subtracted from fish $\dot{M}O_2$ during this experiment. During the habituation period, chambers were flushed with clean, well-oxygenated, temperature-controlled sea water. This allowed for habituation to occur whilst preventing the accumulation carbon dioxide and other metabolites, as well as excretory products, which may influence oxygen consumption (Steffensen 1989). Oxygen concentrations were monitored throughout a single 30 minute experimental period using a Firesting contactless oxygen system (Pryoscience). Measurements were taken approximately every five minutes for the 30 min experimental period. Oxygen concentration never fell below 80% during this time.

Fish were given at least 1 h after $\dot{M}O_{2Routine}$ estimation before $\dot{M}O_{2Max}$ measurements were taken. To determine $\dot{M}O_{2Max}$, fish were transferred to an upright circular swim chamber with a diameter of 145 mm (Nilsson et al. 2007; Donelson & Munday 2012; Seebacher et al. 2014). Water current inside the cylinder was created using a magnetic stirring bar inside the chamber and stir plate placed below the cylinder and water bath. The speed of the magnetic stir bar was increased slowly until the fish could sustain a maximal swimming speed while maintaining its position in the water column and without making (presumably anaerobic) lunge movements. During this time, the oxygen concentration in the water was measured every second for a minimum of 5 min. Directly after $\dot{M}O_{2Max}$ measurements were taken, the wet weight of each fish was measured to the nearest mg. $\dot{M}O_{2Max}$ and $\dot{M}O_{2Routine}$ (mg O_2 consumed kg^{-1} h^{-1}) were calculated for each fish using the recorded fall in oxygen and fish wet weight.

Determination of critical thermal limits

The critical thermal limit (CT_{max}) of *A. polyacanthus* from the Torres Strait was calculated by rapid environmental temperature increase to estimate the temperature at which locomotory activity became disorganised independent of fish death (Cox 1974; Becker & Genoway 1979). Fish used in this experiment had experienced the average present-day Torres Strait thermal regime under lab conditions for approximately 10 months (June 2012 to April 2013). On commencement of trials, water temperature was increased from the average summer temperature of 30°C at a rate of 1°C h^{-1}. Pilot

studies indicated this rate was the fastest rate at which loss of equilibrium could be observed independently of the death of the fish, without a significant time lag in temperature increase. Water temperature was maintained within ±0.3°C of the desired temperature. Loss of equilibrium was defined as the point where the fish lost the ability to remain dorso-ventrally upright and could not regain this ability. CT_{max} was measured for six replicate fish as variance was low, and the mean and standard error was calculated.

Data analysis

Prior to statistical analysis measures of $\dot{M}O_{2Routine}$, $\dot{M}O_{2Max}$ and aerobic scope for each species were log-transformed and a homogeneity of slopes model was examined to ensure that the relationship between metabolic output and fish mass was consistent across treatments. All measures were then standardised to the average fish body mass using a residuals plot of log metabolic rate (mg O_2 h^{-1}) vs log fish mass (g). Differences in aerobic measures between treatments were compared for the adjusted values using a two-way nested ANOVA, with treatment (25, 26.5 and 28 ˚C during winter and 30, 31.5 or 33˚C during summer) and exposure (acute, chronic) as fixed factors with exposure nested within treatment. Although the same fish were tested across seasons, a repeated measures test could not be conducted as individual fish were not tracked throughout the study. To deal with this, data collected during summer and winter were analysed separately, reducing the chance of type 1 error caused by an inflated degrees of freedom. Where necessary, a Fisher LSD post-hoc analysis was used to determine significant differences between specific treatment groups. Adjusted values were converted to mg O_2 h^{-1} for presentation in figures by taking the inverse log. If acclimation had occurred, it was expected that metabolic performance would be significantly improved in fish that developed at higher temperatures, when compared with the performance of the acutely exposed developmental control line at those higher temperatures.

Temperature quotients (Q_{10}) were calculated to describe the magnitude of any change between treatments using the equation Q_{10} = (R2/R1)^[10/(T2-T1)], where R is mean oxygen consumption and T is a corresponding treatment temperature. This measure

characterises the rate of change that a 10˚C increase in temperature imparts on metabolic rate. A Q_{10} of 1.0 indicates thermal independence, whereas a value greater than 1.0 indicates increasing thermal dependence. Values less than 1.0 indicate a negative or inverse thermal dependence. Where there was no significant effect of exposure time on metabolic rate the combined average of chronic and acute exposure was used to calculate Q_{10}.

A Cox proportional hazards survival analysis was applied using S-Plus (TIBCO Software Inc., Palo Alto, USA) to identify significant differences in fish mortality from the time that water temperature began to increase after the winter low (September 2012), with fish standard length as a covariate. The survival analysis was performed for the periods both before and after metabolic testing was carried out to determine both the effect of temperature alone on fish survival, as well as the effect of temperature in combination with maximal exercise (swim chamber testing) as a secondary stressor. For periods where mortality of the control group was zero, a dummy censored value was added to the final day of each of the three temperature treatments in order to deal with the effects of convergence and allow for interpretation of the Wald test.

2.4 Results

Metabolic response to temperature

During winter, water temperature had a significant impact on $\dot{M}O_{2Routine}$ (mg O_2 h^{-1}; $F_{2,68}$ = 11.35, P < 0.001; Fig. 2.1a), however this effect was not influenced by exposure time (Exposure (Treatment): $F_{2,68}$ = 2.61, P = 0.08; Fig. 2.1a). This suggests that acclimation did not occur during the 10 month experimental period. Between the current-day control treatment and the +1.5˚C treatment group there was a significant increase in mean $\dot{M}O_{2Routine}$ (Post hoc: P < 0.001; Q_{10} = 5.95), however no significant difference in was observed between the +1.5˚C and +3˚C treatment groups (P > 0.05; Q_{10} = 0.95). During summer, a significant nested term indicated that the $\dot{M}O_{2Routine}$ of fish differed significantly between temperature treatments dependant on length of exposure (Exposure (Treatment): $F_{1,50}$ = 4.94, P < 0.05; Fig. 2.1a). Fish that were only acutely exposed to 31.5˚C had a significantly lower $\dot{M}O_{2Routine}$ than those in the chronic exposure treatment group (Post hoc: P < 0.05). Between the current-day control treatment and

the chronic +1.5˚C treatment group there was a significant increase in mean $\dot{M}O_{2Routine}$ ($P < 0.001$; $Q_{10} = 7.22$) whilst similarly to winter, no significant difference in $\dot{M}O_{2Routine}$ was observed between the chronic +1.5˚C and chronic +3˚C treatment groups (Post hoc: $P > 0.05$; $Q_{10} = 1.15$).

Water temperature had a significant impact on $\dot{M}O_{2Max}$ during winter, however this time the effect was influenced by exposure time (Exposure (Treatment): $F_{2,79} = 3.42$, $P = 0.04$; Fig. 2.1b). Between the current-day control treatment and the chronic +1.5˚C treatment group there was a significant increase in mean $\dot{M}O_{2Max}$ (Post hoc: $P < 0.001$; $Q_{10} = 8.45$) but no significant difference was observed between the current-day control treatment and the acute +1.5˚C treatment group (Post hoc: $P > 0.05$; $Q_{10} = 2.33$). The acute and chronic +1.5˚C treatment groups were not significantly different from their respective +3˚C treatment group ($P > 0.05$; $Q_{10} = 1.12$ and 0.60, respectively). In contrast, during summer water temperature had a significant impact on $\dot{M}O_{2Max}$ ($F_{2,48} = 21.32$, $P < 0.001$; Fig. 2.1b), which was not influenced by exposure time (Exposure (Treatment): $F_{1,48} = 0.13$, $P > 0.05$; Fig. 1b). $\dot{M}O_{2Max}$ increased significantly from 30˚C (+0˚C) to 31.5°C (+1.5°C) (Post hoc: $P < 0.001$; $Q_{10} = 7.69$), but then fell sharply from 31.5 to 33°C (+3°C; $P < 0.001$; $Q_{10} = 0.01$) to a level that was significantly lower than the 30.0°C control treatment ($P < 0.001$; $Q_{10} = 0.26$; Fig. 2.1b).

Finally, during winter, absolute aerobic scope was not influenced by temperature treatment ($F_{2,56} = 2.11$, $P > 0.05$; Fig. 2.1c). In summer trends were similar to those seen for $\dot{M}O_{2Max}$, where water temperature had a significant impact on aerobic scope ($F_{2,42} = 21.72$, $P < 0.001$; Fig. 2.1c), not influenced by exposure time (Exposure (Treatment): $F_{1,42} = 0.07$, $P > 0.05$).This again indicated no reversible acclimation of this trait. As with $\dot{M}O_{2Max}$, aerobic scope increased from 30°C to 31.5°C (Post hoc: $P < 0.05$; $Q_{10} = 8.08$), then decreased significantly at 33°C ($P < 0.001$; $Q_{10} = 0.0004$; Fig. 2.1c) to a level that was just 41.67% of aerobic scope at 30°C and 30.46% of the aerobic scope attained at 31.5°C.

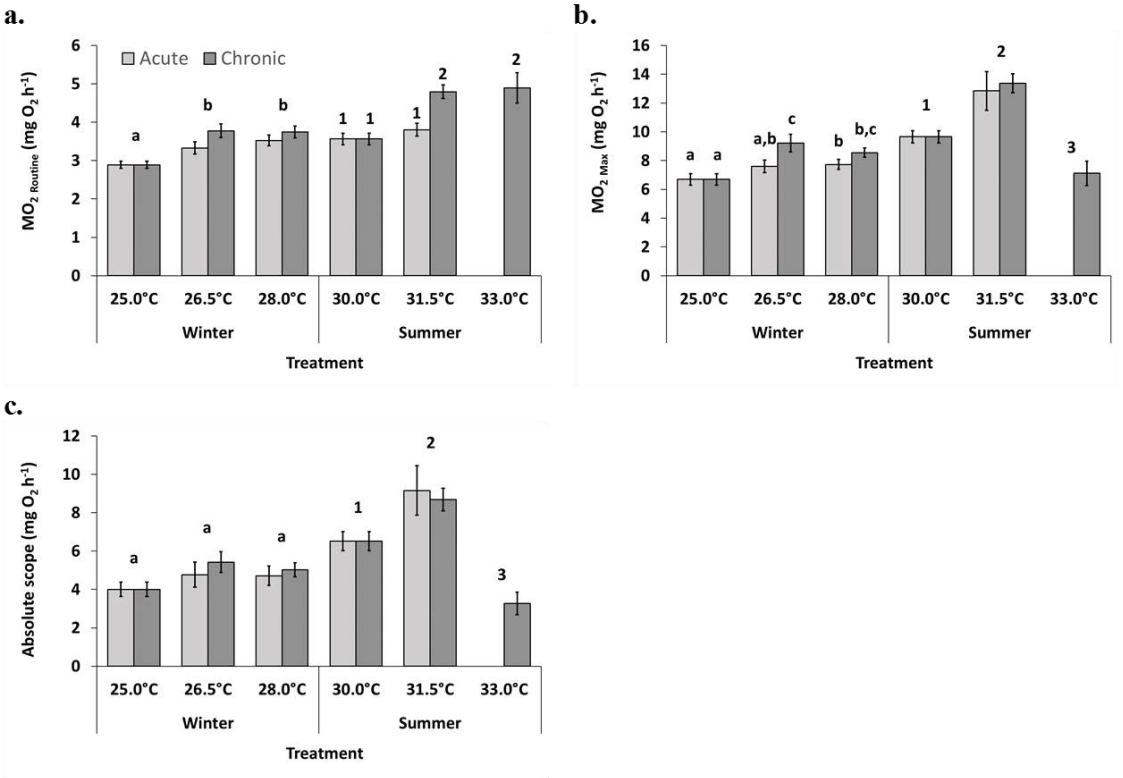

Figure 2.1. Mean (± SE) routine (a) and maximum (b) oxygen consumption and absolute scope (c) of *Acanthochromis polyacanthus* during the austral winter and summer. Temperature treatments tested were current average ocean temperatures (25.0 and 30.0˚C), +1.5°C (26.5 and 31.5˚C) and +3°C (28.0 and 33.0˚C). Acute (one week) exposure to each treatment is represented by the light grey columns and chronic exposure by dark grey. Letters indicate significant differences between treatment groups during winter where exposure time was not significant, or between individual treatments where it was; whilst numbers indicate significant differences during summer.

Fish survival in relation to temperature

No mortality was observed until 100 days after the last day of winter temperatures, which was one month after summer temperatures were reached (Fig. 2.2). Significantly higher mortality was observed for fish maintained at +3°C, compared with fish at current-day +0°C and +1.5°C (Cox proportional analysis: Z = 2.02, P = 0.04). Survival within the +1.5°C temperature treatment was not significantly different from the current-day +0°C control (Z = 0.21, P = 0.84). The majority of mortality occurred at approximately 30 days after summer temperatures were achieved when the number of

fish remaining within the +3°C treatment decreased by 20% within a period of 10 days. The survival of fish was significantly correlated to their standard length (Z = -2.44, P = 0.02), with smaller fish surviving better.

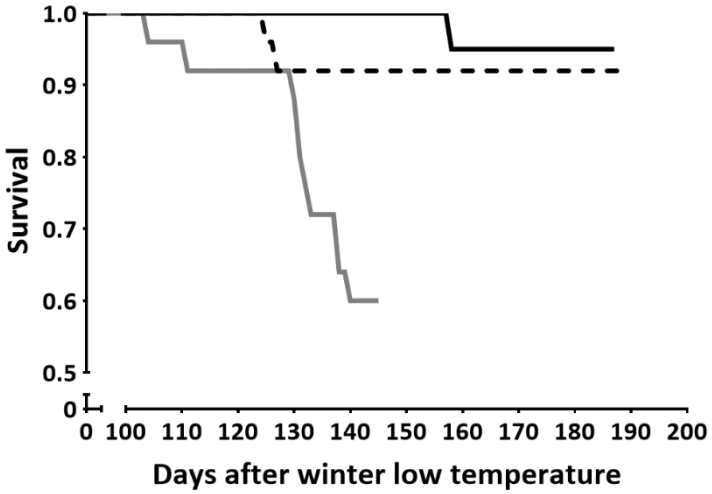

Figure 2.2. Proportion of fish remaining in each temperature treatment in relation to the number of days having passed since temperatures began to increase from the winter low and up until the point at which the experiment was terminated at the beginning of metabolic testing. The +0˚C treatment group is represented by the solid black line, +1.5˚C by the broken black line and +3˚C by the solid grey line. Data before the summer maximum was achieved is not shown on the graph as there were no deaths during this time. Due to high mortality, fish at +3˚C were sampled after day 145.

Survival during the week after metabolic testing was significantly poorer for fish in both the +1.5 and +3°C treatments than the current-day +0°C (Fig. 2.3; z = 2.67, P = 0.008 and Z = 2.32, P = 0.02 respectively). However, survival between the +1.5 and +3°C treatments was not significantly different (Z = 0.51, P = 0.61). Both elevated temperature treatments followed a similar trend of consistent mortality throughout the week after metabolic testing, with each population experiencing approximately 50% mortality by the end of the week. Fish standard length did not have an effect on survival after post metabolic testing (Z = 0.98, P = 0.33).

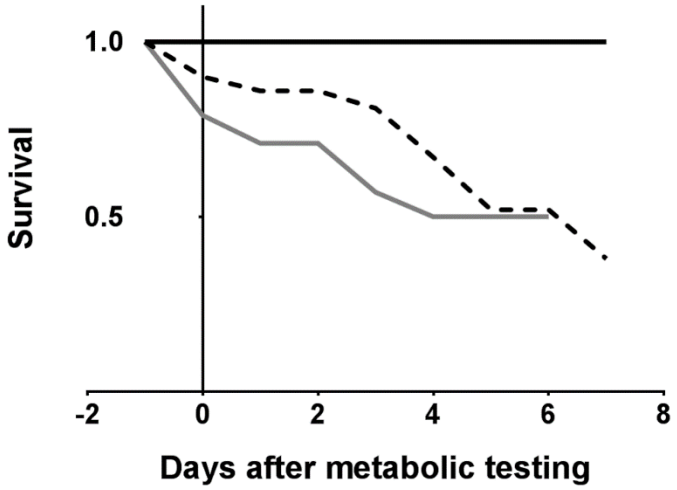

Figure 2.3. Proportion of fish remaining in each temperature treatment in relation to the number of days post metabolic testing. The +0˚C treatment group is represented by the solid black line, +1.5˚C by the broken black line and +3˚C by the solid grey line. Day -1 represents the day prior to testing and day 0 is day of testing. Mortality was recorded for one week, or until the proportion of fish remaining reached 50% or below.

Critical thermal limit

The CT_{max} of *A. polyacanthus* maintained at current-day +0˚C temperatures, ranged from 36.8 to 37.5˚C (Mean = 37.07°C, +/-SE = 0.11°C). This is on average 7˚C higher than the average summer temperatures they experience and 5.73˚C above the maximum temperature that Torres Strait reefs have experienced in the past 10 years.

2.5 Discussion

For this low-latitude population of *A. polyacanthus*, sustained increases in water temperature had little effect on the aerobic capacity of fish during the winter months. In contrast, aerobic scope was greatly reduced during the summer when water temperatures were +3°C above current ocean temperatures (33˚C). Aerobic scope was highest during summer at 31.5˚C, which might indicate the species' thermal optimum. However, mortality after a secondary stressor (short periods of maximal exercise via metabolic testing) was significantly higher at this temperature, indicating that a restriction of aerobic capacity may not to be the first indicator of declining fish health when ecologically relevant circumstances are considered for this population. Generally,

metabolic measures did not differ between fish that were acclimated to elevated temperatures for one week and those that experienced high temperatures for approximately 10 months. Exceptions were an improvement in $\dot{M}O_{2\ Max}$ at 26.5 ˚C under chronic temperature exposure and an improved $\dot{M}O_{2\ Routine}$ at 31.5 ˚C under acute temperature exposure. Mortality at +3˚C was also more gradual under chronic temperature exposure. As differences in oxygen consumption did not translate to a substantial difference in aerobic scope between chronic and acute temperature exposure, results suggest that the capacity for reversible acclimation to elevated temperatures in this population is minimal. Given that significant mortality was observed at +3˚C above current day temperatures, and at +1.5˚C when a secondary stressor was introduced, this low-latitude population may be extremely vulnerable to increasing water temperature and adults appear to have limited capacity to adjust to climate change.

Temperature has consistently been found to have a significant effect on the metabolic scope of *A. polyacanthus* (Nilsson et al. 2009; Donelson & Munday 2012; Rummer et al. 2014a). At higher latitudes (central and southern GBR; Heron Island, Palm Island and Lizard Island), a decrease in aerobic scope with increasing temperature has regularly been observed, however this is generally only significant between current day temperatures and 1 – 1.5˚C above current day averages (Nilsson et al. 2009; Donelson & Munday 2012). After this point, scope has been shown to remain largely unchanged at subsequent higher temperatures. In contrast, Torres Strait fish in this study were able to maintain aerobic scope until 31.5˚C (+1.5˚C), after which point scope decreased dramatically. Only one other study has examined the effect of increased temperature on aerobic scope at latitudes lower than the central GBR. Rummer and colleagues (2014a) examined the effect of increased temperature on the aerobic capacity of a variety of damselfish and cardinalfish species in PNG (2˚35,765'S; 150˚46,193'E) at summer temperatures. For both locations *A. polyacanthus* aerobic scope was driven primarily by $\dot{M}O_{2\ Max}$. This resulted in a trend of increasing scope between the lowest two temperature treatments in both locations, before a decrease in scope to 33˚C.

The coral reef fish tested in this study are currently living only 3˚C below their thermal limit for chronic exposure to higher temperature. Previous research on the same species

has shown that short-term exposure (1-2 weeks) to water temperatures of 34˚C resulted in significant mortality in populations from Papua New Guinea (PNG; Rummer et al. 2014a) and Central GBR (Zarco-Perelló et al. 2012), compared to 33˚C in the present study, despite differing thermal regimes between these locations. These similarities suggest that the chronic thermal limit of this species is stable between populations, irrespective of latitude. Previously this lack of variation in thermal limits across latitude has only been observed in terrestrial ectotherms (Addo-Bediako et al. 2000; Deutsch et al. 2008).

Mortality levels of fish maintained at 3°C above the current average temperature during the summer also correlated with a dramatic loss of aerobic capacity, indicating that failure of oxygen delivery to tissues may be been associated with the observed rapid rise in mortality. Specifically, there is mounting evidence to suggest that it is likely to be a limitation on heart function, which delivers oxygenated blood to the tissues, ultimately leading to organism death under increased temperatures (Farrell 2002; Farrell 2009; Iftikar & Hickey 2013; Muñoz et al. 2014). The most commonly cited mechanism of heart failure is a limitation on maximum heart rate, which results in oxygen deficit to the myocardial tissue, known as myocardial hypoxia (Farrell 2002; Farrell 2009; Muñoz et al. 2014). Under hypoxic conditions (caused by increased exercise or higher temperatures) more oxygen is extracted from the venous blood supply by the skeletal muscle tissue before it can reach the heart, causing an oxygen deficit and subsequent arrhythmia and bradycardia as heart rate fails to meet oxygen demand in the tissues (Farrell 2002). Recent research is also beginning to show that myocardial hypoxia could additionally be preceded or further exacerbated by cardiac mitochondrial dysfunction, as mitochondrial cells in the heart also suffer damage due to heat stress, ischemic damage, and oxidative stress, particularly in species from a thermally stable environments (Iftikar & Hickey 2013; Iftikar et al. 2014).

Our study supports previous findings that tropical marine fish have limited capacity to undergo reversible acclimation as adults (Nilsson et al. 2009; Nilsson et al. 2010; Gardiner et al. 2010; Rummer et al. 2014a). However, in earlier investigations the ability to reversibly acclimate has only been tested over significantly shorter time scales (longest 22 days; Nilsson et al. 2010). We maintained adults for approximately 10

months at elevated temperatures and still found no improvement in metabolic attributes, with the exception of an improvement in $\dot{M}O_{2\ Max}$ at 26.5 ˚C during winter under chronic temperature exposure. This limited ability to cope at temperatures outside of their normal range is attributed to the narrow thermal range experienced by tropical organisms. Compared with temperate species, tropical reef fish are subjected to limited temperature variations (Sunday et al. 2011; Rummer et al. 2014a). In contrast, reversible thermal acclimation is more commonly observed for temperate fishes (Fry & Hart 1948; Johnson & Bennett 1995; Wilson et al. 2007), likely due to the naturally wider thermal range in their habitat. Surprisingly, some polar fishes which also experience a narrow thermal range, may have more capacity for reversible acclimation than theory predicts, and have been shown to acclimate to increased temperatures under relatively short time periods (longest 8 weeks; Seebacher et al. 2005; Podrabsky & Somero 2006; Franklin et al. 2007; Seebacher et al. 2015). Too few low-latitude fish species have been tested to know whether exceptions also exist in this environment, but at least for the species tested to date there is limited evidence. It is possible that with further testing and the inclusion of species from more thermally variable niches (e.g., reef flats) some capacity for reversible acclimation may yet be revealed. For those species that are not able to acclimate and that additionally display a low tolerance for warming, a high risk of population extirpations due to rapid climate change exists (Deutsch et al. 2008).

Based on metabolic and survival data for this study, populations are unlikely to persist past 33˚C, and as a result CT_{max} does not give a reliable indication of thermal sensitivity. Observed mortality due to chronic exposure to elevated water temperature occurred at 4˚C lower than the critical thermal maximum (37.07˚C). Based on these measures, low-latitude populations will be far more vulnerable to the temperature changes associated with ocean warming than predicted by CT_{max}. Studies that use CT_{max} as their only method of predicting the effect of future increased temperatures (e.g., Mora & Ospina 2001) should be interpreted with caution, as this method commonly overestimates a species' ability to deal with climate change (Pratchett et al. 2015). CT_{max} was not considered for acclimated fish in this study, however it is expected that acclimation would not have further increased the temperature at which CT_{max} occurred,

based on the results obtained for metabolic data (thermal optimal of aerobic scope did not change with acclimation).

Additional stress caused by maximum exercise increased the mortality rate of fish at +1.5˚C above current-day temperatures. In the wild a secondary stressor could include a vast range of biotic and abiotic factors. The effects of climate change are expected to be coincident with additional stressors such as changes in ocean chemistry and food availability, as well as additional non climate-related anthropogenic effects (Harley et al. 2006). Fish health after a secondary stressor is likely to be more indicative of their capacity to deal with higher temperatures in nature as stressors do not tend to occur in isolation. Counter to theory (Pörtner 2001; Pörtner & Knust 2007), the restriction of whole-animal aerobic scope was not the first symptom of declining fish health at temperatures above an organism's thermal optimum when a secondary stressor was incorporated; enhanced aerobic scope was observed at 31.5˚C where a secondary stressor caused mortality to increase. Therefore, it is likely that a restriction of cardiac performance occurred before a decrease in aerobic scope could be observed. When even a moderate amount of exercise is performed at high temperature, organism death can result due to a crash in cardiac output before a decrease in maximum aerobic performance (Farrell 2002). This drop in cardiac performance has been shown to precede a decrease in aerobic scope in salmonid species because as warming approaches the fish's thermal optimum, the increase in cardiac output of a resting fish escalates more rapidly than the increase in maximum cardiac output with exercise (Farrell 2009). Until very recently, the OCLTT theory has remained relatively unchallenged. However, with the growing popularity of metabolic studies as a means of classifying an organism's response to a changing thermal environment the ecological relevance of the theory is becoming more commonly tested and in some cases contradiction of the OCLTT theory has been observed (Clark et al. 2011; Clark et al. 2013; Donelson et al. 2014; Gräns et al. 2014; Norin et al. 2014). The results of this study may fit more closely with the multiple optima theory, however, testing of a greater array of physiological measures is required to confirm this.

Given the vulnerability to higher temperatures shown in this study, the capacity for acclimation and adaptation to a rapidly changing environment will be crucial for these

coral reef fishes if they are to persist at the higher temperatures associated with climate change (Munday et al. 2013). Adaptation may occur through the selection of favourable genotypes whilst acclimation occurs through non-genetic modifications. Due to the short time scale of climate change, and the closeness of low-latitude fish populations to their thermal limit, an understanding of modifications other than adaptation, such as developmental (within a generation) and trans-generational (across multiple generations) acclimation will be particularly important, as these changes are capable of operating over climate change relevant time scales. Developmental and trans-generational thermal acclimation has previously been shown to occur in some higher latitude coral reef species, including *A. polyacanthus* (Donelson et al. 2011a,b; Donelson & Munday 2012; Donelson et al. 2012). The next step in this research will be to determine whether low-latitude reef fishes have the potential for these types of acclimation, given that they seem to be on the cusp of their thermal tolerance limit. However, even if fish are able to developmentally acclimate, there may be other trade-offs which could affect fish health. For example, Donelson et al. (2011a) showed reduced growth in thermally acclimated fish.

This long-term study is consistent with the hypothesis that low-latitude species may be particularly vulnerable to thermal elevations associated with CO_2-induced climate change. Future research should consider the extent to which the response seen in this research can be generalised for all low-latitude populations of coral reef fish. *A. polyacanthus* is a direct developer with no pelagic larval phase (Kavanagh 2000), and this lack of a larval dispersal stage whilst beneficial for carrying out lab-based studies, may also be result in findings obtained for this species being conservative. This is due to a higher probability of genetic local adaptation, potentially meaning that *A. polyacanthus* has a lower potential for thermal plasticity relative to other reef fish species. Another important next step in the study of low-latitude organisms will be to investigate their capacity to developmentally or transgenerationally acclimate to ocean temperatures predicted with climate change, or to adapt through genetic selection. Previous research has shown that corals are potentially amongst the marine organisms under greatest threat from climate change and that they are likely to have already exceeded their capacity to adapt to higher ocean temperatures (Hoegh-Guldberg 1999).

Certain fish populations may also be at the limit of their thermal capacity, and may be similarly vulnerable to changing ocean temperatures.

Chapter 3: Impacts of increased ocean temperatures on a low-latitude coral reef fish: evidence for multiple thermal optima

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3.1 Summary

Increased temperatures are expected to significantly affect the physiological performance of ectotherms, particularly in tropical locations where seasonal temperature variation can be low. Many studies consider only single traits when measuring the impacts of thermal stress on organismal health, and as a result, may not obtain the best estimate of how an organism will cope under future conditions. The present study investigated the effects of elevated temperatures on oxygen consumption rates, haematological parameters and tissue health for a low-latitude population of coral reef damselfish. *Acanthochromis polyacanthus* were collected from Torres Strait (10°31-46'S, 142°20-35'E) and maintained at current average ocean temperatures (+0 °C; seasonally cycling), +1.5 °C and +3 °C higher than present day temperatures for 10 months. Aerobic performance indicated a thermal limit to metabolic function at +3 °C (33 °C), following an increase in aerobic capacity at +1.5 °C (31.5 °C). Neither the haematological parameters nor gill histology showed the same improvement in performance at 31.5 ˚C. There was a strong trend towards poorer gill tissue health with increasing temperature as aneurismal dilations in the gills became larger and more numerous. Findings from this study suggest thermal specialisation in this low-latitude population of reef fish and a variation in thermal performance, depending on the physiological trait. Of the measures considered in this study it appears that gill histopathology may provide the best indicator of thermal tolerance, as it provided the first indicator of a decline in organism health and corresponded with mortality observations from previous research.

3.2 Introduction

Temperature increases associated with ocean warming are projected to be greater toward the poles and less toward the tropics (Stocker et al. 2013). Despite this, tropical species are expected to be the most sensitive to projected ocean warming due to the higher trajectory of change in these environments and the thermally stable climate in which organisms have evolved (Janzen 1967; Deutsch et al. 2008; Tewksbury et al. 2008, Burrows et al. 2011). Climate change is expected to impose significant behavioural, ecological and physiological changes on organisms (Walther et al. 2002). These changes will impact whole organism performance, which – for ectotherms – can be represented using a thermal reaction norm (Angilletta 2009). A thermal reaction norm is a normally distributed curve that graphically represents organism performance as a function of temperature for a given trait and provides an indication of an organism's thermal range. Performance increases with increasing temperature, until it reaches a thermal optimum after which performance begins to decrease. The thermal reaction norm for tropical species is narrower than that of temperate species, again reflective of their naturally narrower thermal range (Deutsch et al. 2008; Tewksbury et al. 2008; Sunday et al. 2011). As a result, some tropical organisms appear to live at the edge of their thermal tolerance, even at present day temperatures (Stillman 2003; Kellermann et al. 2012; Rummer et al. 2014a), and may be unable to cope with even small temperature increases in the future. Within the tropics different thermal environments exist, and so the sensitivity of populations is likely to vary throughout. Lower latitude populations are expected to be more sensitive to elevated temperatures than populations from higher tropical latitudes (Nguyen et al. 2011; Rummer et al. 2014a).

Predicting the shape of an organism's thermal reaction norm and subsequently its thermal optimum and maximum can provide important information on the likely persistence of that population under climate change scenarios. Difficulty in making such predictions lies with selecting the performance measure, or traits, that are most meaningful to whole organism performance and therefore individual and even biological fitness. Aerobic scope is a commonly considered performance metric for these types of studies. Aerobic scope describes the oxygen available – above basic maintenance of the organism – for important ecological activities such as reproduction,

foraging, and predator avoidance (Pörtner & Knust 2007). The oxygen- and capacity-limited thermal tolerance (OCLTT) hypothesis predicts that all measures determining the fitness of an organism are causally linked to aerobic capacity (Pörtner 2001; Pörtner & Farrell 2008). This hypothesis assumes that for ectotherms, restriction of whole-animal aerobic scope is one of the first symptoms of a negative effect on organism health associated with temperatures outside the optimal thermal range. This is due to insufficient uptake, transport, and delivery of oxygen (Pörtner 2001; Pörtner & Farrell 2008). The obvious value of the OCLTT theory is that a single measure could be used to make predictions about population level responses to climate change (Pörtner & Knust 2007; Eliason et al. 2011). Recently though, this hypothesis has been applied to a wider range of populations and thermal environments, sometimes with conflicting results (Clark et al. 2011; Clark et al. 2013; Donelson et al. 2014; Gräns et al. 2014; Norin et al. 2014; Norin et al. 2015; Farrell 2016; Drost et al. 2016; **Chapter 2**).

Given that the OCLTT theory has not been found applicable in all cases, an alternative hypothesis may be that there is no single optimal temperature for organism function, but instead that different physiological processes have different thermal optima (Hadfield 1966; Bustard 1967; Du et al. 2000; Clark et al. 2013). The multiple optima hypothesis states that any physiological driver could be a limiting factor on organism health and that the trait most related to individual fitness may vary among species, life stage, or thermal challenge (Clark et al. 2013). As well as determining direct causes of organism mortality, considering multiple traits and multiple optima could have the added advantage of identifying other indirect or sub-lethal effects of a stressor on organism health, which may be equally important to the organism's functional niche. The multiple optima hypothesis has not been as well tested as the OCLTT theory, possibly because of the additional time required to test multiple measures, or because there is no widely accepted suite of traits that are commonly tested.

Even within the limitations of oxygen uptake and transport there are a range of measures other than oxygen consumption that could be examined which may help to determine an organism's thermal optima and/or maxima. As the respiratory organ primarily responsible for oxygen uptake, the gills are likely to play a critical role in dealing with temperature stress and resulting oxygen deficiencies (Tzaneva et al. 2011).

As a result, changes to gill morphology may be expected with temperature stress. Past studies have described significant changes in gill structure in response to hypoxia and temperature stress in a number of fish species (Sollid et al. 2005; Sollid & Nilsson 2006; Nilsson 2007; Mitrovic et al. 2009; Tzaneva et al. 2011). Sollid and Nilsson (2006) hypothesised that in highly plastic species, gill remodelling is likely to occur in response to a changing oxygen demand based on the levels of variability in the availability of oxygen in the environment. Species such as the crucian carp (*Carassius carassius*) experience seasonal hypoxia and the capacity to change the area of the gill surface is a major evolutionary advantage (Sollid & Nilsson 2006). The amphibious mangrove killifish (*Kryptolebias marmoratus*) are also able to remodel their gills, this time in response to moving between aquatic and terrestrial habitats and as a result are able to maintain aerobic fitness over both environments (Ong et al. 2007; Turko et al. 2012).

Other ways to modulate oxygen transport during stress events such as those caused by increased temperature can occur at the level of the blood, as blood haemoglobin [Hb], which is encapsulated within the red blood cells (RBCs) is responsible for transporting 98-99% of the oxygen in blood. In times of thermal stress, oxygen demand within an organism increases and this can subsequently result in hypoxia if the oxygen transport system is not able to sufficiently meet increased demand (Cocking 1959; Brett 1971). Compounding this problem, the oxygen content of water also decreases with increasing temperature and this can reduce oxygen uptake by the blood at the gills (Farrell 2002). Oxygen availability in water can be much more variable than in air, and so between species both the oxygen-carrying capacity of the blood (Hb levels) and the oxygen affinity of Hb itself may be adapted to suit life history (Wells et al. 1989). There is also evidence that these characteristics can vary within a species in response to short-term changes in the environment (Weber 1982; Wells et al. 1989). One of the primary ways that an organism is able to increase oxygen carrying capacity of the blood is through contraction of the spleen, which holds a reserve of RBCs, thus releasing more Hb into circulation (Bonga 1977).

The above are of course only a limited selection of measures that could be considered in response to temperature stress, but due to their critical role in oxygen delivery they were selected for analysis in this study. In the present study we compare the effect of

climate change relevant increases in ocean temperature on a range of physiological measures in a low-latitude population of a coral reef fish, the spiny chromis damselfish (*Acanthochromis polyacanthus*) from Torres Strait (far northern Great Barrier Reef; GBR). Average temperatures for this location range from approximately 25 °C in winter to 30 °C in summer; a seasonal variation of only 5 °C. We predicted that there would be some variation in response to increased temperatures among physiological traits. Despite this variation, we hypothesised that the effects of elevated temperatures on whole organism aerobic scope should provide the earliest indicator of a decline in organism health at increased temperatures, in accordance with the OCLTT hypothesis. Whole animal metabolic performance, haematological parameters (including spleen weight), and tissue health were compared for fish maintained at average temperatures for the collection location, as well as +1.5 °C and +3 °C above a naturally cycling average for a period of 10 months. By comparing the thermal optimum for each trait, we were able to determine if a single physiological measure could provide sufficient information to indicate the thermal optimum of the whole organism, or if a multiple trait approach is required.

3.3 Methodology

Fish collection and temperature treatments

Adult spiny chromis damselfish were collected from three reef locations – Dugong Reef, Twin Cays and Kagar Reef (10°31-46'S, 142°20-35'E) – in Southern Torres Strait, the northernmost part of the GBR, during December 2011. Fish were transported by plane to aquarium facilities at James Cook University (Townsville, Queensland Australia) where they were maintained as pairs, each in 60 L tanks containing a shelter (half of a terracotta pot) and connected to a system providing recirculating filtered, UV-sterilized seawater. The mean (±SE) mass of *A. polyacanthus* was 26.54 ± 0.80 g, with a maximum size up to 45.32 g. Pairs were fed *ad libitum*, one to two times per day using commercial fish pellets (INVE NRD G12). All pairs were maintained at the average summer water temperature for the collection location (30 °C; Australian Institute of Marine Science (AIMS) sea surface temperature database;

http://data.aims.gov.au/aimsrtds/datatool.xhtml?site=921&param=water%20temperature).

In June 2012, pairs were randomly assigned to one of three temperature treatments (10-15 pairs per treatment). Treatment temperatures were: 1) current average ocean temperatures for the collection locations (control; +0 °C; 25.0 °C – 30.0 °C, seasonally cycling), 2) 1.5 °C above current average ocean temperatures (+1.5 °C), and 3) 3 °C above current average ocean temperatures (+3 °C). Temperatures were adjusted over a 7-day period (<0.5 °C per day to summer temperatures as seasons were offset in aquarium environment) until target temperatures were reached at which point fish were maintained for 10 months to test the chronic effects of increased water temperature. All measurement of physiological metrics was carried out at summer temperatures, as previous work has shown that this is when temperature has the most significant impact on fitness for this population (**Chapter 2**).

Whole animal oxygen consumption rates

All fitness measures were carried out in summer, when the effects of temperature on the fish were expected to be greatest. To determine the effects of increased temperature on aerobic performance of *A. polyacanthus*, routine and maximum oxygen consumption rates ($\dot{M}O_{2\ Routine}$, $\dot{M}O_{2\ Max}$) were measured as described in **Chapter 2**. Briefly, $\dot{M}O_{2\ Routine}$ and $\dot{M}O_{2\ Max}$ were measured for all fish (n = 22, 18 and 14 for treatments +0, +1.5 and +3 °C, respectively) at their mean summer (February/March 2013) temperatures. Measurements were then used to calculate absolute aerobic scope ($\dot{M}O_{2\ Max}$ - $\dot{M}O_{2\ Routine}$) for each fish. Metabolic data is previously published in **Chapter 2**, however has been simplified and reanalysed for the purpose of this manuscript.

Blood collection and haematology

One week following respirometry trials, fish were terminally sampled. In order to minimise the effects of handling time and associated stress response in the fish, blood was sampled immediately upon netting a fish from its holding tank (<10 s). Blood was drawn from the caudal artery/vein using a 25 G hypodermic needle into a 1 mL

heparinized syringe. Blood haemoglobin [Hb] was determined using the HemoCue® (Hb 201 System, Australia Pty Ltd) with 10 µl of whole blood and was reported as grams per 100 ml using a calibration curve according to Clark and colleagues (2008) and corrected for tropical reef species (Rummer et al. 2013). Haematocrit (Hct) was determined by centrifuging 60 µl of whole blood in heparinised microcapillary tubes for 3 min at 17 000g and calculated as the ratio of packed red blood cells to total blood volume (reported as a percentage). Both [Hb] and Hct were used to calculate the mean corpuscular or cell haemoglobin concentration (MCHC; n = 6 fish per treatment except for [Hb] where n was 6, 8 and 7 for treatments +0, +1.5 and +3 °C, respectively).

Tissue samples and preservation

Immediately after blood was drawn fish were euthanized by severing the spinal cord prior to tissue sampling. The wet weight (nearest 0.01g) and standard length (SL, in mm) of each fish was then recorded. Then, the second and third gill arches were removed and preserved in 4% phosphate buffered formaldehyde (PBF) and subsequently transferred to ethanol after ~48 hours for histological analyses. The spleen was also removed, weighed, and then snap frozen in liquid nitrogen. All frozen samples were transferred to -80 °C and stored until analysis.

Histology

Gill samples were grouped into cassettes according to treatment temperatures. Samples (n = 5-10 fish per treatment) were dehydrated through a series of graded ethanol (EtOH) concentrations (Shandon Southern Duplex Processor BS5), embedded in paraffin wax blocks (Shandon Histocentre 3, Thermo Electron Corporation) and sectioned using a microtome (Microm HM 325) into 4 µm sections. Then, all tissue samples were stained with standard Haematoxylin and Eosin (H and E).

Gill tissue was cut parallel to the long axis of the filament and as the gill block orientation was not consistent and thus the number of observable primary lamellae varied, a standardised subsample was quantified for each sample. To do this, sections from each gill arch were randomly selected and digitally photographed (Olympus BX43 and Olympus camera DP27) up to a maximum of five times (less if total preserved area was

captured in a lower number of images) at 200x magnification. Gill aneurisms and dilations were noted and if present, counted (average number per standardised section of gill arch), and measured (widest section). Common stress response indicators such as cell proliferations, mucus, or inflammation were also recorded when present.

Calculations and statistical analyses

Prior to statistical analysis measures of $\dot{M}O_{2\ Routine}$, $\dot{M}O_{2\ Max}$ and aerobic scope for each fish was log-transformed and a homogeneity of slopes model was examined to ensure that the relationship between metabolic output and fish mass was consistent across treatments. All measures were then standardised to the average fish body mass using a residuals plot of log metabolic rate (mg O_2 h^{-1}) vs log fish mass (g). Differences in aerobic measures between the three temperature treatments were compared for the adjusted values using a one-way ANOVA, with temperature treatment (+0, +1.5 and +3 °C) as the fixed factor.

Temperature quotients (Q_{10}) were calculated for all aerobic measures among temperature treatments using the equation $Q_{10} = (R_2/R_1)^{[10/(T_2-T_1)]}$, where R is mean oxygen consumption and T is corresponding treatment temperature. The Q_{10} characterises the rate of change that a 10 °C increase in temperature imparts on a physiological or biochemical process such as metabolism. A Q_{10} of 1.0 indicates thermal independence, whilst larger values indicate thermal dependence. Values less than 1.0 indicate a negative or inverse thermal dependence.

Differences in haematological parameters, spleen weight and gill morphology among the three temperature treatments were compared using a one-factor ANOVA with temperature treatment (+0, +1.5 and +3 °C) as the fixed factor.

All statistical analyses were carried out using Statistica (StatSoft Inc., Tulsa, USA), and all assumptions were examined with residual analysis and transformed when necessary to meet the assumptions of normality and homogeneity of variance. Where necessary, Tukey's Post-hoc means comparisons were used to identify the nature of significant effects found by ANOVA.

3.4 Results

Whole animal oxygen consumption

$\dot{M}O_{2\ Routine}$ (mg O_2 kg^{-1} h^{-1}) of fish differed significantly between temperature treatments ($F_{2,46}$ = 12.65, P < 0.001). Between the current-day control treatment and the +1.5 °C treatment group there was a significant increase in $\dot{M}O_{2\ Routine}$ (Post hoc: P < 0.001; Q_{10} = 7.22), however no significant difference in $\dot{M}O_{2\ Routine}$ was observed between the +1.5 °C and +3 °C treatment groups (Post hoc: P > 0.05; Q_{10} = 1.15).

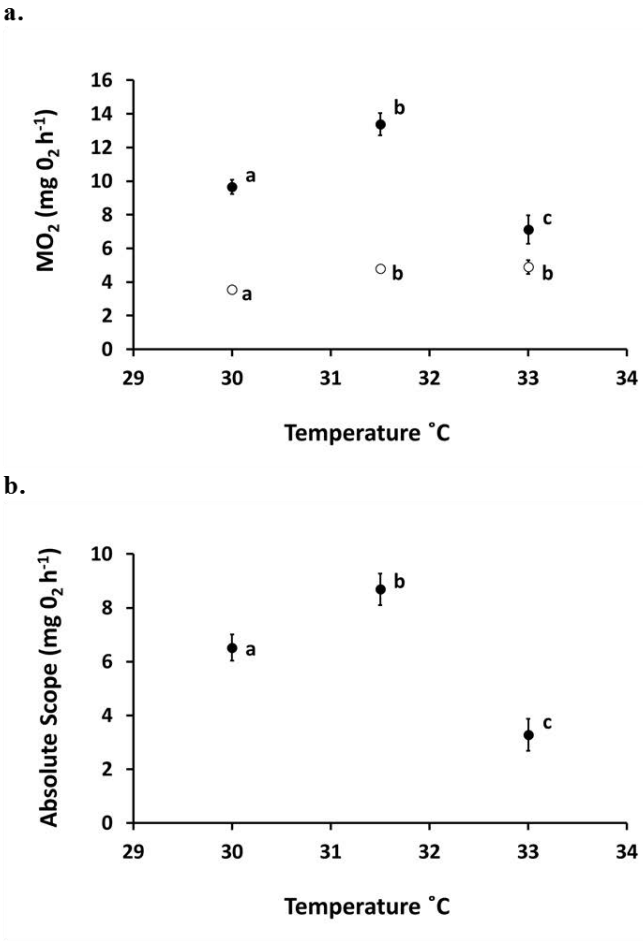


Figure 3.1. Routine (open circles) and maximum (closed circles) oxygen consumption rates (a) and absolute aerobic scope (b) (means ± SE) for *A. polyacanthus* during the austral summer and winter. Temperature treatments included average ocean temperatures (25 and 30 °C) for the collection site as well as +1.5 °C (26.5 and 31.5 °C) and +3 °C (28.0 and 33.0 °C). Letters indicate significant differences among treatment groups.

$\dot{M}O_{2\ Max}$ also differed between treatments for *A. polyacanthus* ($F_{2,44}$ = 23.92, P < 0.001; Fig. 3.1a). $\dot{M}O_{2\ Max}$ increased significantly from 30 °C (+0 °C, control) to 31.5 °C (+1.5 °C; Post hoc: P < 0.001; Q_{10} = 8.82), but then fell sharply from 31.5 to 33 °C (+3 °C; P < 0.001; Q_{10} = 0.01) to a level that was significantly lower than $\dot{M}O_{2\ Max}$ values measured in fish at the control (P < 0.001; Q_{10} = 0.26; Fig. 3.1a).

Changes in aerobic scope were largely driven by changes in $\dot{M}O_{2\ Max}$ and were again dependant on temperature treatment ($F_{2,38}$ = 21.27, P < 0.001; Fig. 3.1b). Aerobic scope increased in fish from 30 °C to peak at 31.5 °C (Post hoc: P < 0.05, Q_{10} = 6.78), which appeared to be the thermal optimum for the fish in this study. Aerobic scope then significantly decreased in fish from 31.5 to 33 °C (Post hoc: P < 0.001; Q_{10} = 0.0004) to a level that was just 35.39% of the control (30 °C) aerobic scope and 27.12% of the aerobic scope for fish maintained at 31.5 °C.

Haematology

Both [Hb] and Hct followed the same trend with increasing temperatures as aerobic scope (i.e., decreasing at 33 °C; $F_{2,20}$ = 7.23, P < 0.01 and $F_{2,17}$ = 18.06, P < 0.001, respectively; Fig. 3.2). There was no significant difference between values from the control fish (30 °C) and the +1.5 °C fish (31.5 °C) for either measure (Post hoc: P > 0.05), but both values significantly declined from the +1.5 °C when compared to fish from the +3 °C (33 °C) treatment group (P < 0.05). There was no significant difference in MCHC between any of the treatments ($F_{2,17}$ = 1.95, P = 0.18).

There was a significant decrease in spleen somatic index with temperature (SSI; $F_{2,13}$ = 5.10, P = 0.02; Fig. 3.3). SSI was significantly lower at +3 °C when compared to fish from the control (30 °C; Post hoc: P < 0.05), indicating a smaller spleen size relative to fish weight at this temperature.

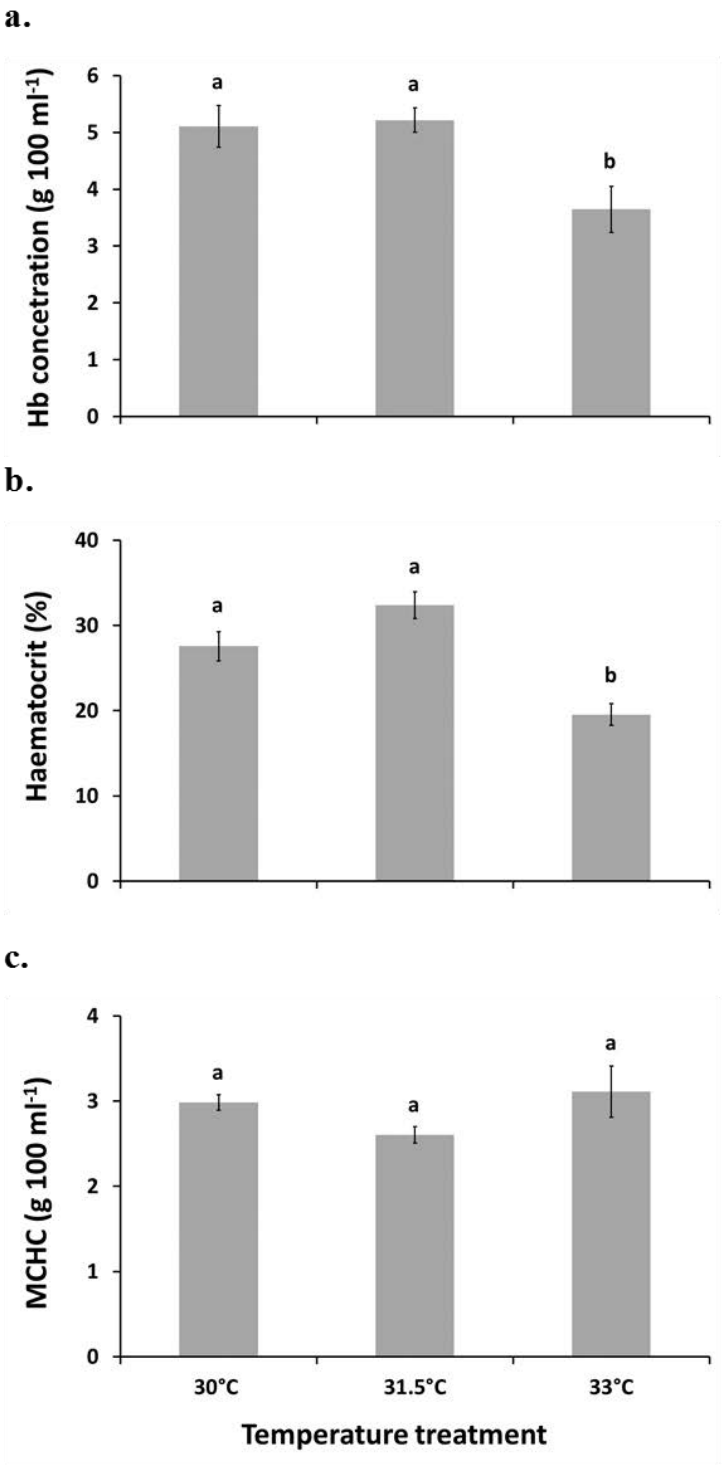


Figure 3.2. Haematological parameters for *A. polyacanthus* sampled during the summer months at current average ocean temperatures (30 °C), +1.5 °C (31.5 °C) and +3 °C (33.0 °C): (a) Haemoglobin concentration (g 100 ml^{-1}), (b) haematocrit (% packed red blood cells to total blood volume), and (c) mean cell haemoglobin concentration (MCHC, g 100 ml^{-1}). Letters indicate significant differences among treatment groups.

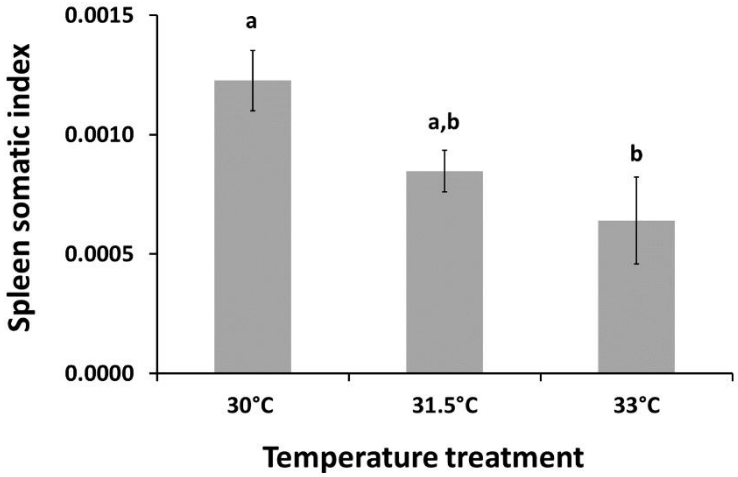
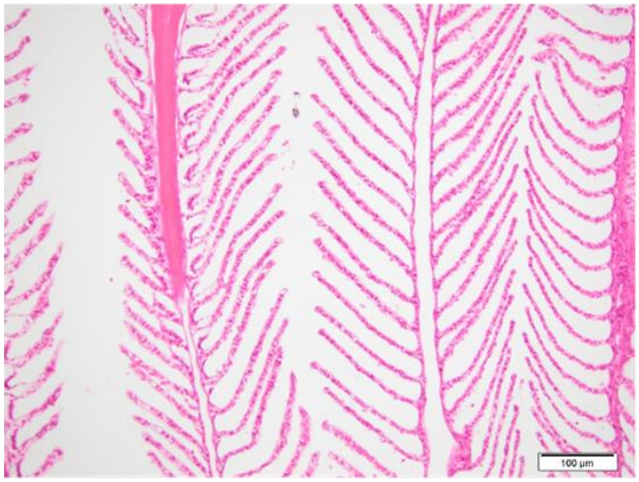


Figure 3.3. Spleen-somatic index (means ±SE) for *A. polyacanthus* sampled during the summer months at current average ocean temperatures (30 °C; n = 5), +1.5 °C (31.5 °C; n = 8) and +3 °C (33.0 °C; n = 3). Letters indicate significant differences among treatment groups.

Histological analysis

Aneurysmal dilations were the most striking feature in the gill tissue, particularly at higher temperatures (Fig. 3.4). The number and diameter of aneurysms increased with increased water temperatures. This trend was significant for the number of aneurisms per standardised section of gill arch ($F_{2,20}$ = 4.67, P < 0.05; Fig. 3.5a), as there were significantly more aneurisms per gill arch in fish from the warmest treatment temperature (33 °C) when compared to control fish (Post hoc: P < 0.05). The number of aneurisms in the gills from fish maintained 31.5 °C was highly variable, ranging from between 2 to 178 aneurisms per gill arch, and the mean was not significantly different than that of either the control or 33 °C treated fish (Post hoc: P > 0.05 in both cases). Although not significant ($F_{2,17}$ = 3.50, P = 0.056; Fig. 3.5b), there was a trend for the mean width of the aneurisms to increase with increasing temperature. In addition to the quantified data, it was apparent that fusion of dilations and concentric fibrin formation encircling the endothelium (recanalization) were common in fish reared at 33 °C, which is indicative of chronicity. Other commonly noted gill abnormalities such as specific cell proliferations, mucus, or inflammation, however, were not apparent in any of the samples.

a.



b.

c.

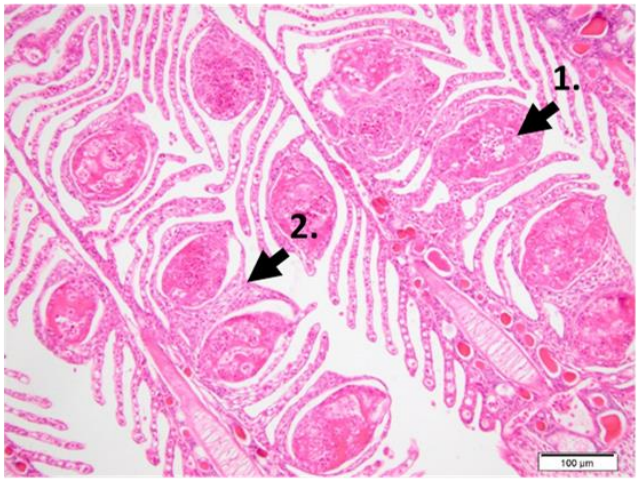


Figure 3.4. Micrographs of *A. polyacanthus* gill sections following prolonged exposure to a) average ocean temperatures (30˚C) for where fish were collected, b) +1.5 ˚C (31.5 ˚C), and c) +3 ˚C (33.0 ˚C). Images are 200x magnification, and scale bars in the lower right corner of each image are 100µm. Fibrous remodelling / re-canalization of aneurisms in the +3 ˚C treatment group are indicated by arrow 1, and enlargement and fusion of secondary lamellae are indicated by arrow 2.

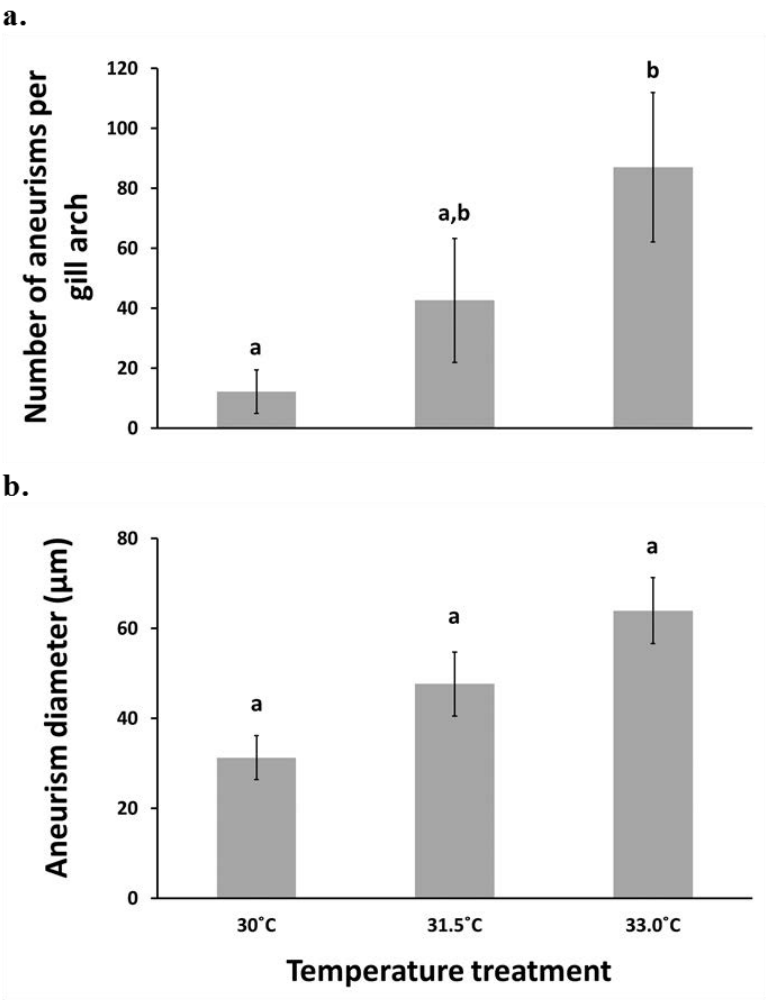


Figure 3.5. Frequency (a) and size (b) of gill aneurisms (means ±SE) for *A. polyacanthus* sampled during the summer months at average ocean temperatures (30 °C; n = 6) for where fish were collected, +1.5 °C (31.5 °C; n = 7) and +3 °C (33.0 °C; n = 7). Letters indicate significant differences among treatment groups.

3.5 Discussion

Elevated temperatures affected each physiological and morphological trait considered in this study, but trends in performance were not consistent across all indicators of fitness. While both aerobic scope and haematological measures revealed a decline in performance after temperatures exceeded 31.5 °C, gill histopathology began to show a trend towards declining performance at this temperature. Performance thresholds in aerobic capacity and haematological parameters were similar to mortality thresholds observed in previous research (Rummer et al. 2014a; **Chapter 2**). Results from

histopathology however, aligned more closely with mortality thresholds observed when a secondary stressor (e.g. maximal exercise) was introduced in previous studies of *A. polyacanthus* at low-latitudes (**Chapter 2**). Post exercise, these studies showed a significant increase in mortality at 31.5 °C. Of the measures examined, morphological changes in the gills appeared to provide the first indication of a decline in fish health and were subsequently the best gauge of thermal tolerance from the measures considered for this low-latitude population.

Elevated temperatures of 33 °C resulted in a significant decline in the aerobic scope of *A. polyacanthus*. Aerobic scope was maximised at 31.5 °C, which is 1.5 °C above the average summer temperature experienced by this population. When temperatures were increased by 3 °C above the average summer temperature (33 °C), aerobic scope was significantly reduced to the lowest level observed over all testing temperatures. Previous work on this population associated significant (>50%) mortality with this drop in aerobic performance at 3 °C above average summer temperatures (**Chapter 2**). Based on measurements of aerobic scope alone, it appears that fish performance was maximised 31.5 °C. However, this may be a best-case scenario for this population as **Chapter 2** also showed that when a secondary stressor (e.g. short periods of maximal exercise) was imposed on fish living at 31.5 °C, mortality rates significantly increased. This suggests that there may be attributes other than aerobic scope that respond to elevated temperatures before a decrease in aerobic scope is observed, and these could provide an earlier indication of temperature stress, before significant mortality is experienced.

When gill histopathology was considered, gill health at +1.5 ˚C was not found to be significantly different from either the +0 or the +3 ˚C temperature treatments. This indicates that performance at +1.5 ˚C was more similar to performance at +3 ˚C for measures of gill health than for measures of aerobic performance, potentially providing an earlier indication of a decline in fish health. Gill histopathology did however reveal some surprising trends. Typically when analysing damage to gill tissue, gill health will be ranked based on a predetermined set of stages (e.g., Flores-Lopes & Thomaz 2011; Salamat et al. 2013), but these stages of degradation were not found in the current study. Primary and secondary lamellae did not show common, less severe

histopathological changes such as specific cell proliferations, mucus, or inflammation, however aneurismal dilations were present. An aneurysm is an irreversible change, so any gills with this feature would ordinarily be classified as in an advanced stage of damage. This is the first time to our knowledge that gill aneurysms have been observed in response to temperatures above a species' optimum. Aneurysmal injuries are common in response to pollutants such as pesticides or heavy metals (van den Heuvel et al. 2000; Cengiz & Ünlü 2002; Simonato et al. 2008; Oliva et al. 2009), which is unsurprising as the gills are a first point of contact with pollutants. One review suggests that an increase in plasma cortisol and catecholamines in response to stress may contribute to gill tissue degradation in some instances of pollutant exposure (Wendelaar Bonga 1997). Whilst this may be a contributing factor, we expect that oxygen deficiency and related increases in blood pressure are likely to be the primary cause of aneurysms observed in our study.

Although there has been no previous evidence of aneurysmal injury to gill tissue in response to temperature stress, past research has described significant changes in gill structure (namely surface area) in response to hypoxia and temperature stress for a number of fish species (Sollid et al. 2005; Sollid & Nilsson 2006; Nilsson 2007; Mitrovic et al. 2009; Tzaneva et al. 2011). The population of *A. polyacanthus* examined in the present study are from a stable thermal environment with no evidence for regular changes in oxygen availability. Based on this, morphological gill remodelling may not be expected, at least for discrete genetic subpopulations occupying low latitudes. Bowden et al. (2014) tested this by taking measurements of the secondary lamellae from *A. polyacanthus*, as well as four other closely related coral reef fish species collected in Papua New Guinea. Fish were exposed to increased temperatures for periods of 12-14 days, but overall no significant relationship between increased temperatures and changes in gill morphology were observed. Remodelling is energetically costly and so it could be expected that many species show limited flexibility in this trait. No capacity to remodel the gills in order to optimise oxygen uptake may mean that fish are unable to cope with extended periods of temperature stress, possibly leading to the occurrence of aneurysms in the present study. Evidence for repair (recanalization) of aneurysms

was indicative of chronicity, suggesting that insufficient uptake of oxygen had been an ongoing problem at higher temperatures.

Similarly to measures of aerobic scope, elevated temperatures of 33 °C resulted in a significant decline measures of [Hb] and Hct for the *A. polyacanthus* population considered in this study. Decreases in [Hb] and Hct at 33 °C indicate that there was either a decrease in the number of RBCs in circulation, a decrease in the size of the RBCs, or possibly a decrease in the Hb per cell. The former is supported because there was no change in MCHC between treatments. Four possible causes for this decrease in [Hb] and Hct were considered. The first was that RBCs were being reabsorbed by the spleen. This was thought to be unlikely, as it would restrict the transportation and uptake of oxygen throughout the fish, a response that would be counterproductive during stress. The spleen also showed a significant decline in relative mass with increasing temperatures, indicating that it is more likely that the spleen released more RBC into circulation, rather than reabsorbing them (Wells et al. 1989). The second possible explanation was that an increase in the volume of the primary circulation occurred, possibly through water being pushed into circulation from muscle tissue. This scenario was also deemed to be unlikely as marine fish typically dehydrate during stress (Wendelaar Bonga 1997). Higher temperature reduces the viscosity of the blood and so potentially reduces pumping costs (Graham & Fletcher 1983; Randall & Brauner 1991). Hct and Hb could therefore have been reduced as the result of a temperature-driven viscosity effect, however again the decline in SSI does not support this proposition as spleen relative mass should not be affected by a change in blood viscosity. The final possibility, and the hypothesis considered most likely in this study, was that RBCs were being re-distributed into the secondary vascular system (SVS), which normally contains only plasma (Kampmeier 1969).

The SVS was initially thought to function in a similar way to the mammalian lymphatic system (Kampmeier 1969; Yaniv et al. 2006; Isogai et al. 2009), however further studies have challenged this hypothesis, providing evidence of a connection to the arterial system and thus describing the vessels as a secondary vascular system in form and function (Vogel 1981; Vogel & Claviez 1981; Steffensen & Lomholt 1992). Jensen et al. (2009) and Rummer et al. (2014b) both show that during exercise and in some cases

hypoxia, RBCs are able to pass from the primary vascular system (PVS) to the SVS. Normally, the input vessels from the PVS to the SVS are too small to allow RBC to pass through under resting conditions, but Rummer and colleagues (2014b) suggest that the anastomoses open during stressful conditions thus permitting RBCs to flow into the SVS. The current study is the first to suggest that elevated temperatures can also trigger this pathway. Benefits of allowing RBCs into the SVS may include reducing pressure on the heart, buffering ionic or osmotic changes in the PVS, or enhancing oxygen uptake across the skin (cutaneous respiration); all of which would assist in the event of thermal stress (Rummer et al. 2014b). The mechanism that causes vascular relaxation and allows RBCs to pass into the SVS is likely related to increased production of nitric oxide (NO), which is catalysed by nitric oxide synthase (NOS: Jensen et al. 2009). Numerous physiological and pathophysiological processes have been linked to NO, and NO production has been shown to increase during stress (Bolli 2001). Although much of the research to date on NO and associated enzymes has been on mammalian models, a review of studies on ectothermic vertebrates indicates that NO has evolved as a major cardiac modulator in these species also; although some of the enzymes involved may differ slightly (Imbrogno et al. 2011). NO could potentially link to a number of important processes involved in dealing with temperature stress, and therefore warrants further investigation.

Each of the measures examined in this study relate to the uptake and transport of oxygen, however no one measure provides a definitive causal mechanism for the fish mortality at higher temperatures recorded for this population by Rodgers et al. (**Chapter 2**). In some fish that died prior to metabolic testing, rupturing of gill aneurysms was predicted to be the mechanism driving mortality because, in several cases, bleeding from the gills was observed at time of death. This symptom was not seen for all fish however and so was thought to provide cause of death in only some instances. It is possible that another mechanism, not examined in this study, could provide a more definitive early indication of declining fish health under temperature stress. A growing body of evidence suggests that this mechanism may be heart function (Farrell 2002; Farrell 2009; Muñoz et al. 2014). Limitation of maximum heart rate, resulting in ischemia injury through deficit of oxygen to the myocardial tissue (myocardial hypoxia) is commonly cited as the mechanism driving heart failure (Farrell 2002; Farrell 2009;

Muñoz et al. 2014). Heart rate fails to meet oxygen demand in the tissues when a higher volume of oxygen is extracted from the venous blood supply by the skeletal muscle before it can reach the heart, subsequently causing an oxygen deficit leading to arrhythmia and bradycardia (Farrell 2002). In some cases this decline in heart function has been observed to precede a decline in aerobic scope (Farrell 2009). Myocardial hypoxia could be further exacerbated or preceded by cardiac mitochondrial dysfunction, as mitochondrial cells in the heart have also been shown to suffer damage due to heat stress, ischemic damage, and oxidative stress, particularly in species from a thermally stable environment (Iftikar & Hickey 2013; Iftikar et al. 2014). A cascade of physiological symptoms could result from this central problem of heart failure, including some of those reported in this study. If the heart is working harder to uptake more oxygen from the gills, it may follow that gill tissue is unable to support the increased blood pressure as the heart attempts to meet oxygen demand, resulting in symptoms such as the aneurysms observed here.

Regardless of the causal mechanism of fish death, slight differences in thermal performance across the measures examined in this study provided support for the multiple optima hypothesis. A decline in aerobic scope was not the primary indicator of declining organism health at temperatures above the thermal optimum of *A. polyacanthus* examined in this study, and thus the OCLTT hypothesis was not supported in this instance. There is now a growing body of literature that provides examples of situations where the OCLTT hypothesis is not applicable (Clark et al. 2011; Clark et al. 2013; Donelson et al. 2014; Gräns et al. 2014; Norin et al. 2014). One of the reasons why this hypothesis may fail is that the measuring of oxygen consumption as an indirect method of estimating adenosine triphosphate (ATP) production and is likely to provide only a partial representation of energy metabolism (Brand 2005; Salin et al. 2015; Schulte 2015). Whilst oxygen consumption can provide a good indicator of flux through the mitochondrial electron transport chain, it is a less reliable indicator of the amount of ATP produced per unit of oxygen consumed, as this conversion can vary significantly dependant on a range of individual and environmental factors including temperature (Schulte 2015). Elevated temperatures can disrupt the stability (proton leak rate) of mitochondrial membranes, reducing the amount of ATP produced per unit of oxygen

consumed (Salin et al. 2015; Schulte 2015). It follows that this effect may be particularly detrimental to interpretation of metabolic data when temperatures are elevated at or around the region of an organism's thermal limits. Temperature can also control the cells demand for energy and an organism must balance the efficiency of ATP production with the potential for oxidative stress and the production of reactive oxygen species, which is higher at more efficient levels of oxygen conversion (Salin et al. 2015). The way in which an organism navigates such trade-offs may differ over time as its demand for energy changes with food availability or life history stage (Salin et al. 2015).

Conclusions

Elevated temperatures have wide ranging implications for the fitness of low-latitude fish populations, and therefore the capacity for acclimation and adaptation will be essential for these fish populations to persist under future climate change scenarios. Previous work has already given evidence for a low capacity for reversible acclimation both in this population specifically, and in other populations of coral reef fish (Nilsson et al. 2009; Nilsson et al. 2010; Gardiner et al. 2010; Rummer et al. 2014a; **Chapter 2**), but further studies should examine this capacity over multiple generations. It is also important to note that different life stages may be affected differently by thermal stress, and so impacts over various ontogenetic stages should also be considered. Our study shows that for the population considered, temperature driven oxygen limitation has serious implications for fish health, and may ultimately be the cause of increased mortality at higher temperatures. The gills were the first of the organs considered to display these effects. The issue of considering multiple fitness measures is complex and would clearly benefit from further research. Our study has shown that by expanding the number of measures used to quantify the physiological impacts of elevated temperature, a much better understanding of how an organism may respond to a stressor can be obtained. Based on our results we suggest that studies using single response measures should be cautiously interpreted in a longer term climate change context.

Chapter 4: Thermosensitive period of sex determination in the coral reef damselfish *Acanthochromis polyacanthus* and the implications of projected ocean warming

This chapter was prepared for submission to *Coral Reefs*

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4.1 Summary

For many species that possess thermosensitive sex determination, warming associated with climate change has the potential to significantly alter the balance of gender in the population. Whether or not environmental temperature effects sex determination is determined by the both the absolute temperature and when during development thermal conditions are experienced. We explored the importance of exposure timing during early development for the coral reef fish, *Acanthochromis polyacanthus*, by increasing water temperature 1.5˚C or 3˚C above the summer average (28.5˚C) at different stages of development. We also measured the effect of treatment temperature on fish size and condition, in order to provide a gauge of how the thermal threshold for ESD may compare with thresholds for other commonly considered physiological metrics. Increasing grow-out temperature from 28.5 ˚C to 30 ˚C had no effect on the sex ratio of offspring, however an increase to 31.5 ˚C produced a strong male bias (average ~90%). The thermosensitive period for this population lasted up to 60 days post-hatching, with the bias in sex ratio greater the earlier that fish were placed into warm conditions. These results suggest that average summer temperatures will need to increase by ~3˚C before there is an effect on sex ratios in this population. Temperatures high enough to bias the sex ratio are likely to be seen first during late summer (January and February) and would affect clutches produced late in the breeding season. There was no change to fish condition in response to the temperature, however the two higher temperature treatments produced significantly smaller fish at sampling, indicating that other physiological effects of temperature may be observed at lower temperatures. Clutches produced early in the season could buffer the population from

a skewed sex ratio, as their development will remain below the thermal threshold; however, continued ocean warming could mean that clutches produced earlier in the breeding season would also be affected in the longer-term. A skewed sex ratio could have negative impacts by reducing the number of females in the breeding population.

4.2 Introduction

Environmental sex determination (ESD) is a widespread phenomenon, most commonly documented among reptiles, amphibians and fish (Charnov & Bull 1977; Janzen & Paukstis 1991; Baroiller & D'Cotta 2001; Angelopoulou et al. 2012). Organisms that display ESD have the potential to develop into either sex, but environmental factors can affect either the determination of sex during embryogenesis or the differentiation of the testis or ovaries from the undefined gonad later in life (Hayes 1998; Baroiller & D'Cotta 2001). In species where ESD occurs during embryogenesis, incubation temperature, maternal physiology, or cytoplasmic environment may act on the genes directing sex determination (Korpelainen 1990; Baroiller & D'Cotta 2001). When the ESD influences differentiation, a primary sex may be predetermined at fertilisation, but whether or not offspring develop into that sex is dependent on the presence or absence of an environmental cue that occurs during development (e.g. social sex determination; Korpelainen 1990; Baroiller & D'Cotta 2001). Causes, timing and factors involved in ESD vary both between and within taxa, especially in nematodes, crustaceans and reptiles (Korpelainen 1990). Environmental factors responsible for ESD are diverse and may include changes in temperature, pH, photoperiod, nutrition, parasite loading or the presence of conspecifics, to name a few (Korpelainen 1990).

Climate change related temperature increases could potentially drive gender bias in the future due to ESD (Janzen 1994; Hawkes et al. 2007; Laloë et al. 2014). Some organisms have developed ESD as an adaptive response to situations where environment affects the relative fitness of the sexes differently (Charnov & Bull 1977; Conover 1984). Other organisms that do not gain any adaptive advantage from ESD typically only display a gender bias when temperatures are outside of their normal thermal range and are likely to have a greater genetic affect on sex ratio (Charnov & Bull 1977). In these species, increased temperatures associated with climate change could create a gender bias

away from the species' optimal sex ratio, causing detrimental effects on the population by reducing the effective breeding population (Milner-Gulland et al. 2003; Wright et al. 2012).

Temperature is the most prevalent environmental determinant of gender in vertebrates, including many fish species, and one of the most important environmental influences on biological rates and processes generally (Ciofi & Swingland 1997; Baroiller & D'Cotta 2001, Korpelainen 1990). The most common response in fish to increasing environmental temperature is an increase in the male to female ratio (Baroiller et al. 1999; Ospina-A´lvarez & Piferrer 2008). This trend has been observed in a wide range of freshwater and marine species (reviewed in Baroiller & D'Cotta 2001). Less common is an increase in female to male ratio with rising temperature (Craig et al. 1996; Patiño et al. 1996), or the production of either monosex male or female populations at high and low temperature extremes with a balanced ratio in-between (Yamamoto 1999). In fish, these less common trends may be artefacts of other, non-temperature related impacts on sex ratio (Ospina-A´lvarez & Piferrer 2008).

Changes in sex ratio that occur gradually with changing temperature are often associated with what is known as "pure" temperature sex determination; where the sex of an individual is indifferent until determined by incubation temperature post-conception (Bull 1983). In organisms where a gender bias is only observed outside of the normal thermal range, a genotype x environment (G X E) interaction is expected to be more likely (Charnov & Bull 1977; Valenzuela & Lance 2004). These G X E interactions occur due to an instability in genetic sex determination caused by temperatures outside of a species' normal thermal range (Valenzuela & Lance 2004). A range of mechanisms have been proposed linking temperature to sex ratio for various taxa. Navarro-Martin and colleagues (2011) proposed an underlying epigenetic mechanism to sex ratio bias, showing that elevated temperatures increase DNA methylation of the aromatase promoter, preventing aromatase gene expression and subsequently resulting in male-biased sex ratios. Ramsey and Crews (2009) suggest that estrogen production is inhibited at male-producing temperatures, leading to the absence of aromatase and upregulation of testis-specific genes.

For a shift in sex ratio to occur, increased temperatures must be present at a specific time during development, known as the thermosensitive period (TSP). The timing and duration of this period can vary between taxa and species. In fish, the TSP sometimes occurs within the embryonic period (Wang & Tsai 2000), but more commonly precedes or coincides with the end of the larval period, before the onset of gonad differentiation (Valenzuela & Lance 2004). For species where a 1:1 sex ratio occurs within the normal range of temperatures, the timing of any peaks in temperature will be critical in determining whether a change in sex ratio is observed. This is because for these species temperatures must be outside of the organism's normal thermal range during the TSP to have an effect on offspring sex ratio.

Understanding the TSP of an organism will help to predict when projected climate change associated temperature increases are likely to have the greatest effect on sex ratio. To understand the full impact of climate change related temperature increases on sex ratio, it is not sufficient to know that elevated temperatures will lead to a gender bias. Given current and projected rates of warming, only parts of an organism's reproductive season may be affected by temperatures high enough to cause a gender bias, depending on the timing of reproduction, the temperature at which ESD occurs and the length of the TSP (Yntema & Mrosovsky 1982; Schwanz & Janzen 2008).

In this study we determine the TSP for a common coral reef damselfish, the spiny chromis (*Acanthochromis polyacanthus*). This species has previously been shown to deviate from 0.5 towards a male biased sex ratio when exposed to increased temperatures within the first three months of life (Donelson & Munday 2015). We exposed juveniles to temperatures 1.5 and 3˚C higher than seasonal average temperatures at different stages of development. This allowed us to determine the period within which temperature can influence sex determination for this population. We aimed to expand on previous knowledge of ESD in this species by determining the period of plasticity for sex determination. The effect of treatment temperature on fish size and condition was also measured in order to provide a gauge of how the thermal threshold for ESD may compare with thresholds for other commonly considered physiological metrics for this species. This information will improve predictions on how projected temperature increases will effect breeding populations in the future and will

also provide an indication of the order in which some key processes may be affected by increasing temperature.

4.3 Methodology

Study species

A. polyacanthus is a brooding tropical damselfish, with complete larval development occurring during embryogenesis (Doherty et al. 1994). This coral reef fish forms monogamous pairs and breeds primarily during the summer months (October – February; Robertson 1973; Thresher 1983). Adults provide parental care to their eggs and offspring typically remain with their parents for approximately 30-45 days post hatching (Kavanagh, 2000). *A. polyacanthus* is widely distributed throughout the Indo Pacific (15°N–26°S and 116°E–169°E), as far north as the Philippines and Indonesia, through to north-eastern Australia and Melanesia (Randall et al. 1997). Previous work by Donelson and Munday (2015) has shown that conspecific density and relative size of individuals does not control sex determination in this species.

Broodstock and hatching conditions

Adult breeding pairs used to obtain offspring for this experiment were taken from breeding stock at the James Cook University Marine Aquarium Research Facility Unit, Queensland, Australia. Pairs were collected from the Orpheus Island region (18°37'06"S 146°29'37"E) on the central Great Barrier Reef (GBR) and maintained at the long-term weekly average temperature for that location (22.3-29.1 ˚C) on a seasonal cycle (Australian Institute of Marine Science sea surface temperature database; http://data.aims.gov.au/aimsrtds/datatool.xhtml?from=1980-01-01&thru=2016-01-21&period=MONTH&aggregations=AVG&channels=2107,1769). Five breeding pairs were used, and when a clutch was produced the newly hatched juveniles were removed from the parental tank and assigned to their experimental treatments within 6 hours.

Thermal effects to sex determination

Two experimental temperature treatments were tested; +1.5 ˚C (30.0 ˚C) and +3 ˚C (31.5 ˚C) above current-day summer (December) temperatures. Treatments were

chosen to represent a moderate and a high level end of century warming scenario and to allow for comparison with previous research on similar populations. A single-shift design similar to Valenzuela and Lance (2004) was used to determine the length of the TSP. Immediately upon removal from the adult tanks, each clutch was split evenly between the two temperature treatments (+1.5 and +3 ˚C) and then further distributed between four shift groups and a control group (two tanks per family, per treatment). All fish commenced rearing at the control temperature (28.5 ± 0.4 ˚C) and were then raised to their assigned temperature treatment after 0 (G0), 10 (G10), 30 (G30) or 60 (G60) days post hatching (dph). By exposing groups to elevated temperatures at different dph, it was possible to determine the length of the TSP. An additional control group from each family remained at the population's mean summer temperature (28.5 ˚C) for the entire duration of the experiment in order to measure the sex ratio at present-day temperatures. For the purpose of analysis this control group was identified as G90. On each shift day a small sample (n = 2-4) of juveniles was taken from each family and euthanised by an overdose of seawater and clove oil mixture, then fixed in 4 per cent phosphate-buffered formaldehyde solution in preparation for length and weight measurements. At 90 dph the gender of all fish was determined by the shape of the external urogenital papilla using a dissection microscope, the number of males and females were recorded and a sample was again taken for weight and length analysis (Donelson & Munday 2015). Throughout the duration of the experiment fish survival averaged 93%, or 97% within the experimental period (from shift day to day 90), after accounting for sampled fish.

Length and weight measurements

Sampled juveniles were removed from the fixative within 48 h of preserving, blotted dry and then weighed to the nearest 0.01 g. Juveniles were photographed in a lateral position against a scale grid (Canon PowerShot G16). Standard length (SL) was estimated for each fish from the digital photograph using image analysis software (ImageJ). SL was estimated three times and an average of the three values was taken for statistical analysis.

Data analysis

Differences in the proportion of males produced among temperature treatments and shift days were compared using a generalized linear model (GLM) with a logit link function and binomial distribution. Differences in sex ratios were compared between treatment groups and against the present-day control group (G90), with family included as a covariate. The goodness of fit (dispersion) was checked by examining Pearson residuals prior to analysis. GLM were completed using SPSS (IBM, Armonk, USA).

To determine whether grow out temperature had a cost to growth and body condition, the relationship between SL and weight was plotted for fish that were maintained at the control (28.5 ˚C), 30 ˚C and 31.5 ˚C treatments for the full 90 days. A power function was fitted to each group as this best described the relationship between standard length and weight. Fish weight and SL were both logged for statistical analysis and the effect of SL and control temperature on fish weight was analysed with an ANCOVA, with treatment as the grouping variable and log weight dependent on log SL. The average end length and weight were also compared between grow out temperatures using a one-way factorial ANOVA with temperature treatment as the fixed factor. A Fisher LSD post hoc test was used to determine differences between treatments where a significant effect of treatment temperature was found. Assumptions for both tests were examined with residual analysis prior to interpretation and homogeneity of slopes was tested prior to the ANCOVA. All ANOVA and ANCOVA statistical tests were carried out using Statistica (StatSoft Inc., Tulsa, USA).

4.4 Results

The proportion of males present was influenced by a significant interaction between testing temperature and shift day (Wald = 109.74, df = 9, $P < 0.001$; Fig. 4.1). Fish reared at 30 ˚C did not exhibit a change in sex ratio compared with the control (G90) and produced close to a 1:1 sex ratio, regardless of shift day ($P > 0.05$ in all cases). In contrast, fish reared at 31.5 ˚C produced a significantly higher proportion of males than the control for shift days G0 ($P < 0.001$; ~90% male), G10 ($P < 0.001$; ~80% male) and G25 ($P < 0.001$; ~70% male). Fish shifted at G60 did not exhibit a sex ratio significantly different from the control group ($P = 0.44$). Family did not significantly influence the sex

ratio of *A. polyacanthus* offspring in this study with all families possessing similar gender bias for a given shift time (Wald = 2.20, df = 1, P = 0.14).

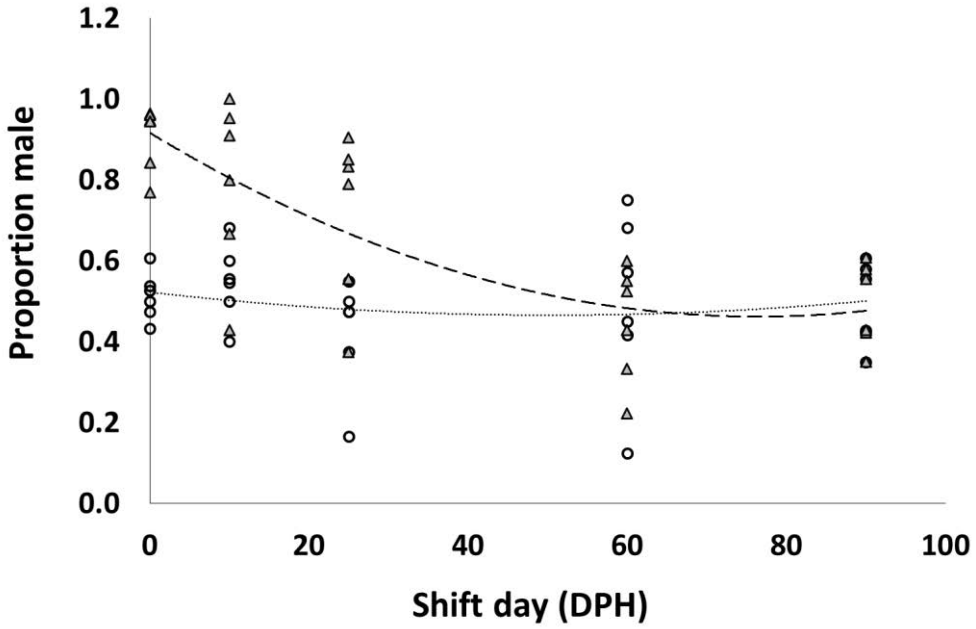

Figure 4.1. Proportion of males in the 30 ˚C (open circles) and 31.5 ˚C (closed triangles) temperature treatments in relation to shift day. Each point represents the mean proportion of males within one family group for a given temperature treatment and shift day. Polynomial curves have been fitted to the data as they best describe the relationship between proportion male and shift day.

For fish grown out at each of 28.5, 30 and 31.5 ˚C for the full 90 days, treatment temperature had a significant effect on average final fish weight (28.5 = 1.21 ± 0.07 g, 30 = 0.88 ± 0.07 g, 31.5 = 0.86 ± 0.06 g; $F_{2,105} = 20.96$, $P < 0.001$) and length (28.5 = 33.99 ± 0.80 mm, 30 = 29.96 ± 1.08 mm, 31.5 = 29.71 ± 0.78 mm; $F_{2,105} = 22.49$, $P < 0.001$). Fish that developed at 28.5 ˚C were significantly heavier and longer than those that developed at either 30 or 31.5 ˚C (Post hoc: $P < 0.001$ in all cases), but there was no significant difference in fish weight or length between the two higher temperature treatments ($P>0.05$). Despite the differences in final weight, there was no effect of treatment on body condition at 90 days, i.e length to weight relationship (Fig. 4.2; $F_{2,104} = 0.12$, $P = 0.87$).

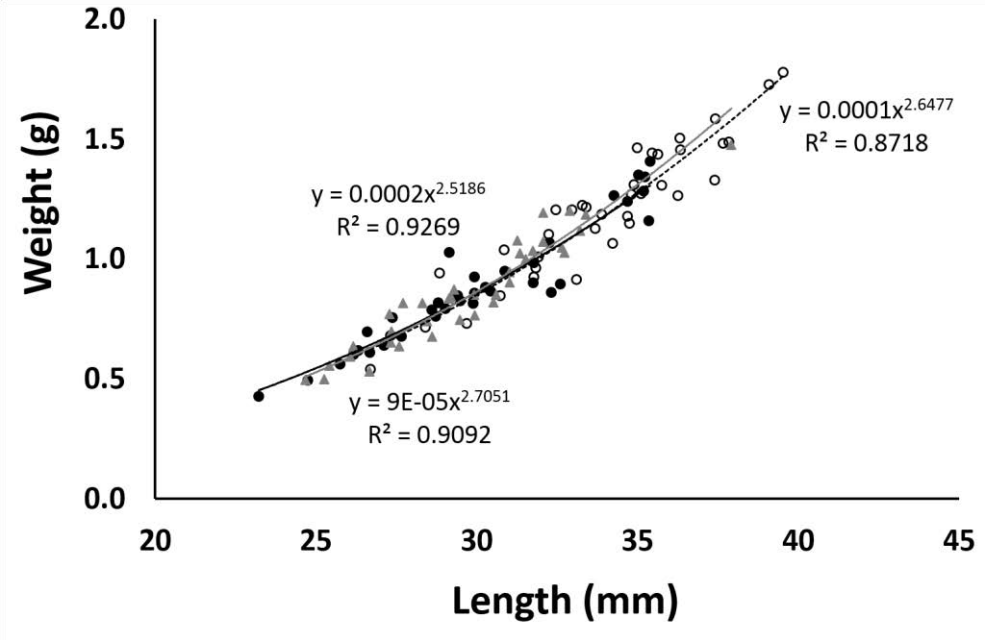

Figure 4.2. Weight of fish after 90 days in present-day (open circles, dashed black line of best fit), 30 ˚C (closed circles, solid black line of best fit) and 31.5 ˚C (triangles, solid grey line of best fit) temperature treatments. Lines of best fit are power functions**.**

4.5 Discussion

For the population of *A. polyacanthus* considered in this study, an increase in grow-out temperature to 30 ˚C did not cause the sex ratio of offspring to differ significantly from the control; however, when temperatures were further increased to 31.5 ˚C a significant increase in the proportion of males was observed. The TSP persisted until 25-60 DPH and fish that experienced increased temperatures from 60 DPH onwards possessed a normal sex ratio. Whilst no change in sex ratio was seen for fish that developed at 30 ˚C, this temperature did have a significant impact on fish weight, with fish that developed at higher temperatures for the full 90 days being lighter than controls.

Both this study and Donelson and Munday (2015) found an increase in the proportion of males produced at 31.5 ˚C. Although this temperature is well above this study population's average summer temperature, it could be expected to occur regularly by the end of the century. Average daily temperatures for this population exceeded 30 ˚C on 7.02% of days during summer for the collection location and maximum daily temperature was greater than 30 ˚C on 16.06% of days. On no occasions did

temperatures reach or exceed 31.5 ˚C in records dating back over the last 10 years (excluding 2007 for which no data was recorded; AIMS sea surface temperature database; Fig. 4.3). The peak spawning period for *A. polyacanthus* occurs during the summer months (October – February; Robertson 1973; Thresher 1983) and the later part of the spawning season (January/February) corresponds with maximum temperatures experienced by this population (Fig. 4.3). For this reason, under future climate conditions clutches hatched towards the end of the breeding season, in January and February, are likely to be the first to experience the effects of temperature on sex ratio, with a bias towards the production of males. Temperatures at this time of year would need only to increase 2-3 ˚C above the current average to produce a sex ratio bias of up to 90 % males. Figure 4.3 indicates the portion of the breeding season likely to be effected under a range of future warming scenarios, from conservative to extreme. If temperatures were to increase by 4 ˚C, almost the entire breeding season would be effected by a strong male bias. Even though at present day temperatures the months of November and December are generally cooler, our results suggest that clutches produced at this time could still be vulnerable to biased sex ratios in the future under temperature increases of only 2-3 ˚C, because the TSP could extend up to approximately 60 DPH. However, the sex ratio bias decreased with increasing time between hatching and temperature increase, and therefore, any sex ratio effects would likely be less in juveniles hatched earlier in the season. Consequently, clutches produced early in the season may initially provide a buffer at the population level were operational sex ratio, or the ratio at which sufficient numbers of males and females are available to reproduce, is maintained despite higher temperatures creating a biased sex ratio towards the end of the breeding season.

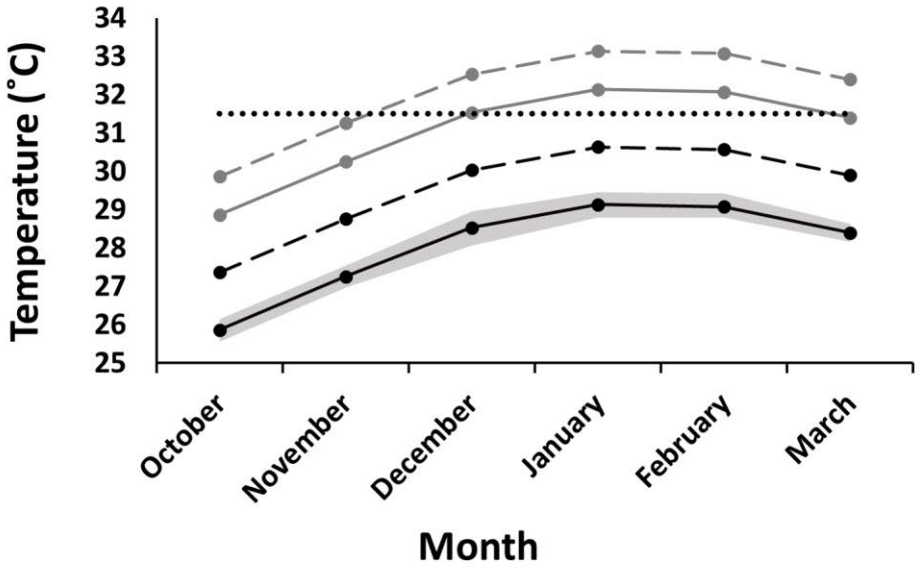

Figure 4.3. Average daily temperatures for the central Great Barrier Reef collection location (solid black line), +1.5 ˚C (dashed black line), +3 ˚C (solid grey line), and +4 ˚C (dashed grey line). Average maximum and minimum range for current average temperatures is shaded. The dotted black line indicates 31.5 ˚C, the temperature at which a significant male bias in the population is observed. Data was obtained from the Australian Institute of Marine Science (AIMS) sea surface temperature database; http://data.aims.gov.au/aimsrtds/datatool.xhtml?from=1980-01-01&thru=2016-01-21&period=MONTH&aggregations=AVG&channels=2107,1769 for Orpheus Island, Cattle Bay, data loggers 2 and 4 at a depth of 6.5 m.

No increase in the proportion of males was observed for offspring grown out at 30 ˚C in this study, regardless of when exposure occurred. In contrast, Donelson and Munday (2015), did see an increase in the proportion of males produced at 30 ˚C, but the bias in sex ratio was not as strong as when fish were reared at 31.5 ˚C (72% males produced at 31.5 ˚C compared with 66% at 30 ˚C). The borderline nature of the 30 ˚C treatment group in terms of the thermal range experienced by these populations may explain why this difference between studies has occurred, as even slight differences in thermal tolerance between parental populations would be more likely to drive a difference in response where temperatures are at a tipping point. Previous work also found that parents which developed at 30 ˚C for one generation were able to completely restore a

50/50 sex ratio via transgenerational acclimation, whilst those developed at 31.5 ˚C only achieved partial restoration even after two generations (Donelson & Munday 2015). This further demonstrates the borderline nature of the 30 ˚C temperature treatment, as well as the significant consequences of the 31.5 ˚C treatment.

The TSP observed in this study falls close to or within the range of TSP previously recorded for a large number of other fish species. TSP can be highly variable, however a large body of literature has recorded a strong effect of temperature on sex ratio from approximately 10 days to 1 month post fertilisation on a range of species including, but not limited to, cichlids (*Oreochromis niloticus*; Baroiller et al. 1995), channel catfish (*Ictalurus punctatus*; Patiño et al. 1996), silversides (*Odontesthes bonariensis* and *Patagonina hatcheri*; Strüssmann et al. 1997; Baroiller & D'Cotta 2001) and tilapia (*Oreochromis niloticus*; Baroiller & Toguyeni 1996). This critical period is also when the greatest influence of temperature on sex ratio was observed for *A. polyacanthus* in the present study. Other species such as the Atlantic silverside (*Menidia menidia*) have been reported as having a much longer TSP, up to 111 days (Conover & Fleisher 1986), however these longer periods of TSP are not as common in the literature and may be an artefact of experimental conditions. TSP may vary with temperature increase or decrease experienced, and greater temperature manipulations are likely to affect sex ratio at times that smaller changes in temperature will not (Mrosovsky & Pieau 1991).

Our results suggest that either the earliest stages of development are the most plastic in terms of sex ratio (Römer & Beisenherz 1996; Wang & Tsai 2000; Koumoundouros et al. 2002), there is a cumulative effect of increased temperature on sex ratio (Bull & Vogt 1981; Koumoundouros et al. 2002) or that a combination of both is occurring in this population (Koumoundouros et al. 2002). Fish that were transferred to the highest temperature treatment at birth and therefore spent the longest amount of time in warmer temperatures produced a strongly biased sex ratio, with an average of 90% male offspring. Percentage of male offspring decreased by ~10% with subsequent shift days G10 and G25 and was reduced even further at G60 to a level not significantly different from the control. Further studies in which a shift-twice design is implemented and fish are moved to a higher temperature at a specified point in development and then back down to control temperatures after a period of exposure to the treatment

temperature could help to further define whether timing or length of exposure is more important for the fish species tested in this study (Valenzuela & Lance 2004). None of the observed effects on sex ratio are expected to be due to selective mortality (Réale et al. 1996; Torres & Drummond 1997; Tecot et al. 2013; Székely et al. 2014)., as >90% survival was observed for all groups in this study

Whilst there was no change to fish condition in response to the temperature treatments tested, a significant effect on fish size at sampling was recorded within the two higher temperature treatments, producing significantly smaller fish. Decreased growth in juvenile *A. polyacanthus* at 30 ˚C, where there was no effect on sex ratio, provides evidence that different thermal thresholds exist for various biological processes, and that these processes will be affected by different levels of projected warming. The 30 ˚C temperature treatment had no effect on sex ratio in this study, however there was significant negative effect on weight of *A. polyacanthus* grown out at this temperature for the full 90 days, equal to that seen at 31.5 ˚C. This finding is similar to that of Munday and colleagues (2008b) who also saw a trend towards reduced growth of *A. polyacanthus* at higher temperatures, however the effect of temperature is stronger in this study, possibly because of the earlier life stage considered. At the temperatures expected to influence sex ratio for this species an additional direct threshold to reproductive output has also been observed, with a cessation of reproduction observed after two generations spent at 31.5 ˚C (Donelson & Munday 2015). It follows that if temperatures increase to 31.5 ˚C, reproduction in this population will be affected both by a reduction in the effective breeding population and an inability of fish to reproduce at these temperatures. Other physiological effects of temperature may however be observed at lower temperatures, as the reduction in fish size at 30 ˚C shows in this study.

This study has shown that by the end of the century, with 3 ˚C warming, biased sex ratios could be seen in offspring produced throughout the entire summer breeding season in this population of *A. polyacanthus*. Furthermore, these changes to sex ratio will occur at temperatures to which previous research has shown that fish have a limited capacity to acclimate. Donelson and Munday (2015) showed that even after two generations at 3 ˚C above current ocean temperatures, only a partial restoration of sex

ratio could be achieved, suggesting a limitation to transgenerational plasticity when developmental temperature is substantially increased. Prior to the end of the century, clutches produced early in the season could buffer the population from a skewed operational sex ratio, as their development will remain below the thermal threshold. However, if environmental warming leads to temperatures above the thermal threshold for biased sex ratios for extended periods of the breeding season there is the potential for negative effects on the population due to a decline in the number of breeding females, possibly in combination with declines in reproductive capacity of those females that remain.

Chapter 5: Estimating the capacity for low-latitude coral reef fishes to developmentally acclimate to increased ocean temperatures

This chapter was prepared for submission to *Conservation Physiology*

Authors: G. G. Rodgers, J. M. Donelson, P. L. Munday and M. I. McCormick

5.1 Summary

Low-latitude species are expected to be especially vulnerable to the effects of projected warming due to their narrow thermal range. For this reason, the ability to acclimate to higher temperatures is both extremely important and theoretically less likely for these populations. In this study we tested the capacity for developmental thermal acclimation of metabolic rate in low-latitude populations of three coral reef damselfishes: *Acanthochromis polyacanthus*, *Pomacentrus moluccensis* and *Pomacentrus wardi*. Newly settled juveniles were collected from reef locations in Torres Strait (142°20' to 142°35' and 10°31' to 10°46') and reared for 3 months at the current-day summer average (30 ˚C) and at 1 or 2 ˚C above the average (31 and 32 ˚C), consistent with climate change projections for the end of this century. Routine and maximum oxygen consumption were directly measured and subsequently used to estimate net aerobic scope for each fish at both their developmental temperature and at the two remaining temperatures after acute exposure. The effect of developmental temperature on fish weight was also considered. Ability to acclimate metabolic rate to increased temperatures differed among species, with the greatest capacity observed for *P. wardi*. For *A. polyacanthus* and *P. moluccensis* acclimation occurred at a similar temperature to what has been observed for the same species in more southern locations. When considered in relation to the summer average temperature for each location though, acclimation was found to occur within a much more limited range for northern populations. This indicates that although there is some scope to deal with climate change relevant temperature increases in low-latitude populations, they are still more vulnerable to temperature increases than their more southern counterparts.

Acclimation capacity was associated with the life history and habitat traits of each species, leading to the conclusion that these correlations could place scientists in a better position to make generalised predictions about the effects of climate change should we consider the ecological niche of the study species.

5.2 Introduction

Temperature increases projected to occur over the next century as a result of global climate change could present one of the greatest challenges to the persistence of biodiversity on Earth (Sala et al. 2000; Thomas et al. 2004). Temperature increases are expected to be greater towards the poles and less at the equator, however tropical species could be the most sensitive to rising temperatures, due to the thermally stable climate in which these organisms have evolved and the higher trajectory of change projected for these environments (Janzen 1967; Deutsch et al. 2008; Tewksbury et al. 2008, Burrows et al. 2011). Many tropical organisms appear to exist at the edge of their thermal tolerance, even at present day temperatures (Stillman 2003; Kellermann et al. 2012; Rummer et al. 2014a). Within the tropics a range of thermal environments exist and so the sensitivity of populations is likely to vary throughout. Lower latitude and equatorial populations could be more sensitive to elevated temperatures associated with climate change than populations from higher tropical latitudes, given that thermal stability generally increases towards the equator (Nguyen et al. 2011; Bowden et al. 2014; Rummer et al. 2014a; Pintor et al. 2015). Increased temperatures are expected to impose significant behavioural, ecological and physiological challenges on low-latitude populations (Walther et al. 2002). Projecting the fate of these populations with respect to climate change requires not only an understanding of how organisms will be affected by warming, but also how populations will respond to environmental change over time.

Aside from geographic re-distribution, the two primary mechanisms which may allow organisms to persist under future projected climate conditions are adaptation and acclimation (Angilletta 2009; Sunday et al. 2014). Genetic adaptation occurs through the selection of favourable genotypes over multiple generations, whilst acclimation involves changes to physiological, behavioural or morphological traits through phenotypic plasticity (Reusch 2014; Sunday et al. 2014). Acclimation has recently

become a topic of particular interest in climate change research because it does not rely on the passing on of genes from one generation to the next, therefore changes can occur over faster time scales than for genetic adaptation (Crozier & Hutchings 2014; Reusch 2014). Acclimation can either increase the breadth of temperatures within which an organism is able to function, or cause a shift in an organism's thermal optimum. The occurrence of such changes relies on a trade-off between physiological fitness and the cost of acclimation (Gabriel & Lynch 1992). The benefits of maintaining a wider thermal tolerance curve are expected to be greater in a heterogeneous environment than in a stable thermal environment and therefore the likelihood of acclimation is also predicted to be higher in these locations (Gabriel & Lynch 1992).

Adult organisms may acclimate to environmental change via short-term, regulated responses to environmental variation, referred to as reversible acclimation and commonly associated with species that live in heterogeneous environments (Angilletta 2009). Alternatively, conditions experienced during ontogeny can illicit an irreversible response to a stimulus known as developmental acclimation, which then goes on to influence performance in later life (Donelson et al. 2011a; Scott & Johnston 2012). Evidence for reversible acclimation as a mechanism for coping with climate change is variable and largely dependent on environmental heterogeneity (Pörtner 2001; Steffensen 2002; Nilsson et al. 2009; Rummer et al. 2014a; **Chapter 2**), whereas developmental acclimation has been shown to improve performance in response to increased temperatures for a wide range of animals (West-Eberhard 2003). Scott and Johnston (2012) found that zebrafish embryos exposed to temperatures at the extremes of the fish's thermal range displayed reduced thermal sensitivity in swimming performance later in life at their exposure temperature, even after long grow out periods back in common conditions. Similarly, Donelson et al. (2011a) found that damselfish reared during the first three months of life at 3 °C above average summer temperatures exhibited a smaller effect of higher temperatures on metabolic rate when compared with fish reared at control temperatures.

Reef fishes represent a large group within the ectotherms and many have wide geographical ranges, from mid-latitudes to biodiversity hotspots close to the equator (Bellwood & Wainwright 2002, Mora et al. 2003). As mentioned, populations which

inhabit environments that typically have a narrow thermal range are expected to be more vulnerable to change than those from more heterogeneous thermal environments and so low-latitude populations may be at greatest risk from climate change (Stillman 2003; Tewksbury et al. 2008; Somero 2010). Low-latitude populations have spent long periods of time evolving in thermally stable habitats and so may have lost important regulatory mechanisms, critical for dealing with change (Somero 2010), so as a result acclimation capacity may also be limited. In most tropical species observed to date, an extremely limited capacity for reversible acclimation has been observed, even after periods of as long as 10 months at higher temperatures (Hop & Graham 1995; Van Dijk et al. 1999; Pörtner 2001; Steffensen 2002; Nilsson et al. 2009; Nilsson et al. 2010; Gardiner et al. 2010; Rummer et al. 2014a; **Chapter 2**). Available research has shown that some coral reef fish populations from the central and southern Great Barrier Reef (GBR) that display no capacity for reversible acclimation can in fact thermally acclimate when exposed to increased temperatures either early in life or over multiple generations, despite their narrow thermal range (Donelson et al. 2011a,b; Grenchik et al. 2013; Donelson et al. 2014). This capacity has never been investigated for low-latitude populations of coral reef fish.

Very little of the current research on the acclimation capacity of tropical reef species extends to near equatorial populations, in fact only a few studies have been conducted at latitudes of lower than ~18 degrees and all of these take into account only reversible acclimation (Bowden et al. 2014; Rummer et al. 2014a; **Chapter 2**). This research supports the hypothesis that near equatorial populations of coral reef fish are currently living at or above their thermal optimum, and that they are more susceptible to the effects of increased temperature than southern tropical populations. In populations of reef fishes from Papua New Guinea (PNG) and Torres Strait, high levels of mortality were recorded with temperature increases of ~ 3-4 ˚C above the summer average, whereas similar increases in temperature result in decreased physiological performance, but not increased mortality in populations on the southern and central areas of the GBR (Rummer et al. 2014a; **Chapter 2**).

Metabolic performance is commonly used to estimate organism fitness when investigating acclimation capacity (Donelson et al. 2011a; Rummer et al. 2014a; **Chapter**

2). Resting or routine and maximum metabolic rates, with subsequent calculation of aerobic scope, provide an estimate of the aerobic capacity available for an organism to carry out ecologically relevant activities such as foraging, reproduction and predator evasion (Pörtner & Knust 2007). Declines in metabolic performance may occur at temperatures above those within a species normal range due to an insufficient uptake, transport, and delivery of oxygen, as the circulatory and ventilatory systems of aquatic species struggle to keep pace with increased oxygen demands at higher temperatures (Pörtner 2001; Pörtner & Knust 2007; Pörtner & Farrell 2008). In addition to the strong evidence linking changes in metabolic performance to decreased fitness at temperatures outside of an organism's thermal optimum, measurement of these traits also has the benefit of being a non-lethal study method. The ability to test a subject multiple times at different temperatures is of great advantage in climate change research and has also contributed to the popularity of this method.

The present study compares the potential for developmental acclimation of three low-latitude coral reef fish species to temperatures projected to occur by the end of this century. To do this, analysis of aerobic scope for three common damselfish species collected from Torres Strait was used to determine fitness after three months of developmental exposure to the current-day summer average temperatures (30 ˚C; control) or to 1 or 2 ˚C above the average. Comparisons of juvenile performance for fish reared at different temperature treatments and then tested at both their developmental temperature and at the remaining temperatures after an acute exposure of 7 days was used to indicate this species' capacity for developmental acclimation. If acclimation had occurred, it was expected that metabolic performance would be significantly improved in fish at their developmental temperature, when compared with the performance of an acutely exposed fish from one of the other developmental groups. It is likely that developmental acclimation would result in a reduction of baseline oxygen consumption (routine metabolism) and an increase aerobic scope (an increase in energetic potential for non-maintenance processes). Given that the results of past studies on reversible acclimation indicate that low-latitude fish are currently living extremely close to their thermal tolerance threshold, it was predicted that increased temperature may have severe effects on these populations

and that they would have a low capacity for developmental acclimation. As the species selected for this study were closely related, it was also expected that they would display similarities in their ability to acclimate to higher temperatures.

5.3 Methodology

Fish collection and treatment allocation

Three common species of juvenile damselfish (*Acanthochromis polyacanthus*, *Pomacentrus moluccensis* and *Pomacentrus wardi*) were collected for this study from two reefs, Dugong and Kagar, in Southern Torres Strait (142°20' to 142°35'E and 10°31' to 10°46'S), during December of 2013. For all fish the smallest size class possible was collected in order to maximise the likelihood of collecting fish capable of undergoing developmental acclimation. This is expected to occur within the first three months of life, based on previous studies (Donelson et al. 2011a). Fish were transported to aquarium research facilities in Townsville, Australia where they were maintained in conspecific groups of up to five fish in tanks of 40 to 60 L. During this time fish were maintained at the average summer ocean temperature for the collection location (30.0 ˚C; Australian Institute of Marine Science (AIMS) sea surface temperature database; http://data.aims.gov.au/aimsrtds/datatool.xhtml?site=921&param=water%20temperature). Fish were fed ad libitum, once per day using commercial fish pellets (INVE NRD 5/8).

After habituation to the aquarium environment (three weeks), all fish were randomly assigned to one of three temperature treatments. Temperatures were adjusted to the target temperature over a 7-day period (0.2 - 0.3 ˚C per day; 43 – 66 fish per developmental treatment, per species). The three temperature treatments were: 1) current average summer ocean temperatures for the collection locations as determined from the AIMS sea surface temperature database (control; +0 ˚C; 30.0 ˚C); 2) 1 ˚C higher than current average ocean temperatures (+1 ˚C, 31.0 ˚C); and 3) 2.0 ˚C higher than current average ocean temperatures (+2 ˚C, 32.0 ˚C). The treatment temperatures of +0 ˚C and +1 ˚C are already naturally experienced for at least short periods in Torres Strait. The +2 ˚C treatment temperature is greater than this population naturally experiences, but provides data comparable with similar studies conducted for more southern

populations and is within projected climate change conditions for this location. Fish were maintained at treatment temperatures for 3 months.

Metabolic response to temperature

To determine the effect of temperature on aerobic performance, routine oxygen consumption ($\dot{M}O_{2\ Routine}$) and maximum oxygen consumption ($\dot{M}O_{2\ Max}$) were directly measured and subsequently used to estimate net aerobic scope of each fish. All fish were first tested at their grow-out treatment temperature. In addition, all fish were also tested at the remaining two treatment temperatures after a habituation period of one week (7 days; Fig 5.1). Rather than testing an entire developmental group at one of the remaining temperatures and then the other, each group was split into two so that half could be tested at each of the two remaining temperatures. Groups were then swapped and tested at the remaining temperature. This experimental design aimed to ameliorate any effects that testing order may have had on the results. The additional testing after short term exposure to non-developmental temperatures was designed to establish differences in oxygen consumption due to acute temperature change from changes due to possible developmental acclimation. If acclimation had occurred, it was expected that metabolic performance would be significantly improved in fish at their developmental temperature, when compared with the performance of acutely exposed fish from a different developmental line. This experimental design also determines through temperature back-crossing whether or not fish are able to return to normal function (or continue at an improved level) when temperatures are lowered again, hence giving some indication of the advantages or disadvantages of plasticity in a variable environment for these populations.

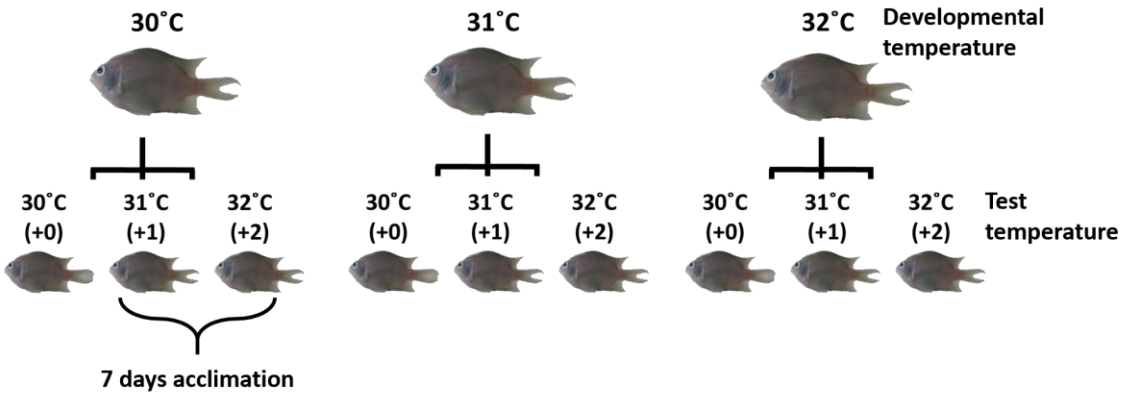

Figure 5.1. Experimental design demonstrating fish grown out in control (30 °C) or elevated (31 and 32 °C) thermal conditions and tested at their developmental temperature and at the remaining temperature treatments after one week of (acute) exposure.

Respirometry measurements of routine oxygen consumption ($\dot{M}O_{2\ Routine}$) were carried out using closed chamber static respirometry (Sinclair et al. 2006; Seebacher et al. 2014). This type of respirometry was chosen due to the large number of replicate samples that were required to be carried out within a limited timeframe. Previous work has shown that for damselfish this type of respirometry can provide reliable results in which trends in the data are not distorted by the absence of a mixing device (Rodgers et al. 2016 **Appendix 1**). This is because damselfish are sufficiently active to provide mixing of the respiration chamber and have not been shown to exhibit diurnal trends in oxygen consumption. Food was withheld for 24 hours prior to testing to prevent specific dynamic action increases in oxygen consumption associated with digestion. In order to measure $\dot{M}O_{2\ Routine}$, each fish was placed inside a respirometer of volume 590 or 1190 ml for *A. polyacanthus* (2.81 ± 0.16 g SE, maximum 6.71 g at testing), 295 or 590 ml for *P. moluccensis* (0.63 ± 0.04 g SE, maximum 1.49 g) and 295, 590 or 1190 ml for *P. wardi* (1.82 ± 0.15 g SE, maximum 4.66 g). Chambers were then submerged for 1 h in a water bath in order to allow the fish to habituate. A pilot study which examined the oxygen consumption of each species subsequent to being introduced to the respirometry chambers showed that oxygen consumption was not significantly different at 1h after introduction to the respiration chambers from all successive measurements taken up to seven hours (Repeated measures ANOVA with time as the dependent variable; $F_{6,18}$ =

0.46, P > 0.05, $F_{7,21}$ = 0.49, P > 0.05 and $F_{7,21}$ = 0.35, P > 0.05 for *A. polyacanthus, P. moluccensis* and *P. wardi*, respectively). Therefore it was determined that 1h was sufficient time to recover from any handling stress that may have elevated $\dot{M}O_2$ in this study. During the habituation period, chambers were flushed with clean, well-oxygenated and temperature-controlled sea water in order to prevent the accumulation of carbon dioxide and other metabolites, as well as excretory products, which may have influenced oxygen consumption (Steffensen 1989). After this time chambers were sealed and oxygen concentrations were monitored throughout a single 30 minute experimental period. Measurements were taken approximately every five minutes during this time using a Fibox fibre optic oxygen system (PreSens). Oxygen concentration was never allowed to fall below 80% during this time.

Fish were given at least 1 h to recover after $\dot{M}O_{2\ Routine}$ estimation before $\dot{M}O_{2\ Max}$ measurements were taken. To determine $\dot{M}O_{2\ Max}$, fish were transferred to an upright circular swim chamber (volume 807.78 ml; Nilsson et al. 2007; Donelson & Munday 2012; Seebacher et al. 2014). Water current inside the cylinder was created using a magnetic stirring bar and stir plate. The chamber was placed inside a water bath in order to maintain water temperature throughout the trial. The speed of the magnetic stir bar was increased slowly until the fish could sustain a maximal swimming speed while maintaining its position in the water column and without making (presumably anaerobic) lunge movements. During this time, the oxygen concentration in the water was measured every second for approximately 5 min.

The wet weight and standard length of all fish was recorded after metabolic testing was carried out and $\dot{M}O_{2\ Routine}$ and $\dot{M}O_{2\ Max}$ (mg O2 consumed h^{-1}) were calculated for each fish using the recorded fall in oxygen. Net aerobic scope was then calculated by subtracting $\dot{M}O_{2\ Routine}$ from $\dot{M}O_{2\ Max}$ for each fish. Differences in each of these aerobic measures between the three developmental and testing temperature treatments were compared for each species.

Data analysis

Prior to statistical analysis measures of $\dot{M}O_{2\ Routine}$, $\dot{M}O_{2\ Max}$ and aerobic scope for each species were log-transformed and a homogeneity of slopes model was examined to

ensure that the relationship between metabolic output and fish mass was consistent across treatments. All measures were then standardised to the average fish body mass for each measure using a residuals plot of log metabolic rate (mg/h) vs log fish mass. Residual values were converted back to mg O_2 h^{-1} by taking the inverse log. Differences in aerobic measures between treatments were compared separately for each species using a split-plot (mixed-design) analysis of variance (ANOVA), with testing temperature as a repeated measure and developmental temperature as a fixed factor. Where necessary, a Fisher LSD post-hoc analysis was used to determine significant differences between specific treatment groups.

Fish weight after three months grow out at each of the developmental temperatures was compared using a one-factor ANOVA, with developmental temperature as the fixed factor. Again a Fisher LSD post-hoc analysis was used to determine significant differences between treatment groups.

All statistical tests were carried out in Statistica (StatSoft Inc., Tulsa, USA). Assumptions were examined with residual analysis prior to interpretation and significance was accepted to a level of 0.05.

5.4 Results

Acanthochromis polyacanthus

Statistical analysis of $\dot{M}O_{2\ Routine}$ revealed a significant interaction between developmental and testing temperature for *A. polyacanthus* ($F_{4,72} = 3.38$, $P < 0.05$; Fig 5.2a). When tested at +0 ˚C, the 31 ˚C developmental group generally displayed a lower $\dot{M}O_{2\ Routine}$ than both the 30 and 32 ˚C developmental groups. This improved performance was significant when compared to the 30 ˚C developmental control (Post hoc: $P < 0.05$). For both the 30 and 32 ˚C developmental groups $\dot{M}O_{2\ Routine}$ was consistent between the +0 and +1 ˚C testing temperatures (Post hoc: $P > 0.05$ for both developmental groups). For the 31 ˚C developmental group, $\dot{M}O_{2\ Routine}$ increased significantly between the +0 and +1 ˚C testing temperatures (Post hoc: $P > 0.01$) to a level that did not differ significantly from the other developmental treatments (Post hoc: $P > 0.05$ compared with both 30 and 32 ˚C developmental groups). Fish that

developed at 30 and 32 ˚C displayed an increased $\dot{M}O_{2\ Routine}$ with increasing testing temperature from +1 to +2 ˚C (Post hoc: $P < 0.001$). The 31 ˚C developmental group maintained $\dot{M}O_{2\ Routine}$ between +1 and +2 ˚C (Post hoc: $P > 0.05$) and again displayed a lower $\dot{M}O_{2\ Routine}$ than both the 30 and 32 ˚C developmental groups, which was significant when compared to the 30 ˚C developmental group (Post hoc: $P = 0.02$). The lower $\dot{M}O_{2\ Routine}$ produced for fish that developed at 31 ˚C when tested at +0 and +2 ˚C suggests some evidence of acclimation of $\dot{M}O_{2\ Routine}$ for this group.

No significant interaction between developmental and testing temperature was observed for $\dot{M}O_{2\ Max}$ ($F_{4,70} = 1.15$, $P > 0.05$), nor was there a significant effect of developmental temperature on oxygen consumption ($F_{2,35} = 1.37$, $P > 0.05$). In contrast, testing temperature did have a significant effect on $\dot{M}O_{2\ Max}$ for *A. polyacanthus* ($F_{2,70} = 8.28$, $P < 0.001$; Fig. 5.2b). $\dot{M}O_{2\ Max}$ was significantly higher overall when fish were tested at +1 and +2 ˚C, compared with when fish were tested at +0 ˚C (Post hoc: $P < 0.05$ for both comparisons).

Aerobic scope was driven by a combined effect of $\dot{M}O_{2\ Routine}$ and $\dot{M}O_{2\ Max}$, however no significant interaction between testing temperature and developmental temperature was observed ($F_{4,66} = 1.05$, $P > 0.05$). A significant individual effect of testing temperature was recorded ($F_{2,66} = 5.96$, $P < 0.01$; Fig. 5.2c) and aerobic scope was highest when fish were tested at +1 ˚C (Post hoc P = when compared with +0 and +2 ˚C respectively). This trend appeared strongest in fish that developed at 32 ˚C however no significant effect of developmental temperature was recorded ($F_{2,33} = 1.25$, $P > 0.05$). Overall these results suggest no effect of developing at 31 ˚C on aerobic scope and a trend towards reduced aerobic capacity for fish developed at 32 ˚C.

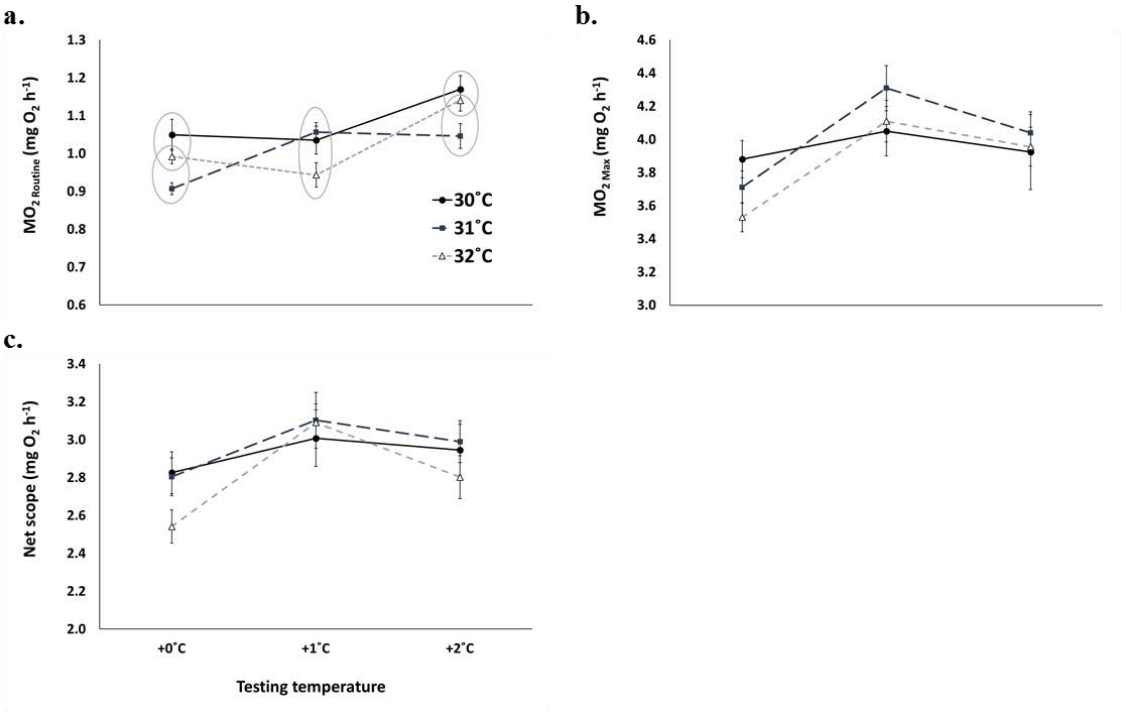

Figure 5.2. Routine oxygen consumption (a), maximum oxygen consumption (b) and net aerobic scope (c; means ± SE) for *Acanthochromis polyacanthus* after development at 30 (current average ocean temperatures), 31 and 32 ˚C. Developmental temperature is indicated in the legend (solid black line 30 ˚C , dark-grey dashed line 31 ˚C and light-grey dashed line 32 ˚C) whilst testing temperature is given on the x-axis. Where a significant interaction between test temperature and developmental temperature occurs, ovals have been used to indicate significant groupings of developmental treatments within a test temperature.

Pomacentrus moluccensis

There was no interaction between developmental and testing temperature for $\dot{M}O_{2\ Routine}$ of *P. moluccensis* ($F_{4,76}$ = 1.28, P = 0.28; Fig. 5.3a), nor was there any individual effect of developmental or testing temperature on oxygen consumption ($F_{2,38}$ = 2.66, P = 0.08 and $F_{2,76}$ = 0.92, P = 0.40 respectively). Despite this, similarly to *A. polyacanthus*, fish that developed at 31 ˚C displayed a trend towards equal or improved performance in $\dot{M}O_{2\ Routine}$ when compared with fish that developed at other temperatures, this time when tested at +1 ˚C in particular. This is reflected in the P value for the effect of developmental temperature on $\dot{M}O_{2\ Routine}$ which was close to significant (P = 0.08) and suggests that some enhanced performance may be present for fish developed at 31 ˚C.

A significant interaction between developmental and testing temperature was present for $\dot{M}O_{2\ Max}$ of *P. moluccensis* ($F_{4,80}$ = 6.40, P < 0.001; Fig. 5.3b). When tested at +0 ˚C, the 31 ˚C developmental group displayed a significantly higher $\dot{M}O_{2\ Max}$ than both the 30 and 32 ˚C developmental groups (Post hoc: P < 0.05 in both cases). $\dot{M}O_{2\ Max}$ increased with testing temperature for both the 30 and 32 ˚C groups and oxygen consumption was significantly higher when tested at +1 ˚C in comparison to when fish were tested at +0 ˚C (Post hoc: P < 0.01). $\dot{M}O_{2\ Max}$ was consistent for the 31 ˚C developmental group between +0 and +1 ˚C (Post hoc: P > 0.05) and was not significantly different from either the 30 or 32 ˚C developmental groups at +1 ˚C (Post hoc: P > 0.05). The 30 ˚C developmental group maintained a consistent $\dot{M}O_{2\ Max}$ between +1 and +2 ˚C (Post hoc: P > 0.05). The 31 ˚C developmental group produced a higher $\dot{M}O_{2\ Max}$ when tested at +2 ˚C compared to when tested at both +0 (Post hoc: P < 0.05) and +1 ˚C (P < 0.01). At +2 ˚C $\dot{M}O_{2\ Max}$ for the 31 ˚C group was not significantly different from the control developmental group (Post hoc: P < 0.05). At +2 ˚C both the 30 and 31 ˚C developmental groups produced a significantly higher $\dot{M}O_{2\ Max}$ than the 32 ˚C group (Post hoc: P < 0.01 in both cases). The increased $\dot{M}O_{2\ Max}$ of the 30 and 31 ˚C developmental groups when tested at higher temperatures suggests that they may not have yet reached their thermal optimum at current day temperatures.

Aerobic scope was driven primarily by trends in $\dot{M}O_{2\ Max}$ and produced an interaction between developmental and testing temperatures ($F_{4,74}$ = 4.62, P < 0.01; Fig. 5.3c). When tested at the +0 ˚C the 31 ˚C developmental line again performed better than both the 30 ˚C and 32 ˚C developmental groups (Post hoc: P < 0.05 in both cases). Aerobic scope increased with testing temperature for the 30 ˚C developmental group, and performance was significantly higher when tested at both +1 ˚C (Post hoc: P < 0.01) and +2˚C (P < 0.0001) compared with +0 ˚C. The 31 ˚C developmental group also produced a higher aerobic scope when tested at +2 ˚C than when tested at either +0 or +1 ˚C (Post hoc: P < 0.01 and 0.05, respectively). Fish developed at 32 ˚C again had lower aerobic scope than other developmental groups when tested at their own developmental temperature (Post hoc: P < 0.01 in both cases).

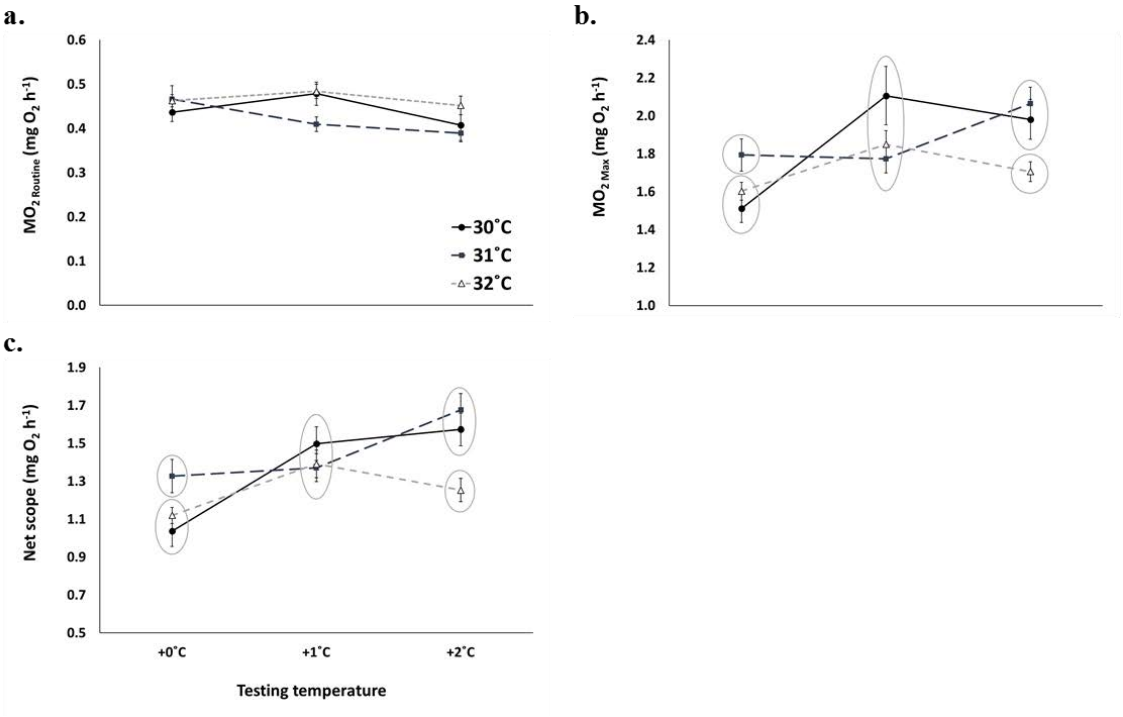

Figure 5.3. Routine oxygen consumption (a), maximum oxygen consumption (b) and net aerobic scope (c; means ± SE) for *Pomacentrus moluccensis* after development at 30 (current average ocean temperatures), 31 and 32 ˚C. Developmental temperature is indicated in the legend (solid black line 30 ˚C , dark grey dashed line 31 ˚C and light grey dashed line 32 ˚C) whilst testing temperature is given on the x-axis. Where a significant interaction between test temperature and developmental temperature occurs, ovals have been used to indicate significant groupings of developmental treatments within a test temperature.

Pomacentrus wardi

No significant interaction between developmental and testing temperature was present for $\dot{M}O_{2\,Routine}$ of *P. wardi* ($F_{4,76}$ = 1.96, P > 0.05; Fig. 5.4a) and there was no significant individual effect of testing temperature ($F_{2,76}$ = 1.49, P > 0.05). Developmental temperature did however have a significant individual effect on $\dot{M}O_{2\,Routine}$ ($F_{2,38}$ = 5.91, P < 0.01). Fish that developed at 32 ˚C had a higher $\dot{M}O_{2\,Routine}$ than other developmental groups (Post hoc: P < 0.01 when compared with both the 30 and 31 ˚C developmental groups). This result appears particularly strong for fish tested at +0 and +2 ˚C.

No significant interaction between developmental and testing temperature was present for $\dot{M}O_{2\ Max}$ ($F_{4,82}$ = 1.58, P < 0.05; Fig. 5.4b). Again testing temperature did not have a significant individual effect on oxygen consumption ($F_{2,82}$ = 2.20, P > 0.05), however the effect of developmental temperature was significant for this species ($F_{2,41}$ = 8.45, P < 0.001). Fish that developed at 32 ˚C performed better than both the 30 (Post hoc: P < 0.001) and 31 ˚C (P < 0.01) developmental groups. This result appears to be driven by the particularly high performance of this group when tested at +1 ˚C compared with the other developmental groups.

Aerobic scope followed the same trend as $\dot{M}O_{2\ Max}$ and again there was no significant interaction between developmental and testing temperature ($F_{4,72}$ = 1.39, P > 0.05; Fig. 5.4c) or of testing temperature ($F_{2,72}$ = 1.09, P > 0.05), but there was a significant effect of developmental temperature ($F_{2,36}$ = 4.67, P < 0.05). The increase in $\dot{M}O_{2\ Routine}$ observed for the 32 ˚C developmental group was strongly outweighed by the high performance of this group in terms of $\dot{M}O_{2\ Max}$ and again, fish that developed at 32 ˚C performed the best. This time though, performance increased with increasing developmental temperature and fish that developed at 32 ˚C only performed significantly better than those that developed at 30 ˚C (Post hoc: P < 0.001), with fish that developed at 31 ˚C displaying an intermediate aerobic scope, not significantly different from either the 30 ˚C (P = 0.12) or 32 ˚C (P = 0.13) developmental groups.

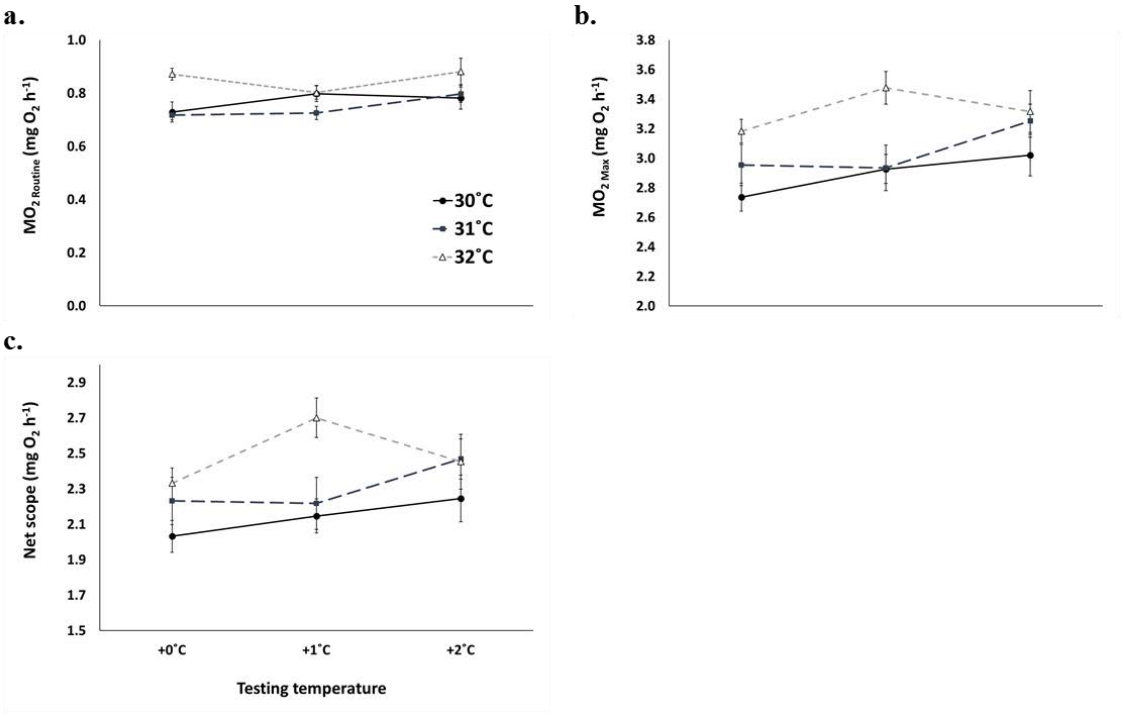

Figure 5.4. Routine oxygen consumption (a), maximum oxygen consumption (b) and net aerobic scope (c; means ± SE) for *Pomacentrus wardi* after development at 30 (current average ocean temperatures), 31 and 32 ˚C. Developmental temperature is indicated in the legend (solid black line 30 ˚C , dark grey dashed line 31 ˚C and light grey dashed line 32 ˚C) whilst testing temperature is given on the x-axis.

Fish weight

No significant effect of developmental temperature on fish weight was observed for *A. polyacanthus* ($F_{2,62}$ = 1.71, P = 0.19; Fig. 5.5a). Despite this, there was a strong trend in the data towards a decline in fish weight with increasing temperature. Fish weight within the 30 ˚C developmental group was extremely variable and this is likely to be why no significant difference in fish weight between groups was recorded. For this species, average fish weight was 3.09, 2.88 and 2.40 g for 30 ˚C, 31 ˚C and 32 ˚C developmental groups, respectively. Developmental temperature had a significant impact on the weight of *P. moluccensis* ($F_{2,59}$ = 3.67, P = 0.03; Fig. 5.5b). Fish grown out at 30 ˚C and 31 ˚C were not significantly different in size (Post hoc: P = 0.89) and weighed on average 0.56 and 0.55 g respectively. Both groups were significantly smaller than fish that developed at 32 ˚C (Post hoc: P < 0.05), which weighed on average 0.79 g. Developmental temperature did not have a significant effect on fish weight for *P. wardi*

($F_{2,57}$ = 0.11, P = 0.90; Fig. 5.5c). The average weight reached for this species was between 1.7 - 2.0 g.

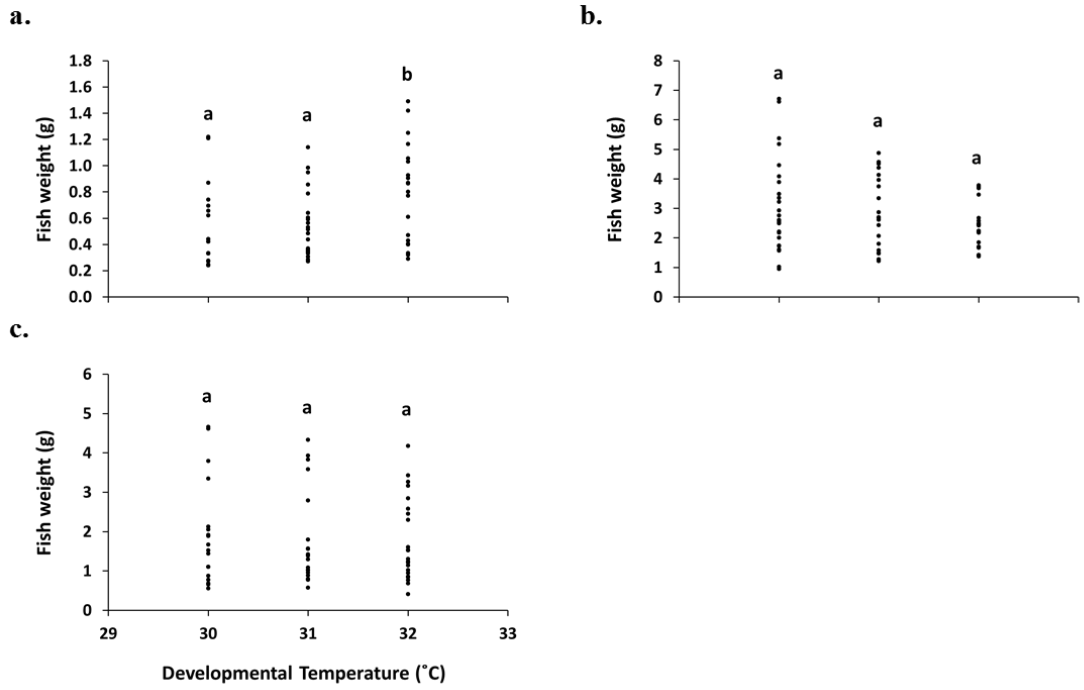

Figure 5.5. Weight of *Acanthochromis polyacanthus* (a), *Pomacentrus moluccensis* (b) and *Pomacentrus wardi* (c), after developing for three months at each temperature treatment. Temperature treatments tested were current average ocean temperatures (30 ˚C), +1 °C (31 ˚C) and +2 °C (32 ˚C). Letters indicate significant differences between treatment groups.

5.5 Discussion

Species considered in this study showed a variable capacity to acclimate to increased temperatures. *A. polyacanthus* displayed very little capacity for developmental acclimation to either of the higher temperatures. Trends in $\dot{M}O_{2\ Max}$ and aerobic scope did however reveal a reduction in performance (lower aerobic capacity) for fish that developed at 32 ˚C. A lower $\dot{M}O_{2\ Routine}$ for fish that developed at 1 ˚C higher than average summer temperatures when tested back at control temperatures was the only evidence of possible developmental acclimation for this species. *P. moluccensis* saw an increase in the $\dot{M}O_{2\ Max}$ and aerobic scope for fish that developed at 30 ˚C (control) with increasing test temperature, indicating that at present day temperatures this species

may not yet have reached its thermal optimum. Similarly to *A. polyacanthus*, *P. moluccensis* exhibited a trend towards a lower $\dot{M}O_{2\ Routine}$ in fish that developed at 31 ˚C, this time when tested at its own developmental temperature, however this trend was again not significant. $\dot{M}O_{2\ Max}$ drove trends in aerobic scope for *P. moluccensis* and when this species developed at 31 ˚C both measures were maintained at control levels when tested at their developmental temperature or at +2 ˚C, and increased relative to the control when tested at +0 ˚C. Developing at 32 ˚C reduced $\dot{M}O_{2\ Max}$ and aerobic scope to a level lower than both the 30 ˚C and 31 ˚C developmental groups when tested at +2 ˚C (developmental temperature). Finally, *P. wardi* showed the strongest evidence for developmental acclimation of all species. Although there was an increase in $\dot{M}O_{2\ Routine}$ for fish that developed at higher temperatures, fish that developed at 32 ˚C had a higher $\dot{M}O_{2\ Max}$ and aerobic scope than both the 30 ˚C and 31 ˚C developmental groups. This trend was strongest in fish tested at +1 ˚C. This evidence underscores that some species may have an ability to acclimate their aerobic capacity to tolerate the elevated temperatures predicted to occur as we head towards the next century, even at low-latitudes, while others are likely to be adversely affected.

The response of *A. polyacanthus* and *P. moluccensis* reared under ambient conditions (30 ˚C) to elevated temperatures was comparable to previous studies that have also examined the thermal tolerance of metabolic attributes for near equatorial reef fishes (Rummer et al. 2014a; **Chapter 2**). All three studies showed only a small change in $\dot{M}O_{2\ Routine}$ over the same temperature range for *A. polyacanthus*, however both of the previous studies saw a peak in $\dot{M}O_{2\ Max}$ at ~ 31 ˚C that was not observed in the present study. This difference may be because previous studies investigated thermal tolerance in older, more mature fishes. Both the present study and that of Rummer and colleagues (2014a) also described little difference in $\dot{M}O_{2\ Routine}$ between 30 and 32 ˚C for *P. moluccensis*. $\dot{M}O_{2\ Max}$ for this species increased with increasing temperature in both studies, providing further support to the theory that this species has not yet reached its thermal optimum at current day temperatures. No comparable data is currently available for *P. wardi*.

For the species which showed the least capacity for acclimation, *A. polyacanthus*, increasing developmental temperature to 32 ˚C produced a strong trend towards

reduced offspring weight. This trend supports the findings of previous studies (Munday et al. 2008b; Donelson et al. 2010), who also saw reduced growth at higher temperatures for this species. Trends differed for *P. moluccensis,* where the smallest offspring were produced at temperatures most strongly associated with developmental acclimation. This result could indicate that for *P. moluccensis* there is a physiological cost to metabolic acclimation during development. A cost of developmental acclimation has also observed by Donelson and colleagues (2011a), who hypothesise that energy used for somatic growth may be redirected towards acclimation during development at times when the benefits of acclimation outweigh these physiological consequences. Despite showing the greatest potential for developmental acclimation, *P. wardi* did not show any physiological cost of acclimation, suggesting a high level of thermal plasticity.

Studies of developmental acclimation for *A. polyacanthus* and *P. moluccensis* have only previously been conducted at higher latitudes and have primarily examined the acclimation capacity of $\dot{M}O_{2\ \text{Routine}}$. Although differences in $\dot{M}O_{2\ \text{Routine}}$ between developmental treatments were not found to be statistically significant for *P. moluccensis* this study, when results were compared for both *A. polyacanthus* and *P. moluccensis* between this study and those conducted on the central and southern regions of the GBR (Donelson et al. 2011a; Donelson et al. 2012; Grenchik et al. 2013), the temperatures at which a trend towards improved performance was observed were similar across studies (Fig. 5.6). *A. polyacanthus* was shown to display a lower $\dot{M}O_{2\ \text{Routine}}$ at 31 ˚C in both the present study and that of Donelson and colleagues (2011a), and at 31.5 ˚C by Donelson colleagues (2012), with both of the previous studies conducted on fish collected from the Palm Island region, 8 degrees further south on the central GBR compared to the Torres Strait population considered in this study. Evidence for developmental acclimation of $\dot{M}O_{2\ \text{Routine}}$ in *P. moluccensis* was seen at 30.5 ˚C for fish collected from the central GBR, compared with 31 ˚C in the present study (Grenchik et al. 2013). Although the temperatures at which acclimation was most likely to occur were similar, average summer temperatures in the Palm Island region are approximately 28.5 ˚C, whereas for the population considered in this study the summer average was 30 ˚C. Consequently, improved performance occurred at a higher temperature relative to the ambient for the Palm Island population. Specifically, central GBR species appear to be

acclimating to temperatures ~ 2 ˚C above their summer average, compared to half that for near equatorial fish. When fish from the Torres Strait region experienced a 2 ˚C increase in developmental temperature, they began to display indicators of a loss of performance at lower and/or developmental temperatures, indicating a physiological cost to having developed at this temperature. These findings are concerning for near equatorial populations, as they suggest that these species will be able to cope with much smaller temperature increases than their more southern counterparts in the future. An increase in the sample size considered for near equatorial fish could help to draw out these trends further, as there was a high degree of individual variation seen in this study.

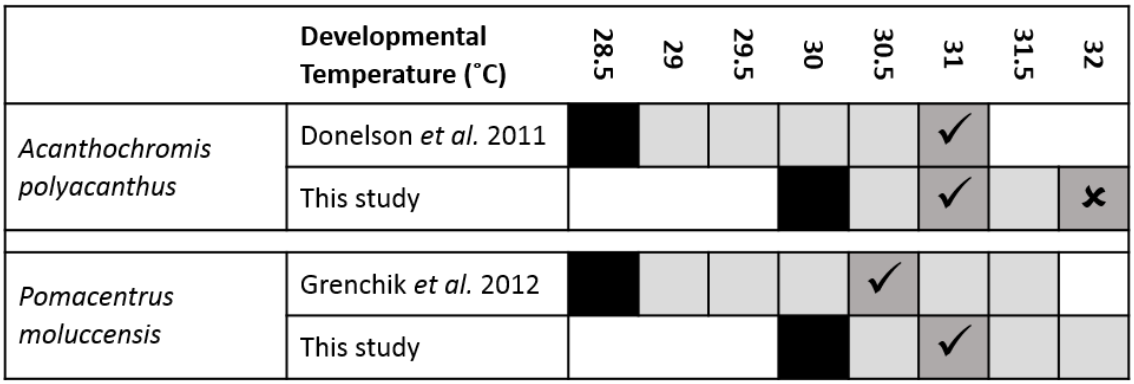

Figure 5.6. Comparison of ṀO2 Routine acclimation temperatures observed for populations of *Acanthochromis polyacanthus* and *Pomacentrus moluccensis* considered in this study with those recorded for studies conducted on central and southern Great Barrier Reef populations. Black boxes indicate the average summer temperature for that population, dark grey boxes with a tick symbol indicate beneficial acclimation, dark grey boxes with a cross symbol indicate a negative effect of that developmental temperature and light grey boxes indicate either that that temperature was within the range of experimental temperatures but was not specifically tested or that there was no change in performance from the control at that temperature.

Evidence for developmental acclimation in *P. moluccensis* in this study only resulted in an improvement in aerobic performance above that obtained by the developmental control fish when tested at lower temperatures (+0 ˚C). Whilst this is an example of acclimation, these changes are unlikely to be useful under future climate change scenarios. For this reason, as well as being the species which showed the greatest

capacity for acclimation, *P. wardi* may also be the only species of the three considered in this study to gain a true advantage in coping with projected temperature increases through developmental acclimation. Given that acclimation of *P. moluccensis* at 31 ˚C also occurred in conjunction with a decrease in fish weight, the net effect of acclimation for this species may in fact be negative. Often acclimation is assumed to have a beneficial impact on overall organism fitness (Huey & Berrigan 1996), however further consideration of trade-offs in whole organism fitness and environmental conditions may reveal that the beneficial acclimation hypothesis does not apply in all circumstances (Leroi et al. 1994; Hoffmann 1995; Huey & Berrigan 1996; Huey et al. 1999). Additionally, species examined in this study appeared to increase the breadth of their thermal tolerance via developmental acclimation rather than shift their thermal optimum, as performance was generally maintained at higher temperatures and improved at control temperatures when fish developed at warmer temperatures. This seems counterintuitive as maintaining a wide thermal tolerance curve is thought to be an energetically costly strategy, and acclimation of the thermal optimum is expected to occur more regularly, particularly for populations in a relatively stable thermal environment (Gabriel & Lynch 1992). Counter to theory though, past literature shows that in practice, thermal optimum appears to be highly conserved in many species and so the results of this study are in fact not unusual in light of much of the previous research (reviewed in Angilletta 2009).

Whilst the three species considered in the present study are closely related, they each showed different responses to identical temperature increases, suggesting that researchers should be cautious how we generalise the impacts of climate change, even within a family. Information on the life history of the species may aid our interpretation. For the fish considered in this study, each has a slightly different ecological role and life history strategy. *A. polyacanthus* showed the lowest capacity for developmental acclimation and is the only one of the three species without a pelagic larval phase (Thresher et al. 1989; Doherty et al. 1994). This restricts distribution, causing the juvenile and parental environment to be the same, and consequently results in genetically distinct sub-populations (Planes et al. 2001). This restriction on distribution is likely to also restrict the thermal range experienced by a population and could to lead

to thermal specialisation. *P. moluccensis* and *P. wardi* each have larval dispersal phases of ~20 days, so are less likely to develop into distinct sub-populations (Thresher et al. 1989; Jones et al. 2010), thus a generalist approach could be favoured in these species. Both also have ranges that extend further south than that of *A. polyacanthus*, occurring as far south as central New South Wales, Australia (~ 33 °S; Randall et al. 1997; Kuiter 2000), requiring adaptation to a wider range of thermal environments. *Pomacentrus wardi* showed a stronger capacity for developmental acclimation and less consequence of developing at higher temperatures in comparison to *P. moluccensis*. A closer examination of the habitat of these species reveals some important differences between the two. *P. moluccensis* is a live coral associated species throughout its life and is found at a depth range of 1-12 m (Randall et al. 1997; Feary et al. 2007). In contrast, *P. wardi* is a habitat generalist. This species often inhabits areas interspersed with coralline masses and rubble and can be found in what are classically recognised as thermally stressful habitats such as shallow (< 1 m) lagoons and reef flat, through to more thermally stable environments on the outer reef slope (to 20 m; Doherty 1983; Randall et al. 1997; Robertson & Lassig 1980). The differences between these two species represent differences in the level of thermal heterogeneity each might experience upon settlement and may explain differences in their capacity for developmental acclimation.

Species considered in this study breed primarily during the summer months (October – February; Robertson 1973; Thresher 1983). It is estimated that fish collected for this study during December were approximately 1 month old at time of capture, based on the average size of juveniles and the time of year that collection was carried out. It is possible however that fish may have been up to 2-3 months of age. If fish were greater than 1 month of age at collection, this study may provide a conservative estimate of the potential for developmental acclimation for these species and a greater capacity for acclimation may have been observed in fish exposed to higher temperatures earlier in development or at hatching. As the three species were of a similar size at capture it is anticipated that they recruited as part of the same breeding pulse. It was therefore deemed unlikely that any differences in acclimation capacity between species were due to age differences.

This study considers oxygen consumption as an estimate of metabolic performance, however increased temperatures will of course influence many biological processes within an organism (Cossins & Bowler 1987). Previous research suggests that for at least one of the species considered in this study, different physiological performance indicators have different thermal optima (**Chapter 3**). For this reason, future work should consider the acclimation potential of other important fitness measures in order to gain further understanding of the effects of climate change on these low-latitude species. Developmental acclimation is also not the only means by which fish may alter their ability to cope with elevated temperatures. Transgenerational thermal acclimation, where parents influence the phenotype of their offspring by non-genetic means, has previously been shown to occur for a range of fish species, including some higher latitude populations of coral reef fish, including *A. polyacanthus* (Donelson et al. 2012; Salinas & Munch 2012; Shama et al. 2014) and so like developmental acclimation may also be possible in near equatorial populations. In fact, the benefits of transgenerational acclimation may be even greater than those produced under developmental acclimation (Donelson et al. 2012).

Acclimation will play an important role in species' ability to cope with increased temperatures into the future. This study has shown that, despite existing in a thermally stable environment, near-equatorial fish may have at least some capacity for developmental acclimation. This capacity is however much lower than for populations in higher latitude environments, is potentially associated with significant physiological trade-offs and differs among species. These differences are likely to be dependent on species' life history and ecological niche. Further consideration of acclimation over multiple generations and species' capacity for genetic adaptation will be important for realistic predictions of how low-latitude coral reef fish may respond to future elevation in temperature expected to occur through climate change.

Chapter 6: General Discussion

Theory suggests that species existing in low-latitude environments are likely to be the most sensitive to future projected climate change due to the narrow thermal range naturally experienced at these latitudes and the high trajectory of change predicted to occur at these locations (Janzen 1967; Deutsch et al. 2008; Tewksbury et al. 2008, Burrows et al. 2011). Within the tropics themselves a range of thermal environments exist, and so population sensitivity is likely to vary throughout. Low-latitude populations are expected to be more sensitive to elevated temperatures than populations from higher tropical latitudes, particularly if dispersal is limited and population connectivity is low (Nguyen et al. 2011; Rummer et al. 2014a). This thesis represents the most in-depth examination to date of the physiological response of near-equatorial coral reef fish populations to projected change, and considers for the first time the ability of these fish to acclimate to change over much longer time scales than have previously been considered. By exploring the physiological effects of increased temperature on fishes collected from Torres Strait, this study reveals that the impacts of a warming ocean are complex and differ dependant on the performance trait measured. This research also confirms that many low-latitude populations are currently living close to their thermal maximum even at current day temperatures, but that they possess a capacity for acclimation to warmer temperatures that is likely to be largely dependent on ecological niche and life history. These findings challenge the assumption that low-latitude organisms have a limited capacity to deal with a changing climate; however they also reveal the vulnerability of these populations to change when compared with higher-latitude counterparts.

Thermal tolerance and physiological effects of temperature

The few studies conducted to date suggest that tropical reef fishes exhibit a limited thermal range and a low capacity for reversible acclimation (Nilsson et al. 2009; Nilsson et al. 2010; Gardiner et al. 2010; Rummer et al. 2014a). Research in this thesis was the first to examine the capacity for reversible acclimation in an adult population of near equatorial reef fish over periods of more than one month (longest acclimation period prior was 22 days; Nilsson et al. 2010). Even after spending extended periods (10

months) at higher temperatures, the study population showed no improvement in physiological performance (**chapter 2**). The limited capacity for acclimation to temperatures above what is normally experienced in these populations can be attributed to the narrow thermal range experienced by tropical organisms, particularly those existing closer to the equator, and supports the theory that these low-latitude populations are already operating close to their thermal maximum, even at current day temperatures (Stillman 2003; Kellermann et al. 2012; Rummer et al. 2014a). For *A. polyacanthus*, the thermal limit exhibited in this thesis is likely to represent a species limit rather than simply a population limit, as although conducted over a shorter time span, research on the same species in Papua New Guinea showed decreases in performance at similar temperatures to those seen in the present study, indicating no additional thermal tolerance with a decrease in latitude (Rummer et al. 2014a). This is unsurprising as in Torres Strait, populations of *A. polyacanthus* are already existing extremely close to the upper limit of the thermal range experienced by this species (total temperature span approximately 20–31°C).

Similar relative increases in water temperature produced different effects on aerobic fitness for near-equatorial fish populations considered in this thesis, when compared with the same species at higher latitude tropical locations (**chapters 2 & 3**). At higher latitudes (central and southern GBR; Heron Island, Palm Island and Lizard Island), a decrease in aerobic scope with increasing temperature is generally observed between current day temperatures and 1 – 1.5 ˚C above current day averages (Nilsson et al. 2009; Gardiner et al. 2010; Donelson & Munday 2012). After this point, scope remains largely unchanged at subsequent higher temperatures, although generally these populations have not been tested to temperatures as high as those considered in this study of low-latitude populations. Torres Strait populations were able to maintain aerobic scope until 31.5˚C (1.5˚C above current day average), however after this point scope decreased dramatically and significant mortality resulted. Despite differences between populations in response to temperature increases throughout the thermal range, the chronic thermal limit of this species was stable between populations, irrespective of latitude, as short term exposure to 34 ˚C has been shown to result in significant

mortality of this species in all studies conducted to date (Zarco-Perelló et al. 2012; Rummer et al. 2014a; **chapter 2**).

Evidence that the chronic thermal limit is similar for this species irrespective of latitude suggests that the thermal reaction norm for *A. polyacanthus* is likely to have a similar upper limit among all populations. However, the strong decline in aerobic performance between the thermal optimum at 31.5 ˚C and significant temperature related mortality at 33 ˚C in low-latitude populations, while higher-latitude populations saw a more gradual decline in performance with temperature, suggests that the shape of thermal reaction norms may differ between populations. Despite having a similar thermal maximum, the thermal reaction norm may be more highly skewed towards the left for low-latitude populations, while higher latitude tropical populations may possess more of a bell shaped curve. Further research which tests high-latitude populations at a greater range of temperatures is required to confirm this.

Pioneering research on the physiological impacts of increased temperatures on ectotherms accepts that restriction of whole-animal aerobic scope is one of the first symptoms of a negative effect on organism health (Pörtner 2001; Pörtner & Farrell 2008). The oxygen- and capacity-limited thermal tolerance (OCLTT) hypothesis predicts that all measures determining organism fitness are causally linked to aerobic capacity (Pörtner 2001; Pörtner & Farrell 2008). Despite this, recent studies, as well as the research conducted in this thesis, show that the theory is not widely applicable across all ectotherm species or populations (Clark et al. 2011; Clark et al. 2013; Donelson et al. 2014; Gräns et al. 2014; Norin et al. 2014; Norin et al. 2015; Farrell 2016; Drost et al. 2016; **chapters 2 & 3**). Increased temperatures of 1.5-3 ˚C above average for *A. polyacanthus* had a significant effect on all physiological and morphological traits considered in **chapters 2-4.** Thermal tolerance however was not consistent across all indicators of fitness. Survival, metabolic performance, haematological measures and sex ratio (although considered for a higher latitude population) all showed a decline in performance at temperatures 3 ˚C above current day averages, whilst histological analysis of gill tissue and survival after a secondary stressor (maximal exercise) both saw declines in fitness prior to other physiological measures, at 1.5 ˚C above current day temperatures. The findings of this thesis provide support for the multiple optima

hypothesis, which states that no single optimal temperature for organism function exists, but instead that different physiological processes have different thermal optima (Hadfield 1966; Bustard 1967; Du et al. 2000; Clark et al. 2013). It was further determined that for the population and life stage of *A. polyacanthus* considered in this study, that gill health provided the best indicator of fitness from the measures considered as it provided the earliest indication of a decline in organism fitness in response to higher temperatures. Of course different life stages and populations may be affected differently by thermal stress (Clark et al. 2013), and so critical indicators of organism fitness may vary by location or ontogenetic stage and this should be considered in future research.

Aerobic scope was not the primary indicator of declining organism health in *A. polyacanthus* (**Chapter 2 & 3**), however, it is expected that problems relating to oxygen transport and uptake provided a significant if not the definitive cause of fish mortality in this study. Each of the physiological changes observed at higher temperatures point to an attempt to maximise blood flow and increase the amount of oxygen taken up by the fish. Increases in blood pressure to facilitate higher oxygen demand are likely to be the primary cause of aneurysms present in the gills of fish, and movement of blood into the secondary vascular system (SVS) is also predicted to have increased oxygen uptake via cutaneous respiration and subsequently reduced pressure on the heart (Jensen et al. 2009; Rummer et al. 2014b). Changes in gill tissue showed a strong response to increased temperatures; the strongest of the physiological measures considered in this thesis (**Chapter 3**). The development of gill aneurisms is likely to have serious consequences given that the gills are the organ primarily responsible for oxygen uptake. In several highly plastic species, which typically experience high variation in oxygen availability, studies have described significant changes in gill structure in response to hypoxia (Sollid et al. 2005; Sollid & Nilsson 2006; Nilsson 2007; Mitrovic et al. 2009; Tzaneva et al. 2011), however no such capacity is expected for the fish considered here. It is unsurprising that species from environments where there is no evidence of variable oxygen availability should lack a well-developed capacity to remodel the gills, as this is an energetically costly process and should only be expected under circumstances only

where there is a strong evolutionary advantage (Sollid & Nilsson 2006; Ong et al. 2007; Turko et al. 2012).

Temperature-related mortality has not previously been observed with temperature increases as small as those used in this thesis (**Chapters 2 and 3**); only 1.5 ˚C outside of the species' normal thermal range. Fish mortality occurred at these temperatures in response to short periods (approximately 5 min) of maximal exercise. Although mortality at such low temperatures was not purely due to temperature stress, fish health after a secondary stressor is likely to be more indicative of their capacity to deal with higher temperatures in nature as stressors do not tend to occur in isolation. The effects of climate change will impact organisms in conjunction with additional climate-related stressors such as changes in ocean chemistry and food availability; as well as non-climate-related anthropogenic effects such as overfishing, habitat destruction, pollution and coastal development (Harley et al. 2006). It is therefore important to understand how combined stressors will interact to effect organisms because, as demonstrated in **chapter 3**, these interactions can have significant negative implications. Understanding these interactions will be an important next step in the current research on low-latitude populations. Past studies often have assumed that the effect of multiple stressors is simply an accumulation of impacts (Bryant et al. 1998; Sanderson et al. 2002; Halpern et al. 2007, 2008; Ban & Alder 2008). However, a meta-analysis of 171 studies each measuring more than one anthropogenic stressor in marine systems found that additive, antagonistic and synergistic effects are all common (Crain et al. 2008). In combination with increased temperatures, ocean acidification makes up the second of the two biggest threats from climate change on marine organisms (Hoegh-Guldberg et al. 2007, Doney et al. 2009). In studies that have examined the interactive effects of these two stressors on marine fish, an additive or synergistic effect has been observed on aerobic performance (Munday et al. 2009; Gräns et al. 2014), reproduction (Miller et al. 2015) and foraging and food consumption (Nowicki et al. 2012), whilst no interaction has been observed for growth (Gräns et al. 2014). These findings highlight the need for multi-stressor research to be conducted over a range of latitudes.

At temperatures where mortality occurred in low-latitude fish without the presence of a secondary stressor, the cause was predicted to be rupturing of gill aneurysms because, in these incidences, bleeding from the gills was observed at time of death. This obvious trauma to the gills was however only reported for some fish and so heart failure was identified as a possible alternative cause of death. Heart failure was not examined in this thesis but has been shown to be the primary cause of mortality in fish exercising even moderately at high temperatures - prior to a decrease in maximum aerobic performance (Farrell 2002). Ischemia injury through deficit of oxygen to the myocardial tissue (myocardial hypoxia) occurs as an organism is unable to meet oxygen demand in the tissues and ultimately results in an oxygen deficit leading to limitation of the maximum heart rate and subsequent arrhythmia and bradycardia (Farrell 2002; Farrell 2009; Muñoz et al. 2014). The symptoms of thermal stress observed in **chapter 3** are likely to be part of a complex cascade of physiological problems leading up to and resulting in the central problem of heart failure.

Nitric oxide (NO) has been identified as a potentially important mediator in physiologically dealing with the symptoms associated with increased oxygen demand at higher temperatures, and is likely to be involved in dealing with temperature stress both in *A. polyacanthus* and in other fish species (**chapter 3**). Given that the heart is potentially an important "missing link" in this research, the synthesis and production of NO could play an critical role in the protection of the heart and other vital organs from ischemia injury, as well as mediating other important processes designed to deal with hypoxia such as the opening of the secondary vascular system (Bolli 2001; Park et al. 2003; Imbrogno et al. 2001). Mammalian models have shown that NO preconditioning to a stressor is possible (Bolli 2001; Dawn & Bolli 2002; Park et al. 2003), and Imbrognao and colleagues (2001) hypothesise that the potential for NO-elicited plasticity in cardiac performance may be greater in fish and amphibians than in endothermic species. Longer periods of growth into adulthood for ectotherms and subsequent rapid replacement of myocardial tissue may allow for NO driven cardiac adjustments in response a stressor not be possible in endotherms (Imbrognao et al. 2001). This type of plasticity is not well studied or understood though, and prolonged stimuli or excess NO production could also have a deleterious effect on both heart function and other

aspects of organism health, by processes such as apoptosis (Imbrognao et al. 2001; Dimmeler & Zeiher 1997). For these reasons, future studies may benefit from considering the role of NO in the ability of ectothermic species to cope with climate change induced stressors.

Developmental acclimation

Given the potential effects of increased temperatures on low-latitude coral reef fish populations as outlined in **chapters 2-4**, it can be concluded that the ability to acclimate to warmer temperatures will be critical for these organisms. Acclimation can occur by either widening the thermal reaction norm of a population, so that an increased performance breadth is achieved; or by shifting the mode of the curve so that the thermal optimum occurs at a higher temperature (Gabriel & Lynch 1993; Angilletta 2009). These changes to the thermal performance curve are energetically costly, and thus a trade-off between physiological fitness and the cost of acclimation exists (Gabriel & Lynch 1992). In the low-latitude populations considered in this thesis, within generational thermal variation is low and so acclimation of the thermal optimum would be expected in preference to a widening of the thermal reaction norm (Gabriel & Lynch 1992). Between generations thermal variation is also typically low, and so it may be expected that these populations are unlikely to have developed a capacity for developmental acclimation, based on their thermally stable habitat (Gabriel & Lynch 1992; Angilletta 2009).

In this thesis I show for the first time that counter to theory, some populations of low-latitude coral reef fish are likely to possess a capacity for developmental acclimation to increased ocean temperatures (**chapter 5**). This capacity for developmental acclimation was highly species dependent, even for members of the same family (**chapter 5**). The life history and ecological niche of species may be a good predictor of acclimation capacity and should be taken into greater consideration in future studies of acclimation both within and across generations (**chapter 5**). Previous studies have suggested that consideration of ecological traits will aid in predicting the vulnerability of a species or population to a warming environment (Williams et al. 2008), however it is likely that consideration of ecological parameters will also help to determine a species' capacity

for acclimation. In this thesis it was shown that species with a larval dispersal stage and thus a less predictable settlement environment might possess a higher capacity for developmental acclimation than those without a larval dispersal stage (e.g. *A. polyacanthus*; Thresher et al. 1989; Doherty et al. 1994; Jones et al. 2010; **chapter 5**). Dispersive species are more likely to experience variation in temperature among generations, a trait which past literature has identified as increasing the likelihood of developmental acclimation (Gabriel & Lynch 1992; Angilletta 2009). Beyond this, habitat specialisation could also play an important role as in this study the more generalist species (*P. wardi*; Doherty 1983; Randall et al. 1997; Robertson & Lassig 1980) displayed a greater capacity for acclimation when compared with a habitat specialist (*P. moluccensis*). This is again likely to relate to the predictability of the thermal environment that juveniles will enter, as *P. moluccensis* tends to occupy a more narrow depth range. Further studies which consider a greater range of species are required to verify this hypothesis.

By comparing the results of this thesis with recent literature considering the capacity for developmental acclimation of damselfish species at higher latitudes, we found that *A. polyacanthus* and *P. moluccensis* both show signs of developmental acclimation at similar absolute temperatures regardless of latitude (Donelson et al. 2011a; Donelson et al. 2012; Grenchik et al. 2013; **Chapter 5**). Although the temperatures at which acclimation occurs are similar, average summer temperatures in the Palm Island region, where previous research on these species has been conducted, are approximately 1.5 ˚C lower than the summer average in Torres Strait, where fish considered in this thesis were collected. Therefore improved performance occurs at a higher temperature relative to the ambient for higher latitude populations. Central GBR species are able to acclimate to temperatures ~ 2 ˚C above their summer average, compared to half that for fish from near-equatorial populations. These findings are concerning for near equatorial populations, as they suggest that although these species may be able to acclimate to higher temperatures in some cases, they will not be able to cope with temperature increases as large as their more southern counterparts. Based on these results we might expect that less local adaptation occurs than predicted in these

populations, as large differences in physiological performance were not observed with latitude.

The capacity for different traits to acclimate to projected future temperatures will most likely determine which processes provide the ultimate barriers to persistence. The capacity for populations to survive under a particular thermal regime will be governed by how temperature negatively affects the most sensitive life-history/physiological trait. Variation in acclimation ability of traits has been observed in previous literature. For example, sex ratio shows a low capacity for acclimation when compared with metabolic traits in central GBR *A. polyacanthus*, and therefore could be a limiting factor to organism persistence for those populations (Donelson et al. 2012; Donelson & Munday 2015). In **chapter 5** I only consider the potential for developmental acclimation of aerobic performance to higher temperatures. Future studies should examine the potential for acclimation of other important physiological measures in order to gain a more holistic understanding of the effects of projected climate change on these low-latitude populations and to assist in predicting which physiological measures are most likely to limit persistence.

Transgenerational acclimation

While not tested in this thesis, transgenerational plasticity may also alter an organism's ability to cope with elevated temperatures. Transgenerational plasticity, where parents influence the phenotype of their offspring by non-genetic means, has previously been shown to restore growth and aerobic performance in a number of fish species under increased temperatures, including *A. polyacanthus* (Donelson et al. 2012; Salinas & Munch 2012; Shama et al. 2014). Indeed, the benefits of transgenerational acclimation have been shown for *A. polyacanthus* from the central GBR to be even greater than those produced under developmental acclimation (Donelson et al. 2012). For this reason, transgenerational acclimation may provide low-latitude species with a greater capacity for dealing with change than first thought.

Rapid transgenerational acclimation has been shown to fully restore performance in some traits in as little as two generations (Donelson et al. 2012), whilst others at least saw partial restoration within this timeframe (Shama et al. 2014). This rapid recovery of

traits provides a potential mechanism for dealing with changes to thermal regime over climate change relevant time scales. Despite the benefits of transgenerational acclimation there may also be some significant drawbacks for this type of coping mechanism. Restoration of one trait with transgenerational acclimation my come at a physiological cost in another area (Salinas & Munch 2012), and these costs may then be passed onto subsequent generations. If offspring do not experience an environment similar to that of their parents (i.e. due to larval dispersal), then acclimation can be maladaptive (Munday 2014). Trade-offs may also occur across life stages, where enhanced performance in one life stage may have negative effects on other life stages (Munday 2014). These trade-offs are likely to limit the capacity of some traits for thermal acclimation.

Thermal theory and low-latitude populations

Evolutionary theory suggests that species or populations which live in thermally stable environments should optimise performance over a narrow range of temperatures and trade-off their ability to respond to environmental change (Huey & Hertz 1984; Somero et al. 1996). This thesis shows however, that although populations existing within thermally stable regions may be living close to their thermal limits as predicted, they may still have a surprising capacity for thermal acclimation, compared to what is predicted by theory. Acclimation capacity did appear more limited in species whose populations had a higher probability of genetic local adaptation (eg. *A. polyacanthus*) and there were other physiological trade-offs associated with acclimation for some species, however the generalised assumption that low-latitude populations should have no capacity to acclimate to a changing environment was not supported by this study.

Polar fishes are another group for which despite existing in a relatively stable thermal environment, a capacity for acclimation has been observed in some populations. In these populations, fish are able to reversibly acclimate to increased temperatures over relatively short time periods (longest 8 weeks; Seebacher et al. 2005; Podrabsky & Somero 2006; Franklin et al. 2007; Seebacher et al. 2015), whereas for near equatorial fish exposure to higher temperatures had to occur within a critical time window. Compared to polar fish, the ability for acclimation that was observed in this thesis was

relatively limited, however it is possible that with further testing and the inclusion of species from more thermally variable niches (e.g., reef flats) and over multiple generations, a further capacity for acclimation could be revealed. Regardless, both this study and the above mentioned for polar fish, show that the capacity for a species or population to respond to environmental change cannot be assumed only based on the thermal stability of the environment that it exists within.

Concluding remarks

This thesis begins to break down the physiological costs of increased ocean temperatures for fishes existing in stable thermal environments. By examining the long-term effects of temperature on fish health we begin to understand the mechanisms behind increased mortality observed in these populations, when compared with those from more heterogeneous environments. From this thesis important research pathways have been identified, such as the need to better understand the effect of temperature on cardiac function in these populations. It was found that simply testing a single measure of organism health or a single life stage is unlikely to provide an accurate reflection of an organism's ability to deal with a changing climate. Exposure to higher temperatures during the developmental period gave some species a distinct advantage in dealing with warmer temperatures and in the future multi-generational research may see this advantage extended even further. This thesis represents the most comprehensive analysis of the effects of projected temperature increases on low-latitude coral reef fish to date and is the only study to examine the acclimation capacity of fish at these latitudes. Further research is still needed to build on these findings and create a more holistic understanding of how these extremely vulnerable fish populations may persist in a warming environment, and how they can be better managed into the future.

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Appendix 1: Experimental methods in aquatic respirometry: the importance of mixing devices and accounting for background respiration.

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A.1 Summary

There is an increasing trend in the fish biology literature towards using static respirometry techniques without the inclusion of a mixing mechanism and without accurately accounting for the influence of microbial (background) respiration rates. This paper quantifies the effect that these approaches have on the oxygen consumption rates ($\dot{M}_{O_2}$) measured from juvenile barramundi *Lates calcarifer* (mass = 20.3 ± 0.8 g) and adult spiny chromis damselfish *Acanthochromis polyacanthus* (22.0 ± 2.5 g). Background respiration changed consistently and in a sigmoidal manner over time in the treatment with a mixing device (inline recirculation pump), whereas attempts to measure background respiration in the non-mixed treatment yielded highly variable estimates of $\dot{M}_{O_2}$ that were probably artefacts due to the lack of water movement over the oxygen sensor during measurement periods. This had clear consequences when accounting for background respiration in the calculations of fish $\dot{M}_{O_2}$. Exclusion of a mixing device caused a significantly lower estimate of $\dot{M}_{O_2}$ in both species and reduced the capacity to detect differences between individuals as well as differences within an individual over time. There was evidence to suggest that the magnitude of these effects was dependent on the spontaneous activity levels of the fish; as the difference between mixed and non-mixed treatments was more pronounced for *L. calcarifer* (sedentary) than for *A. polyacanthus* (more spontaneously active). It is clear that respirometry setups for sedentary species must contain a mixing device to prevent oxygen

stratification inside the respirometer. While more active species may provide a higher level of water mixing during respirometry measurements and theoretically reduce the need for a mixing device, the level of mixing cannot be quantified and may change with diurnal cycles in activity. To ensure consistency across studies without relying on fish activity levels, and to enable accurate assessments of background respiration, it is recommended that all respirometry systems should include an appropriate mixing device.

A.2 Introduction

The use of physiological measures to explain broad scale issues in ecology and conservation biology is growing in popularity throughout the scientific community (Wikelski & Cooke 2006; Clark et al. 2012; Seebacher & Franklin 2012; Cooke et al. 2013). By examining physiological responses to environmental stressors, scientists are better equipped to establish important mechanistic links between environmental conditions and whole organism performance. As humans continue to put greater pressure on the environment, now more than ever it is important for scientists to be able to understand the impacts of external stressors on an organism (Wikelski & Cooke 2006).

With the increased call for this type of research comes a desire to refine techniques for measuring the physiological performance of organisms. A popular method for quantifying the response of fish to a stressor is to measure rates of oxygen consumption ($\dot{M}_{O_2}$) (Fry 1947; Brett 1971; Steffensen 1989; Clark et al. 2013; Cooke et al. 2014; Svendsen et al. 2016). The diversity of methods used by such studies is increasing as scientists from different disciplines become interested in animal metabolism (e.g., Norin & Clark 2016). Resting $\dot{M}_{O_2}$ is typically measured in static, intermittent-flow respirometers, as fish are not required to swim in order to maintain position in the water column and should therefore exhibit low rates of oxygen consumption (Clark et al. 2013). Nevertheless, there exists substantial variation in equipment and techniques in studies using static respirometry. Often these respirometers contain some form of mixing mechanism to ensure sufficient mixing of water and oxygen levels within the chamber (Keys 1930; Steffensen 1989). Mixing can be achieved with submersible pumps

incorporated either inside the chamber or as part of a closed-circuit recirculation loop. Alternatively, some studies use a magnetic stir plate and stir bar to create water movement within the respirometer (Nilsson et al. 2007). The rate of flow created by a mixing device should be sufficient to ensure oxygen tension is homogenous throughout the entire respiration chamber, but not so great that the fish is disturbed by the flow or forced to swim against it (Clark et al. 2013). It is expected that calculations of $\dot{M}_{O_2}$ may be erroneous in chambers that do not contain a mixing device, as the mixing depends solely on the movements of the fish. A lack of sufficient fish movement will result in oxygen stratification within the respirometer and oxygen measurements that are not representative of the entire water volume, thus leading to incorrect calculations of $\dot{M}_{O_2}$ (Steffensen 1989; Clark et al. 2013; Svendsen et al. 2016).

When respirometry techniques are applied in ecological research, logistical challenges arise associated with the desire to carry out experiments in field locations or conduct studies which require large sample sizes. In this type of research there is an increasing trend towards using static respirometry techniques without the inclusion of a mixing device (Donelson et al. 2011b; Donelson & Munday 2012; Miller et al. 2012; Seebacher et al. 2013; Handelsman et al. 2013; Seebacher et al. 2014a, Seebacher et al. 2014b). It is important to understand how the exclusion of a mixing device will affect the results of a study. The standardisation of methods is an important aspect of experimental design and if erroneous results are produced using inappropriate methods, comparisons within and between studies become misleading.

This paper seeks to quantify the effect that the exclusion of a mixing device has on $\dot{M}_{O_2}$ data measured from fishes. Additionally, this study investigates the implications of using different methods for accounting for microbial (background) respiration during calculations of fish $\dot{M}_{O_2}$. These methods are varied and, at times, either not specified or not applied at all (Urbina & Glover 2012; Enzor et al. 2013; Dwyer et al. 2014; Klinger et al. in press), but can be critical in particular circumstances such as warm environments or when the respirometer system has a large surface area to volume ratio. The present study is one of the first to investigate these issues explicitly (but see some aspects of Dalla Via (1983) and Clark et al. (2013)).

A.3 Methodology

Animals and holding conditions

Two fish species (adult spiny chromis damselfish *Acanthochromis polyacanthus* Bleeker 1855 and juvenile barramundi *Lates calcarifer* Bloch 1790) were used in this study, sourced from stocks maintained at James Cook University Marine Aquarium Research Facility Unit and the Australian Institute of Marine Science, Queensland, Australia. Juvenile *L. calcarifer* were selected for this study as they were a very similar size to the adult *A. polyacanthus* and so could be tested in the same sized respirometers. The mean (±SE) mass of *L. calcarifer* and *A. polyacanthus* was 20.31±0.81 g and 22.03±2.53 g, respectively. Throughout the experimental period, *A. polyacanthus* were maintained in individual tanks or in pairs, while *L. calcarifer* were stocked at a density of 5-8 fish per tank (30 l). All tanks were continuously supplied with flow-through filtered seawater (salinity 35 ppt) at a temperature of 28 ± 1 °C (actual range). Feeding was once daily with NRD Aquaculture Nutrition commercial pellets (*A. polyacanthus*; www.inveaquaculture.com) or Ridley Aqua-Feed Marine Float pellets (*L. calcarifer*; www.ridley.com.au). For each species, 6-8 replicate fish were used for each treatment group. A total of n = 12 *A. polyacanthus* and n = 14 *L. calcarifer* were used. All fish were moved into the experimental laboratory a minimum of one week prior to commencement of the project to allow for acclimation to the indoor aquarium environment.

Respirometry setup and experimental design

Eight transparent, static respirometers (cylindrical, ~3 l polyethylene terephthalate (PET) plastic jars; length 210 mm, diameter 140 mm narrowing to 110 mm for 20 mm where the lid was attached) were set up within a large reservoir bath equipped with continuous aeration and supplied with filtered flow-through seawater at 28 ± 1 ^{o}C (actual range). The respirometers were darkened on the outside with black plastic to prevent external disturbances, although three ~10 mm strips were left uncovered to allow light to penetrate into the chambers. The respirometers included a closed-circuit recirculation loop (~10 mm internal diameter, narrowing to ~6 mm at the inlet and

outlet tubes) with an inline pump (max. 300 l h^{-1} submersible power head with flow reduced to ~150 l h^{-1}; www.eheim.com) attached to the outside of the lid section. The inlet tube on the recirculation loop pulled respirometer water from the immediate underside of the lid, while the outlet tube extended through the lid and to the far end of the respirometer to ensure good mixing. These inline pumps were switched on and off when necessary to produce treatments of mixing and non-mixing, respectively. Each respirometer was also connected to a flush system (~50 l h^{-1} per respirometer) regulated by a timer, where flushing occurred for 20 min every 40 min and excess water flowed out of an elbow fitting in the lid of the respirometer that extended above the water surface of the reservoir bath. As with the outlet tube on the recirculation loop, the outlet tube from the flush pump extended to the opposite end of the respirometer to the lid in order to maximise flush efficiency. Oxygen consumption rates ($\dot{M}_{O_2}$) were measured as the rate of decline in respirometer oxygen concentration during the 20 min between flush cycles, as measured using a contactless oxygen sensor spot system at 0.5 Hz (Firesting O_2, PyroScience, www.pyro-science.com) where the sensor spot material was attached to the inside of a clear window towards the top of each respirometer chamber. The 20/20 min measurement/flush cycle produced clear slopes of oxygen concentration during the measurement period without allowing respirometer oxygen levels to fall below ~75% air saturation. The oxygen sensor system was calibrated using manufacturer's specifications prior to every experiment while the recirculation and flush pumps were running and after the system had been cleaned.

Background respiration

Before commencing trials on fishes, long term changes in background $\dot{M}_{O_2}$ patterns were quantified under both mixed and non-mixed conditions. All components of the system were first bleached for 1 h in order to minimise the level of biological activity present in the experimental setup. The system was then drained, rinsed and wiped down with fresh seawater multiple times before refilling the reservoir bath and respirometers for the commencement of background respiration measurements. All recirculation pumps were first switched on to remove any bubbles in the respirometer system, and then the lids of the respirometers were connected and four of the

recirculation pumps were switched off to create the non-mixed treatment. The system was then left to run for ~40 h with measurements of background $\dot{M}_{O_2}$ occurring every 20 min as described above. An identical protocol was conducted 2 d later, but with the opposite configuration for the recirculation pumps that were switched on or off, to give a total of eight replicate trials for each of the mixed and non-mixed treatments.

Fish respiration

Food was withheld for 48 h prior to conducting respirometry on fish to ensure that the $\dot{M}_{O_2}$ measurements were not influenced by specific dynamic action (the energy used in the digestion, absorption and assimilation of a meal; Clark et al. 2010; Chabot et al. 2016a). The respirometers were cleaned as detailed above and all recirculation pumps were switched on to assist with clearing air bubbles from the respirometers. The oxygen sensor system was calibrated as described above. Background respiration was checked at this point and was negligible in all trials. Seven fish of one species were moved individually from their holding tanks to the respirometers carefully using a hand net. One respirometer was randomly chosen to remain empty in order to conduct parallel measurements of background respiration. Once all respirometers were sealed (~ 5 min), 3-4 of the recirculation pumps were switched off (never the empty respirometer) and recording commenced. $\dot{M}_{O_2}$ recordings continued on a 20/20 min measurement/flush cycle for ~20 h at which point all recirculation pumps (except for the empty respirometer) were switched to the opposite configuration for the following ~20 h. That is, fish that were in the mixed (pump on) treatment for the first 20 h changed to the non-mixed (pump off) treatment for the subsequent 20 h, and vice versa. This allowed for both treatments to be tested over almost a full daily cycle once accounting for logistical tasks between trials (e.g., cleaning respirometers, catching new fish, etc). By staggering the start times between trials, all hours of the day were captured for each species and treatment group. Following the ~40 h trial, fish were removed, weighed and returned to their holding tanks (individually numbered to prevent reuse of the same fish), and each respirometer was resealed for 2-3 measurements of background respiration while the recirculation pump was in each of the on and off configurations. Identical protocols were conducted on four occasions (using different fish each time) to

achieve desired sample sizes for each of the two species under each of the recirculation pump configurations: *n* = 6 *L. calcarifer* started with pump on, *n* = 8 *L. calcarifer* started with pump off (plus two parallel background trials with pump on); *n* = 6 *A. polyacanthus* started with pump on, *n* = 6 *A. polyacanthus* started with pump off (plus two parallel background trials with pump on).

Spontaneous activity of A. polyacanthus in respirometers

Video observations were conducted on a separate subset of *A. polyacanthus* at 28 ^{o}C to determine if activity levels within the respirometers were influenced by having the recirculation pump switched on or off (*n* = 8 in each group). Techniques and respirometers were the same as detailed above, except that in these trials the chambers were transparent and dividers were placed between the chambers so that fish could not see each other. Drop sheets were also hung around the experimental tanks so that there was no outside influence on fish behaviour. The chambers were kept transparent (rather than largely darkened, as detailed above) so that a webcam placed above the tanks could record fish activity. Behaviour in each chamber was recorded for one minute at different points after respirometer entry (0.5, 1, 2 and 3 h).

Upon review of the video footage, individual *A. polyacanthus* were assigned a behaviour ranking from 1 to 5 for each one minute recording. Behaviour was classified as 1) fish stationary in chamber, 2) fish showing some on-the-spot movement but not moving around the chamber, 3) fish displaying slow, controlled swimming, occupying up to half the chamber area, 4) fish displaying slow, controlled swimming, occupying the entire chamber, or 5) fish displaying fast, erratic swimming behaviour. Generally, fish only displayed one behaviour during the observation period, however if more than one behaviour was observed the higher ranked behaviour was recorded as this was assumed to have a greater effect on metabolic rate. One person conducted all of the behaviour analyses to minimise variability in interpretation, yet that person was kept blind to the treatment groups in the video footage to remove any possibility of bias.

Data analysis and statistics

Text files from the Firesting O_2 system were formatted and loaded into LabChart (ADInstruments, www.adinstruments.com) for analysis of the slopes of oxygen concentration over time, with subsequent calculations of $\dot{M}_{O_2}$ performed in Excel (Microsoft Corporation, WA, USA). Slopes could be easily visualised in LabChart such that data with the most linear slope could be selected for calculations of $\dot{M}_{O_2}$ (always >10 min of slope data). Mass-specific $\dot{M}_{O_2}$ (µmol min^{-1} kg^{-1}) was calculated according to equation 2 of Clark et al. (2013). Background respiration (µmol min^{-1}) was calculated using the same equation but with the omission of fish volume and fish mass. Respirometer volumes used in the calculations were adjusted between mixed (3.108 l) and non-mixed (3.077 l) treatments to account for the small amount of additional water (31 ml) contained in the recirculation loop when the recirculation pumps were switched on (it was assumed that no water circulated through this loop when the recirculation pump was switched off). To account for any slight deviations in temperature during a trial (±1 ^{o}C throughout the entire experimental period), all $\dot{M}_{O_2}$ data were corrected to 28 ^{o}C using a Q_{10} of 2.2.

Three different analytical methods were used to assess and visualise the impact of background respiration on fish $\dot{M}_{O_2}$ measurements. These different analytical approaches were taken in order to be as thorough and transparent as possible in the presentation of the data. First, it was assumed that background respiration was non-existent and thus nothing was subtracted from the fish $\dot{M}_{O_2}$ values. Second, the initial (always zero) and final measurements of background respiration in each respirometer were used from the 'Fish Respiration' experiments and a linear change in background respiration was assumed over the ~40 h trials (these linear changes in background values were different for mixed and non-mixed configurations, and thus the subtracted background value depended on both time and pump configuration). Third, the mean changes in background respiration calculated for a given time point from the dedicated 'Background Respiration' experiments (see Fig. A.1) were subtracted from fish $\dot{M}_{O_2}$. The latter was chosen to ensure consistency in the calculations, which would not have been possible if the continuous background measurements conducted in parallel with

the 'Fish Respiration' experiments were used because only the mixed treatment was assessed for background respiration during those trials. Nevertheless, the $\dot{M}_{O_2}$ data from the parallel background trials were qualitatively and quantitatively similar to those obtained from the dedicated background trials and thus the conclusions of the study remain the same.

Statistical tests were conducted using R software (www.r-project.org) and SigmaPlot 11 (www.sigmaplot.com). Comparisons of $\dot{M}_{O_2}$ between pump treatments (mixed vs. non-mixed) at 0-10 h after respirometer entry were conducted separately for each species using a two-way repeated measures ANOVA (time and pump treatment as factors) with the Holm-Sidak method for multiple comparisons, where $\dot{M}_{O_2}$ data were log-transformed to satisfy assumptions of equal variance (Fig. A.2). Comparisons of $\dot{M}_{O_2}$ between pump treatments as a function of time of day were conducted separately for each species using mixed effects models, where pump treatment was a fixed factor, time of day was included as a continuous variable, and fish identity was treated as a random factor. The model was also run using time of day as a categorical variable to allow any treatment effects to be identified at each time point. For the analyses based on time of day, the first 5 h of data for each trial were excluded in order to remove any confounding effects of handling stress (see Fig. A.2). Differences in activity levels during the video monitoring trials were tested using a one-way ANOVA.

A.4 Results

Background respiration

Background respiration changed consistently over time in the mixed treatment, with values remaining close to zero for around 30 h before trending upwards as microbial load in the respirometers began to increase (Fig. A.1). The coefficient of determination (R^2) for the slopes of oxygen concentration over time were always approximately zero at the beginning of the background trial (i.e., no discernible slope) and increased to 0.975±0.004 and 0.383±0.220 (mean±SD) after 40 h for the mixed and non-mixed treatments, respectively. Background respiration in the non-mixed respirometers was much less predictable and consistent, beginning to deviate substantially from zero after

about 10 h and reaching peak values at 19-23 h that were 23-times higher (based on means) and 23-times more variable (based on SE) than those obtained for the mixed treatment at the same time points. Background respiration in the non-mixed group trended back down between 24-33 h to end at values similar to those obtained for the mixed group at 34-40 h (Fig. A.1). It is highly likely that these trends in background respiration in the non-mixed treatment are artifacts of having no water movement in the respirometers between flush cycles, and therefore the term 'apparent background respiration' is used as necessary herein to describe the background measurements for the non-mixed treatment.

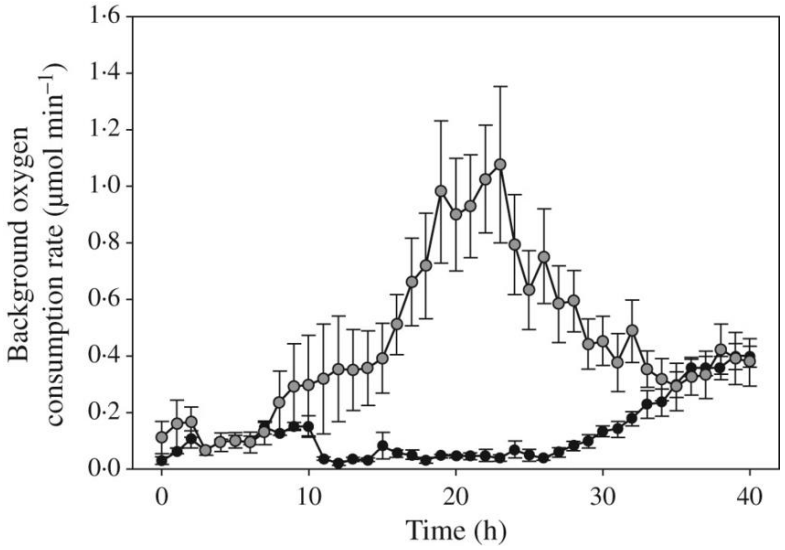

Figure A.1. Background respiration data from empty respirometers (i.e., containing no fish) where the recirculation pump was either switched on (black circles; n = 8 trials) or switched off (grey circles; n = 8 trials).

Fish respiration after respirometer entry

Given that background respiration did not deviate substantially between mixed and non-mixed treatments until ~10 h (Fig. A.1), direct comparisons of fish $\dot{M}_{O_2}$ were possible between mixed and non-mixed treatments in the first 10 h without the confounding effects of subtracting treatment-specific background respiration values (Fig. A.2). Oxygen consumption rates fell steadily after entry to the respirometers as the fish recovered from handling stress (Fig. A.2). *L. calcarifer* seemed to reach a low plateau 2-3 h after entry (Fig. A.2 A), whereas *A. polyacanthus* took longer and did not appear

to reach a plateau until 5-6 h after entry (Fig. A.2 B). While the qualitative patterns in fish $\dot{M}_{O_2}$ between mixed and non-mixed treatments were similar over the first 10 h of trials, the $\dot{M}_{O_2}$ values obtained from the non-mixed treatment were consistently and significantly lower than those obtained from the mixed treatment in both species (two-way repeated measures ANOVA: $F_{1, 153} = 18.4$, $P = 0.001$ for *L. calcarifer*; $F_{1, 131} = 6.8$, $P = 0.026$ for *A. polyacanthus*).

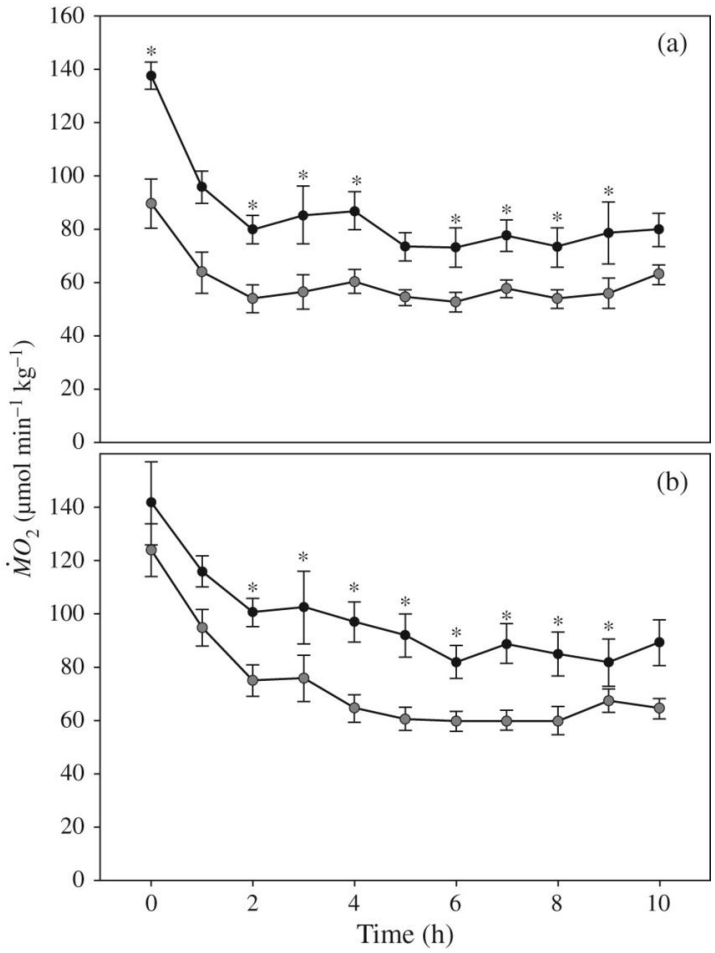

Figure A.2. Oxygen consumption rates ($\dot{M}_{O_2}$) of (A) *L. calcarifer* and (B) *A. polyacanthus* following entry into respirometers. Black circles are data from respirometers where the recirculation pump was switched on, while grey circles are data from respirometers where the recirculation pump was switched off (n = 14 *L. calcarifer* and n = 12 *A. polyacanthus*). Data have not been adjusted to account for changes in background respiration over time because background respiration was negligible for the first few hours of measurements (see Fig. A.1). Data are means ± SE. Significant differences between pump treatments within time points are indicated with asterisks.

Fish respiration over 24 hours

There were visible and immediate changes in the slopes of oxygen decline in the respirometers as the treatment was switched from mixed to non-mixed, and vice versa, and these produced notable changes in the calculations of $\dot{M}_{O_2}$ (Fig. A.3). All $\dot{M}_{O_2}$ data were aligned with time of day (after excluding the first 5 h of data post-entry to the respirometers) to allow more complete comparisons between pump treatments (Fig. A.4).

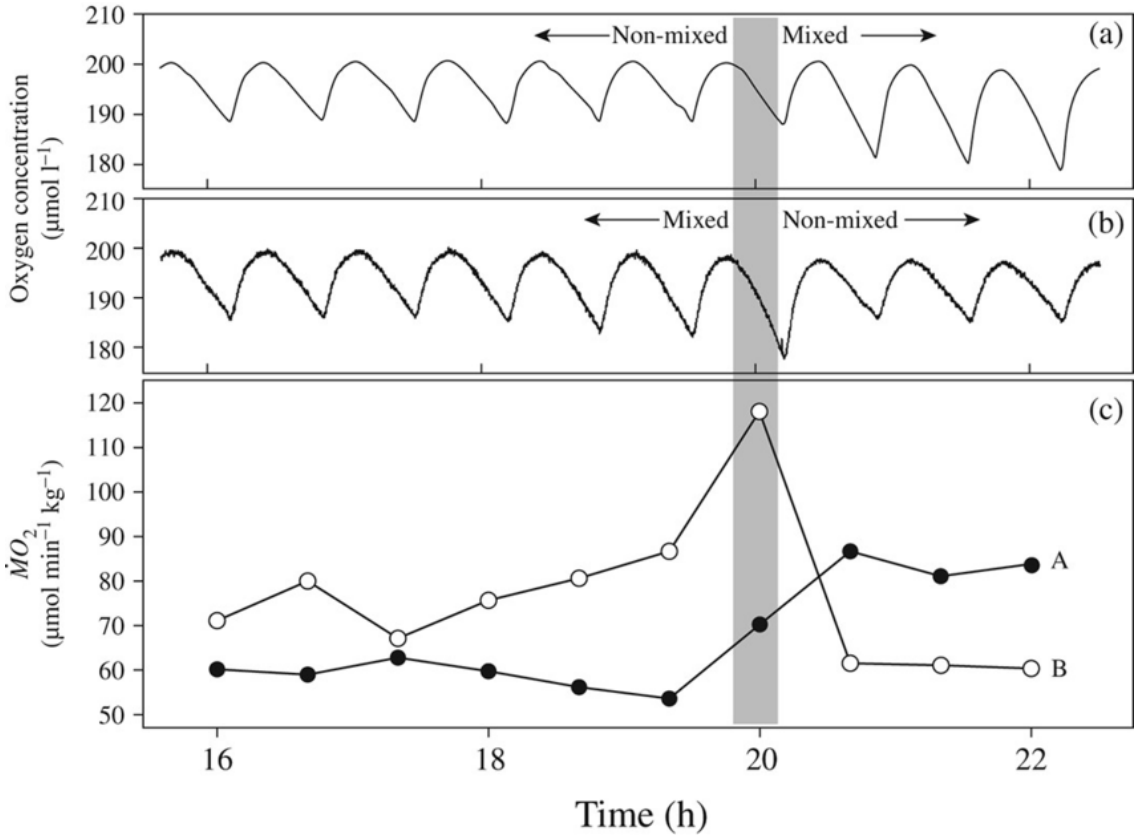

Figure A.3. Representative raw traces of respirometer oxygen levels over time for *L. calcarifer*, where (A) shows the changes as the respirometer was transitioned from a non-mixed to a mixed configuration by switching on a recirculation pump (indicated by grey rectangle), and (B) shows the changes as the respirometer was switched from a mixed to a non-mixed configuration. Upward deflections in oxygen indicate when the automatic flush pump switched on. (C) shows the corresponding calculations of $\dot{M}_{O_2}$ based on (A) and (B), where background respiration was subtracted assuming a linear change over time (see Materials and Methods).

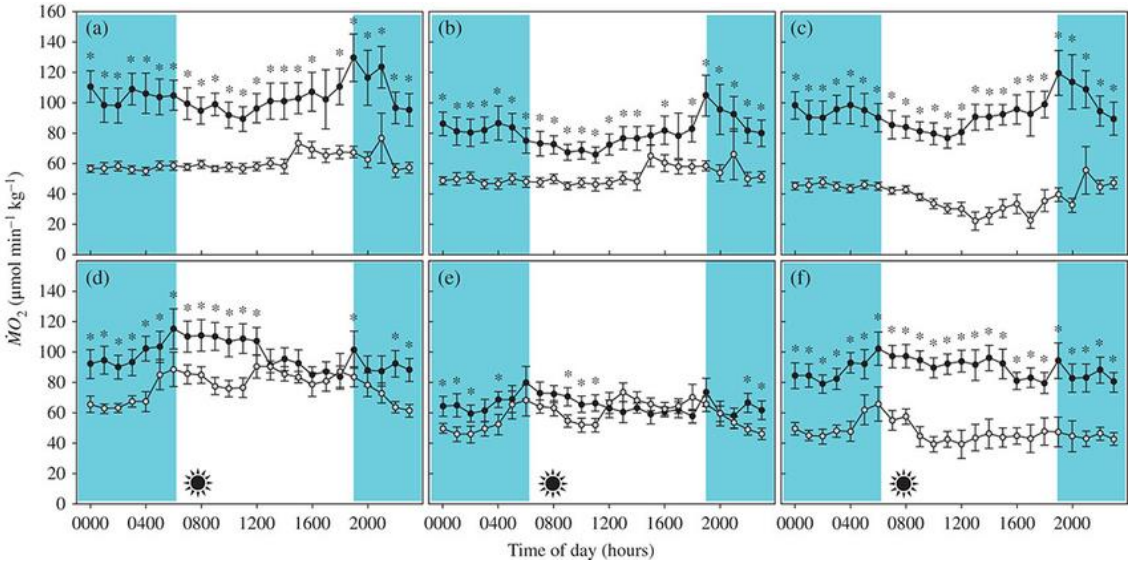

Figure A.4. Oxygen consumption rates ($\dot{M}_{O_2}$) of (A-C) *L. calcarifer* and (D-F) *A. polyacanthus* as a function of time of day (dark hours shaded). Data are means ± SE. Black circles are data from respirometers where the recirculation pump was switched on (mixed), while grey circles are data from respirometers where the recirculation pump was switched off (non-mixed; n = 14 *L. calcarifer* and n = 12 *A. polyacanthus*). Data in (A) and (D) have not been corrected for background respiration, data in (B) and (E) have been corrected assuming a linear change in background respiration between the beginning and end of the experiment (different values for 'pump on' vs. 'pump off' treatments), and data in (C) and (F) have been corrected using the mean measured changes in background respiration that occurred over time throughout the experiments (see Fig. A.1; different values for 'pump on' vs. 'pump off' treatments). Significant differences between pump treatments within each time point are indicated with an asterisk.

For *L. calcarifer*, the mixed (pump on) treatment consistently gave $\dot{M}_{O_2}$ values that were higher than the non-mixed (pump off) treatment, regardless of whether background respiration was ignored (Fig. A.4 A; $F_{1, 563} = 838.7$, $P < 0.0001$), subtracted in a linear fashion (Fig. A.4 B; $F_{1, 563} = 488.0$, $P < 0.0001$), or subtracted in a non-linear fashion based on the dedicated background respiration trials detailed above (Fig. A.4 C; $F_{1, 563} = 859.0$, $P < 0.0001$). For each method of subtracting background respiration, time of day had a significant effect ($F_{23, 563} = 2.2$, $P = 0.0014$, $F_{23, 563} = 3.3$, $P < 0.0001$ and F_{23},

$_{563}$ = 6.3, *P* < 0.0001 in Figs. A.4 A-C, respectively). No significant interaction was observed for *L. calcarifer* between treatment and time of day when either background respiration was ignored or when a linear trend was assumed, indicating that differences among treatments did not vary with time ($F_{23, 563}$ = 1.1, *P* = 0.3489 and $F_{23, 563}$ = 1.2, *P* = 0.2737, respectively). In contrast, when mean measured changes in background respiration were used to correct *L. calcarifer* $\dot{M}_{O_2}$, a significant interaction between treatment and time of day was observed (Fig. A.4 C; $F_{23, 563}$ = 5.3, *P* < 0.0001).

For *A. polyacanthus*, there was again a significant effect of treatment (mixed vs. non-mixed) on $\dot{M}_{O_2}$ for all three methods of accounting for background respiration ($F_{1, 423}$ = 212.8, *P* < 0.0001, $F_{1, 423}$ = 34.1, *P* < 0.0001 and $F_{1, 423}$ = 513.0, *P* < 0.0001 for Figs. A.4 D-F, respectively). Time of day also had a significant effect for all methods ($F_{23, 423}$ = 4.4, *P* < 0.0001, $F_{23, 423}$ = 2.2, *P* = 0.0011 and $F_{23, 423}$ = 1.7, *P* = 0.0209). However, the differences between the mixed and non-mixed treatments were generally less pronounced than for *L. calcarifer*, especially when background respiration was corrected using a linear function (Fig. A.4 E). When background respiration was ignored, a significant interaction was recorded between treatment and time of day (see Fig. A.4 D; $F_{23, 423}$ = 2.3, *P* = 0.0006). No significant interaction was observed for *A. polyacanthus* when a linear change in background respiration was assumed (Fig. A.4 E; $F_{23, 423}$ = 1.1, *P* = 0.2893), nor when mean measured changes in background respiration were used to correct fish $\dot{M}_{O_2}$ (Fig. A.4 F; $F_{23, 423}$ = 0.6, *P* = 0.9550).

Fish activity in respirometers

An additional observation from Fig. A.4 is that the mixed treatment generally had more variability around the mean. This suggests either that the fish in the mixed treatment were less relaxed or that the non-mixed treatment failed to detect real differences in $\dot{M}_{O_2}$ between individuals. Routine observations of the fish in the respirometers (1-2 observations per day) suggested that the activity levels of the fish in each treatment were identical; *L. calcarifer* were observed to sit calmly at the rear of the respirometers in both treatments, while *A. polyacanthus* were more active in general but did not behave differently between treatments. These observations were further supported by

the semi-quantitative video analysis of *A. polyacanthus* activity levels, where no significant differences were detected between the mixed and non-mixed treatments at any of the measured time points after respirometer entry (one way ANOVA: $F_{1, 62}$ = 203.33, *P* = 0.473; Fig. A.5).

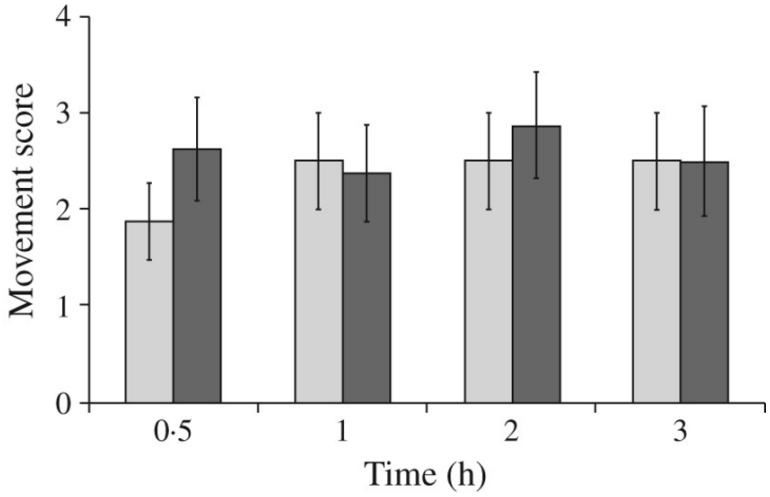

Figure A.5. Movement of *A. polyachanthus* within respiromters as a function of time after respirometer entry in mixed and non-mixed respirometers (n=8 in each group). Activity levels were ranked from 1-5 as indicated in the Materials and Methods. Values are means ± SE.

A.5 Discussion

Handling stress upon respirometer entry

There was clear evidence of handling stress in both species once they were placed into respirometers, a response typical for fishes (Holeton 1974; Caulton 1978; Davis & Schreck 1997; Chabot et al. 2016b). While the qualitative trends in $\dot{M}_{O_2}$ were similar between treatments, within a species the values in the mixed treatment were consistently higher than those from the non-mixed treatment. The difference in the highest recorded $\dot{M}_{O_2}$ (time = 0 h) between treatments was more pronounced for the sedentary *L. calcarifer* (which remained relatively still upon respirometer entry) than for the more active *A. polyacanthus* (which was consistently active upon respirometer entry).

Species differences in the time it took for $\dot{M}_{O_2}$ to plateau once fish were placed into respirometers highlight the importance of conducting species-specific pilot studies if resting levels of $\dot{M}_{O_2}$ are desired. Different species and different sized fish have different acclimation times after respirometer entry (Holeton 1974; Chabot et al. 2016b), and within a species the time to acclimation may vary between systems due to slight and often unavoidable variations in methods. Dissimilarities will occur between systems based on a range of factors including how often fish have been handled prior to experimentation, the methods used to catch/handle the fish prior to respirometer entry, and the relative size of the respirometer compared with fish size (Holeton 1974; Caulton 1978; Davis & Schreck 1997). Past studies on a range of fish species have reported times to acclimation from as little as one hour, up to several hours or overnight (Clark et al. 2010; Obermüller et al. 2010; Donelson et al. 2011a; Couturier et al. 2013). It is generally recognised in the aquatic physiology community that overnight recovery times should be provided as standard practice.

Effects of subtracting background respiration – mixed vs. non-mixed

While the patterns of background respiration over time were consistent and expected in respirometers with a mixing mechanism, those in respirometers without a mixing mechanism were more random and difficult to interpret. Perhaps the best explanation for the large increase in apparent background respiration between 10 and 23 h in the non-mixed treatment is that microbial populations were able to build up on the oxygen sensor spot in the absence of any water circulation to dislodge them (it is unclear why the flow from the flush pump between $\dot{M}_{O_2}$ measurements did not fill this role). It remains speculative why the apparent background respiration in the non-mixed treatment trended back down again after 23 h and ended at a similar level as the mixed treatment. A similar trend where the microbial growth curve overshoots and then returns to a lower level was also observed by Dalla Via (1983), however that study also was not able to offer an explanation of the mechanism behind this trend. It is possible that these extreme deviations would not occur in the respirometer when a fish is present because the fish would provide at least some water movement during periods

between flush cycles, but of course it is impossible to measure background respiration under this scenario.

The length of time over which background respiration remains negligible in a study varies between lab setups, dependent on factors such as temperature, how the respirometers are cleaned, the material from which the respirometers are constructed, the surface area to volume ratio of the respirometers, and the biological load of the water (e.g., freshwater versus seawater, tropical versus temperate; Clark et al. 2013). For example, respirometry studies on cold water species generally experience less microbial interference than in tropical systems (Genovesi et al. 2011). The data from the present study emphasise the importance of understanding the patterns of microbial build-up in a respirometry system when trying to accurately quantify the $\dot{M}_{O_2}$ of aquatic organisms.

The three methods of accounting for background respiration used in this study highlight the effect that different methods can have on a range of aspects relating to how data are interpreted. Failing to make any correction for background respiration has obvious disadvantages. Whilst this method may not have a significant effect on the data over the first several hours of data collection if the equipment was initially sterilised, subsequent measurements may be largely driven by microbial respiration, especially in warm water, in respirometers where surface area is large relative to volume, or where fish size is relatively small compared with respirometer size.

When assuming a linear increase in background respiration, $\dot{M}_{O_2}$ can be predicted by taking a measurement of background respiration only for a short time at the beginning and the end of each trial, as has been applied for decades and continues today (e.g., Collins et al. 2013; McLeod et al. 2013; Norin et al. 2014). Comparison with data collected from empty respirometers running for the full 40 h reveals that although not ideal, subtracting background respiration in a linear fashion from mixed chambers should cause only a marginal underestimate of fish $\dot{M}_{O_2}$ between 10-35 h and is not likely to severely distort trends in calculated fish $\dot{M}_{O_2}$ under the conditions of the present study (Fig. A.1). Despite this, subtle patterns in the $\dot{M}_{O_2}$ data such as diurnal

variation may be diminished and thus not detected when using this method. The pattern of apparent background respiration in the non-mixed chambers is such that assuming a linear trend in background respiration would miss the large peak in apparent background respiration measured for this treatment at 10-30 h into recording. This is reflected in the calculations of fish $\dot{M}_{O_2}$, as traces are drawn closer together when using a linear background subtraction due to similar first and last readings between treatments (Figs. A.4 B, A.4 E).

Based on the present comparisons, it is likely that the most accurate representation of fish $\dot{M}_{O_2}$ can be obtained by subtracting background respiration in a non-linear fashion based on dedicated background respiration trials or parallel background trials alongside fish $\dot{M}_{O_2}$ measurements. Given that the purpose of conducting trials over 24 h or longer is to provide precise measures of baseline (standard) metabolic rate or to detect somewhat subtle trends in the data such as diurnal variation in $\dot{M}_{O_2}$, it seems logical that the method least likely to mask these measurements and trends is chosen. Having said that, there are assumptions in all methods of accounting for background respiration (e.g., applying the background dynamics of one respirometer to all other respirometers), and the magnitude of background respiration can vary substantially between study systems (e.g., higher in tropical systems), so it is important that all studies conduct the necessary experiments to understand the dynamics of background respiration in their respirometry systems prior to embarking on studies of fish respiration (see Svendsen et al. 2016).

Although the highest level of resolution can be produced by dynamically accounting for background respiration, it was difficult to apply this method in the present study when treatments were switched within a chamber during the course of the experiment. To account for the different levels of background respiration between treatments, mean background respiration was applied to each time point dependent on the length of time that the experiment had been running. This method of applying mean background respiration does not account for how background respiration was affected by the previous treatment. A similar problem is encountered when assuming a linear trend in background respiration and then switching between mixing and non-mixing treatments

midway through the trial, however in the present study these issues were more pronounced when dynamically accounting for background respiration because of the substantial temporal differences in the mixed vs. non-mixed background respiration assessments (Fig. A.1). Clearly, most respirometry studies do not switch between mixing and non-mixing treatments during a trial, and so several of the abovementioned issues will not be present when dynamically accounting for background respiration.

Fish respiration over 24 hours

Regardless of whether background respiration was ignored, subtracted in a linear fashion, or subtracted in a non-linear fashion based on background respiration trials, the mixed treatment consistently produced a higher and more variable $\dot{M}_{O_2}$ than the non-mixed treatment. There are two possible explanations for why this trend was observed: (1) the fish in the mixed chambers were more stressed and thus consumed oxygen at a higher and more variable rate, or (2) oxygen stratification occurred in the non-mixed chambers such that water oxygen tension was not homogeneous throughout the total water volume of the respirometers, giving the perception of reduced variability between individuals as well as within an individual over time. It is concluded that these differences in $\dot{M}_{O_2}$ between treatments, and the variability within and between individuals within a treatment, were due to the presence or absence of mixing within the respirometers rather than due to any increased stress or discomfort caused by the inclusion of a recirculation pump.

This conclusion is supported by the results of the video analyses (Fig. A.5), which showed no difference in activity levels between the mixed and non-mixed treatment groups, indicating that no one group was more active than the other (i.e., the recirculation pump did not increase $\dot{M}_{O_2}$ via additional swimming activity). Notably, the video activity analysis also indicated that the initial elevation in $\dot{M}_{O_2}$ upon respirometer entry (Fig. A.2) was due to the repayment of an oxygen debt acquired during the handling procedure rather than being associated with elevated levels of fish activity in the initial stages of respirometer confinement. A comparison of the two treatment groups immediately after entry to the respirometers (where background respiration

was negligible) provides additional evidence that stratification was the cause of the difference in $\dot{M}_{O_2}$ between treatments. If it was indeed stress that caused the higher $\dot{M}_{O_2}$ in the mixed treatment group, it would be expected that at time 0 (Fig. A.2) the handling process would have elicited the same metabolic response in the fish regardless of whether they were placed into a respirometer that had circulating water or not (i.e., both treatments experienced identical handling stress). Instead, the mixed treatment still obtained a higher $\dot{M}_{O_2}$ than the non-mixed treatment immediately after respirometer entry. Notably, the difference between treatments was generally more pronounced for *L. calcarifer* than for *A. polyacanthus* when the data are examined based on time of day (Fig. A.4). It is probable that this species difference eventuated because the sedentary *L. calcarifer* was not as successful at providing any appreciable mixing of the respirometer water in the absence of a dedicated mixing device. Although time of day also had a significant effect on $\dot{M}_{O_2}$, this response seemed to be largely driven by laboratory conditions when lights were switched on or off and would require further investigation to precisely characterise diurnal patterns. Nevertheless, it is notable that the daily changes in $\dot{M}_{O_2}$ were largely masked in the non-mixed treatment, particularly for *L. calcarifer* (Fig. A.4). Moreover, it is important in this context not to assume that "less variable" equals "more accurate", as it is well-known that resting or routine $\dot{M}_{O_2}$ can change dramatically on a diurnal cycle (see Moran et al. 2014) and can also vary up to 3-fold between individuals of the same species (Burton et al. 2011).

Conclusions and recommendations

The data presented in this study illustrate that experimental methods, both in the laboratory and when conducting statistical analyses, can have a significant impact on the output of respirometry studies. This research has highlighted the fact that variables within a system can contribute to variation in the data despite careful laboratory practice. The present study has also shown that the movement of a fish can influence oxygen mixing characteristics in respirometers that do not contain a mixing device. Given these results, it can be concluded that pilot studies are essential in determining the functioning of a particular experimental set up and careful consideration should be given to the design of experimental protocols. To ensure consistency between studies

and remove errors associated with unquantifiable mixing activities by the fish, it is recommended that respirometers be equipped with a mixing device. Additionally, researchers should be aware of pitfalls in the various methods for subtracting background respiration. In the context of the present study, it is recommended that background respiration is continuously measured in an empty respirometer in parallel with fish $\dot{M}_{O_2}$ measurements, particularly if there is a potential for highly influential bacterial growth such as that which occurs in warm environments and in respirometry systems with high surface area to volume ratios.

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