Hypoxia and thermal tolerance in New Zealand triplefin fishes

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Marine Science, The University of Auckland, 2019.

Abstract

Temperature and the availability of oxygen (O_2) each have a profound influence on the metabolism of fish and play a key role in shaping the distribution and abundance of species. All fishes are exposed to at least some fluctuation in each of these environmental parameters, but few species are subjected to the extreme acute changes in O_2 availability and temperature faced by fishes inhabiting intertidal rock pools. This thesis, with a focus on intertidal species, explores the physiological responses of New Zealand triplefin fishes to variability in O_2 availability (hypoxia and hyperoxia) and increased temperature (acute and chronic exposure).

Decreased O_2 availability (hypoxia) is common in rock pools and challenges the aerobic metabolism of fishes living in these habitats. In Chapter 2, the critical O_2 tension (P_{crit}) - a measure of hypoxia tolerance - was compared between two intertidal and two subtidal triplefin fishes endemic to New Zealand. The intertidal species had a lower P_{crit} than the subtidal species indicating adaptations to meet O_2 demands of maintenance metabolism at lower O_2 tensions. While maintenance metabolism (measured as standard metabolic rate; SMR) did not show a major functional difference between species, the intertidal species had higher maximal rates of O_2 consumption ($\dot{M}O_{2,max}$) and higher aerobic metabolic scope (MS). The high O_2 extractive capacity of the intertidal species was associated with increased blood O_2 carrying capacity (i.e. higher Hb concentration); additionally, intertidal species had higher mass-specific gill surface area and thinner gill secondary lamellae that collectively conveyed a higher capacity for O_2 flux across the gills. The specialist intertidal species also had higher glycogen stores in both white muscle and brain tissues, suggesting greater potential to generate ATP anaerobically and survive in rock pools with O_2 tensions less than P_{crit}. Overall, Chapter 2 shows that the superior hypoxia tolerance of intertidal New Zealand triplefin species is not linked to a minimisation of basal metabolic demand (SMR), but is instead associated with a maximisation in the O_2 extractive capacity of the cardiorespiratory system (i.e. $\dot{M}O_{2,max}$, MS, Hb concentration and gill O_2 flux) and glycolytic tissue stores.

Environmental stressors often occur simultaneously or in quick succession, but how animals respond to multiple stressors is not well studied or understood. Acute heat shock has previously been shown to improve subsequent low O_2 (hypoxia) tolerance in an intertidal fish species, a process known as cross-tolerance, but it is not known whether this is a widespread phenomenon. As acute heat and hypoxic stress tend to occur out of phase in intertidal rock pools, Chapter 3 specifically examined whether a New Zealand rock pool specialist, the triplefin fish *Bellapiscis medius*, exhibits hypoxic cross-tolerance (i.e. longer time to loss of

equilibrium (LOE) and lower critical O_2 saturation (S_{crit}) under hypoxia) after recovering from an ecologically relevant heat shock. Non-heat shock controls had a median time to loss of equilibrium (LOE_{50}) of 54.4 min under severe hypoxia (7% of air saturation) and a S_{crit} of 15.8% air saturation. However, contrary to expectations, treatments that received an initial 8 or 10°C heat shock showed a significantly shorter LOE_{50} in hypoxia (+8°C = 41.5 min; +10°C = 28.7 min) combined with no significant change in S_{crit} (+8°C =17.0% air saturation; +10°C =18.3% of air saturation). No evidence of heat shock induced cross-tolerance in *B. medius* was, therefore, found because acute exposure to peak temperatures resulted in an impaired tolerance to hypoxia. This is cause-for-concern because climate change will increase the frequency and intensity of heat shock events in rock pools rendering *B. medius* less able to cope with multiple stressors across successive low tides.

Daytime low tides that lead to high temperature events in stranded rock pools often co-occur with algal mediated hyperoxia as a result of strong solar radiation. Recent evidence shows MS can be expanded under hyperoxia in fish but so far this possibility has not been examined in intertidal species despite being an ecologically relevant scenario. Furthermore, it is unknown whether hyperoxia increases the upper thermal tolerance limits of intertidal fish and their ability to withstand extreme high temperature events. Therefore, Chapter 4 measured the metabolic response (mass-specific rate of oxygen consumption [$\dot{M}O_2$]) to thermal ramping (21-29°C) and the upper thermal tolerance limit (CT_{max}) of two intertidal triplefin fishes (*B. medius* and *Forsterygion lapillum*) under hyperoxia and normoxia. Hyperoxia increased $\dot{M}O_{2,max}$ and MS of each species at ambient temperature (21°C) and also after thermal ramping to elevated temperatures such as those observed in rock pools (29°C). While hyperoxia did not provide a biologically meaningful increase in upper thermal tolerance of either species (>31°C under all conditions), the observed expansion of MS at 29°C under hyperoxia could potentially benefit the aerobic performance, hence the growth and feeding potential etc., of intertidal fish at non-critical temperatures. That hyperoxia does not increase upper thermal tolerance in a meaningful way is cause for concern, as climate change is expected to drive more extreme rock pool temperatures in the future; this could present a major challenge for these species.

Intertidal fish species face gradual chronic changes in temperature and greater extremes of acute thermal exposure through climate induced warming. As sea temperatures rise, it has been proposed that whole animal performance will be impaired through oxygen and capacity limited thermal tolerance (OCLTT, reduced aerobic metabolic scope-MS) and, on acute

exposure to high temperatures, thermal safety margins may be reduced due to constrained acclimation capacity of upper thermal limits. Using the New Zealand triplefin fish (*F. lapillum*), Chapter 5 addressed how performance in terms of growth and metabolism (MS) and upper thermal tolerance limits would be affected by chronic exposure to elevated temperature. Growth was measured in fish acclimated (12 weeks) to present and predicted future temperatures, and metabolic rates were then determined in fish at acclimation temperatures and with acute thermal ramping. In agreement with the OCLTT hypothesis chronic exposure to elevated temperature significantly reduced growth performance and MS. However, despite the prospect of impaired growth performance under warmer future summertime conditions, an annual growth model revealed that elevated temperatures may only shift the timing of high growth potential and not the overall annual growth rate. While the upper thermal tolerance (i.e. critical thermal maxima) increased with exposure to warmer temperatures and was associated with depressed metabolic rates during acute thermal ramping, upper thermal tolerance did not differ between present and predicted future summertime temperatures. This suggests that warming may progressively decrease thermal safety margins for hardy generalist species and limit the available habitat range of intertidal populations.

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A number of other people have contributed to the research described here and I would like to thank them: Peter Browne and Errol Murray contributed to the design and construction of the respirometry set-ups; John Atkins designed the respirometry software; Peter Browne, Errol Murray, Peter Schlegal, Paul Caiger, Craig Norrie and Esther Stuck helped with fish collection; Lisa Wallace and Andreas Kunzmann helped perform some of the respirometry presented in Chapter 2; Jules Devaux assisted with the glycogen assays in Chapter 2; Andreas Kunzmann critically revised the manuscript presented in Chapter 2.

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Abbreviations

ADP = Adenosine di-phosphate

ANOVA = Analysis of variance

ASR = Aquatic surface respiration

ATP = Adenosine tri-phosphate

BM = Body mass

CO_2 = Carbon dioxide

CT_{max} = Critical thermal maximum

$FADH_2$ = Reduced flavin adenine dinucleotide

GTP = Guanosine triphosphate

Hb = Haemoglobin

Hb-O_2 = Haemoglobin bound oxygen

Hct = Haematocrit

HSI= Hepatosomatic index

Hsps= Heat shock proteins

LDH = Lactate dehydrogenase

LOE= Loss of equilibrium

LOE_{50}= Median time to loss of equilibrium

MMR = Maximum metabolic rate

$\dot{M}O_2$ = Mass-specific oxygen consumption

$\dot{M}O_{2,max}$ = Maximum post-exercise oxygen consumption

MRD = Metabolic rate depression

MS = Aerobic metabolic scope

NADH = Reduced nicotinamide adenine dinucleotide

NAD^+ = Oxidised nicotinamide adenine dinucleotide

O_2 = Oxygen

OCLTT = Oxygen and capacity limited thermal tolerance

OEC = Oxygen equilibrium curve

PCO_2 = Carbon dioxide partial pressure

P_{crit} = Critical oxygen tension

PFK = Phosphofructokinase

PK = Pyruvate kinase

PO_2 = Oxygen partial pressure

RBC = Red blood cell

RMR = Routine metabolic rate

$RMR_{max\ TR}$ = Maximum routine metabolic rate during thermal ramping

SA = Surface area

SGA = Sectioned gill arch

SGR =Mass-specific growth rate

SL = Gill secondary lamellae

SMR = Standard metabolic rate

SST = Sea surface temperature

TCA= Tricarboxylic acid

T_{critMS} = Upper critical temperature where metabolic scope is zero

T_{optMS} = Temperature optimum for metabolic scope

THR = Total hypoxia response

UQ = Ubiquinone

WGA = Whole intact gill arch

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Nature of contribution by PhD candidate	Perfromed the majority of the respirometry, all gill morphometric analysis, and all biochemical assays. Carried out all data analysis, wrote the draft manuscript and contributed to editing the final version of the manuscript.
Extent of contribution by PhD candidate (%)	80%

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Lisa Wallace	Carried out some of the respirometry to determine Pcrit
Andreas Kunzmann	Carried out some of the respirometry to determine Pcrit and edited the draft manuscript
Neill Herbert	Edited the draft manuscript

Certification by Co-Authors

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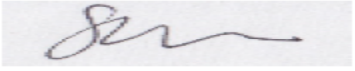
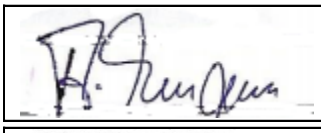
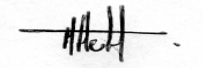
Lisa Wallace		19/12/2018
Andreas Kunzmann		19/12/2018
Neill Herbert		19/12/2018

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Extent of contribution by PhD candidate (%)	90%

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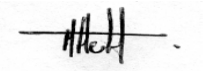
Neill Herbert		19/12/2018

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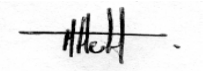
Neill Herbert		19/12/2018

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Nature of contribution by PhD candidate	Perfromed all of the experimental work. Carried out all data analysis, wrote the draft manuscript and contributed to editing the final version of the manuscript.
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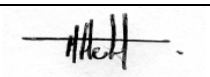
Neill Herbert		19/12/2018

CHAPTER 1: GENERAL INTRODUCTION

1.1 Metabolism

Fish, like all organisms, must match energy supply and demand in order to survive and perform the necessary requirements of life. Energy demand manifests at the whole animal level in processes such as swimming, growth, reproduction etc., but is ultimately driven by cellular energy demand, predominantly due to the energetic costs of protein synthesis and the maintenance of trans-membrane ion gradients (Rolfe and Brown, 1997). The energy demanding processes of living cells are driven by the "free energy" derived from the hydrolysis of adenosine tri-phosphate (ATP) - the so called energy currency of the cell. Thus, the struggle for survival is ultimately reliant on the ability of an organism to match ATP supply to the ATP demand of various life processes.

Non-photosynthetic eukaryotic organisms produce ATP in one of two ways: (1) aerobically (dependent on O_2) and (2) anaerobically (independent of O_2). Aerobic ATP production occurs within mitochondria, which are specialised organelles dispersed throughout the cell's cytoplasm. Mitochondria consist of an outer-membrane and an extensively folded inner-membrane (the cristae), which creates two intra-mitochondrial compartments (Frey and Mannella, 2000). The first compartment, the intermembrane space, separates the outer and inner membranes, while the matrix occupies the space within the cristae (Frey and Mannella, 2000). Aerobic ATP production involves three key steps: (1) glycolysis, (2) the tricarboxylic acid (TCA) cycle, and (3) oxidative phosphorylation. Glycolysis occurs in the cells cytoplasm and converts glucose to pyruvate while generating two ATP molecules. The enzymes of the TCA cycle, which are located within the mitochondrial matrix, then consume pyruvate to form reduced nicotinamide adenine dinucleotide (NADH), reduced flavin adenine dinucleotide ($FADH_2$), ubiquinone (UQ), guanosine triphosphate (GTP), and carbon dioxide (CO_2) (Voet and Voet, 1990). The monomeric units of lipids and proteins (fatty acids, glycerol and amino acids) can also be consumed in the TCA cycle but they do not enter via the glycolytic pathway. NADH, $FADH_2$, UQ, and GTP then enter the electron transport chain - a series of four large enzyme complexes (the electron transport chain) located in the inner mitochondrial membrane where oxidative phosphorylation occurs (Tymoczko et al., 2011). Ultimately, the transport of electrons across these protein complexes to O_2 pumps protons from the mitochondrial matrix

(a region of low H^+) across the inner membrane to the intermembrane space (a region of high H^+) (Voet and Voet, 1990). Oxygen is required for aerobic metabolism because it serves as the final electron acceptor at the end of the electron transport chain. The energy of the electrochemical gradient generated by proton pumping then drives the phosphorylation of ADP to ATP via another protein complex (ATP synthase) embedded in the inner mitochondrial membrane (Tymoczko et al., 2011). Overall, aerobic metabolism of a molecule of glucose via the steps of glycolysis, the TCA cycle, and oxidative phosphorylation yields around 38 ATP molecules (Voet and Voet, 1990). Oxidative phosphorylation, however, is not the only means by which cells can generate ATP, and it is reliant on the presence of sufficient O_2 to operate. Thus, if O_2 availability is insufficient to allow a cell to meet its ATP demand via oxidative phosphorylation, then ATP demand must be met anaerobically via glycolysis. This can occur, for example, in muscle cells during intense activity when ATP demand is high, and O_2 has been depleted. Glycolysis, as noted above, is the initial step of aerobic metabolism where glucose is converted to pyruvate by glycolytic enzymes in the cytoplasm - a process yielding just 2 ATP molecules for every molecule of glucose. In anaerobic conditions, however, pyruvate does not enter the TCA cycle and instead undergoes homolactic fermentation, a series of reactions where lactate dehydrogenase (LDH) catalyses the reduction of NADH by pyruvate to yield NAD^+, lactate, and an associated proton (Voet and Voet, 1990). Although glycolysis is a far less efficient pathway for ATP production than complete aerobic oxidation of glucose, the enzymes of glycolysis are in such high concentrations that they can produce ATP at a faster rate than oxidative phosphorylation (Voet and Voet, 1990). However, as the function of glycolytic enzymes are inhibited by pH below 7, ATP production via anaerobic glycolysis can only be maintained for short time periods due to the acidification resulting from homolactic fermentation of pyruvate (Voet and Voet, 1990). Thus, while organisms rely on a combination of aerobic and anaerobic metabolism to meet ATP demands at any one precise moment, it is the highly efficient aerobic pathway which allows long term survival.

The reliance on oxidative phosphorylation to efficiently meet ATP demand means that complex aerobic organisms, such as fish, have evolved specialised cardiorespiratory systems for the uptake and delivery of O_2 from the environment to mitochondria. In water breathing fishes, this involves the transfer of O_2 from water to blood across the gills, and the subsequent delivery of O_2 carried in the blood to the respiring cells via the circulatory system. The following section will provide a brief description of the processes and components of the

cardiorespiratory system that facilitate the transit of O_2 from water to the respiring cells in water breathing fishes.

1.2 The oxygen cascade in water breathing fishes

In fish, the pathway for O_2 delivery to cells begins at the gills, which are located within the buccal cavity, and are the primary site of O_2 uptake. Water laden with O_2 enters the buccal cavity by active ventilatory movements of the buccal and opercular pumps, or by ram ventilation where the mouth of the fish remains open when swimming or station holding in a fast moving current (Steffensen, 1985). The ventilatory flow of water through the buccal cavity can vary by up to 30 fold in response to changes in environmental gas tensions and metabolic demands (Perry and Wood, 1989). Adjustments in ventilatory flow rates come about through changes in breathing frequency (i.e. opercular beat frequency) and ventilation stroke volume (i.e. ventilation amplitude) in active ventilators (Perry and Wood, 1989); as well as transitions between different modes of ventilation (i.e. active to ram ventilation) in ram ventilators (Steffensen, 1985). The gills themselves consist of two sets of four gill arches, which are situated bilaterally on either side of the pharynx. Attached to each gill arch are two rows of gill filaments, which are secondarily folded so that on each side of a filament is a row of thin, disc shaped protrusions known as secondary lamellae. The large number and shape of the secondary lamellae greatly increase the respiratory surface area available for gas exchange. Some patterns of gill morphometric characteristics have been related to the respiratory needs of different fish species (Wilson and Laurent, 2002). In general, active free-swimming fish species (e.g. tuna) have large gill surface area and a short water-blood O_2 diffusion distance (i.e. thin secondary lamellae); whereas, more sluggish bottom-dwelling species have relatively smaller gill surface area and greater water–blood O_2 diffusion distance (Hughes and Morgan, 1973). In terms of the characteristics of the individual gill components, more active species tend to have a greater number of filaments, a larger number of secondary lamellae per mm of gill filament, and a relatively small surface area of individual secondary lamellae (Hughes and Morgan, 1973). As water passes over the surfaces of the gills, O_2 diffuses down its concentration gradient from water (high PO_2) into blood (low PO_2) filling the vascular core of the secondary lamellae. The flow of water across the gills is always counter-current to the direction of blood flow allowing efficient O_2 uptake as a gas pressure gradient between blood and water is maintained over the entire surface of the secondary

lamellae (Wilson and Laurent, 2002). Some O_2 can also be taken up via the skin, but in the majority of fishes this is only sufficient to meet the metabolic demands of the skin itself and does not contribute to the systemic O_2 supply (Glover et al., 2013).

Oxygen diffuses into blood within the gills, where it rapidly binds with haemoglobin (Hb) molecules in the red blood cells. The O_2 carrying capacity of the blood is determined by both Hb concentration and the oxygen-binding properties of Hb. The shape of the oxygen equilibrium curve (OEC) defines the oxygen binding properties of Hb. OECs represent the relationship between the partial pressure of O_2 (PO_2) and the fraction of Hb-bound O_2; in fish, these range from hyperbolic to sigmoidal in shape (Wells, 2009). Hyperbolic OECs denote a low Hb binding cooperativity, where O_2 is loaded and unloaded effectively from the blood over a broad range of PO_2; whereas, a sigmoidal OEC denotes high Hb binding cooperativity, where O_2 loading or unloading to the blood occurs effectively over a narrow range of PO_2 on the steep part of the OEC (Wells, 2009). In addition to binding cooperativity, the Hb-O_2 binding affinity also influences the loading and unloading of O_2 in the blood. Hb-O_2 binding affinity is expressed as the P_{50} or the PO_2 at which 50% of Hb is saturated with O_2. The OECs of fish species with a low Hb P_{50} are left shifted (e.g. catfish); the Hb of these species is saturated more with O_2 at lower PO_2, which aids O_2 loading at the gills, particularly at reduced environmental PO_2 (hypoxia) (Wells, 2009). On the other hand, a relatively higher Hb P_{50} favours unloading of O_2 from the blood, which enhances the delivery of O_2 to the tissues in active species with high respiratory demands (e.g. rainbow trout) (Wells, 2009). The Hbs of some fish species also show strong Bohr effects where low pH causes a right shift in the OEC, which aids O_2 unloading from the blood at the tissues. The acidic conditions for the Bohr effect are generated by the build-up of CO_2 and lactate, and associated protons in the working tissues. The acidic right hand shift of the OEC can be so pronounced in some species that Hb does not saturate fully with O_2 even at high PO_2 - a situation that indicates the presence of the Root effect. The large drop in Hb-O_2 saturation at low pH associated with a Root effect allows oxygen to pass from the blood to the swim bladder and retina despite large opposing gas pressures (Brittain, 1987).

Hb-bound O_2 in the blood reaches the respiring tissues via the vascular circulatory system. Upon entry to the capillary networks that surround the working tissues, O_2 is released from Hb, and it then diffuses down a concentration gradient from the blood to the cytoplasm of the cells. This O_2 then diffuses down another concentration gradient and enters the mitochondria, where it is used to drive oxidative phosphorylation and ATP production.

Overall, it is estimated that approximately 90% of O_2 consumed by animals is mitochondrial driven, and that around 80% of mitochondrial O_2 consumption is coupled to ATP synthesis (Rolfe and Brown, 1997). Thus, the delivery of O_2 from the environment to mitochondria can be depicted as a cascade of O_2 transfers occurring down a concentration gradient of PO_2 and across multiple levels of biological organisation (Fig. 1.1).

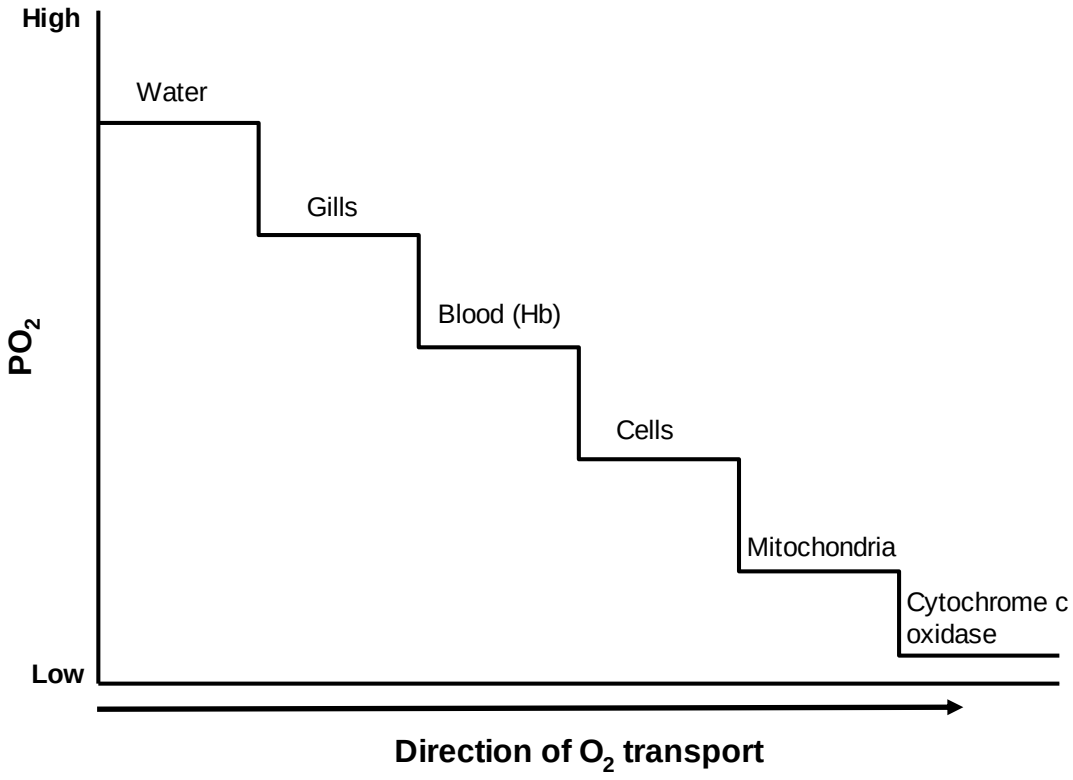


Figure 1.1 The oxygen (O_2) cascade in water breathing fishes. Active ventilation passes water with a high oxygen partial pressure (PO_2) over the gill surfaces. O_2 then diffuses down a pressure gradient across the gill secondary lamellae and enters the blood where it rapidly binds to red blood cell associated haemoglobin (Hb). Hb-bound O_2 is then transported to working tissues through the vascular network. O_2 is released from Hb within the capillary networks surrounding working tissues and subsequently diffuses into cytoplasm of cells. O_2 that has entered cells then diffuses from the cytoplasm into the mitochondria where it binds to cytochrome c oxidase. In the mitochondria O_2 acts as a final electron acceptor in the electron transport chain and is essential for ATP generation via oxidative phosphorylation.

While the O_2 cascade allows the transfer of O_2 from the environment to the mitochondria, it does not dictate the rate at which O_2 is delivered to respiring tissues. When the respiratory demands of tissues increase (e.g. in muscle during swimming), O_2 must be delivered at a greater rate in order to prevent cellular O_2 depletion and a limitation of ATP

production. Greater O_2 delivery to tissues in fish is achieved mostly through a coordinated increase in both cardiac output and gill ventilation rate. Higher cardiac output serves to increase gill perfusion thereby enhancing gill O_2 conductance, while the increased ventilatory water flow rates increase the mean blood-to-water PO_2 gradient (Perry et al., 2009) and reduces the unstirred boundary layers next to the lamellae, decreasing the O_2 to blood diffusion distance (Wood and Perry, 1985). Elevated cardiac output also increases the rate at which blood passes around the vascular system, hence delivering O_2 to the tissues at a faster rate. Bohr effects can also enhance O_2 delivery to working tissues at times of high respiratory demand, and blood O_2 carrying capacity can be increased by the release of stored erythrocytes due to splenic contractions, red blood cell swelling and losses of plasma water content (Wood and Perry, 1985). Overall, these physiological adjustments allow fish to meet changes in the metabolic demands of their tissues, which will be reflected by the mass-specific rate of "whole organism" O_2 consumption ($\dot{M}O_2$).

1.3 Oxygen consumption as a measure of metabolic rate in fish

Understanding the rate of energy use (i.e. metabolic rate) in an organism is useful as it provides key biological insights across multiple levels of organisation (Brown et al., 2004). There is currently no suitable method available to directly assess ATP turnover in a whole fish, thus indirect measurements of metabolism must be utilised in order to assess energy turnover. The "gold standard for quantifying the fire of life" is direct calorimetry which measures the heat liberated by an animal's metabolism through both aerobic and anaerobic pathways (Kaiyala and Ramsay, 2011). However, due to the relatively low metabolic heat production of ectothermic animals and high heat capacity of water, calorimeters with adequate sensitivity to measure heat production in fish are not yet readily available or affordable. Recently, a design for a highly sensitive and relatively affordable calorimeter was published (Regan et al., 2013), which has subsequently been used to measure metabolic heat production in gold fish and three spine sticklebacks (Regan et al., 2017a; Regan et al., 2017b). Despite its promise the use of this calorimeter presents several major difficulties for assessing metabolic rate. In particular, thermal equilibration of the calorimeter requires 18 h before heat production can be accurately measured and this must be carried out in a climate controlled room with extremely stable temperatures (Regan et al., 2013). This requirement for a long period of thermal equilibration prevents the implementation of many experimental

manipulations; for example, it would not be possible to measure the metabolic rate of a fish under acute temperature change using a calorimeter of this design. Furthermore, this calorimeter design requires that animals are euthanized at the conclusion of each experiment in order to measure the baseline heat signal (Regan et al., 2013); from an ethical standpoint this may be difficult to justify. Due to the difficulties associated with performing direct calorimetry, indirect calorimetry methods, most often the measurement of O_2 consumption ($\dot{M}O_2$), have become the predominant approach for assessing metabolic rate in fish (Nelson, 2016). While $\dot{M}O_2$ measurement is convenient and widely used, this approach is not without its limitations. Firstly, $\dot{M}O_2$ does not reflect energy utilisation associated with anaerobic ATP production, which can be significant under certain conditions (e.g. hypoxia) and, secondly, the different oxycaloric coefficients associated with shifts in metabolic fuel use (e.g. carbohydrate vs protein catabolism) are unaccounted for by measurements of O_2 consumption alone (Nelson, 2016). Throughout this thesis $\dot{M}O_2$ is used as an indirect proxy measure of energy utilisation with these caveats in mind.

1.4 The aerobic metabolic scope framework

The ATP produced by metabolism supports the energetic demands of the multitude of life supporting activities an organism must carry out in order to survive and reproduce. In fish, the energy provided via aerobic pathways to support these activities can be conceptualised under the aerobic metabolic scope (MS) framework, which was initially proposed by Fry and colleagues as the "scope for activity framework" (Fry, 1947; Fry, 1971; Brett, 1976), but has more recently been developed further by others (Pörtner, 2001; Claireaux and Lefrancois, 2007; Pörtner, 2010; Claireaux and Chabot, 2016; Pörtner et al., 2017). The MS framework partitions the total aerobic metabolic capacity of an organism into two fractions: (1) the portion of aerobic capacity that supports basal homeostatic functions of cells, tissues and organ systems, and (2) the portion of aerobic capacity beyond basal maintenance requirements that is available to support additional aerobic activities an organism must carry out in order to survive long term and reproduce (e.g. feeding, digestion, predator avoidance, growth, spawning etc.). There are two key parameters used by physiologists to determine MS: firstly, standard metabolic rate (SMR) represents the energy costs of basal maintenance metabolism in an unfed, rested, inactive animal (Chabot et al., 2016), and secondly, maximum metabolic rate (MMR/$\dot{M}O_{2,max}$) represents the maximum

capacity for energy utilisation of aerobic metabolic pathways (Norin and Clark, 2016). SMR and MMR are most often determined in fish by the measurement of O_2 consumption in either a rested (SMR) or strenuously exercised (MMR) individual (Clark et al., 2013; Chabot et al., 2016; Norin and Clark, 2016). MS is defined as the difference between SMR and MMR and it is within the bounds of MS that organisms perform the additional aerobic activities (e.g. growth, feeding, predator avoidance, reproduction etc.) that support long term survival, reproductive fitness and performance (Claireaux and Lefrancois, 2007). The attractiveness of the concept of MS is that it provides a mechanistic link between the environmental factors that shape metabolism and whole organism performance. Under the MS framework environmental factors set the availability of MS through effects on SMR and MMR, and are divided into one of five categories: controlling, limiting, masking, lethal and directive. Claireaux and Lefrancois (2007) describe these environmental factors as follows: the main controlling factor is temperature which affects the rate of the biochemical reactions of metabolism and, therefore, has a profound influence on SMR and MMR; limiting factors (e.g. O_2 availability) can disrupt O_2 supply to working tissues, restricting MMR and constraining aerobic capacity; masking factors (e.g. salinity) increase the costs of baseline homeostatic functions increasing SMR, while lethal factors (e.g. pollutants) can impair aerobic metabolism and eventually lead to mortality if exposure is severe; finally, directive factors (e.g. photoperiod) cause animals to shift to habitats and physiological states that may be more optimal for metabolism. Through integration of the effects of environment on metabolism, it is proposed that MS provides a "metric of an organism's ability to cope with environmental demands", as well as a mechanistic understanding of the emergent properties of organisms in different environmental conditions (Claireaux and Lefrancois, 2007).

1.5 The influence of oxygen availability on metabolism in fish

The most important limiting factor of fish metabolism is the availability of O_2 (Claireaux and Chabot, 2016). This is because the efficient production of ATP from metabolic fuels (e.g. carbohydrate, lipid, amino acids) via oxidative phosphorylation can only be completed if there is sufficient O_2 available to mitochondria. In the absence of O_2 ATP can still be produced via anaerobic glycolysis, but this pathway is around 19 times less efficient than oxidative phosphorylation and only utilises glycogen/glucose as a fuel source which may be in limited supply. Moreover, anaerobic glycolysis can only be sustained for relatively

short periods due to acidosis resulting from lactate production. Thus, the capacity of a fish's metabolism to convert the energy in metabolic fuels to a form that can be utilised by cells (ATP) can be severely limited in situations where O_2 supply to tissues is constrained. The O_2 content of water, dependent on salinity and temperature, is between 20-40 times less by volume than air and O_2 diffuses approximately ten thousand times slower in water than air (Graham, 1990). Moreover, depleted dissolved O_2 levels (hypoxia) are a common occurrence in a wide range of aquatic habitats (Diaz and Breitburg, 2009). The relatively low solubility of O_2 in water, slow O_2 diffusion rate, and propensity for O_2 depletion make aquatic environments (in comparison to terrestrial environments) a challenging place for organisms to obtain sufficient O_2 to drive aerobic metabolism. The variability in O_2 availability in aquatic environments can be placed into one of four categories: (1) anoxia – a situation where there is no biologically available O_2, (2) hypoxia – a spectrum of severe to mild low O_2 availability that impacts normal physiological function and behaviour, and places limits on aerobic metabolism; the impacts of a given level of hypoxia are strongly dependent on the hypoxia tolerance of the fish species in question and what is considered hypoxic for one species may have no effect on another, (3) normoxia – a situation where water is fully air saturated and well oxygenated, and (4) hyperoxia – a situation of higher than normal water O_2 levels where water becomes super-saturated with O_2 due to the photosynthetic activity of algae, phytoplankton and aquatic plants.

The influence of O_2 availability on aerobic metabolism in fish is elegantly depicted within the MS framework (Farrell and Richards, 2009a; Claireaux and Chabot, 2016). As O_2 levels decline below normoxia aerobic capacity is increasingly constrained due to a limitation on MMR and there is a corresponding reduction in MS (Fig. 1.2). While moderate hypoxia is survivable, the restriction on MS can be associated with a reduced capacity to perform aerobically demanding activities such as swimming (Jones, 1971; Fitzgibbon et al., 2007; Dutil et al., 2007) and digestion (Jordan and Steffensen, 2007; Wang et al., 2009). Moreover, restricted MS under hypoxia is associated with reduced somatic growth rates in a number of fish species (Chabot and Claireaux, 2008; Wang et al., 2009) and less energy is available to be invested in reproduction (Wu, 2009). The constraint on MMR continues with further declines in O_2 availability until the point where MMR becomes equal to SMR and MS is nil (Fig. 1.2). The intersection between MMR and SMR is defined as the critical O_2 level (P_{crit}) and marks the point where O_2 uptake is only sufficient to meet the demands of maintenance metabolism (i.e. SMR) (Cook et al., 2011; Claireaux and Chabot, 2016). If O_2 availability is

reduced further than P_{crit} then O_2 uptake falls below the rate associated with SMR, and the organism transitions from an O_2 regulating to an O_2 conforming state (Farrell and Richards, 2009a). It is important to point out here that if the energetic demands of the organism are elevated above SMR, then the transition to an O_2 conforming state will occur at a higher level of O_2 availability (i.e. the P_{crit} is pushed higher, see Fig. 1.2 P_{crit} for RMR). Under exposure to O_2 levels below P_{crit} the O_2 uptake of an organism is not sufficient to meet even maintenance energetic requirements via aerobic metabolism; in this situation energy balance is severely compromised and death can rapidly ensue in hypoxia sensitive species. The capability of an animal to survive below P_{crit} is dependent on its ability to maintain energy balance in the face of severely limited aerobic metabolism. In fish, maintenance of energy balance at critically low O_2 availability is achieved by the recruitment of anaerobic ATP production (i.e. anaerobic glycolysis and creatine phosphate hydrolysis) and/or a reduction in energetic demands through metabolic rate depression (MRD) (Richards, 2011). The ability of fish to utilise each of these physiological mechanisms (i.e. anaerobic glycolysis and MRD), however, is highly species dependent and, consequently, there is a high degree of interspecific variation in the ability of fish to survive exposure to critically low O_2 availability.

The influence of O_2 availability on aerobic metabolism in fish has been best studied across the normoxia to severe hypoxia/anoxia spectrum, whereas the influence of hyperoxia on aerobic capacity has received little attention. While the assumption has been that most fish species have evolved to maximise aerobic capacity under normoxia (Claireaux and Chabot, 2016), a recent study showing a two-fold increase in MS under hyperoxia in European perch (*Perca fluviatilis*) clearly demonstrates this assumption is not always valid (Brijs et al., 2015). Overall, there are few studies which have assessed the influence of hyperoxia on aerobic capacity in fish, and its potential effects on MS requires greater consideration, particularly in species which routinely face hyperoxia under natural conditions.

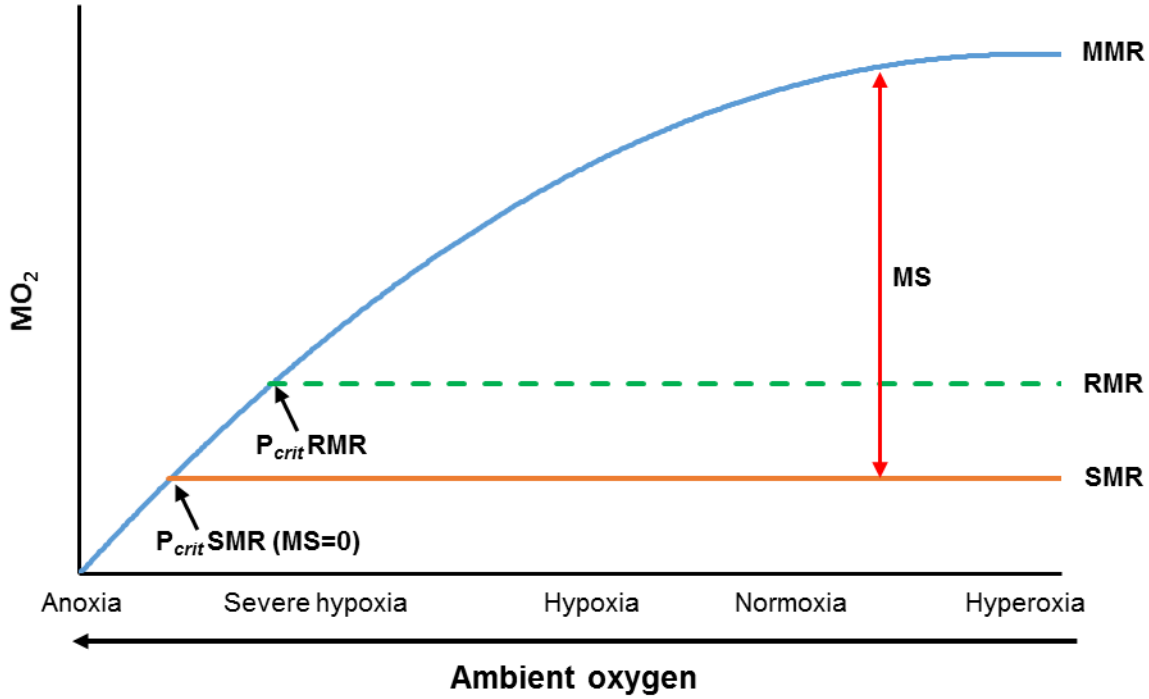


Figure 1.2 The influence of oxygen avaialbility on aerobic metabolism ($\dot{M}O_2$) as depicted within the metabolic scope (MS) framework (Claireaux and Chabot, 2016). The difference between maximum metabolic rate (MMR) and standard metabolic rate (SMR) is redcued as ambient oxygen (O_2) avialbility declines and, as a consequnce, MS becomes progressively constrained with falling O_2 levels. The ambient O_2 availibility where MMR becomes equal to SMR represents the critcal O_2 tension/partial pressure (P_{crit} SMR). At P_{crit} SMR there is zero scope for excess activity (i.e. MS is nil) and further declines in O_2 availbility result in a transition from an O_2 regulating to an O_2 confroming metabolic state. The ability to survive exposure to O_2 levels below P_{crit} is dependent on the capacity of an organism to maintain energetic balance through recruitment of anaerobic metabolism and minimisation of energy expenditure (e.g. metabolic rate depression). If the metabolic rate of an organism is elevated above SMR during exposure to hypoxia the P_{crit} is pushed to a higher ambient O_2 availibility (see P_{crit} for routine metabolc rate [RMR]).

1.6 How do fish cope with hypoxia?

Fish have independently evolved hypoxia tolerance numerous times (Hochachka and Lutz, 2001) and have developed a wide array of adaptations to cope with low O_2 availability in aquatic environments (Bickler and Buck, 2007; Diaz and Breitburg, 2009). While the ultimate purpose of hypoxia responses is to maintain energy balance, the particular physiological, biochemical and behavioural mechanisms fish utilise to achieve energy balance under low O_2 conditions is diverse, and there is no 'one size fits all' approach. In an effort to make sense of this diversity, a recent review proposed that the suite of mechanisms utilised by a particular fish species in response to low O_2 availability is related to the severity

and duration of hypoxic events in that species native habitat (Mandic and Regan, 2018). These authors introduced the concept of the 'total hypoxia response' (THR) to characterise the overall hypoxia coping strategy employed by a fish species. The THR was defined by Mandic and Regan (2018) as the combination of three metabolic modes used by an animal to cope with hypoxia: (1) sustained utilisation of aerobic metabolism, (2) increased utilisation of anaerobic metabolism, and (3) utilisation of metabolic rate depression (MRD). These three broad categories of hypoxia responses will be used to outline the physiological, biochemical and behavioural mechanisms and adaptations which allow fish to cope with hypoxia. While this system provides a useful means to distinguish the wide array of responses to hypoxia observed in fish, it should be kept in mind that the THR of a particular species is never reliant on responses from just one category.

1.6.1 Sustained utilisation of aerobic metabolism under hypoxia exposure

This category of responses refers to the different mechanisms and adaptations which allow fish to continue to meet energetic demands via aerobic metabolism under hypoxia exposure. The vast majority of fish species, and maybe in fact all (Svendsen et al., 2018), are O_2 regulators, meaning they can maintain a constant $\dot{M}O_2$ under at least moderate hypoxia exposure. This means that almost all fish species, hypoxia tolerant or intolerant, make physiological adjustments that allow them to continue to meet energetic demands aerobically to at least some level of reduced O_2 availability. A distinction, however, can be made between hypoxia tolerant and hypoxia intolerant species based on the severity of hypoxia to which they can maintain a constant $\dot{M}O_2$. This ability is measured as the P_{crit}, which identifies the PO_2 where $\dot{M}O_2$ falls below either the SMR or routine metabolic rate (RMR) under progressive hypoxia exposure (Rogers et al., 2016). The P_{crit} for SMR is analogous to the point where SMR and MMR intersect and MS falls to zero under hypoxia in the MS framework (Claireaux and Chabot, 2016). Although there are exceptions, such as species which are hypoxia tolerant but show $\dot{M}O_2$ conformity at relatively high PO_2 due to MRD (Scott et al., 2008; Speers-Roesch et al., 2012), species with low P_{crit} are generally considered to be more hypoxia tolerant than species with high P_{crit} (Mandic et al., 2009; Richards, 2011; Speers-Roesch et al., 2013; Mandic et al., 2013). Low P_{crit} is an advantage as it allows a transition to anaerobic metabolism or MRD, both of which are associated with significant costs (Regan et al., 2017a; Mandic and Regan, 2018), to be delayed or even completely

avoided in habitats which become periodically hypoxic (Mandic et al., 2009). The drive to take advantage of any available O_2 is exemplified by a recent study which shows that goldfish, among the most hypoxia tolerant of all fishes and known for their ability to depress metabolism, continue to prioritise aerobic metabolism over MRD under hypoxia exposure in all but near anoxic conditions (Regan et al., 2017a). Mandic and Regan (2018) identified sustained utilisation of aerobic metabolism under hypoxia as a key component of the THR of fish species from habitats where hypoxia is moderate (e.g. lakes with pockets of mild hypoxic water), where hypoxia is severe but of short duration (e.g. estuaries and rock pools), or where behavioural responses can access higher O_2 availability during hypoxia exposure (e.g. at the air water interface).

Species with a low P_{crit} are able to take up more O_2 under hypoxic conditions which denotes a higher capacity to extract O_2 from their environment. Thus, it is through adaptations or mechanisms relating to the individual components of the cardiorespiratorycascade that hypoxia tolerant fishes maintain low P_{crit} (Richards, 2011). A well-studied example of fish that rely on low P_{crit} (i.e. sustained utilisation of aerobic metabolism even under severe hypoxia) for hypoxia tolerance are the intertidal sculpins. Intertidal sculpins have lower P_{crit} and survive severe hypoxia for longer than closely related subtidal sculpins (Mandic et al., 2013). A low P_{crit} in the intertidal species is achieved due to a high Hb-O_2 binding affinity (i.e. low red blood cell P_{50}), high intracellular levels of allosteric modulators of Hb-O_2 binding in red blood cells, large mass-specific gill surface area, high cytochrome c oxidase affinity for O_2, and a relatively low basal metabolic rate (Mandic et al., 2009; Richards, 2011; Lau et al., 2017). Collectively these adaptations within the cardiorespiratory cascade, undoubtedly along with others not yet identified, allow intertidal sculpins to continue to rely on aerobic metabolism even under severe hypoxia. Cyprinids (carps and goldfishes) are another example of fish which can sustain aerobic metabolism even under severe hypoxia. Goldfish, for example, can rely almost entirely on aerobic metabolism even under near anoxic conditions due to extremely high Hb-O_2 binding affinity (Burggren, 1982; Regan et al., 2017a). Cyprinids also have the ability to remodel their gills, increasing the surface area available for gas exchange, when exposed to hypoxic waters (Nilsson, 2007).

Another response to hypoxia seen in fish is the near ubiquitous increase in ventilatory water flow (hyperventilation) across the gills due to both increased frequency and amplitude of buccal pumping (Perry et al., 2009). Hyperventilation increases the mean water to blood PO_2 difference and reduces the unstirred boundary layer next to the lamellae surfaces, both of

which enhance branchial O_2 transfer and minimise the extent of reductions in arterial PO_2 in the face of declining environmental PO_2 (Perry et al., 2009). Hyperventilation also causes a respiratory alkalosis due to the equilibration of arterial blood CO_2 partial pressure (PCO_2) with the lower PCO_2 of water surrounding the gills (Gilmour, 2001; Perry et al., 2009). The resulting increase in red blood cell pH can increase O_2 binding affinity by negating the Bohr effect, thereby aiding O_2 uptake (Wells, 2009). In addition to hyperventilation, some fishes also respond to hypoxia by performing aquatic surface respiration (ASR) where better oxygenated water is accessed by ventilating in the surface layer of a water body; this includes in some cases inspiring bubbles of air, which are then held within the buccal cavity (Chapman and Mckenzie, 2009). Beyond ASR, other fishes respond to hypoxia by breathing air; either storing gulped air in specialist air breathing organs, or spontaneously emerging from water to breathe air across the skin, gills and branchial chambers (Chapman and Mckenzie, 2009). The purpose of all these behavioural responses is to access more O_2 when O_2 availability in water is becoming restricted. Another major physiological response used by fish to maintain constant $\dot{M}O_2$ under hypoxia is to increase the perfusion of gill lamellae with blood (Nilsson, 2007). Increased gill perfusion enhances branchial O_2 transfer and comes about through complex adjustments to heart rate, cardiac stroke volume, cardiac output, systemic and branchial vascular resistance and arterial blood pressure (Gamperl and Driedzic, 2009). The release of the catecholamine hormones adrenaline and noradrenaline is another common response to hypoxia in fish and serves to increase blood O_2 carrying capacity (Reid et al., 1998). Binding of adrenaline to beta receptors on the red blood cell membrane activates the Na^+/K^+ exchanger which leads to an increase in intracellular pH and enhances Hb-O_2 binding affinity by not activating the Bohr effect. These changes also induce red blood cell swelling, which dilutes intracellular concentrations of the allosteric modulators of Hb-O_2 binding (the nucleoside triphosphates ATP and GTP), further increasing Hb-O_2 binding affinity. Adrenaline also stimulates splenic contractions, which enhances blood O_2 carrying capacity due to the release of stored red blood cells to the primary circulation (Wells and Weber, 1990).

In addition to the multitude of physiological and biochemical adaptations which facilitate stable $\dot{M}O_2$ under hypoxia there are also a number of behavioural responses which allow fish to either reduce O_2 demands in hypoxia, or avoid hypoxia if it is escapable (Chapman and Mckenzie, 2009). Atlantic cod (*Gadus morhua*), for example, minimise energetic demands when exposed to hypoxia by reducing swimming speed (Schurmann and

Steffensen, 1994; Herbert and Steffensen, 2005). Some fish also select cooler temperature under hypoxia exposure, which decreases energetic demands and enhances branchial O_2 transfer due to a higher Hb-O_2 binding affinity (Schurmann et al., 1991; Schurmann and Steffensen, 1994; Petersen and Steffensen, 2003). In active pelagic fish species, the general behavioural response to hypoxia is an increase in activity and swimming speed (Domenici et al., 2000; Herbert and Steffensen, 2006; Cook and Herbert, 2012). Higher swimming speeds and activity levels are thought to aid the movement of fish away from hypoxic waters to areas with more favourable respiratory conditions (Herbert and Steffensen, 2006).

1.6.2 Increased utilisation of anaerobic metabolism and metabolic rate depression

If hypoxia becomes too severe (despite the myriad of physiological, biochemical and behavioural responses at their disposal) even hypoxia tolerant fish species can reach a point where they are unable to maintain energy balance through aerobic metabolism alone. This point is recognised as the level of hypoxia where $\dot{M}O_2$ falls below the O_2 consumption rate associated with the fish's basal energetic demand (i.e. the P_{crit} for SMR or RMR). To survive below P_{crit}, both hypoxia tolerant and hypoxia intolerant fish species recruit anaerobic metabolism to maintain energy balance. Anaerobic metabolism allows fish to produce ATP independently of O_2 via glycolysis and therefore continue to meet cellular energy demands in O_2 limited environments. While the eventual recruitment of anaerobic ATP production under hypoxia is ubiquitous among fishes, the length of time different species can survive O_2 tensions below P_{crit} is highly variable. For example, the Nile perch (*Lates niloticus*) survives only 5 min exposure to anoxia at 20°C (Schofield and Chapman, 2000), whereas, dependent on temperature, the crucian carp (*Carassius carassius*) can survive days to months of anoxia (Vornanen et al., 2009). Moreover, among 11 closely related sculpin species there was a greater than 20 fold difference in the length of time each species survived a severe hypoxia exposure below P_{crit} (Mandic et al., 2013). Three principle factors are thought to determine how long a fish can survive below P_{crit}: (1) the potential for glycolytic flux, (2) tolerance of the end-products of anaerobic glycolysis (e.g. lactate, H^+), and (3) the ability to reduce energetic demands through MRD (Richards, 2011; Mandic and Regan, 2018). Hypoxia intolerant species recruit anaerobic metabolism under severe hypoxia, but they do not survive for long periods because they quickly exhaust endogenous fermentable fuel stores or succumb to respiratory acidosis. Fermentable fuels are quickly exhausted in hypoxia

intolerant species because they may have only small amounts of stored fuels (e.g. glycogen) available to begin with, and cellular ATP demands are not reduced upon O_2 limitation (Boutilier and St-Pierre, 2000). The most hypoxia tolerant species, on the other hand, maintain large tissue stores of fermentable fuels and supress cellular ATP demand through MRD when exposed to severe hypoxia. The hypoxia tolerant cyprinids (crucian carp and goldfish), for example, maintain the highest tissue glycogen stores of all fishes (Richards, 2009) and can reduce cellular ATP demand via an up to 80% depression of metabolic rate when exposed to severe hypoxia/anoxia (Vornanen et al., 2009; Richards, 2009; Regan et al., 2017a). Crucian carp and goldfish are also able to convert lactate to ethanol in their muscle tissues, which prevents the severe lactic acidosis which would normally be seen in fish when relying on anaerobic ATP production for extended time periods (Vornanen et al., 2009). This combination of large endogenous fermentable fuel stores, MRD and ethanol production allows these species to rely on anaerobic ATP production for extended periods of time in extremely O_2 limited environments. Mandic and Regan (2018) identified prolonged use of anaerobic ATP production in combination with MRD as important components of the THR of species which occupy environments where hypoxia is severe and of a long duration (e.g. eutrophic winter freeze lakes, swamps and at the centre of oceanic oxygen minimum zones).

1.7 The influence of temperature on metabolism in fish

Temperature affects the kinetics of all chemical reactions, including the biochemical reactions of metabolism. Molecules move faster when they are heated and therefore collide more frequently and with higher energy. This means that at higher temperatures a greater number of collisions between molecules occur with enough energy for a reaction to take place and, as a consequence, reaction rates increase. Fish, like all ectotherms, do not maintain a stable body temperature; accordingly, the rate of the biochemical reactions that govern their metabolism is strongly dependent on environmental temperature. Temperature, therefore, has a profound influence on the metabolic rate of fish and strongly influences species distribution and abundance (Schulte, 2015). There is a vast literature that addresses the biochemical mechanisms and adaptations that fish use to cope with both cold and warm temperatures. This includes assessments of the influence of temperature on individual enzymes, cellular and sub-cellular (mitochondrial) metabolism and whole animal metabolic rate (Schulte, 2015). It is not the intention here to outline all of these different temperature adaptations and instead

the following section will focus on the influence of temperature on whole animal metabolic rate, with particular reference to how the influence of temperature on metabolism is integrated within the metabolic scope framework in fish.

1.7.1 The influence of temperature on metabolic scope

The primary controlling factor of the metabolism of fish is temperature (Claireaux and Lefrancois, 2007). Temperature has a significant influence on both SMR and MMR, and therefore also strongly dictates the availability of MS at different temperatures. In the classical view, SMR rises exponentially with increasing temperature, whereas, after initially increasing, MMR plateaus at a moderate temperature, and may even decrease at high temperatures in some species (Fig. 1.3A). This pattern of change in SMR and MMR with increasing temperature leads to a bell-shaped distribution of MS availability, with a distinct optimal temperature range where the availability of MS peaks, and gradually declining MS availability at temperatures either side of the optimal range (Fig. 3B). Under the MS framework the availability of MS defines the aerobic capacity accessible to the fitness supporting processes and activities an animal carries out beyond maintenance requirements (e.g. growth, feeding, reproduction etc.) (Claireaux and Lefrancois, 2007; Claireaux and Chabot, 2016). This notion, in conjunction with observations of declining MS availability at temperatures either side of an optimal range, has led to the hypothesis that impaired whole animal performance at high and low temperatures is driven by constraints on MS in aquatic ectotherms. This idea, which originates from Fry's scope for activity framework (Fry, 1947; Fry, 1971), has been thoroughly developed over the past decade as the hypothesis of oxygen and capacity limited thermal tolerance (OCLTT) (Pörtner, 2001; Pörtner and Knust, 2007; Pörtner and Farrell, 2008; Pörtner and Peck, 2010; Pörtner et al., 2017). The OCLTT first gained prominence, but then attracted controversy, because the theory proposes to lay out a cause and effect understanding of how environmental warming due to climate change will impact whole animal performance in aquatic ectotherms such as fish (Pörtner and Farrell, 2008; Clark et al., 2013; Schulte, 2015).

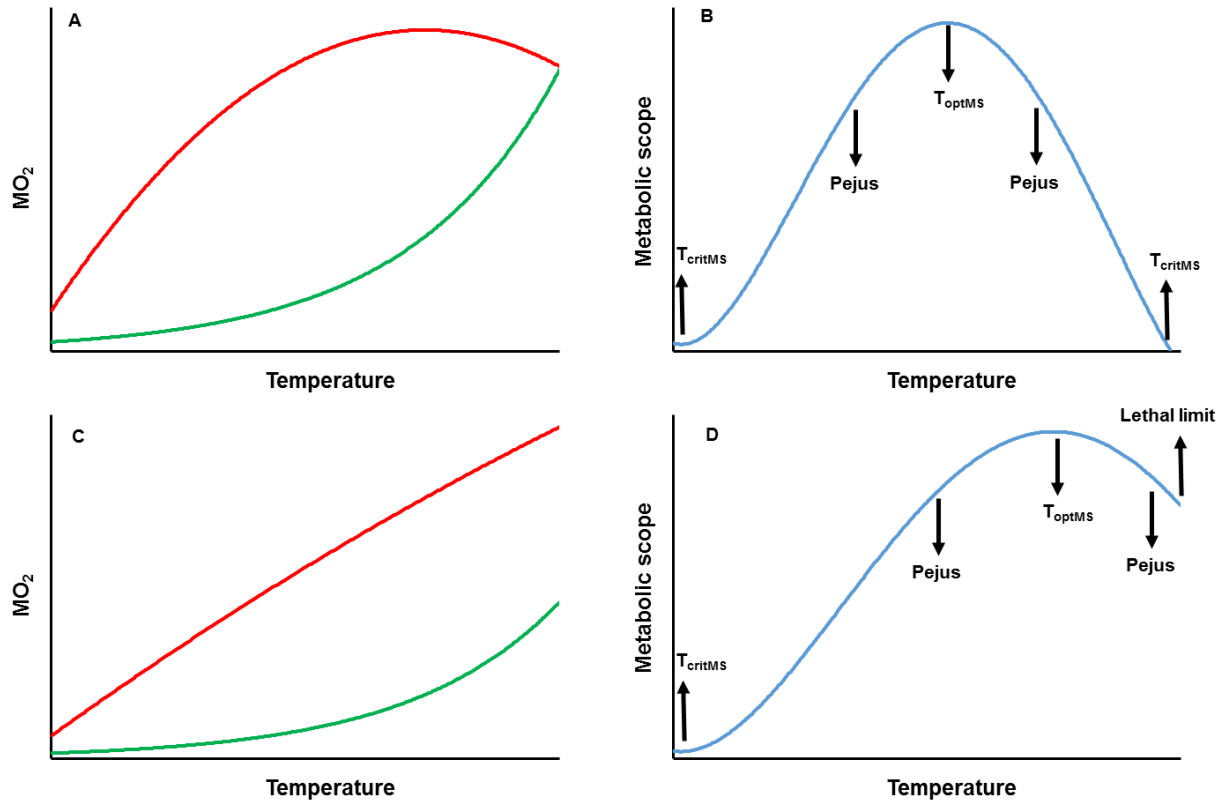


Figure 1.3 The influence of temperature on metabolic scope (MS). Panel A: under the oxygen and capacity limited thermal tolerance (OCLTT) framework standard metabolic rate (green line) increases exponentially with higher temperature while maximum metabolic rate (red line) plateaus and then declines at high temperature. Panel B: this pattern results in a bell shaped distribution of MS availability across a species thermal window with a temperature optimum where MS peaks (T_{optMS}), upper and lower pejus temperatures where MS begins to decline and upper and lower critical temperatures (T_{critMS}) where MS is nil and survival is time limited. Panel C: in some fish species maximum metabolic rate does not plateau or decline at high temperatures. Panel D: in species where maximum metabolic rate does not plateau at high temperatures the T_{opt} for MS is close to the upper lethal thermal limit.

1.7.2 Oxygen and capacity limited thermal tolerance

The primary tenant of the OCLTT hypothesis is that animals operate within a thermal window which is defined by the availability of scope for aerobic performance (i.e. the availability of MS) (Pörtner and Knust, 2007; Pörtner and Farrell, 2008; Pörtner and Peck, 2010; Pörtner et al., 2017). According to the OCLTT hypothesis the thermal window of an animal consists of an optimal range where MS peaks, upper and lower 'pejus' temperatures which mark the onset of a decline in MS, and upper and lower critical temperatures where MS falls to nil (Pörtner and Farrell, 2008) (Fig. 1.3B). The OCLTT proposes that the performance of an animal (i.e. the ability to grow, reproduce, forage etc.) is causally linked to

the availability of MS across the thermal window. Thus, within the OCLTT framework, animal performance is expected to peak across the optimal temperature range for MS, begin to decline above and below the 'pejus' temperatures, and eventually be abolished at critical temperatures where there is no available MS for excess aerobic activities. Beyond the upper critical temperature increasing basal energetic demands can only be supported through anaerobic ATP production and survival is time limited (Pörtner and Farrell, 2008). In regards to the cause of MS limitation above the upper 'pejus' temperature the OCLTT places the blame on a limited capacity of the cardiorespiratory system to take up and supply O_2 to working tissues (i.e. a plateau in MMR). The inability of the cardiorespiratory system to match O_2 supply and demand results in a "temperature dependent O_2 limitation" and tissue hypoxemia (Pörtner and Peck, 2010). The validity of the OCLTT as a 'unifying theory' of thermal tolerance in fish has been keenly debated over the past five years (Clark et al., 2013; Pörtner and Giomi, 2013; Farrell, 2013; Pörtner, 2014; Jutfelt et al., 2014; Schulte, 2015; Farrell, 2016; Jutfelt et al., 2018; Pörtner et al., 2018). Experimental studies investigating the relevance of OCLTT in fish have tested two main hypothesis laid out in the OCLTT framework: (1) that performance (e.g. growth, reproduction, swimming etc.) is correlated with MS in animals exposed to temperatures encompassing the optimal to 'pejus' range, and (2) that the upper critical thermal limits of fish are set by a limited capacity of the cardiorespiratory system to supply O_2 to tissues (i.e. a temperature dependent O_2 limitation and tissue hypoxemia).

There has been some evidence in support of a link between MS and whole animal performance in fish. In an early attempt to validate Fry's scope for activity concept, Brett (, 1976), found that a decline in MS above 15°C was paralleled by a decline in sustained swimming speed in fingerling sockeye salmon (*Oncorhynchus nerka*). However, as growth rate declines precipitously above 20°C in sockeye salmon but MS only moderately so, there was no strong association between MS and growth performance (Brett, 1976). Across a temperature range of 7°C to 30°C both MS and maximal swimming speed increased in juvenile European sea bass (*Dicentrarchus labrax*) (Claireaux et al., 2006). In Atlantic salmon (*Salmo salar*) acclimated to 3, 8, 13, 18 or 23°C MS peaked at 23°C but maximal swimming performance peaked at 18°C; moreover, fish held at 23°C suffered 20% mortality and had poor condition factors (Hvas et al., 2017). In the eelpout (*Zoarces viviparous*), Pörtner and Knust (2007) showed that temperatures associated with restricted MS closely match environmental temperatures beyond which growth performance and abundance

decrease in the wild. Eliason et al. (2011) showed that the optimum temperature for MS matched historic river temperatures in the wild ranges of different populations of sockeye salmon and suggested that population-specific thermal limits are set by limitations on aerobic performance. Khan et al. (2014) demonstrated a strong correspondence between the temperature optimums for MS and growth rate in hapuku (*Polyprion oxygeneios*) reared at 12, 15, 18, 21 and 24°C. In Atlantic halibut (*Hippoglossus hippoglossus*) acclimated to temperatures of 5, 10, 12, 14, 16 and 18°C, MS and cardiovascular function peaked at 16 to 18°C but growth rates declined significantly at the same temperatures (Grans et al., 2014). Norin et al. (2014) found that MS peaks in barramundi (*Lates clacifer*) at temperatures close to those that are acutely lethal and known to severely impair growth performance. It has also been demonstrated in the killifish (*Fundulus heteroclitus*) that MS peaks at temperatures where reproductive and growth performance is suboptimal (Healy and Schulte, 2012b). The common theme of studies which show no support for the OCLTT hypothesis is that MS remains high at temperatures close to the upper critical temperature. The failure of MS to collapse at critically high temperatures usually results from the fact that MMR does not plateau with increasing temperature (Fig. 1.3C&D). A recent meta-analysis demonstrated no clear temperature optimum for MS in 31 out of the 73 data sets available for aquatic ectotherms (Lefevre, 2016). This conclusion dismisses the universality of the OCLTT concept and shows the possibility of a link between temperature dependent whole animal performance and MS must be assessed on a case by case basis.

Whether or not the upper critical thermal limits of fish are set by a limited capacity of the cardiorespiratory system to supply O_2 to the tissues, has been the subject of several investigations. Although it has been demonstrated that there is an increasing contribution of anaerobic metabolism at high temperature in fish exposed to acute warming (van Dijk et al., 1999; Clark et al., 2008; Devor et al., 2016), it is not clear, as stated in the OCLTT framework, that this results from a limited capacity of the cardiorespiratory system to supply O_2 to tissues. Brijs et al., (2015) found that, despite approximately doubling MMR, experimental manipulations of tissue O_2 supply through hyperoxia (excess ambient O_2) had no influence on thermal tolerance limits in European perch. In the same study, dramatic reductions in blood O_2 carrying capacity via experimentally induced anaemia also had no effect on thermal tolerance limits. There was also no effect of hyperoxia on upper thermal tolerance limits in two species of Antarctic notothenoids (Devor et al., 2016), an Antarctic eelpout (*Pachycara brachycephalum*) (Mark et al., 2002), and the common killifish (Healy

and Schulte, 2012a). In European seabass, experimentally induced anaemia reduced upper thermal tolerance limits by only 0.7°C, and there was no correlation between individual levels of haematocrit and upper thermal tolerance values (Wang et al., 2014). It has also been demonstrated in several fishes that substantial reductions in MS through hypoxia have no influence on upper thermal tolerance limits (Ern et al., 2016a; Ern et al., 2017). Overall, these experimental findings and the observation that some fish species maintain high MS at temperatures close to their critical limits (Norin et al., 2014; Jensen et al., 2017), strongly contradicts the role of O_2 supply limitation as the primary driver of upper thermal tolerance in fish.

1.8 Oxygen availability and temperature in intertidal rock pools

This thesis examines the metabolic response of intertidal fishes to changes in the primary limiting (O_2 availability) and controlling (temperature) factors of metabolism. Intertidal fishes are an interesting group in which to examine the effects of O_2 availability and temperature on metabolism because, in rock pool habitats, each of these environmental factors can fluctuate widely over a tidal cycle (hours). For example, Richards (2011) reported changes in O_2 availability from near anoxia to over 400% air saturation and temperature fluctuations of 10°C within a 12h period in high intertidal rock pools on the coastline of British Columbia. Thus, while rock pools provide a refuge for organisms (e.g. fish which would otherwise succumb to desiccation, hyperthermia or an inability to breathe air in a terrestrial climate) they must also be considered one of the most physiologically challenging habitats on planet Earth. The magnitude of change in a particular physico-chemical parameter in a rock pool is dependent on interactions between a number of abiotic and biotic factors. The following section therefore takes the time to characterise how oxygen and temperature fluctuate in rock pools and, as this thesis focuses on New Zealand intertidal triplefin fishes, these factors will be described in relation to a semi-diurnal tidal cycle operating within a temperate climate zone.

1.8.1 Oxygen availability

Oxygen availability can fluctuate widely during low tide emersion in rock pools due to the respiratory and photosynthetic activity of rock pool biota. Daytime isolation and emersion of rock pools is often associated with hyperoxic conditions due to the photosynthetic activity of rock pool algae; this may co-occur with significant acute temperature increases depending on the intensity of solar radiation, air temperature, rock pool volume and elevation of the rock pool on the shoreline (Truchot and Duhamel-Jouve, 1980; Morris and Taylor, 1983; Huggett and Griffiths, 1986; Richards, 2011; Legrand et al., 2018). Rock pool pH also increases during daytime emersion due to high photosynthetic activity and a corresponding reduction in CO_2 partial pressure (Truchot and Duhamel-Jouve, 1980). The salinity of rock pools may also increase during daytime emersion, particularly in hot and windy weather. Periods of nocturnal emersion can result in severely reduced O_2 availability (hypoxia) as there is no photosynthetic activity to buffer O_2 depletion resulting from the respiratory activity of rock pool biota (Truchot and Duhamel-Jouve, 1980; Morris and Taylor, 1983; Huggett and Griffiths, 1986; Richards, 2011; Legrand et al., 2018). Nocturnal hypoxia in rock pools tends to occur alongside increased CO_2 partial pressure and a corresponding reduction in pH (Truchot and Duhamel-Jouve, 1980). The magnitude of changes in O_2 availability are strongly dependent on the timing of low tide, with the most extreme levels of hyperoxia and hypoxia occurring when emersion occurs entirely during the day and night respectively (Fig. 1.4) (Truchot and Duhamel-Jouve, 1980). The abundances of algae and animals inhabiting a rock pool also has a significant influence on the magnitude of O_2 fluctuations (Huggett and Griffiths, 1986).

1.8.2 Temperature

Rock pools represent one of the most thermally dynamic habitats in which marine fishes reside. Temperatures can rise well above ambient sea temperature during daytime emersion, before only hours later dropping below ambient sea temperature at night. Abrupt changes in temperature also occur when rock pools are flushed by the incoming tide. Rock pool temperatures increase during daytime emersion due to a transfer of heat energy from warm air and solar radiation to water. Ultimately, for a given rock pool water volume, pools receiving greater energy inputs will be heated to higher temperatures. Thus, the highest rock

pool temperatures occur in summer when air temperatures peak and solar radiation is most intense. For example, Hilton et al. (2008) observed a maximum summer temperature of 27°C, and a maximum winter temperature 20°C in a rock pool inhabited by New Zealand triplefin fish. On top of this seasonal trend, the time of day emersion occurs also influences the extent of temperature change. When low tide is around solar noon, rock pool emersion coincides with peak air temperatures and intense solar radiation due to the sun's high angle of incidence; conditions that can result in large acute rises in temperature (Fig. 1.4A). If, however, high tide is around solar noon temperature increases are muted because emersion occurs in the morning and afternoon when air temperatures are lower and solar radiation is less intense (Fig. 1.4B). The height of a rock pool in the intertidal zone also influences the magnitude of temperature change because it will determine the duration of emersion. Rock pools positioned high in the intertidal zone are isolated for the longest durations between high tides and, therefore, receive greater inputs of heat energy and solar radiation in comparison to lower intertidal rock pools. For example, Hugget and Griffiths (1986) showed that the greatest increases in rock pool temperature occur in rock pools located highest in the intertidal zone along the coast of the Cape Peninsula, South Africa. Hilton et al. (2008) also observed that the highest rock pool temperatures were observed in pools located highest on the shoreline in North Eastern New Zealand. Other factors which influence the magnitude of temperature increases in rock pools include weather (e.g. cloud cover and wind), rock pool depth and shape (i.e. surface area to volume), local fine-scale topography (i.e. rock pool shading), thermal properties of intertidal substratum (i.e. potential for heat transfer from substratum to rock pool water), and the aspect of the shoreline where the rock pool is located. During periods of nocturnal emersion the direction of rock pool temperature change is dependent on whether night-time air temperature is higher or lower than ambient sea temperature. In New Zealand, night air temperatures are typically lower than ambient sea temperature, this means that rock pool temperatures tend to decline or remain relatively stable during periods of nocturnal emersion.

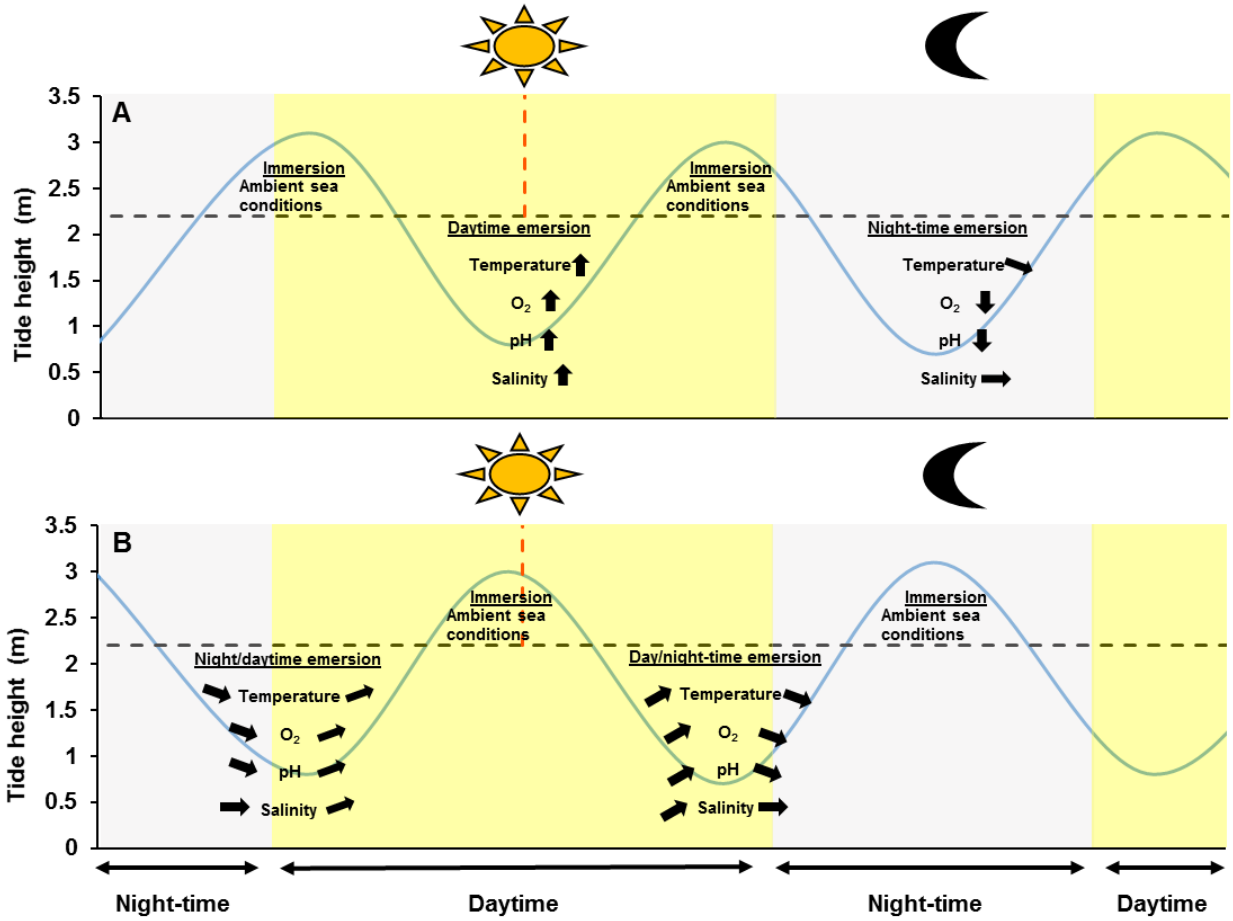


Figure 1.4 The influence of the timing of low tide on physico-chemical conditions in rock pools. Panel A represents the tidal cycle when low tide occurs around solar noon (tidal heights and times are those projected for Auckland, New Zealand on the 4-5thth of January 2019). Panel B has the same tidal heights as A but represents a high tide occurring around solar noon. The black horizontal dashed line represents the position of a hypothetical rock pool on the shoreline. The red vertical dashed line indicates solar noon. Arrows indicate the direction of changes in physico-chemical conditions in rock pool during emersion: upwards= increase, downwards= decrease, horizontal= no change. Yellow shading shows daylight hours and grey shading shows night-time hours. In panel A daytime emersion occurs under hot sunny conditions causing steep increases in temperature, oxygen (hyperoxia due to photosynthesis), pH and salinity. Due to a semi-diurnal tidal pattern the next period of emersion occurs at night when temperature falls, oxygen is depleted (hypoxia) and pH declines. Salinity should remain relatively stable at night if there is not substantial rainfall. In panel C emersion occurs during night and daytime. Despite hot sunny conditions the period of immersion around solar noon prevents large fluctuations in physico-chemical conditions during the day. The period of immersion between 10:30pm and 04:00am prevents large declines in oxygen and pH at night.

1.9 Thesis structure and outline

This thesis examines the physiological response of New Zealand triplefin fishes (Family *Tripterygiidae*), with a primary focus on intertidal species, to changes in O_2 availability and temperature. Triplefins are a family of blennioid fishes with up to 168 species

in 29 genera worldwide (Stewart and Clements, 2015). The New Zealand triplefin species flock is highly diverse consisting of 27 species in 12 genera (Stewart and Clements, 2015). The majority of these species (all but four) form a closely related monophyletic group (Hickey and Clements, 2005) and show distinct habitat partitioning by depth and exposure gradients (Wellenreuther et al., 2007). Previous studies have compared intertidal and subtidal triplefin species in order to identify phenotype-environment correlations of physiological characteristics (Brix et al., 1999; Hickey and Clements, 2003; Hilton et al., 2008; Hilton et al., 2010a) and this thesis, in part, builds upon the work of these authors. Moreover, another group of closely related fishes, the sculpins (Family *Cottidae*), that also show species partitioning into intertidal and subtidal habitats, have provided a useful system in which to examine physiological, biochemical and behavioural mechanisms of hypoxia tolerance (Mandic et al., 2009; Mandic et al., 2009; Richards, 2011; Mandic et al., 2013). Each chapter of this thesis is presented in a standalone publication format and addresses specific aims and hypotheses. These aims and hypothesis are outlined in detail within the introduction of each chapter. Therefore, to avoid repetition, only a brief outline of the content of each chapter will be provided here.

In Chapter 2 the hypoxia tolerance (P_{crit}) of intertidal triplefin fishes is determined and compared to that of exclusively subtidal triplefin species. In addition, whole animal metabolic performance (SMR, MMR/$\dot{M}O_{2,max}$, and MS), gill morphometric characteristics, blood O_2 carrying capacity and tissue glycogen stores are compared among intertidal and subtidal species to identify key physiological characteristics of hypoxia tolerant triplefin species. This chapter builds on the previous work of others (Innes and Wells, 1985; Brix et al., 1999; Hilton et al., 2008; Hilton et al., 2010a) and the findings provide novel information about the hypoxia tolerance strategy of intertidal triplefin fishes.

In Chapter 3 the hypoxia tolerance (P_{crit} and loss of equilibrium) of an exclusively intertidal triplefin species is assessed following recovery from acute heat stress. This sequential multiple stressor exposure is ecologically relevant to intertidal fishes as, during certain parts of the tidal cycle, they can face daytime heat stress followed only hours later by night time hypoxia. The findings of this chapter provide insights regarding the hypoxia tolerance of intertidal triplefin fish under environmentally realistic conditions and more broadly contributes to the currently limited understanding of how fish respond to sequential stress exposure in terms of heat and hypoxia.

In Chapter 4 the aerobic metabolic response to co-occurring hyperoxia and acute heat stress is assessed in two intertidal triplefin species. This is an ecologically relevant situation for intertidal fishes as rock pools commonly become hyperoxic during daytime low tides. The upper thermal tolerance limits of each species was determined under hyperoxia and normoxia, and the influence of hyperoxia on gill ventilation during thermal ramping was also assessed. This chapter is the first study to assess the influence of hyperoxia on aerobic performance and upper thermal tolerance limits in an intertidal fish species.

Chapter 5 assesses the influence of chronic warm exposure (12 weeks acclimation) on the aerobic performance (SMR, MMR/$\dot{M}O_{2,max}$ and MS), growth performance and upper thermal tolerance limits of a triplefin species which occupies both intertidal and subtidal habitats. This chapter provides valuable insights into the role of temperature dependent O_2 limitation (i.e. the OCLTT hypothesis) in the chronic and acute thermal tolerance of fish, and important information about how triplefin fish may respond to future warming due to climate change.

CHAPTER 2: PHYSIOLOGICAL MECHANISMS OF HYPOXIA TOLERANCE IN NEW ZEALAND TRIPLEFIN FISHES

Chapter published as: McArley, T. J, Hickey, A. J, Wallace, L, Kunzmann, A, Herbert (2019). Intertidal triplefin fishes have a lower critical oxygen tension (Pcrit), higher maximal aerobic capacity, and higher tissue glycogen stores than their subtidal counterparts. Journal of Comparative Physiology B. 189, 399-411

2.1 Introduction

To carry out the necessary requirements of life, all aerobic organisms need to acquire enough O_2 to drive ATP production via oxidative phosphorylation. Therefore, decreased O_2 availability (environmental hypoxia), which is a common occurrence in a range of aquatic habitats, presents as a challenge for organisms such as fish (Diaz and Breitburg, 2009; Farrell and Richards, 2009b). Intertidal fish can be exposed to hypoxic conditions when rock pool organisms consume O_2 at a faster rate than it can be replaced by either algal photosynthesis or diffusion from air (Truchot and Duhamel-Jouve, 1980; Berschick et al., 1987; Bridges, 1988; Richards, 2011). Thus, hypoxia is prevalent in rock pools at night when there is no photosynthetic activity to buffer O_2 depletion due to respiration (Table 1 and Fig. 1A and B). Well mixed coastal subtidal habitats on the other hand have relatively stable O_2 conditions (Fig. 1C) and, while hypoxia can develop in subtidal habitats (e.g. due to eutrophication and algal blooms or poor mixing), the frequent and large change in O_2 availability typical of rock pools does not occur to the same extent. Notably, comparisons of closely related intertidal and subtidal species have proven a useful system in which to examine physiological mechanisms of hypoxia tolerance in fish (Brix et al., 1999; Mandic et al., 2009; Hilton et al., 2010a; Richards, 2011; Speers-Roesch et al., 2013; Mandic et al., 2013).

Environmental hypoxia represents a continuum of diminishing O_2 availability under which it becomes increasingly challenging for organisms to take up adequate O_2 to meet energetic demands via aerobic metabolism. If O_2 availability is critically low, organisms cannot extract enough O_2 to meet even basal energetic demands aerobically and O_2 independent (anaerobic) ATP production is recruited to maintain energy balance (Richards, 2011). O_2 independent ATP production, however, is relatively inefficient, dependent on a finite pool of endogenous fermentable fuels (e.g. glycogen) and results in the accumulation of

by-products (e.g. ADP, lactate and H^+) which can be deleterious (Richards et al., 2007; Nilsson and Östlund-Nilsson, 2008; Richards, 2011; Speers-Roesch et al., 2013). Thus, a key strategy for hypoxia survival is to continue to meet O_2 demand even at low environmental O_2 tensions (PO_2) (Mandic et al., 2009). In fish, the capacity to meet O_2 demand under hypoxia can be measured as the critical PO_2 (P_{crit}), which is defined as the lowest O_2 tension at which a minimal resting rate of O_2 consumption ($\dot{M}O_2$) can be maintained. In practical terms P_{crit} is the PO_2 at which $\dot{M}O_2$ is forced lower than standard metabolic rate (SMR) (Rogers et al., 2016; Claireaux and Chabot, 2016). Importantly, fish exposed to PO_2 equivalent to 30% of their known P_{crit} accumulate lactate, deplete tissue glycogen and ATP stores and eventually lose equilibrium (Speers-Roesch et al., 2013). In the sculpins, intertidal species with a lower P_{crit} survive extreme hypoxia for longer (Mandic et al., 2013), showing that a low P_{crit} makes an important contribution to overall hypoxia tolerance in at least some intertidal fishes. Theoretically, species with a lower P_{crit} should be more hypoxia-tolerant as a high extractive capacity for O_2 allows energy demand to be met through the efficient aerobic pathway at lower PO_2, thus avoiding or limiting the extent to which a time-limited anaerobic strategy must be relied upon for survival. It should be acknowledged, however, that some fishes can be very hypoxia-tolerant and also have a relatively high P_{crit}. This phenomenon is seen in the Amazonian oscar (*Astronotus ocellatus*) which can survive up to 6 h anoxia at 28°C but has a P_{crit} of 6.1 kPa (Scott et al., 2008). However, rather than relying on a high extractive capacity for O_2, the Amazonian oscar is thought to achieve hypoxia tolerance through a combination of metabolic rate depression and a high tolerance of the end-products of anaerobic metabolism (Scott et al., 2008).

According to Mandic et al. (2009), intertidal sculpins have a lower P_{crit} than their subtidal counterparts, indicating that the ability to meet resting O_2 demand at low PO_2 has been selected for in fishes occupying intertidal habitats with variable O_2 conditions. Mandic et al. (2009) also showed that sculpin species with a low P_{crit} have red blood cells (RBC) with a higher affinity to bind O_2 (low whole RBC P_{50}), a larger mass-specific gill surface area and a lower routine $\dot{M}O_2$ (an estimate of resting O_2 requirements). Thus, it appears that a low P_{crit} requires a high extractive capacity for O_2, which can then also be put to use to meet a low basal requirement for O_2. Therefore, one prediction is that species with a lower P_{crit} will have a cardiorespiratory system with a high capacity to take up O_2 (i.e. a high maximum metabolic rate [$\dot{M}O_{2,max}$]) and also a low SMR (the best estimate of minimal resting $\dot{M}O_2$ requirements in a rested post-absorptive animal). This combination of respiratory characteristics would

mean that species with a lower P_{crit} will also have higher aerobic metabolic scope (MS), representing the difference between $\dot{M}O_{2,max}$ and SMR. MS reflects the capacity of an organism to perform O_2 demanding activities (e.g. growth, activity, feeding etc.) beyond maintenance requirements and a constraint upon MS has been proposed as a crucial determinant of performance in fish facing hypoxia (Claireaux and Lefrancois, 2007; Chabot and Claireaux, 2008; Claireaux and Chabot, 2016). Intertidal rock pool fish with a high MS might therefore avert impaired aerobic performance during rock pool hypoxia (Fig.1 and Table 1), but MS has yet to be compared among intertidal and subtidal fishes.

New Zealand triplefins (Family *Tripterygiidae*) are a highly specious and closely related group of marine blennoid fishes (Hickey and Clements, 2005) which show habitat partitioning by depth across the nearshore coastal environment (Wellenreuther et al., 2007). Comparisons among New Zealand triplefin fishes already show that intertidal species have a lower P_{crit} than subtidal species (Hilton et al., 2008; Hilton, 2010; Hilton et al., 2010a). However, it is possible that the protocols and respirometry methods used in this previous work produced elevated estimates of P_{crit}. For example, fish within these studies were allowed to recover for only 2.5 h within respirometers prior to hypoxia exposure and P_{crit} measurement. The problems associated with the use of a short recovery time when estimating P_{crit} are two-fold. Firstly, a reliable estimate of minimal resting $\dot{M}O_2$ (i.e. SMR) may be precluded and, if this is the case, P_{crit} has to be identified as the breakpoint in $\dot{M}O_2$ during hypoxia exposure using segmented regression. Segmented regression is not recommended for P_{crit} determination because, if for any reason the $\dot{M}O_2$ of fish is elevated at the time of hypoxia exposure (e.g. due to incomplete recovery from handling stress or spontaneous activity), this methodology will overestimate P_{crit} (Claireaux and Chabot, 2016). This problem is exacerbated when making comparisons of hypoxia tolerance among multiple species that may recover from handling stress at different rates, and/or be more or less active within respirometers. Furthermore, previous estimates of P_{crit} in triplefins (i.e. Hilton et al., 2008; Hilton, 2010; Hilton et al., 2010a) were made using closed-system respirometry, which in some cases has been demonstrated to confound $\dot{M}O_2$ measurements due to the accumulation of waste products (e.g. CO_2) within respirometers (Rogers et al., 2016; Snyder et al., 2016). Therefore, in the present investigation, we determined it necessary to re-examine the hypoxia tolerance of triplefin species, but this time using intermittent stop-flow respirometry (Steffensen, 1989) and more established methods for P_{crit} measurement (see Claireaux and Chabot, 2016; Snyder et al., 2016)

In this study the capacity to meet resting O_2 demands under hypoxia (i.e. P_{crit}) was compared among four New Zealand triplefin fish species (*Bellapiscis medius*, *Forsterygion lapillum, F. varium* and *F. malcolmi*). These four species were included as they occupy different habitat depths (intertidal to subtidal; Table. 2) and represented a broad range of P_{crit} for this group (i.e. low, medium and high P_{crit}) in a previous investigation (Hilton, 2010). *Bellapiscis medius* is an intertidal specialist which occupies rock pools located on the shoreline, on average 1.39 m (range 0.31 - 3.39 m) above chart datum (ACD) (Hilton et al., 2008). *Forsterygion lapillum* is an occasional occupant of intertidal rock pools, but is most abundant in relatively shallow (mean depth of occurrence 3.5 m; Table. 2) subtidal habitats. In the intertidal zone *F. lapillum* is less abundant than *B. medius*, however, their distributions, at least in terms of shoreline elevation, do overlap as *F. lapillum* is found in rock pools on average 1.21 m (range 0.19 – 3.09 m) ACD (Wellenreuther 2007). *Forsterygion varium* and *F. malcolmi* are both exclusively subtidal species with mean depth of occurrences of 8 and 11 m respectively (Table. 2). The inclusion of these four species allowed the physiological characteristics of a specialist intertidal species (*B. medius*) to be compared to three other relatively closely related triplefin species which are either occasionally intertidal (*F. lapillum*) or exclusively subtidal (*F. varium* and *F. malcolmi*). Additionally, we could compare the physiological characteristics of the occasional intertidal rock pool inhabitant *F. lapillum* to that of two exclusively subtidal species from the same genus (*F. varium* and *F. malcolmi*). This comparison was advantageous because if the same physiological characteristics distinguishing *B. medius* from the exclusively subtidal species were also seen in *F. lapillum*, this would provide evidence that the observed characteristics of intertidal species result from selection for intertidal conditions rather than phylogeny.

Specifically, the aims of this study were: (1) to re-examine if there are differences in P_{crit} between intertidal and subtidal triplefin fishes, and (2) to identify physiological characteristics associated with P_{crit} among intertidal and subtidal triplefin fishes. To address these aims the P_{crit} for SMR (Claireaux and Chabot, 2016) was measured to establish differences in the ability to meet resting O_2 demands under hypoxia among species. Whole animal metabolic rate (SMR, $\dot{M}O_{2,max}$ and MS) was assessed to determine if a low P_{crit} is associated with a low resting O_2 demand and/or a high extractive capacity for O_2 as shown in other fishes (Richards, 2011). Blood Hb concentration and gill morphometry were also measured as these may be associated with extractive capacity for O_2 and P_{crit} (Mandic et al., 2009). Finally, tissue glycogen was measured in brain, liver and white muscle, as it has been

hypothesised that hypoxia-tolerant fishes have higher endogenous stores of fermentable fuels (Richards, 2011; Mandic et al., 2013).

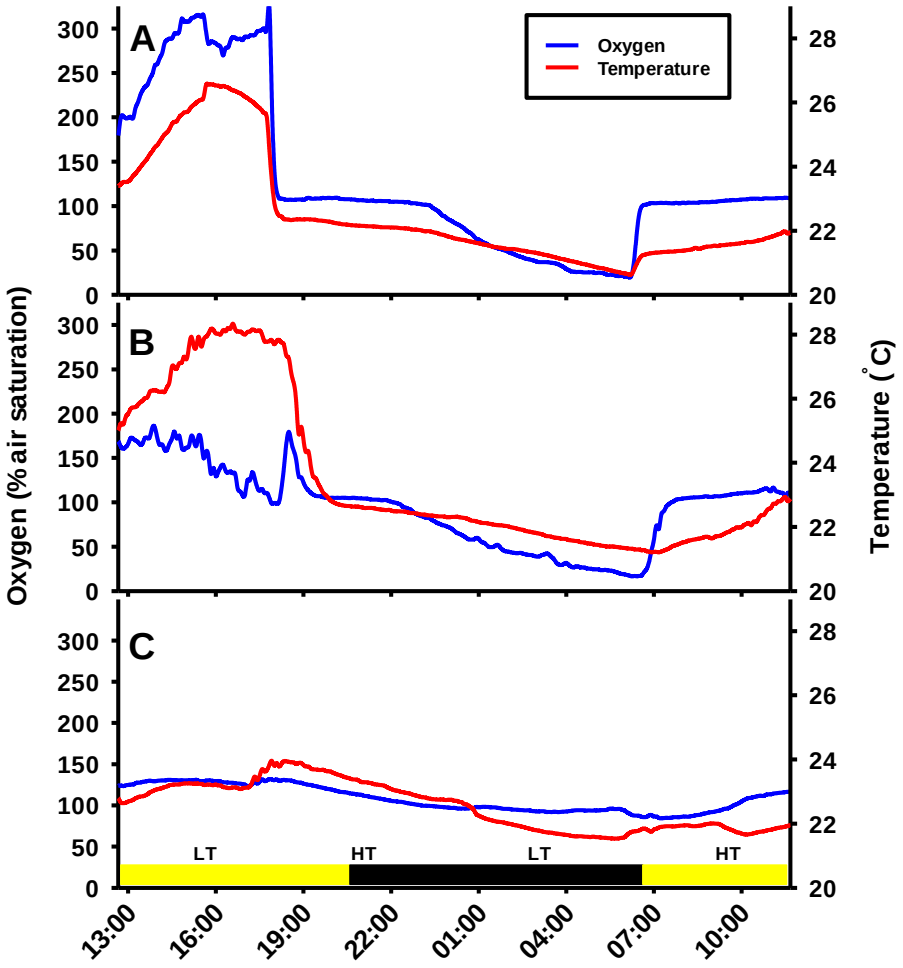


Fig. 2.1 Changes in oxygen availability (% of air saturation) and temperature (°C) in two rock pools inhabited by intertidal triplefin fish (A and B) and at a shallow subtidal site (C). Values represent measurements logged every 5 min between the 5th and 6th of February 2019. LT=low tide, HT=high tide. Yellow and black shaded areas in panel C show daytime and night-time hours respectively. The two intertidal rock pools were located at Goat Island, Leigh, New Zealand (36°16'S, 174°47'E) and the subtidal site was located immediately adjacent to the rock pools at a depth of ~2m.

Table 2.1 Oxygen (O_2) concentration, temperature and fish species observed in 10 rock pools approximately 1 h after low tide at Hatfield's Beach, Auckland, New Zealand (36°34'S, 174°41'E). Values represent spot measurements taken at a mid-level depth in the rock pools between 0530-0630h (night-time low tide 0423h) and 1730-1830h (daytime low tide 1649h) on the 26th of February 2016.

	O_2 content (% air saturation)		Temperature (°C)		Species observed	
Pool	Day	Night	Day	Night	Day	Night
1	109.1	26.3	25.5	22.2	Bm	Bm
2	41.6	10.3	23.9	21.5	Bm, Af	Af
3	95.2	31.7	23.9	21.7	Bm, Af	Bm, Af
4	87.2	16	23.2	21.3	na	Bm
5	87.1	35.6	22.6	20.6	Bm, Fl	Bm, Fl
6	113.2	55.3	22.6	20.3	na	Bm
7	113.1	48.8	22.6	21.9	na	Bm, Fl, Af
8	43.9	13.1	22.7	20.8	na	Bm, Af
9	51.3	9.4	24.6	21.4	na	na
10	196.8	17.3	26.9	21.7	Bm	Bm

Bm= *Bellapiscis medius*, Af= *Acanthoclinus fuscus* (Jenyns, 1842), Fl= *Forsterygion lapillum*

2.2 Methods

2.2.1 Rock pool O_2 measurements

The O_2 content of rock pools occupied by intertidal triplefin fishes was assessed to gain an understanding of the severity of hypoxia that occurs under natural conditions. Two sets of measurements were made: (1) O_2 and temperature were logged at 5 min intervals in two rock pools over a ~24 h period (Fig. 1 A & B) and spot measurements of O_2 and temperature were taken in 10 rock pools at night time low tide and then again in the same rock pools the following day at low tide (Table 2). To make the continuous measurements O_2 loggers (D-Opto O_2 logger, Zebra-Tech Ltd, Nelson, NZ) were placed in rock pools for a period of ~24 h between the 5th and 6th of February 2019. The night and daytime spot measurements were taken on the 26th of February 2016 approximately an hour after low tide and were made at mid depth level in the rock pools. These measurements were made with a Firesting O_2 meter and shielded temperature probe (PyroScience, Aachen, Germany). The species of fish observed in each of the 10 rock pools at the time spot measurements were made was also recorded. The continuous measurements were made in rock pools located at Goat Island, Leigh, New Zealand (36°16'S, 174°47'E) and the spot measurements were made in rock pools located at Hatfields Beach, Auckland, New Zealand (36°34'S, 174°41'E).

Changes in O_2 and temperature over a ~24 h period were also assessed at a shallow subtidal site (~2 m deep) at Goat Island, Leigh, New Zealand (36°18'S 174°47'E). These measurements were taken between the 5th and 6th of February 2019 using the O_2 logger described above.

2.2.2 Experimental animals and laboratory acclimation

Four triplefin species (*B. medius, F. lapillum, F. varium and F. malcolmi*) were collected from the wild for this study. The intertidal triplefin *B. medius* was netted from rock pools and *F. lapillum, F. varium* and *F. malcolmi* were hand netted from subtidal habitats by divers. The animals were housed in 30 L flow-through seawater tanks (air saturated, 200 μm filtered, 35 ppt salinity) at the Leigh Marine Laboratory. Fish were acclimated to normoxia and a temperature of 18˚C (±0.5˚C) for a period of at least 4 weeks prior to experiments and were fed daily on a mixture of crushed aquaculture feed (Skretting, Australia) and pilchard. Food was withheld for a period of 48 h prior to experiments, which were performed under approval of the University of Auckland Animal Ethics Committee (AEC approval number 001441).

2.2.3 General respirometry methods

The mass-specific O_2 consumption rate ($\dot{M}O_2$), reported as mg of O_2 consumed per g of body weight per hour (mg O_2 g^{-1} h^{-1}), was determined using automated intermittent-flow respirometry (Steffensen, 1989). Custom built respirometry chambers (42-210 mL) were held within a 60 L reservoir filled with filtered (1 μm) UV-sterilised seawater, which was heated or chilled to the experimental temperature (~18˚C) by continually pumping the seawater through a 40 L heat exchange tower containing an aluminium coil heat exchanger. The inlet of each chamber was connected to an automated Eheim compact 3000 submersible flush pump (EHEIM GmbH & Co. KG, Germany) which was switched on and off by a relay control unit (USB Power 8800 Pro, Aviosys International Inc, Taiwan) controlled by custom-coded software (Leigh Marine Laboratory). A magnetic stir bar was housed in a recess in the bottom of the respirometry chamber to ensure adequate water mixing and the O_2 concentration of water within the chamber was continuously measured using contactless sensor spots followed by FireSting O_2 meters (PyroScience, Aachen, Germany). The decline in O_2 concentration within a respirometry chamber was used to calculate $\dot{M}O_2$ in repeated measurement cycles according to the equation:

$$\dot{M}O_2 = V\left(\Delta\%\frac{sat}{t}\right)a/M_B \quad ,(1)$$

where V is the respirometry chamber minus fish volume, $\Delta\%\ sat/t$ is the change in O_2 saturation per unit time, α is the solubility coefficient of O_2 (mg O_2 $\%Sat^{-1}$ L^{-1}) in sea water (35 ppt), and M_B is the body mass of the fish in grams (Schurmann and Steffensen, 1997). In all instances of $\dot{M}O_2$ assessment the repeated measurement cycles were interspersed with 1 min periods of flushing in order to fully replace the water volume of the chamber. Background respiration was assessed after the fish was removed from the respirometer, but remained negligible throughout the trials. In all estimates of metabolic rate only $\dot{M}O_2$ values with R^2 of >0.90 for the decline in O_2 per unit of time were used.

Using the procedures above, $\dot{M}O_2$ was measured to establish the standard metabolic rate (SMR), maximum metabolic rate ($\dot{M}O_{2max}$), aerobic metabolic scope (MS) and the hypoxia tolerance (P_{crit}) threshold of fish. (NB. SMR was measured and reported twice as part of two separate procedures. See below).

2.2.4 Measurement of maximum metabolic rate and aerobic metabolic scope

$\dot{M}O_{2max}$ and MS were determined in all species at 18˚C (N=10-11) (*B. medius*: body mass= 2.32 g ± 0.37, *F. lapillum*: body mass=1.98 g ± 0.15, *F. varium*: body mass= 6.1 g ± 0.85, *F. malcolmi*: body mass= 6.97 g ± 0.88). $\dot{M}O_{2max}$ was determined following exhaustive exercise, where fish were continually chased (5 min) in a 30 L tank. An exhaustive exercise protocol has previously been used to elicit $\dot{M}O_{2max}$ values in triplefins (Khan et al., 2014a; McArley et al., 2017) and is best suited for obtaining $\dot{M}O_{2max}$ in benthic species which will not continuously swim in a flume (Clark et al., 2013; Norin and Clark, 2016). Following exhaustive exercise the fish was transferred to a respirometry chamber within 30 s of the conclusion of chasing and repeating 4 min $\dot{M}O_2$ measurement cycles were initiated. $\dot{M}O_{2max}$ was taken as the highest $\dot{M}O_2$ value recorded in any measurement cycle, which in almost all cases was obtained from the first measurement cycle following exhaustive exercise. When the metabolic rate of fish was clearly declining, the measurement period was extended to 8 min and $\dot{M}O_2$ was measured repeatedly for >16 h. During the overnight measurement period fish were left undisturbed in the respirometers and $\dot{M}O_2$ typically recovered to levels around SMR within ~6-8 h. Standard metabolic rate (SMR) was estimated from the mean of the lowest 10% of $\dot{M}O_2$ over this time (Khan et al., 2014b; Norin et al., 2014; McArley et al., 2017). Due to the benthic habit of these triplefin species they remain relatively inactive and

perch in a stationary position on the bottom of the respirometers. This behavioural characteristic means that the lowest $\dot{M}O_2$ values recorded probably related to periods when the fish was in an inactive state and are therefore likely to be a fair representation of SMR. MS was defined as the difference between the mass corrected $\dot{M}O_{2max}$ and SMR (see below) for each individual.

2.2.5 Measurement of critical oxygen tesnion (P_{crit})

P_{crit} was measured in *B. medius* (body mass=3.22 g ± 0.41), *F. lapillum* (body mass=1.72 g ± 0.12), *F. varium* (body mass=6.6 g ± 0.98) and *F. malcolmi* (body mass=4.95g ± 1.16) at 18˚C following a similar protocol to Cumming and Herbert (2016) (N=9-10). In this protocol SMR was once again measured under normoxic conditions, then a progressive hypoxic exposure was used to identify the water O_2 tension at which $\dot{M}O_2$ was no longer maintained above SMR. SMR was determined using automated intermittent flow respirometry (see respirometry methods above), where fish were left undisturbed in respirometers overnight for ~16 hours (~1700h-0900h). During overnight respirometry $\dot{M}O_2$ was repeatedly measured over 7-9 minute cycles and SMR was taken as the mean of the lowest 10% of $\dot{M}O_2$ values. $\dot{M}O_2$ measurements were then made at decreasing levels of water O_2 content (75%, 55%, 40%, 30%, 25%, 20%, 15%, 10%, and 6% of air saturation) and the required water O_2 levels were achieved by bubbling nitrogen into the seawater reservoir supplying respirometers. Three 7-9 min $\dot{M}O_2$ measurements were made at 75%, 55%, 40%, 30%, 25% and 20% of air saturation, and one 7-9 min measurement at 15%, 10% and 6% of air saturation. The progressive decline in O_2 tension to determine P_{crit} was completed in ~3.5 h and the time length of exposure to each O_2 level was the same for each species. To determine P_{crit}, SMR and $\dot{M}O_2$ during progressive hypoxic exposure were first mass corrected (see below), then plotted against water O_2 tension. A linear regression (forced through zero) was then established on $\dot{M}O_2$ values that fell below SMR and P_{crit} was calculated by dividing SMR by the slope of this regression line (i.e. the point where $\dot{M}O_2$ under progressive hypoxia could no longer be maintained above SMR; see Fig. 2) (Schurmann and Steffensen, 1997; Behrens and Steffensen, 2007; Cook et al., 2013; Cumming and Herbert, 2016).

To account for body mass differences between species the $\dot{M}O_2$ values were standardised to the mean body mass of all fish (4 g) using the formula:

$$\dot{M}O_{2(4g)} = \dot{M}O_{2(meas)} \left(\frac{w}{w_{(4g)}}\right)^{(1-A)} \quad ,(2)$$

where $\dot{M}O_{2(4g)}$ is the $\dot{M}O_2$ for a fish with the standardized (corrected) new weight of 4 g, $\dot{M}O_{2(meas)}$ is the measured $\dot{M}O_2$, w is the weight of the fish, $w_{(4\ g)}$ is the standardized body mass of fish set to 4 g and A is the weight exponent describing the relationship between metabolic rate and body weight (Schurmann and Steffensen, 1997). The mass exponent (A) used to correct $\dot{M}O_2$ to body mass was 0.8 (Clarke and Johnston, 1999).

2.2.7 Blood haematology, tissue glycogen and gill morphometry

Haematology, tissue glycogen, and gill morphometry was assessed in *B. medius* (body mass=4.25g ± 0.24), *F. lapillum* (body mass=2.16g ± 0.09), *F. varium* (body mass=10.55g ± 0.88) and *F. malcolmi* (body mass=8.74g ± 0.81) acclimated to 18˚C for a period of 4 weeks (N=8-10). 24 hours prior to sampling, fish were transferred to individual 14 L flow through seawater tanks so that they were not disturbed by the repeated removal of fish from the same common holding tanks. First the fish was netted from its individual holding tank and immediately euthanized by pithing of the brain. The caudal peduncle was then severed and blood was collected by holding the tail of the fish into the base of a heparinised 0.5 mL eppendorf tube. The brain, liver and a sample of white skeletal muscle was then immediately frozen under liquid N_2 and stored at -80°C. The right gill basket was removed and fixed in Bouin's solution for 48 hours and then stored in 70% ethanol. Haemoglobin (Hb) concentration of fresh blood was quantified spectrophotometrically at 540 nm using modified Drabkin's reagent (Wells et al., 2007). Haematocrit (Hct) was determined in 75 mm capillary tubes spun for a period of 10 min in a haemofuge (Haemocentaur, MSE, London, UK). Tissue glycogen content was determined in thawed brain, liver and white muscle which was homogenised in ice cold 0.6 M perchloric acid. Glycogen (in glycosol units) was determined by measuring glucose content of tissue extract with and without prior incubation with amyloglucosidase as per the method of Keppler and Decker (1974). Glucose content of the tissue extract was determined using a commercially available assay kit (D-Glucose HK Assay Kit, Megazyme, Ireland).

Gill morphometry was assessed by light microscopy on intact whole gill arches (WGA) and longitudinally sectioned gill arches (SGA) from the right gill basket. All analysis of gill images was performed using Image J software (U. S. National Institutes of Health, Bethesda, Maryland, USA). Several gill morphometric parameters were measured including: (1) filament number (WGA), (2) filament length (WGA), (3) secondary lamellae (SL) density (WGA), (4) SL basal length (WGA), (5) SL protruding height and thickness (SGA), (6) SL bilateral surface area, and (7) mass-specific gill surface area. Firstly the four gill arches of the right gill basket were separated and photographed under a stereomicroscope. The number of filaments on each gill arch were counted and the length of all filaments in a single row on each arch were measured. The filament length in a single row was then multiplied by two to estimate the total length of filaments on each gill arch. To estimate the total length of gill filaments for the entire gill system (i.e. the right and left gill basket) the total length of filaments measured on each gill arch were summed and then multiplied by two. SL density was estimated by measuring the distance occupied by at least 10 SL on four filaments on each gill arch. The bilateral surface area of SL (mm^2) was calculated as the area of a half ellipse based on measurements of the basal length, protruding height and thickness of SL according to the formula outlined in Matey et al. (2008). SL basal length was measured on different filaments than SL height and SL thickness due to the requirement for sectioning. As such the mean value of each of these parameters for individual fish was used to calculate a global average of SL bilateral SA for each fish. To estimate the basal length of SL individual filaments were removed from the third gill arch and photographed under a stereomicroscope. The width of filaments, where the base of the SL attaches, were then measured at ten evenly spaced distances along their length in order to approximate the basal length of the SL. To measure the thickness and protruding height of SL the whole second gill arch was embedded in paraffin, sectioned longitudinally (5µm) using a microtome, mounted on slides and stained with haematoxylin and eosin. The height and width of 30 SL was then measured from sections where the central venous sinuous of the filament was visible. To estimate the total surface area of the gills the total length of gill filaments was multiplied by SL density to provide an estimate of the total number of SL in the entire gill system. The total number of SL was then multiplied by the SL bilateral surface area to produce an estimate of total gill surface area in mm^2. Total gill surface area was then standardised to body mass and is presented as mm^2 per g of body mass. To take into account the differences in the thickness of the SL between species the maximum O_2 uptake capacity of the gills was determined according to Fick's law:

$$VO_2 = \frac{1}{t} * K * A * dPO_2 \quad ,(3)$$

where VO_2 is the maximum O_2 uptake rate for the gills obtained by morphometric measurements, K is a diffusion constant, *A* is gill area, dPO_2 is the difference between the partial pressure of O_2 on either side of the membrane and *t* is the thickness of the water-blood barrier (Kunzmann, 1990). The thickness of the water-blood barrier (*t*) was taken as half the thickness of the SL and the difference in partial pressure of O_2 between seawater and blood was assumed to be 110 mmHg. *K* was taken as the diffusion coefficient for O_2 in water at 18°C.

2.2.8 Statistics

In all statistical tests significance was accepted at $P<0.05$. $P_{crit,}$ SMR, $\dot{M}O_{2,max,}$ MS, gill morphometric parameters, haematological parameters and tissue glycogen were each compared between species using one-way analysis of variance (ANOVA) with Holm-Sidak *post-hoc* comparisons. Where assumptions of normality and equality of variances were violated, data were either log transformed for the analysis or a non-parametric Kruskall-Wallis ANOVA on ranks test was used. All statistical analysis was carried out using Sigma Plot 13.0 software package.

2.3 Results

2.3.1 P_{crit} and standard metabolic rate in hypoxia tolerance assessed fish

The four species examined showed a similar aerobic response to progressive hypoxia exposure with $\dot{M}O_2$ remaining constant and slightly elevated relative to SMR at higher $PO_{2,}$ before abruptly declining and falling below SMR at lower PO_2 (Fig. 2). A difference among species in the ability to maintain $\dot{M}O_2$ above SMR under hypoxia was confirmed by significant differences in P_{crit} (ANOVA, df=3, F=12.83, P<0.001). *Post-hoc* comparisons showed that *B .medius* had a lower P_{crit} than all other species and *F. lapillum* and *F. varium* had a lower P_{crit} than *F. malcolmi* (Fig. 2). In the fish assessed for hypoxia tolerance (P_{crit}) the SMR of *B. medius* (0.079 mg O_2 g^{-1} h^{-1} ±0.003) was slightly lower than the SMR of *F. lapillum* (0.10 mg O_2 g^{-1} h^{-1} ±0.008), *F. varium* (0.096 mg O_2 g^{-1} h^{-1} ±0.008) and *F. malcolmi* (0.11 mg O_2 g^{-1} h^{-1} ±0.005) (Fig. 2). However, although there appeared to be a difference in

SMR among species (ANOVA, df=3, F=2.95, P=0.046) none of the pairwise *post-hoc* comparisons were significant (P>0.05).

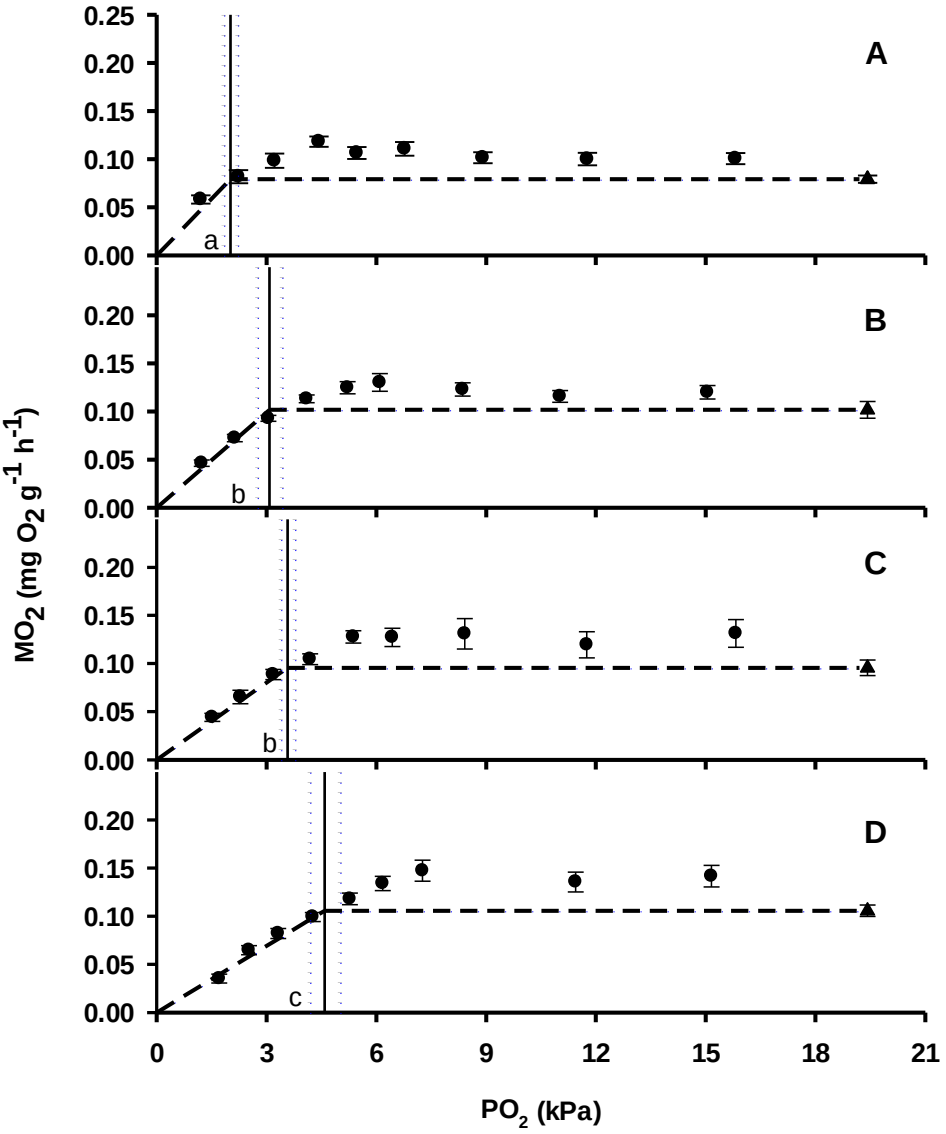


Fig. 2.2 Critical oxygen tension (P_{crit}) and mass-specific oxygen consumption ($\dot{M}O_2$) under progressive hypoxia exposure in *Bellapiscis medius* (A), *Forsterygion lapillum* (B), *Forsterygion varium* (C) and *Forsterygion malcolmi* (D) at 18°C. All values are means ± S.E.M. and $\dot{M}O_2$ values are corrected to a body mass of 4 g (N=9-10). Triangles show standard metabolic rate (SMR) under normoxia, circles show $\dot{M}O_2$ under progressive hypoxia exposure, vertical line shows P_{crit} and vertical dotted line shows P_{crit} S.E.M. The dashed horizontal line shows the break point where $\dot{M}O_2$ falls below SMR (i.e. P_{crit}) and is for illustrative purposes only. Different lower case letters adjacent to the vertical line representing P_{crit} show significant differences in P_{crit} between species (P<0.05). There were no significant differences in SMR among species (see Results).

2.3.2 Maximum metabolic rate and aerobic metabolic scope

In the fish assessed for $\dot{M}O_{2max}$ and MS there were significant differences in SMR among species (Kruskall-Wallis ANOVA, df=3, H=11.59, P=0.009). While *B. medius* appeared to have the lowest SMR of the four species, *post-hoc* comparisons showed the only difference in SMR was between *B. medius* and *F. varium* (Fig. 3A). There were also significant differences in both $\dot{M}O_{2max}$ (ANOVA, df=3, F=20.69, P<0.001) and MS (ANOVA, df=3, F=27.39, P<0.001 between species. *B. medius* had significantly higher $\dot{M}O_{2max}$ and MS than all other species and *F. lapillum* had significantly higher $MO_{2,max}$ and MS than *F. varium* and *F. malcolmi* (Fig. 3B and C).

2.3.3 Gill morphometric parameters

There were significant differences in the number of filaments per gill arch among species (ANOVA, df=3, F=15.31, P<0.001) with *F. lapillum* having fewer filaments than *B. medius, F. varium* and *F. malcolmi* (Table 2). The length of gill filament per unit of body mass was greater in *B. medius* and *F. lapillum* than in *F. varium* and *F. malcomi* (ANOVA, df=3, F=70.38, P<0.001, Table 2) and secondary lamellae density was also greater in *B. medius* and *F. lapillum* than in *F. varium* and *F. malcomi* (ANOVA, df=3, F=123.84, P<0.001, Table 1). The basal length (Kruskall-Wallis ANOVA, df=3, H=24°.01, P<0.001), height (ANOVA, df=3, F=32.98, P<0.001) and thickness (ANOVA, df=3, F=46.81, P<0.001) of secondary lamellae were smaller in *B. medius* and *F. lapillum* than in *F. varium* and *F. malcolmi* (Table 2) and, as a result, the estimated bilateral surface area of the secondary lamellae was also smaller in *B. medius* and *F. lapillum* than in *F. varium* and *F. malcolmi* (Table 2). There was a significant difference in mass-specific gill surface area between species (ANOVA, df=3, F=10.84, P<0.001) with *F. lapillum* having greater mass-specific gill surface than *B. medius, F. varium* and *F. malcolmi* (Table 2). There was some evidence that mass-specific gill surface area decreased with increasing body mass (ANCOVA: covariate, df=1, F=3.99, P=0.057). Therefore, the high mass-specific gill surface area of *F. lapillum* could be due to the comparatively smaller body mass of this species. The maximum rate of O_2 uptake across the gills (gill VO_2) calculated according to Fick's law was significantly higher in both *B. medius* and *F. lapillum* than in *F. varium* and *F. malcolmi* (ANOVA, df=3, F=29.19, P<0.001, Table 2).

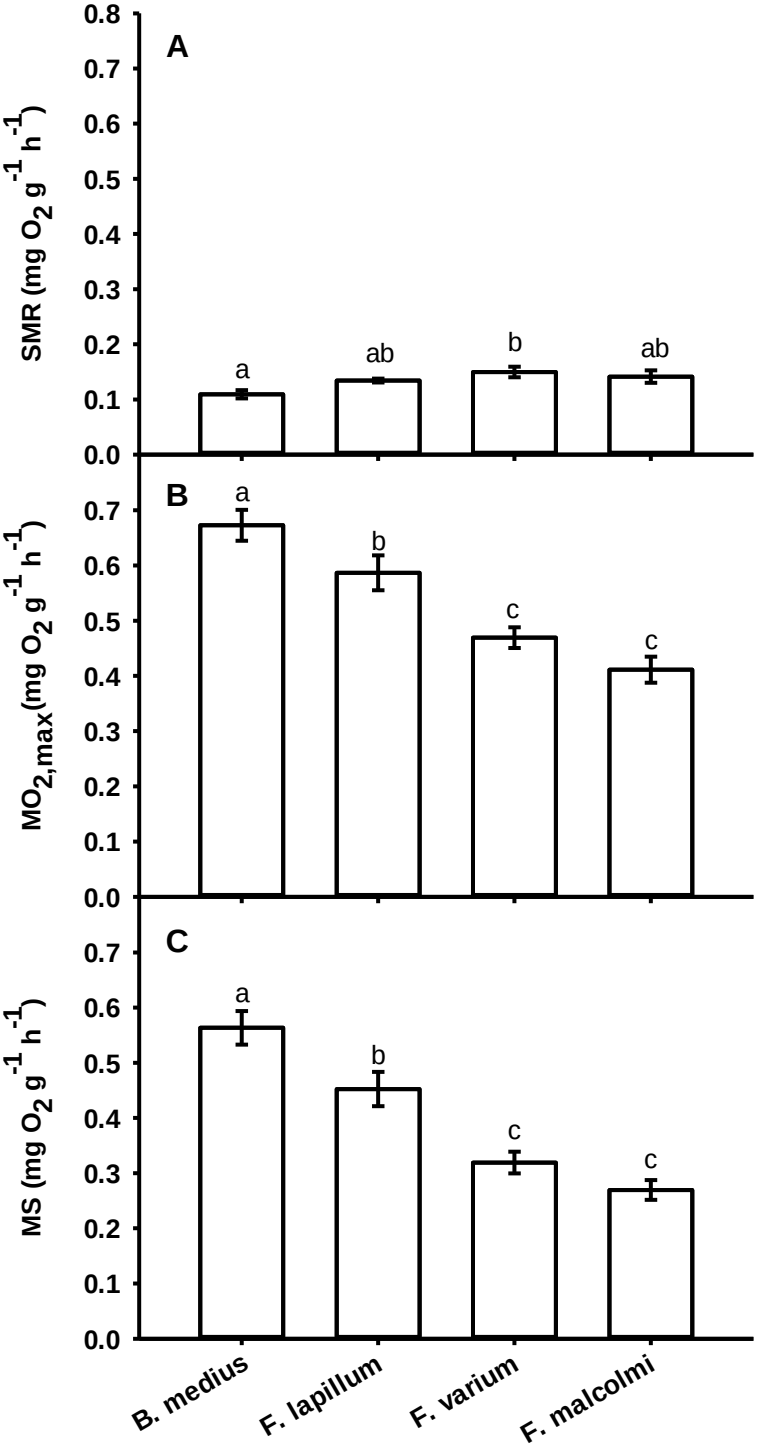


Fig. 2.3 A. Standard metabolic rate (SMR), B. Maximum metabolic rate ($\dot{M}O_{2,max}$), and C. Aerobic metabolic scope (MS[C]) in *Bellapiscis medius, Forsterygion lapillum, Forsterygion varium* and *Forsterygion malcolmi* at 18°C. All values are means ± S.E.M. and corrected to a body mass of 4 g (N=10-11). Lower case letters not shared between bars show significant differences between species ($P<0.05$).

2.3.4 Tissue glycogen stores and haematological parameters

There were differences in brain (ANOVA, df=3, F=7.38, P<0.001) and white muscle (ANOVA, df=3, F=17.04, P<0.001) glycogen stores among species. *B. medius* had a greater amount of glycogen in brain and white muscle than *F. lapillum, F. varium* and *F. malcolmi* and *F. lapillum* had greater white muscle glycogen than *F. malcolmi* (Table 2). There were no differences in the amount of glycogen per gram of liver tissue among species (ANOVA, df=3, F=0.35, P=0.79) but liver size (hepatosomatic index, HSI) was also different among species (ANOVA, df=3, F=6.85, P<0.001, Table 2). Thus, when liver glycogen was expressed per gram of body mass there were differences among species (ANOVA, df=3, F=3.62, P=0.022). While *B. medius* had higher liver glycogen per gram of body mass than all other species only the *post-hoc* comparison with *F. lapillum* was significant (Table 2). Haemoglobin concentration was different among species (ANOVA, df=3, F=6.34, P=0.002) with *B. medius* and *F. lapillum* having a higher haemoglobin concentrations than *F. malcolmi* (Table 2). There was no difference in haematocrit among species (ANOVA, df=3, F=0.55, P=0.65).

2.4 Discussion

2.4.1 The pattern of P_{crit} between intertidal and subtidal triplefin species

B. medius, which appears to be exclusively intertidal and occupies rock pools high on the shoreline (Hilton et al., 2008), has a P_{crit} which is 53%, 77% and 128% lower than *F. lapillum, F. varium,* and *F. malcolmi,* respectively. Lower P_{crit} in intertidal fish species has also been demonstrated among sculpins (Mandic et al., 2009) and the observed pattern mirrors the abrupt change in the prevalence of environmental hypoxia that exists between intertidal and subtidal habitats. *Forsterygion lapillum* was also observed in moderately hypoxic rock pools (Table 1), so the intermediate P_{crit} of this species is consistent with the balance of residing in both intertidal and relatively shallow subtidal habitats. Comparing the two exclusively subtidal species, *F. varium* had a lower P_{crit} than *F. malcolmi,* but this is unlikely to reflect a difference in exposure to hypoxia as these species overlap across depth (Table 2) and utilise almost identical habitat (Wellenreuther et al., 2007). An organism with a low P_{crit} can meet O_2 demand under more severe levels of hypoxia than can an organism with

Table 2.2 Habitat depth, gill morphometry, tissue glycogen and haematological parameters in *Bellapiscis medius*, *Forsterygion lapillum*, *Forsterygion varium* and *Forsterygion malcolmi* (N=8 for gill morphometry, N=10 for tissue glycogen and N=8-10 for haematology).

Trait	*B. medius*	*F. lapillum*	*F. varium*	*F. malcolmi*
Habitat depth range (m)	0-2	0-10	0-30	5-35
Mean depth of occurrence (m)	na	3.5	8	11
Gill morphometry				
Body mass (g)	4.33 (0.29)	2.17 (0.12)	11.28 (1.23)	9.09 (0.94)
Filament number (no. arch)	50.6 (0.89)^{a}	44.5 (0.87)^{b}	54.2 (1.52)^{a}	52.3 (0.85)^{a}
Filament length (mm g BM^{-1})	129.2 (7.7)^{a}	171.7 (7)^{a}	65.1 (5)^{b}	72.2 (3.4)^{b}
SL density (no. mm filament)	26.3 (0.33)^{a}	25.6 (0.48)^{a}	17 (0.4)^{b}	16.7 (0.63)^{b}
SL SA (mm^2)	0.05	0.05	0.14	0.14
SL thickness (µm)	6.9 (0.26)^{a}	7.9 (0.19)^{b}	10.4 (0.21)^{c}	11.2 (0.5)^{c}
SL height (µm)	104.1 (2.5)^{a}	98.9 (2.7)^{a}	146.5 (4.8)^{b}	148.7 (8.4)^{b}
SL basal length (µm)	233.5 (5.4)^{a}	245.5 (4.9)^{a}	401.6 (11.4)^{b}	418.8 (18.4)^{b}
Mass-specific SA (mm^2 g BM^{-1})	364.9 (18.3)^{a}	475.3 (16.8)^{b}	315.9 (30.2)^{a}	328.3 (13.5)^{a}
Gill VO_2 (µM O_2 g^{-1} h^{-1})	114.2 (7.5)^{a}	128.8 (6.9)^{a}	65.5 (6.4)^{b}	63.3 (2.8)^{b}
Tissue glycogen				
Brain glycogen (µM g ww^{-1})	4.47 (0.46)^{a}	3.16 (0.53)^{b}	2.54 (0.14)^{b}	2.39 (0.17)^{b}
WM glycogen (µM g ww^{-1})	52.8 (5.58)^{a}	22.3 (3.35)^{b}	16.8 (2.96)^{bc}	10.2 (2.32)^{c}
HSI	5.77 (0.34)^{a}	3.43 (0.34)^{c}	4.95 (0.45)^{ab}	3.96 (0.45)^{bc}
Liver glycogen (µM g ww^{-1})	375.8 (28)	346.1 (28.6)	360.7 (21.6)	335.6 (37.9)
Liver glycogen (µM g BM^{-1})	22.08 (2.46)^{a}	12.13 (1.79)^{b}	18.07 (2.1)^{ab}	14.4 (2.71)^{ab}
Haematology				
Hb (g L^{-1})	95.5 (2.52)^{a}	91.9 (3.17)^{a}	83.3 (3.68)^{ab}	75.6 (4.62)^{b}
Hct (%)	22.9 (0.84)	21.5 (1.38)	21.4 (1.28)	23.4 (1.92)

Values are means with S.E.M. in parenthesis.
Significant differences ($P<0.05$) between species are shown by lower case letters not shared.
Species depth ranges taken from Hilton (2010) and references therein. Mean depth of occurrence taken from Wellenreuther et al. (2007). Note *B. medius* was not included in the subtidal surveys carried out by Wellenreuther et al. (2007) and this species appears to be exclusively intertidal (Hilton et al., 2008).
no. arch= number of filaments per gill arch, BM=body mass, SL= secondary lamellae, SA= surface area, WM= white muscle, HSI= hepatosomatic index, Hb= haemoglobin, Hct= haematocrit.

a high P_{crit} and the advantages are twofold. Firstly, it is possible a low P_{crit} delays the initiation of anaerobic ATP production to maintain energy balance during a hypoxic event (Mandic et al., 2009; Richards, 2011). Secondarily, among intertidal and subtidal sculpins, a correlation has been demonstrated between P_{crit} and the ability to maintain an upright position (i.e. equilibrium, a proxy for survival) under severe hypoxia (Mandic et al., 2013). This suggests a lower P_{crit} plays a role in allowing intertidal fishes to survive severe hypoxia for

longer periods of time than their subtidal counterparts. Although loss of equilibrium was not directly assessed in the current study it was frequently observed in *F. varium* and *F. malcolmi* at the lowest O_2 levels during P_{crit} determination. Furthermore, in a parallel investigation, we have observed a strong correlation between P_{crit} and time to loss of equilibrium under severe hypoxia among the four species included in the current study (Devaux et al. unpublished data). Thus, for intertidal fishes that reside in hypoxia prone rock pools, a lower P_{crit} appears to make an important contribution to an enhanced ability to survive periods of severe hypoxia exposure.

Previous investigations by Hilton (2010) and Hilton et al. (2010) also found *B. medius* has a lower P_{crit} than subtidal triplefin species. However, P_{crit} in the current study at 18°C was 58-157% lower for all species compared to the data of Hilton and colleagues at 15°C. This likely results from the longer recovery time used in our study (16 h versus 2.5 h) and the use of intermittent flow respirometry for the determination of P_{crit} (Snyder et al., 2016). Estimates of resting $\dot{M}O_2$ in Hilton (2010) and Hilton et al. (2010) were also at least 50% higher compared to SMR in the current study and in the case of *F. varium* were 232% higher. Therefore, when hypoxia exposure was initiated only 2.5 h following entry to respirometers and closed respirometry was used, it is likely the fish under observation by Hilton and colleagues were still recovering from initial handling stress and that this resulted in relatively high estimates of P_{crit} because the fish were not at SMR. However, despite these discrepancies and differences in technique, all studies on triplefins to date show that intertidal species are have a lower P_{crit} than their related subtidal counterparts.

2.4.2 Physiological characteristics associated with P_{crit} in triplefin fishes

Organisms with a high O_2 extractive capacity will be able to meet the O_2 demand of a given aerobic metabolic rate at lower O_2 tensions (Richards, 2011). Therefore, among organisms which have a similar SMR, those with a high extractive capacity for O_2 should be able meet the O_2 demands of resting maintenance metabolism at lower O_2 tensions, and hence have lower P_{crit} (Cook et al., 2011). In the current study, $\dot{M}O_{2,max}$ following exhaustive exercise was assessed to provide an index of the maximum capacity of the cardiorespiratory system of each species to remove O_2 from the environment (i.e. the maximal extractive capacity for O_2). The two intertidal species *B. medius* and *F. lapillum* had higher $\dot{M}O_{2,max}$ than the exclusively subtidal species demonstrating the cardiorespiratory system of the intertidal species has a higher capacity to take up and deliver O_2 to tissues. As there were only

small differences in SMR among species, the high extractive capacity for O_2 of the intertidal species allows minimal resting O_2 demand to be met at a lower O_2 tension and, therefore, their P_{crit} is also lower (e.g. see Cook et al. 2011). A high extractive capacity for O_2 would also lower the limiting PO_2 for other O_2 demanding activities over and above SMR (e.g. digestion, feeding, activity etc). This could benefit intertidal fishes as it may allow them to avoid limitation of aerobic performance for longer periods of time during hypoxia exposure (i.e. to lower O_2 tensions). While the P_{crit} of an animal is likely a function of maximal O_2 extraction capacity, it could also depend on maintenance O_2 demand (assessed as SMR in the current study). The intertidal specialist *B. medius* had a lower P_{crit} and higher $\dot{M}O_{2,max}$ than the other species, but there was little evidence of it also having a lower SMR (Fig. 2 and Fig. 3). It was also clear that *F. lapillum, F. varium* and *F. malcolmi* had an almost identical SMR, but different P_{crit}. As a result, there was no trend between P_{crit} and SMR in the four species examined. Among sculpins, variation in routine O_2 demand (an estimate of resting metabolic rate) also explained a comparatively smaller part of the variation in P_{crit} among species than did variation in characteristics associated with extractive capacity for O_2 (HbO_2 binding affinity and mass-specific gill surface area) (Mandic et al., 2009; Richards, 2011). Thus, the findings of the current study suggest that, like sculpins, the extractive capacity for O_2 plays a more important role than resting demand for O_2 in setting P_{crit} among triplefin fishes.

The observed trend of higher $\dot{M}O_{2,max}$ and lower P_{crit} in the intertidal species may be associated with a range of factors influencing the cardiorespiratory cascade (e.g. blood O_2 carrying capacity, HbO_2 binding affinity, gill morphometric parameters, cardiac output etc.). In the current study, the intertidal species had a higher Hb concentration than subtidal species; this could be involved as a driver of increased $\dot{M}O_{2,max}$ and lower P_{crit} because it would raise blood-O_2 carrying capacity. Indeed, Cook et al. (2011) used phenylhydrazine to pharmacologically induce anaemia (i.e. lower blood O_2 carrying capacity) of a sea bream, which ultimately reduced $\dot{M}O_{2,max}$ and increased P_{crit} compared to sham controls. Secondarily, the O_2 extractive capacity of the gills could also feasibly link high $\dot{M}O_{2,max}$ and low P_{crit} together. For example, in a previous study by Mandic et al. (2009), a high mass-specific gill surface area was associated with a lower P_{crit} among 12 species of sculpins. The results of the current study provide some evidence that mass-specific gill surface area also plays a role in setting P_{crit} among triplefin fish. Mass-specific gill surface area was significantly higher in the occasionally intertidal and shallow subtidal species *F. lapillum* than in the two deeper dwelling exclusively subtidal species *F. varium* and *F. malcolmi*. The relationship between

mass-specific gill surface area and P_{crit} among species, however, was not straight-forward as, despite having the lowest P_{crit}, the intertidal specialist *B. medius* had a lower gill surface area than *F. lapillum* and a not significantly greater gill surface area than the two higher P_{crit} exclusively subtidal species *F. varium* and *F. malcolmi*. However, the secondary lamellae of both *B. medius* and *F. lapillum* were thinner than those of the two exclusively subtidal species, suggesting a shorter diffusion distance for O_2 to reach the red blood cells during gill ventilation. Assuming that half the thickness of the secondary lamellae is representative of the blood O_2 diffusion distance across the secondary lamellae, we estimate that gill VO_2 was approximately double in the intertidal specialist *B. medius* and occasional intertidal occupant *F. lapillum* in comparison to the two exclusively subtidal *Forsterygion* species. Thus, the larger gill surface area and thinner secondary lamellae of the intertidal specialist *B. medius* and occasional intertidal *F. lapillum* provides a high capacity for gill O_2 flux, which likely contributes to both increased $\dot{M}O_{2,max}$ and lower P_{crit} in these species. The relationship between gill VO_2 and P_{crit} among species, however, like that for gill surface area and P_{crit}, was also not straight-forward. There was no difference in gill VO_2 between *B. medius* and *F. lapillum* despite these species having different P_{crit} and there was also no difference in gill VO_2 between *F. varium* and *F. malcolmi* despite differences in P_{crit}. These discrepancies in the relationship between gill morphometric parameters and P_{crit} are likely due to the fact that P_{crit} is a complex whole animal trait influenced by a number of other physiological parameters (e.g. ventilatory capacity, cardiac output, blood oxygen binding affinity) not investigated in the current study.

Predominately as a result of higher $\dot{M}O_{2,max}$ the intertidal specialist *B. medius* and the occasionally intertidal and shallow subtidal dwelling *F. lapillum* had higher MS (the difference between $\dot{M}O_{2,max}$ and SMR) than the deeper dwelling exclusively subtidal species *F. varium* and *F. malcolmi*. The first limitation imposed by hypoxia on aerobic capacity, which occurs at PO_2 well above P_{crit}, is a constraint on $\dot{M}O_{2,max}$ and a reduction in MS (Claireaux and Lagardère, 1999; Claireaux et al., 2000; Lefrançois and Claireaux, 2003; Chabot and Claireaux, 2008; Farrell and Richards, 2009b; Cook et al., 2011; Claireaux and Chabot, 2016). Exposure to increased temperature, as occurs for intertidal fish during the day in rock pools (e.g. Fig. 1), also limits MS because increased basal O_2 demand reduces the difference between SMR and $\dot{M}O_{2,max}$ (Pörtner and Knust, 2007; Schulte, 2015). As aerobic activities (e.g. growth, feeding, activity etc.) must occur within the bounds of MS, it is thought that MS-limiting environmental conditions can cause energy budgeting conflicts

which lead to a loss of whole organism performance (e.g. reduced growth in Atlantic cod under hypoxia) (Chabot and Claireaux, 2008). Potentially, a high MS provides a reserve of aerobic capacity which intertidal fishes can utilise to avoid severe constraints on performance under exposure to hypoxia or high temperature in rock pools. Further studies examining the effects of hypoxia on other aerobic activities (e.g. specific dynamic action, which is probably forced to operate within the bounds of MS) would therefore provide useful insight into whether rock pool fishes utilise high MS to their advantage.

The pattern of MS observed among species in the current study could also result from different regimes of wave exposure between intertidal, shallow subtidal and deeper subtidal habitats. Hickey and Clements (2003) determined that intertidal triplefins are subjected to substantially higher water velocities than those species occupying subtidal habitats, and that water velocities resulting from wave action decline progressively with increasing habitat depth. Thus, the higher MS of the specialist intertidal species *B. medius* and the occasionally intertidal and shallow subtidal dwelling *F. lapillum* could reflect an increased requirement for activity (e.g. station holding) in the high water velocities of intertidal and shallow habitats. The comparatively lower MS of *F. varium* and *F. malcolmi* may reflect a lower demand for activity as the water velocities resulting from wave action will not be as high in the deeper subtidal habitats occupied by these species.

Although a low P_{crit} is beneficial for fish exposed to hypoxia, it does not preclude exposure to O_2 tensions capable of threatening survival. Indeed, intertidal fish are more at risk of exposure to O_2 tensions less than P_{crit} than their subtidal counterparts because environmental hypoxia does not regularly occur in the habitat of the latter. At O_2 tensions below P_{crit}, organisms rely at least partly on O_2 independent ATP production to maintain energy balance. This is evidenced by the accumulation of lactate in the tissues and plasma of fish exposed to O_2 tensions below P_{crit} (Herbert and Steffensen, 2005; Scott et al., 2008; Speers-Roesch et al., 2013). Thus, fish species that reside in hypoxia prone habitats should have large stores of endogenous fermentable fuels such as glycogen (Richards, 2011). The intertidal specialist *B. medius* maintained a higher resting concentration of glycogen in white skeletal muscle and brain tissue than the occasional intertidal occupant *F. lapillum*, and the two exclusively subtidal *Forstyregion* species. Although there was no difference in liver glycogen concentration between species, *B. medius* did have a larger liver relative to body mass. Thus, there was some evidence of a trend for increased liver glycogen stores relative to

body mass in *B. medius*, which fits with the theory outlined above that intertidal fishes may store higher levels of endogenous fermentable fuels to power anaerobic metabolism under hypoxia exposure. Higher tissue glycogen stores in intertidal fishes could also be a response to a more variable food supply, as a limitation in food availability may be a common occurrence in rock pool habitats (Silberschneidera and Booth 2001).

A limitation of the current study is that the intertidal specialist *B. medius* is placed in a different genus than the subtidal species examined. Thus, we cannot rule out the possibility that the observed differences in physiological traits between *B. medius* and the subtidal species are a result of phylogeny rather than adaptation to rock pool conditions. It is noteworthy, however, that *F. lapillum*, a species which also occurs in the intertidal had a lower P_{crit}, and higher $\dot{M}O_{2,max}$, MS, blood Hb concentration, and gill VO_2 than exclusively subtidal species *F. varium* and *F. malcolmi*. Thus, the physiological characteristics of *B. medius* that appear suited to a rock pool existence also differentiated *F. lapillum* from two closely related subtidal species within its own genus. Future studies that include a greater number of species and take phylogeny into account would be beneficial in further elucidating the adaptive value of the physiological traits examined in the current study.

2.4.3 Conclusions

The present investigation found that the specialist intertidal triplefin species *B. medius* possesses a lower P_{crit} than the occasional rock pool inhabitant *F. lapillum,* and the two exclusively subtidal species *F. varium* and *F. malcolmi*. *Forsterygion lapillum* also had a lower P_{crit} than two closely related exclusively subtidal species (*F. varium* and *F. malcolmi*). *Bellapiscis medius* also had a higher $\dot{M}O_{2,max}$ than all three *Forsterygion* species and *F. lapillum* had a higher $\dot{M}O_{2,max}$ than *F. varium* and *F. malcolmi.* These findings demonstrates that an enhanced capacity of the cardiorespiratory system to extract O_2 from water is likely to be an important determinant of P_{crit} in triplefin species which inhabit intertidal rock pools. There was, however, virtually no association between SMR and P_{crit} among the species examined. This finding suggests that among triplefin fishes, a high extractive capacity for O_2 is a comparatively more important determinant of P_{crit} than a low resting demand for O_2. Overall, triplefin species which can extract more O_2 from their environment have a lower P_{crit} and therefore, have the ability to meet aerobic metabolic demands at lower O_2 tensions. A lower P_{crit} is advantageous for an exclusively intertidal species such as *B. medius*, because environmental hypoxia is a routine occurrence in its rock pool habitat. High $\dot{M}O_{2,max}$ also

endows intertidal species with a high MS, and therefore a greater capacity to perform aerobic activities over and above maintenance requirements. Whether high MS in intertidal fish can be utilised to mitigate constraints on aerobic performance under hypoxia and high temperature, would be an interesting avenue for future research.

CHAPTER 3: THE IMPACT OF ACUTE HEAT STRESS ON HYPOXIA TOLERANCE IN THE INTERTIDAL TRIPLEFIN FISH *BELLAPISCIS MEDIUS*

3.1 Introduction

Marine organisms commonly have to contend with fluctuating environmental conditions (e.g. temperature, salinity, and oxygen) severe enough to illicit a physiological stress response. Moreover, environmental parameters rarely occur in isolation so organisms must often endure multiple environmental conditions that fluctuate either simultaneously or sequentially (Gunderson et al., 2016a). The response of organisms to multiple stressors can be complex and is largely dependent on the temporal pattern of exposure between each stressor. For example, when organisms experience multiple stressors simultaneously, or in rapid succession, the combined negative effect is generally worse (synergistic) than the additive effects of the individual stressors acting in isolation (Crain et al., 2008; McBryan et al., 2013; Gunderson et al., 2016a). However, if there is a sufficient period of recovery between exposures, the combined negative effect of the stressors can be equal to the sum of the individual stressor effects (additive), or even reduced (antagonistic) (McBryan et al., 2013; Gunderson et al., 2016a). In the case of an antagonistic response, exposure to one stressor can sometimes increase the tolerance of an organism to a second stressor, a phenomenon known as cross-tolerance (Todgham and Stillman, 2013).

Due to the interaction between semi-diurnal tidal patterns and diel changes in air temperature and solar irradiance, certain physico-chemical parameters in intertidal rock pools tend to fluctuate out of phase and sequentially. Thus, for rock pool organisms such as intertidal fish, cross-tolerance would be a very useful trait as it would offer protection against different environmental stressors across consecutive tidal cycles. For example, temperature and oxygen availability in rock pools fluctuate widely, but when low tides roughly align with the middle of the day and night, peak daytime high temperatures occur out of phase with nocturnal hypoxia (Truchot and Duhamel-Jouve, 1980; Morris and Taylor, 1983; Huggett and Griffiths, 1986; Gunderson et al., 2016a; Legrand et al., 2018; McArley et al., 2018). This is because the conditions associated with high temperature in isolated rock pools in the middle of the day (i.e. strong solar radiation) also promotes algal photosynthesis which buffers against O_2 depletion. On the other hand, severe hypoxia can develop in isolated pools at night

(i.e. only 8-11 h later) when respiring organisms draw down O_2 at a time of comparatively stable temperatures (see Fig.1 McArley et al., 2018).

Rock pool habitats are prone to a bout of extreme high temperature followed by severe hypoxia, but only one study has addressed the tolerance of intertidal fish to this set of conditions (Todgham et al., 2005). Todgham et al. (2005) showed that an intertidal species of sculpin (*Oligocottus maculosus*) had elevated tolerance (cross-tolerance) to both high salinity and hypoxia after recovering from an acute heat shock. With respect to measuring hypoxia cross-tolerance, these authors showed that fish exposed to an initial bout of acute heat shock had greater survival under subsequent exposure to severe hypoxia. It also appeared that cross-tolerance to high salinity could be maintained for quite a long period of time after heat shock (i.e. 8-48h), and that the magnitude of heat shock was also important because high salinity cross-tolerance only developed with a +12°C heat shock and not a +15°C heat shock. Todgham et al. (2005) also assessed the role of heat shock proteins (Hsps) in cross-tolerance, but found no clear association between elevated levels of Hsps and increased tolerance to a secondary stressor. As 8h was the minimum recovery time for cross-tolerance to develop, and this period matched the approximate time between successive periods of low tide emersion in the intertidal zone, it was suggested that a protective effect of cross-tolerance might be relevant for fish under real life conditions (Todgham et al., 2005).

In order to build upon the findings of Todgham et al. (2005), the present study examined cross-tolerance in another specialist intertidal fish (*Bellapiscis medius*, Günther, 1861) and, therefore, assessed whether heat shock induced cross-tolerance is a common response in rock pool fishes. To assess the presence of cross-tolerance in *B. medius*, the hypoxia tolerance of this species was assessed after allowing *B. medius* a ~19 h period of recovery from either a +8°C (21-29°C) or +10°C (21-31°C) heat shock treatment over a 5h ramping period. The +8°C heat shock (21-29°C) was used because peak temperatures of this magnitude have been observed in rock pools inhabited by *B. medius* on hot summer days (McArley et al., 2018). The more extreme +10°C (21-31°C) heat shock was used to gauge how fish would cope with a future climate change scenario, where peak daytime temperatures may become higher in rock pools. Two measures of hypoxia tolerance were also employed. Firstly, the time to loss of equilibrium (LOE) under severe hypoxia (O_2= 7% air saturation) was measured. As time to LOE is a standard proxy measure of hypoxia survival in fish (Mandic et al., 2013; Speers-Roesch et al., 2013) this allowed us to make comparisons to the findings of Todgham et al. (2005) who demonstrated cross-tolerance as lower morbidity

under hypoxia. It was therefore hypothesised that time to LOE would increase in *B. medius* after heat shock. Secondly, this study also measured the critical oxygen saturation (S_{crit}) of *B. medius* after heat shock because, although it is a commonly used indicator of hypoxia tolerance in fish (Rogers et al., 2016; Claireaux and Chabot, 2016), S_{crit} was not measured by Todgham et al. (2005). S_{crit} represents the change in O_2 consumption as fish transition from being an O_2 regulator, to an O_2 conformer under progressive hypoxia exposure (Farrell and Richards, 2009a). Among sculpins, species with a lower S_{crit} showed LOE after longer periods under exposure to severe hypoxia (Mandic et al., 2013). Therefore, under the expectation that *B. medius* would show evidence of cross-tolerance (longer time to LOE), it was hypothesised that S_{crit} would decline after heat shock. This would indicate that heat shock treated fish could maintain basal O_2 consumption at lower O_2 levels, and also provide insight as to whether cross-tolerance to hypoxia operates via a mechanism associated with the cardiorespiratorycascade. Despite the widespread use of S_{crit} as a measure of hypoxia tolerance in fish (Rogers et al., 2016), to our knowledge, the effect of prior heat shock on S_{crit} has never been addressed.

3.2 Methods

All experiments were carried out at the Leigh Marine Laboratory and the fish used were collected from rock pools using hand nets. Fish were housed in 30 L flow-through seawater tanks and acclimated to constant temperature (~21°C), and a 12 h light dark photoperiod for at least four weeks prior to experiments. Food was withheld for a period of 48 h prior to the start of experiments. In all experiments water oxygen content and temperature was monitored using Firesting O_2 meters followed by robust O_2 dipping probes and shielded temperature probes (PyroScience, Aachen, Germany). All statistical analysis was performed using the Sigma Plot 13.0 software package and significance was accepted at $P<0.05$. The experiments were carried out under the approval of the University of Auckland Animal Ethics Committee (AEC approval number 001801).

3.2.1 Experiment 1: Cross-tolerance for survival of severe hypoxia following heat shock

Experiment 1 aimed to determine if a prior heat shock exposure influences the ability of the intertidal triplfein *B. medius* to survive severe hypoxia. The time to loss of equilibrium (LOE) under a constant severe hypoxia exposure (~7% of air saturation) was therefore

measured in three experimental groups, that either did or did not receive an initial heat shock: (1) an ambient temperature (21°C) control group not exposed to heat shock (body mass= 2.13 g ± 0.14, N=12), (2) a group exposed to a +8°C (~21-29°C) heat shock challenge (body mass= 1.95 g ± 0.16, N=12) and (3) a group exposed to a +10°C (~21-31°C) heat shock challenge (body mass= 1.92 g ± 0.15, N=12). There was no difference in body mass of fish in each experimental group (ANOVA, DF=2, F=0.56, P=0.58). The heat shock exposure was carried out in a 50 L aquarium in which seawater was heated with aquarium heaters. At ~09:00, fish were transferred from holding tanks to the thermal ramping tank where they were held at 21°C for an hour. The water temperature was then gradually heated from 21°C, to either 29 or 31°C, over a 5h period (see Appendix 1 for the exact rate of temperature change in each experimental run). The control group was held in the heat shock exposure tank at ambient temperature (21°C) for 5h. After heat shock, fish were transferred to a 50 L tank supplied with ambient temperature (~21°C) flow through seawater. The fish were then allowed to recover overnight for ~19h before being subjected to a hypoxic challenge (~7 % of air saturation, see Appendix 1). Hypoxia was achieved by bubbling seawater with N_2 until ~7% of air saturation was reached (7-10 minutes of continuous bubbling). N_2 was then bubbled in short bursts to maintain O_2 at 7%. Fish were immediately removed from hypoxia once LOE occurred and placed in air saturated seawater to recover. Two groups of 6 fish were run separately for each of the three experimental treatments (i.e. a total of 6 experimental runs; see Appendix 1 for the details of each run). There were no differences in the time to LOE between experimental runs of the same treatment group and data were pooled for subsequent analysis (N=12 per treatment). Median loss of equilibrium time (LOE_{50}) between treatments was assessed using Kaplan-Meier log-rank survival analysis and one-way ANOVA with Holm-Sidak *post-hoc* comparisons (Speers-Roesch et al., 2013).

3.2.2 Experiment 2: Cross-tolerance for hypoxia following heat shock (critical oxygen saturation [S_{crit}])

The purpose of experiment 2 was to determine if a prior heat shock exposure induced changes in the critical oxygen saturation (S_{crit}) point of the intertidal triplefin *B. medius*. S_{crit} was assessed in three experimental groups: (1) an ambient temperature (21°C) control group not exposed to heat shock (body mass= 1.78 g ± 0.08, N=10), (2) a group exposed to a +8°C (~21-29°C) heat shock challenge (body mass= 2.24 g ± 0.24, N=10) and, (3) a group exposed

to a +10°C (~21-31°C) heat shock challenge (body mass= 2.06 ± 0.22, N=8). There were no differences in body mass of fish in each experimental group (ANOVA, DF=2, F=1.65, P=0.21) and the heat shock exposures were implemented following the same protocol as experiment 1. After heat shock, fish were transferred to respirometry chambers to assess S_{crit} (see Chapter 4 for detailed respirometry methods). The fish were first allowed to recover in the respirometers overnight at ~21°C. During this recovery phase, mass-specific oxygen consumption ($\dot{M}O_2$) was measured over repeated 8 min measurement cycles interspersed with 1 min flushing and wait periods. The standard metabolic rate (SMR) was estimated as the mean of the lowest 10% of $\dot{M}O_2$ values recorded in the overnight recovery period (Norin et al., 2014; Khan et al., 2014b; McArley et al., 2017). Following overnight recovery, a run of progressive hypoxia was initiated (~75, 55, 40, 30, 25, 20, 15, 10, 6 % of air saturation) to resolve S_{crit} by identifying the oxygen saturation at which fish could no longer maintain $\dot{M}O_2$ above SMR (Claireaux and Chabot, 2016). Progressive hypoxia exposure began between 18-19.5 h following the conclusion of heat shock and was completed in ~4.5 h (see Appendix 2). Temperature was maintained at ~21°C and N_2 was bubbled into the water reservoir supplying respirometers to achieve each level of hypoxia. Three $\dot{M}O_2$ measurements were made at 75-20 % of air saturation and one measurement was made at 15, 10 and 6% of air saturation. To determine S_{crit}, SMR and $\dot{M}O_2$ at each level of hypoxia were plotted against water oxygen air saturation, and a linear regression forced through zero was performed between $\dot{M}O_2$ values which fell below SMR. The point where the regression line intersected with SMR was taken as the S_{crit} for an individual fish (Schurmann and Steffensen, 1997; Behrens and Steffensen, 2007; Cook et al., 2013; Cumming and Herbert, 2016). S_{crit} in each of the treatment groups was assessed over 3-4 separate experimental runs (see Appendix 2 for the details of each experimental run). SMR and S_{crit} were compared among the control and heat shock treatments using one-way analysis of variance (ANOVA) with Holm-Sidak post-hoc comparisons.

As background oxygen consumption was evident within the respirometry chambers by the end of experimental runs, background respiration for each measurement cycle was back calculated using linear regression and subtracted from $\dot{M}O_2$. The mean level of background respiration was equivalent to 4.7, 5.0, and 6.3 % of $\dot{M}O_2$ in the control, +8°C heat shock and +10°C heat shock treatment groups respectively. There was no difference in the amount of background respiration among treatment groups (ANOVA, DF=2, F=2.18, P=0.13).

3.3 Results

3.3.1 Experiment 1: Loss of equilibrium under a hypoxic challenge following acute heat shock

During severe hypoxia exposure (~7% of air saturation) LOE occurred after 19-85 min in the control group, after 13-60 min in the +8°C heat shock group, and after 6-43 min in the +10°C heat shock group (Fig 3.1). The survival curves for time to LOE were significantly different among treatment groups (Log-Rank test, DF=2, Statistic=19.52, P<0.001). *Post-hoc* comparisons showed the LOE_{50} was significantly shorter in the +8°C heat shock group (LOE_{50}= 41.5 min ±4.84) compared to the control group ((LOE_{50}= 54.4 min ± 11.17), and significantly shorter again in the +10°C heat shock group (LOE_{50}= 28.7 min ± 5.86) compared to both the control and +8°C heat shock group (Fig. 3.1).

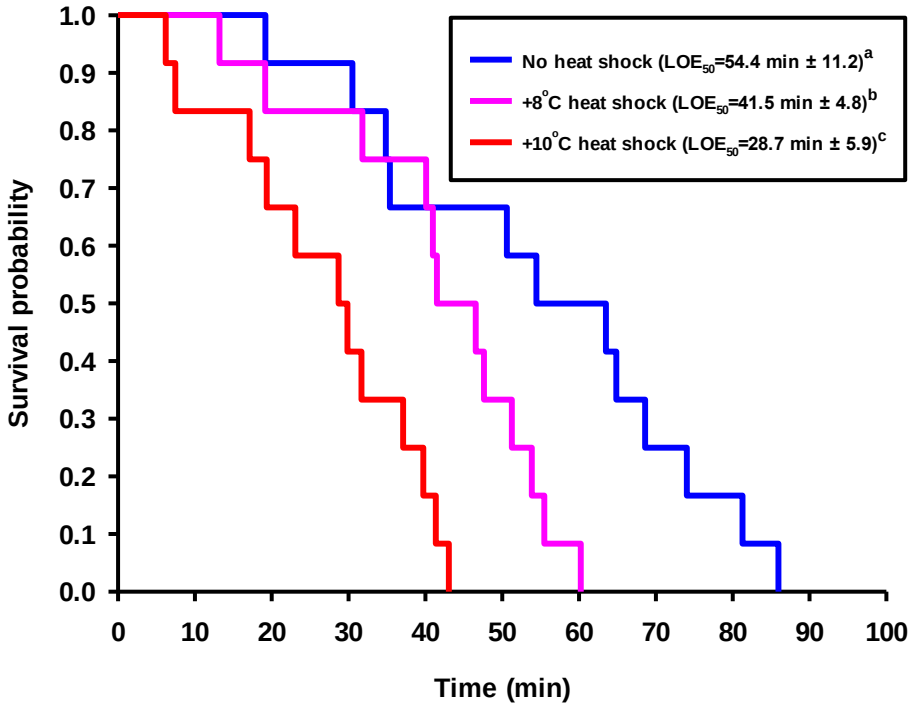


Figure 3.1 Survivorship curves and median time to loss of equilibrium (LOE_{50}) in *Bellapiscis medius* exposed to severe hypoxia (7% air saturation) after 19 h recovery from acute heat shock. Blue line = control group (no heat shock), pink line = +8°C heat shock (21-29°C), red line = +10°C heat shock (21-31°C). Values represent median time to loss of equilibrium ± standard error (N=12). Different lower case letters indicate significant differences (P<0.05) in LOE_{50} among treatment groups.

3.3.2 Experiment 2: Critical oxygen saturation (S_{crit}) following acute heat shock

The $\dot{M}O_2$ of fish was elevated in all treatment groups following entry to respirometers but had fallen to within 1 standard deviation of SMR after 8-11 h recovery. After overnight recovery in the respirometers there was no difference in SMR between the control fish and the fish exposed to an +8°C or +10°C heat shock (ANOVA, DF=2, F=0.184, P=0.83, Fig. 3.2). $\dot{M}O_2$ remained stable in all treatment groups during progressive hypoxia before declining abruptly below SMR between 15 and 20% of air saturation (Fig. 3.2). The S_{crit} of the control fish was 15.8 % of air saturation (± 0.9) but was slightly higher in fish exposed to an 8°C (S_{crit}= 17 % of air saturation ±0.85) or 10°C (S_{crit}= 18.3 % of air saturation ± 1.03) heat shock (Fig. 3.2). There was, however, no significant difference in S_{crit} between any of the treatments (ANOVA, DF=2, F=1.72, P=0.19).

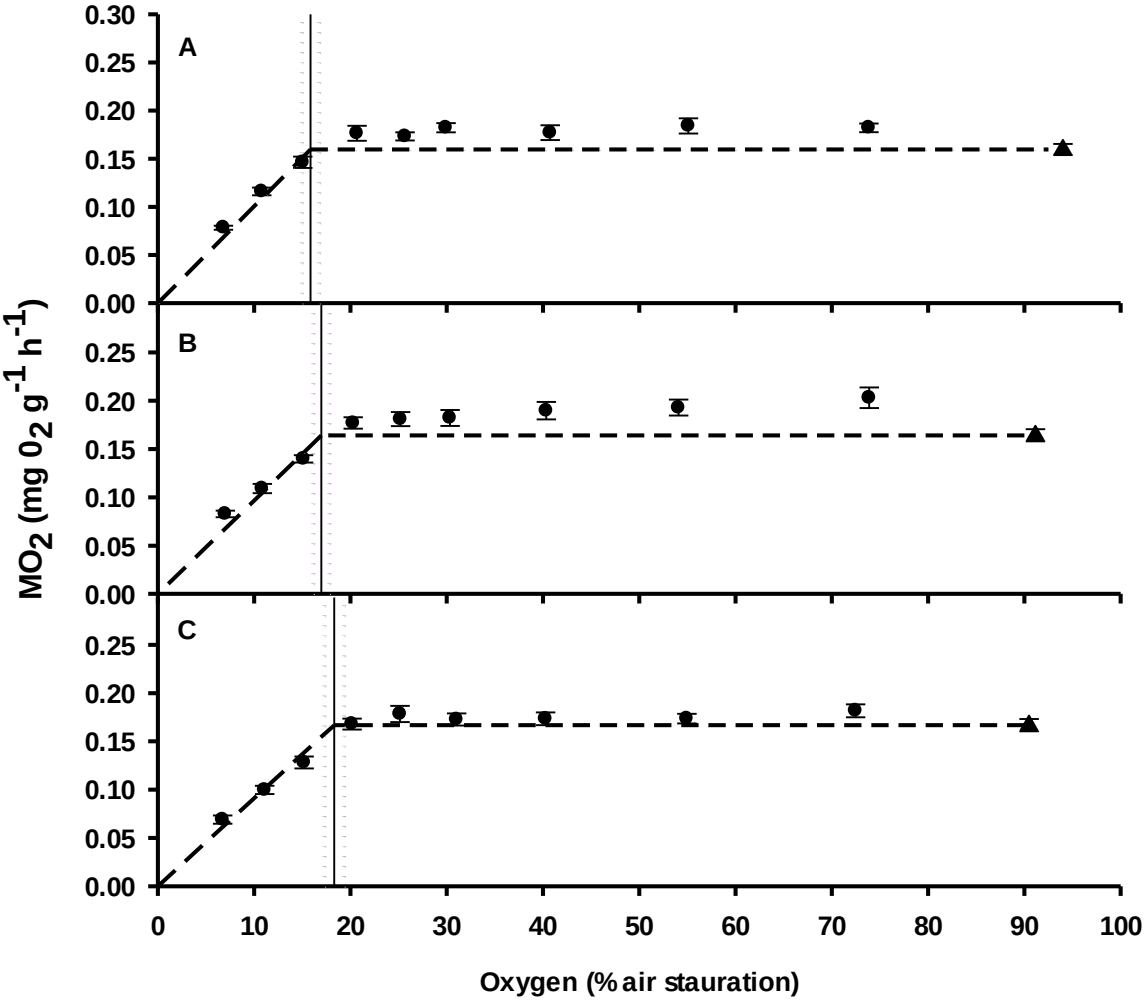


Figure 3.2 Critical oxygen saturation (S_{crit}) and standard metabolic rate (SMR) of *Bellapiscis medius* following recovery from acute heat shock. All values represent mean ± s.e.m. A= control (N=10), B= +8°C heat shock (N=10), C= +10°C heat shock (N=8). Triangles show SMR under normoxia, circles show routine $\dot{M}O_2$ under progressive hypoxia exposure, vertical line shows S_{crit} and vertical dotted line shows S_{crit} standard error. The dashed horizontal line shows the break point between oxygen regulation and oxygen conformation and is for illustrative purposes only.

3.4 Discussion

Knowledge relating to the impacts of multiple stressors is crucial to understanding the tolerance of marine organisms to real life environmental conditions (Crain et al., 2008; McBryan et al., 2013; Gunderson et al., 2016a). The majority of studies have applied stressors simultaneously to assess organismal tolerance to multiple stressors, but this does not always represent ecologically relevant conditions because in many habitats different stressor types occur asynchronously (Gunderson et al., 2016a). Todgham et al. (2005) sequentially

exposed rock pool sculpins to high temperature, then hypoxia, and provided evidence of cross-tolerance, as fish showed improved hypoxia tolerance 8h after recovering from the initial heat shock. Sequential stressor exposures are highly relevant for intertidal fish in rock pools, because critically high temperatures can occur with intense solar radiation during daytime low tides, but during the next low tide cycle (approximately 8-11h later at night) severe hypoxia can develop under cooler night-time conditions (Truchot and Duhamel-Jouve, 1980; Morris and Taylor, 1983; McArley et al., 2018). However, when examining *B. medius*, a rock pool specialist within the family of New Zealand triplefin fishes, we found no evidence of heat shock induced cross-tolerance to hypoxia, a finding that suggests cross-tolerance is not a universal adaptation for intertidal fishes.

Based on evidence of cross-tolerance from the study of Todgham et al. (2005), our specific hypotheses were that the LOE_{50} of *B. medius* under severe hypoxia would increase, and that the S_{crit} would decrease after recovery from acute heat shock (i.e. *B. medius* would be preconditioned). However, neither hypothesis was supported because the LOE_{50} of *B. medius* decreased and S_{crit} showed no significant change. These findings therefore reveal no evidence for cross-tolerance in *B. medius* after acute exposure to peak temperatures observed in rock pools. In fact, the direction of change in LOE_{50} suggested that the hypoxia tolerance of *B. medius* was impaired by an initial heat shock. We cannot, however, discount the possibility that cross-tolerance is induced following exposure to less extreme heat shock (i.e. less than +8°C). Indeed, Todgham et al. (2005) found the tolerance of sculpins to high salinity improved after a +12°C heat shock but not when a +15°C shock was applied. Thus, there may be a heat shock intensity threshold which, if surpassed, prevents the development of cross-tolerance. In the current study the magnitude of heat shock exposures was based on field observations of temperature extremes in rock pools occupied by *B. medius* (McArley et al., 2018). Moreover, fish were subjected to a thermal ramping protocol (i.e. increased temperature over 5h) in order to replicate environmentally realistic heat shock in rock pools, where temperature gradually increases to a peak throughout the duration of low tide emersion. Thus, the concern raised by the current study is that *B. medius* showed a reduced tolerance to hypoxia after heat shock representative of current day scenarios (+8°C heat shock treatment) and hypoxia tolerance was further impaired in near-future warming scenario (+10°C heat shock treatment). This is a cause-for-concern as climate change is predicted to increase the frequency and intensity of heat wave events (Perkins et al., 2012); this may result

in more extreme rock pool temperatures in the near future, rendering *B. medius* less capable of coping with multiple stressors across successive low tides.

The S_{crit} of fish subjected to acute heat shock remained unchanged, clearly demonstrating there is no adjustment in the capacity of *B. medius* to meet basal O_2 demands under low O_2 following overnight recovery from heat shock. S_{crit} is determined by an interaction between the extractive capacity of a fish's cardiorespiratory system for O_2 and basal metabolic demand for O_2 (i.e. SMR) (Mandic et al., 2009; Richards, 2011). Since S_{crit} and SMR were similar across treatments, prior exposure to heat shock neither enhanced, nor hindered the extractive capacity of the cardiorespiratory system for O_2. Yet, despite no differences in S_{crit}, the LOE_{50} of *B. medius* under exposure to severe hypoxia (~7% air saturation) was still reduced after heat shock. It is important to note, however, that severe hypoxia in the current study exposed fish to an O_2 level approximately 50% less than S_{crit}. The ability of an organism to survive hypoxia below S_{crit} partly relies on utilising anaerobic ATP production to maintain energy balance (Richards, 2011). Thus, a reduced ability to survive levels of hypoxia below S_{crit} might reflect either faster energy expenditure and/or lower availability of endogenous fermentable fuel stores such as glycogen. Since there was no difference in SMR between treatment groups a faster rate of energy expenditure in heat shock exposed fish seems unlikely. As blood lactate is raised in fish exposed to acute high temperatures (e.g. Clark, Timothy D. et al., 2008), it therefore seems more reasonable to suggest that heat shock treated fish exposed to hypoxia lost equilibrium faster because tissue glycogen stores were used to maintain energy balance at high temperatures, leaving a diminished pool available when they faced hypoxia ~19 h later.

The studies of Hilton et al. (2008; 2010b) demonstrate a 1.7-2.1 fold increase in the S_{crit} of *B. medius* with an acute increase in temperature from 15°C to 25°C. However, in both these previous studies, hypoxia tolerance was assessed when the 25°C (i.e. +10°C) heat shock was applied, so the large increases in S_{crit} were likely due to elevated metabolic costs at high temperatures. As shown by McArley et al. (2018), an acute +10°C increase in rock pool temperature is only likely to occur during daytime low tides associated with direct solar radiation and high air temperatures, conditions which also promote algal mediated hyperoxia due to photosynthesis. Thus, while the results of Hilton and colleagues demonstrate that *B. medius* have a substantial impairment of S_{crit} when hypoxia and acute heat shock co-occur, this is probably a circumstance rarely, if ever, faced under natural conditions.

3.4.1 Study limitations

Whilst cross-tolerance was not seen in the current study it is important to discuss two limitations in our experimental design that may have impacted our findings. *B. medius* in this study were allowed ~19h to recover from heat shock before facing hypoxia but, in reality, fish undergoing a natural tidal cycle in the wild would experience a shorter time in which to recover from heat shock. For example, in one rock pool inhabited by *B. medius*, nocturnal O_2 levels fell to 20% air saturation approximately 10 h after the peak in daytime temperature (McArley et al., 2018). We therefore cannot discount the possibility that a shorter 10 h recovery period after acute heat shock might reveal cross-tolerance in *B. medius* where it was absent in the current study. In reality it is more likely that a shorter recovery would lead to greater level of cross-sensitivity, so future studies should replicate the natural temporal pattern of multiple stressor exposures with greater precision if a complete, ecologically relevant result is to be formed. A second limitation is that rock pools occupied by *B. medius* also become hyperoxic during acute high temperature events (McArley et al. 2018). This is important because, when facing acute heat stress combined with hyperoxia, the aerobic metabolic scope of *B. medius* is not constrained to the same degree as it is under normoxia (McArley et al., 2018). It is thus possible that an expanded aerobic scope under hyperoxia during heat shock could dampen the requirement for anaerobic metabolism, thus preserving tissue glycogen stores and permitting fish to survive longer during a subsequent hypoxic exposure. We note that Devor et al. (2016) found hyperoxia mitigated the rise in plasma and muscle lactate levels in two Antarctic notothenioid fishes exposed to acute heat shock, but clearly future research is required to resolve this issue fully.

3.4.2 Conclusion

The present study shows that prior exposure to heat shock impairs the subsequent hypoxia tolerance of *B. medius*, thus providing no support for the theory that cross-tolerance is a widespread phenomenon in intertidal fishes. Instead, this species appears to be mildly cross-sensitive to hypoxia after acute heat shock exposure representative of a current day situation. Perhaps more concerning a +10°C heat shock, representing a future climate change scenario, results in a significant sensitisation to hypoxia. Overall, *B. medius* is likely to retain relatively normal levels of hypoxia tolerance under current day conditions, but if heat shock events intensify with climate change as expected, this might substantially reduce the ability of this species to tolerate multiple ecologically relevant stressors.

CHAPTER 4: THE INFLUENCE OF HYPEROXIA ON THE AEROBIC METABOLIC RESPONSE TO ACUTE HIGH TEMPERATURE EXPOSURE IN INTERTIDAL NEW ZEALAND TRIPLEFIN FISHES

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4.1 Introduction

Temperature has pervasive impacts on biological processes and plays an important role in shaping the distribution and abundance of species (Schulte, 2015). All fish face at least some natural fluctuation in environmental temperature, but species inhabiting intertidal rock pools can be exposed to large acute (hours) increases in temperature when daytime low tide coincides with hot terrestrial conditions (Fig. 4.1A). Low tide thermal ramping events in rock pools may ultimately threaten the upper thermal tolerance limits of intertidal fish, but are also associated with sub-lethal impacts including increased energetic demands. These higher energetic demands are reflected in the profound influence of temperature on aerobic metabolism where rates of oxygen consumption typically increase exponentially under acute thermal ramping (e.g. Healy and Schulte, 2012b; McArley et al., 2017). The aerobic metabolic response to thermal ramping has been well characterised in fish but there are a lack of studies investigating the role of aerobic metabolism in thermal tolerance in intertidal fish species, which are routinely exposed to acute high temperatures.

Thermal tolerance has been proposed to be linked to aerobic metabolism in aquatic ectotherms through the influence of temperature on aerobic metabolic scope (MS) (Pörtner and Farrell, 2008; Sokolova et al., 2012; Schulte, 2015; Pörtner et al., 2017). MS is the difference between basal (termed the standard metabolic rate [SMR]) and maximum ($\dot{M}O_{2,max}$) rates of oxygen consumption and theoretically represents the portion of metabolic capacity above and beyond basal requirements (i.e. SMR) that can be directed to aerobic-dependent activities such as growth, feeding, digestion, locomotion and reproduction (Claireaux and Lefrancois, 2007; Clark et al., 2013). As thermal ramping proceeds in rock pools the MS of intertidal fish is expected to fall because higher temperature increases SMR (Schulte, 2015), and the capacity to support aerobically driven activities may then decline at

elevated temperatures. However, for MS to decline under thermal ramping, $\dot{M}O_{2,max}$ has to increase less than SMR across an equivalent temperature increment. In some fish species $\dot{M}O_{2,max}$ may increase more than or in parallel with SMR at higher temperatures, and therefore MS fails to decline because $\dot{M}O_{2,max}$ does not plateau (Healy and Schulte, 2012b; Norin et al., 2014; Lefevre, 2016). In the common triplefin (*Forsterygion lapillum* Hardy 1989), a species known to inhabit rock pools, $\dot{M}O_{2,max}$ did not increase at higher temperature following acclimation to 15, 18, 21 and 24°C (McArley et al., 2017). Furthermore, in the same study, routine $\dot{M}O_2$ under acute thermal ramping to critical thermal limits (>29°C) only marginally exceeded $\dot{M}O_{2,max}$ measured at the lower acclimation temperatures. Therefore, at least for this triplefin species, there may be a limited capacity to increase $\dot{M}O_{2,max}$ at higher temperatures, and this would result in vulnerability to a collapse of MS when exposed to thermal ramping in rock pools.

Although the MS of at least one triplefin species appears constrained at higher temperatures (McArley et al. 2017) these findings were determined under normoxia (100% of air saturation) and this is not always the setting within rock pools. Thermal ramping in rock pools generally occurs on hot sunny days when rates of algal photosynthesis are high and rock pool water can become hyperoxic at this time (see Fig. 4.1B and Berschick et al., 1987; Bridges, 1988; Richards, 2011). Thus, in a thermal ramping event, intertidal fish reside within normoxic water at ambient temperatures as the tide first recedes; then rock pool seawater warms and becomes increasingly hyperoxic until the incoming tide flushes the rock pool. $\dot{M}O_{2,max}$ and MS were approximately doubled under hyperoxia compared to normoxia in European perch (*Perca fluviatilis*) when measured at an acclimation temperature of 23°C (Brijs et al., 2015). Furthermore, in the same study, fish exposed to hyperoxia had a greater capacity to increase routine $\dot{M}O_2$ at temperatures approaching upper tolerance limits (>30°C). It is not known if the effects of hyperoxia seen in perch are widespread in fish but, if present in triplefins, an expected loss of MS under thermal ramping due to constrained $\dot{M}O_{2,max}$ may be compensated for through the permissive effects of hyperoxia. MS could also be protected at high temperature under hyperoxia because the energetic costs of meeting oxygen demands (e.g. ventilatory costs) are diminished by excess oxygen availability (Mark et al., 2002; Pörtner et al., 2017). While environmental hyperoxia in rock pools may therefore offer a metabolic refuge for intertidal fish facing acute thermal stress, this possibility has not been addressed.

Intertidal organisms are assumed to live very close to their thermal tolerance limits (Helmuth et al., 2002). In rock pools inhabited by triplefin fish we have measured temperatures exceeding 29°C in summer (Fig. 4.1A). These temperatures are close to the upper thermal tolerance limit (31.4°C) of *F. lapillum* (McArley et al., 2017) suggesting that, in at least some rock pools, this species would face temperatures close to its tolerable limit. While the exclusively intertidal triplefin (*Bellapiscis medius* Günther 1861) also appears live close to its thermal limits (Hilton et al., 2008), the upper thermal limit of this species is unknown. An inability to match oxygen supply and demand has been proposed as a key determinant of thermal tolerance in aquatic ectotherms (Pörtner and Farrell, 2008; Pörtner, 2010; Pörtner et al., 2017) and, experimental manipulations of tissue oxygen supply using hyperoxia have addressed the upper thermal limits of fish in this context (Mark et al., 2002; Healy and Schulte, 2012a; Brijs et al., 2015; Ekström et al., 2016; Devor et al., 2016). While these studies indicate that factors other than tissue oxygen supply may also set absolute upper thermal limits, hyperoxia did slightly increase the critical thermal maximum (CT_{max}; +0.9°C) of European perch (Ekström et al., 2016). Rock pools are expected to become hyperoxic during thermal ramping events that should approach upper thermal limits, and for this reason, the possibility of increased maximal thermal tolerance under hyperoxia in intertidal fish requires consideration. Given predicted increases in the severity of heat wave events due to climate change (Perkins et al., 2012), and the possibility that intertidal fish already live close to their upper thermal limits, resolving thermal tolerance limits of intertidal fish under environmentally relevant conditions is pertinent.

The aims of this study were to determine: (1) if hyperoxia increases the $\dot{M}O_{2,max}$ and MS of intertidal fish during thermal ramping, (2) if hyperoxia decreases routine $\dot{M}O_2$ (an estimate of basal metabolic costs during rapidly changing temperature) in intertidal fish under thermal ramping, and (3) if hyperoxia increases the upper thermal limits of intertidal fish. To address these aims, the $\dot{M}O_2$ (mass-specific oxygen consumption) and upper thermal tolerance capacities were determined for two New Zealand triplefin fishes under hyperoxia (~200% air saturation) and normoxia (~100% air saturation). This included an exclusively intertidal species (*B. medius*) and a second species (*F. lapillum*), which also occupies intertidal rock pools but is more commonly found in shallow subtidal habitats. To address the first and second aims, MS was measured at 21°C, routine $\dot{M}O_2$ was measured at 21, 24, 26, 28 and 29°C under thermal ramping and MS above routine metabolism was measured at 29°C (see Fig. 2). It was predicted that $\dot{M}O_{2,max}$ and, therefore MS, would be higher under

hyperoxia at 21°C and 29°C due to a limitation of $\dot{M}O_{2,max}$ under normoxia (Brijs et al., 2015). Additionally, routine $\dot{M}O_2$ under thermal ramping was predicted to increase less under hyperoxia than normoxia because of decreased cardiorespiratory costs required to meet oxygen demands. As such, the rate and amplitude of gill ventilation were also measured under thermal ramping to determine if the lower metabolic rates expected in hyperoxia could indeed be attributed to decreased ventilatory costs. To address the third aim (whether hyperoxia increases absolute thermal limits) the upper thermal tolerance limit (CT_{max}) of each species was assessed under hyperoxia and normoxia. CT_{max} was defined as the temperature of equilibrium loss during a standardised (2°C per h) thermal ramping exposure.

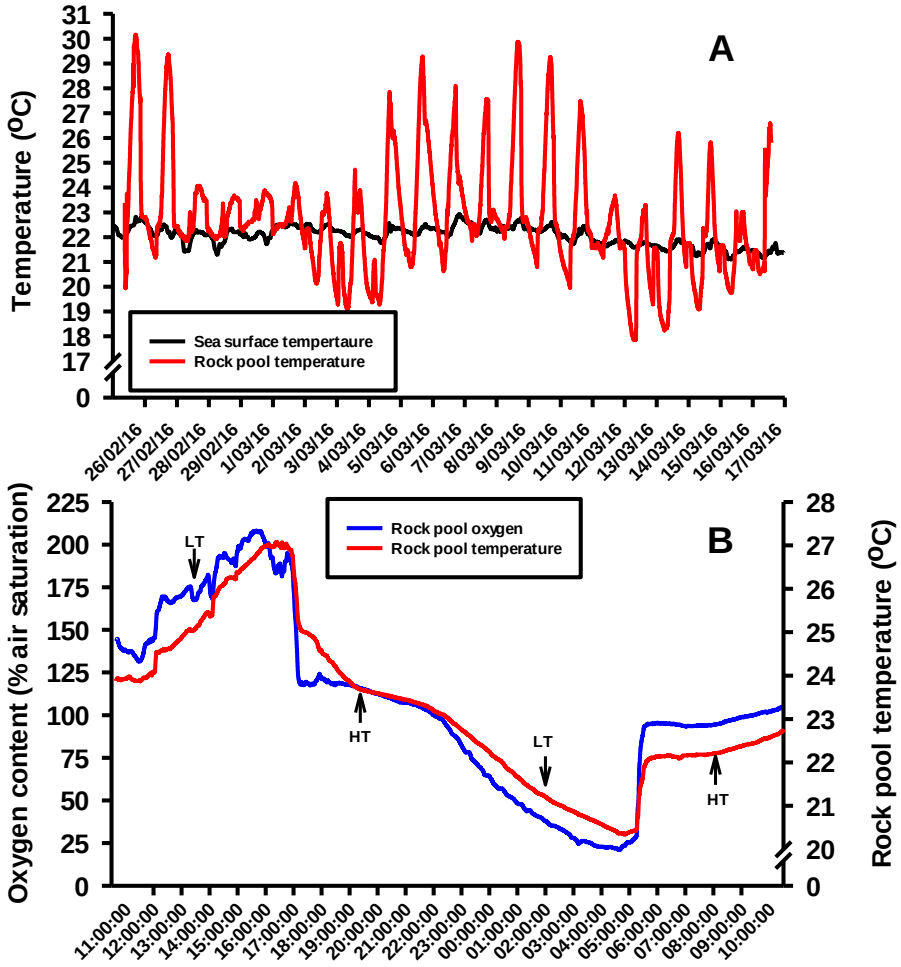

Figure 4.1 Rock pool temperature and dissolved oxygen content. Panel A shows the temperature of a rock pool known to be inhabiated by intertidal triplefin species (red line) over a 3 week period during summer 2016. The rock pool was located in the interidal zone adajcent to Leigh Marine Laboratory in north eastern New Zealand. The black line in panel A shows sea surface temperature over the same 3 week period from coastal waters adjacent to the Leigh Marine Laboratory. Panel B shows water oxygen content (blue line, left axis) and water temperature (red line, right axis) of a rock pool known to be inhabiated by intertidal triplefins over a 24 hour period during summer. Upward facing arrows show the time of high tide and downward facing arrows show the time of low tide.

4.2 Methods

4.2.1 Experimental animals and laboratory acclimation

Fish used in this study were netted from intertidal rock pools (*B. medius*) or shallow subtidal (<5 m) habitats by divers (*F. lapillum*) in the vicinity of Leigh, New Zealand. Animals were housed in 30 L flow through seawater tanks (air saturated, 200 µm filtered, 35

ppt salinity) at the Leigh Marine Laboratory (Leigh, New Zealand) and were acclimated to a 12 hour photoperiod and a temperature of 21˚C (±0.5) for at least 4 weeks prior to experimentation. During the acclimation period fish were fed daily on a mix of crushed aquaculture feed (Skretting, Australia) and pilchard. Food was withheld for a period of at least 48 h prior to the start of experimental protocols. Specific dynamic action is completed in 48 h in triplefin fish (Stobie, 2017), so a stable non-digesting state should have been reached when experimental protocols began. All experimental techniques were performed under approval of the University of Auckland Animal Ethics Committee (approval: 001801).

4.2.2 Experimental treatments and protocols for assessment of $\dot{M}O_2$

Mass-specific oxygen consumption ($\dot{M}O_2$) was assessed in *B. medius* (body mass=1.92g ± 0.13, length=57mm ± 1.6) and *F. lapillum* (body mass=2.13g ± 0.11, length=56.4mm ± 2.7) under conditions of normoxia ~21 kPa (100 % air saturation) or hyperoxia ~42 kPa (200% air saturation) (N=10 for all treatments) and there were no differences in body mass between treatments groups (ANOVA, DF=3, F=1.21, P=0.32). Hyperoxia was achieved by bubbling oxygen into the seawater reservoir supplying respirometers and was maintained at the required level by an oxy-guard control unit (Loligo Systems, Tjele, Denmark). The sequence of $\dot{M}O_2$ measurements that were made in each treatment group is displayed in Fig. 4.2. Assessment of $\dot{M}O_2$ started by transferring fish to a circular 30 L tank filled with normoxic or hyperoxic seawater at 21°C in which they were exhaustively exercised by chasing for a period of 5 min. All fish appeared to be fully exhausted after 5 min (i.e. no longer able to perform burst swimming in response to touch). At the conclusion of chasing the fish were transferred to individual respirometry chambers (see respirometry methods below) supplied with either normoxic or hyperoxic (21˚C) seawater and repeating 4 min $\dot{M}O_2$ measurement cycles were initiated. $\dot{M}O_{2max}$ (21˚C) was taken as the highest $\dot{M}O_2$ value recorded in any measurement cycle which was in all cases the first cycle following exhaustive exercise. A 5 min exhaustive exercise protocol has previously been used to elicit $\dot{M}O_{2max}$ values in triplefins (Khan et al., 2014a; McArley et al., 2017) and is suitable for assessments of $\dot{M}O_{2max}$ in benthic species which will not continuously swim in a flume (Clark et al., 2013; Norin and Clark, 2016). The fish were then left undisturbed in respirometers for a period of ~16 h under conditions of constant temperature (~21˚C) and water oxygen content (normoxia or hyperoxia), and $\dot{M}O_2$ was

measured in repeating 10 min cycles (~100 individual $\dot{M}O_2$ measurements). Standard metabolic rate (SMR) at 21˚C was defined as the mean of the lowest 10% of $\dot{M}O_2$ measurements recorded in the overnight recovery period (Norin et al., 2014; Khan et al., 2014a) (Fig. 4.2). At ~0800 the overnight SMR measurement ceased and a thermal ramping protocol was started where temperature was raised from 21˚C to 29˚C at a rate of ~2˚C per hour. Metabolic rate was measured over 2 measurement cycles (~15 min) at 21, 24, 26, 28 and 29˚C during thermal ramping and the mean of the two measurements was defined as the temperature specific routine $\dot{M}O_2$ (Fig. 4.2). After the final routine $\dot{M}O_2$ measurement at 29˚C the fish was removed from the respirometer and transferred to a 30 L tank filled with normoxic or hyperoxic seawater preheated to 29˚C. The fish was exhaustively exercised by chasing for 5 min then returned to the respirometer for assessment of $\dot{M}O_{2,max}$ over four 3-4 minute measurement cycles. The $\dot{M}O_{2,max}$ at 29˚C was defined as the highest value of $\dot{M}O_2$ recorded over these cycles. Finally, the fish was removed from the respirometer and allowed to recover prior to recording body mass and length. The $\dot{M}O_2$ measurements described above were used to determine true MS (the difference between $\dot{M}O_{2,max}$ and SMR) under normoxia and hyperoxia at 21˚C (Fig. 4.2). True MS could not be determined at 29˚C because fish were not held at this temperature for the prolonged periods required to reliably estimate SMR. The difference between the lowest value of routine $\dot{M}O_2$ at 29˚C and $\dot{M}O_{2,max}$ at 29˚C was used as an approximation of MS following thermal ramping (see Fig. 4.2). In four individuals routine $\dot{M}O_2$ at 29°C was marginally higher than $\dot{M}O_{2,max}$ at 29˚C and these individuals were treated as having zero MS above routine $\dot{M}O_2$. Because MS was defined differently at 21 and 29˚C no statistical comparisons were made between temperatures.

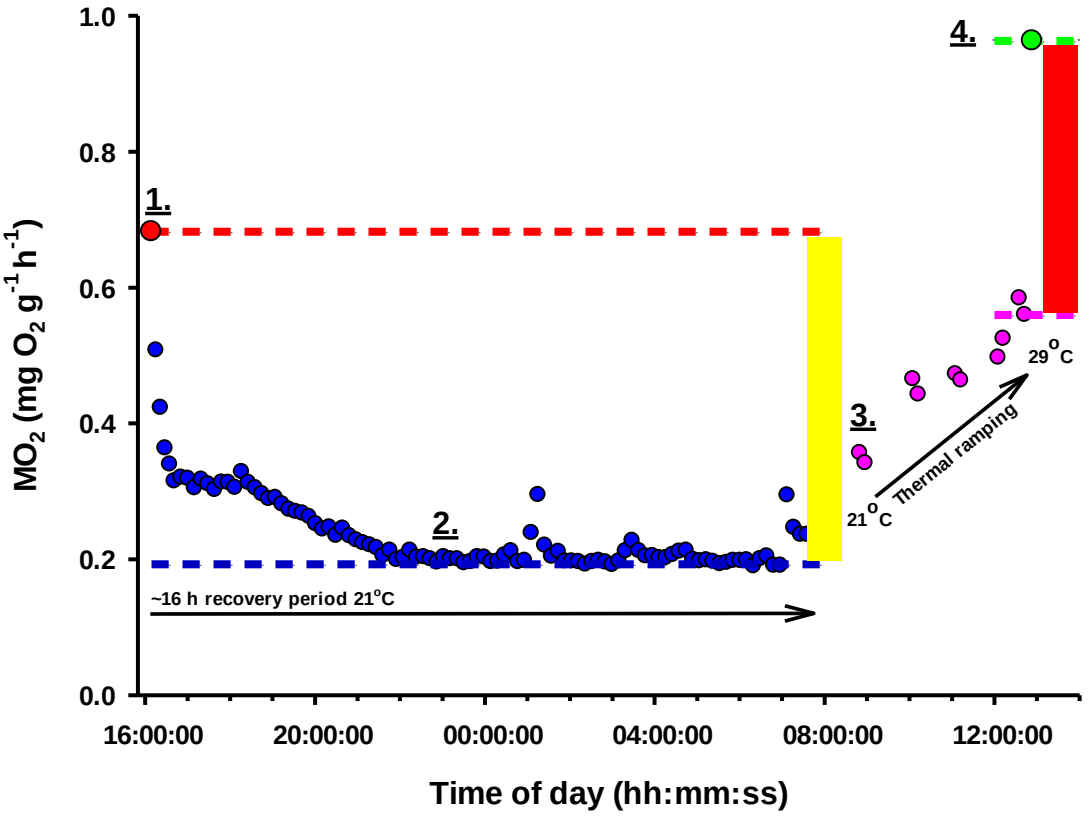

Figure 4.2 Measurements of metabolic rate ($\dot{M}O_2$) made in individual fish. Data points show $\dot{M}O_2$ for an individual *Forsterygion lapillum* under conditions of hyperoxia (200% air saturation). The sequence of measurements shown were made in both *Bellapiscis medius* and *F. lapillum* under conditions of normoxia (100% air saturation) or hyperoxia (200% air saturation). 1. Maximum metabolic rate ($\dot{M}O_{2,max}$) was measured at 21˚C following exhaustive exercise (red circle and broken line), 2. Standard metabolic rate (SMR) was measured at 21˚C over a 16 hour recovery period (blue circles and broken line), 3. Routine $\dot{M}O_2$ was measured at 21, 24, 26, 28 and 29˚C under thermal ramping (pink circles), and 4. $\dot{M}O_{2,max}$ was measured at 29˚C following exhaustive exercise (green circle and broken line). The difference between $\dot{M}O_{2,max}$ at 21˚C and SMR was taken as metabolic scope at 21˚C (yellow box). The difference between $\dot{M}O_{2,max}$ at 29˚C and the minimum routine $\dot{M}O_2$ at 29˚C was taken as the metabolic scope above routine $\dot{M}O_2$ at 29˚C (red box).

4.2.3 Respirometry methods

$\dot{M}O_2$ reported as milligrams of oxygen consumed per gram of body mass per hour (mg O_2 g^{-1} h^{-1}), was determined using automated intermittent-flow respirometry (Steffensen, 1989). Respirometers were constructed of a cylindrical acrylic chamber fitted with an adjustable stopper, which allowed the chamber volume (60-150 mL) to be adjusted to match fish size. The chambers were held in a 60 L reservoir filled with filtered (5 µm) UV-sterilised seawater. The temperature of the reservoir was controlled by continually pumping the

seawater through a 40 L tower containing an aluminium heat exchanger. The inlet of each chamber was connected to an automated Eheim compact 3000 submersible flush pump (EHEIM GmbH & Co. KG, Germany) that was switched on and off by a relay control unit (USB Power 8800 Pro, Aviosys International Inc., Taiwan) controlled by custom-coded software (Leigh Marine Laboratory). An inline pump (modified Eheim compact 3000) was connected to the respirometry chamber in a closed loop to ensure adequate water mixing and the oxygen concentration of water within the chamber was continuously measured using contactless sensor spots and FireSting O_2 meters (PyroScience, Aachen, Germany). The decline in O_2 concentration within a respirometry chamber was used to calculate $\dot{M}O_2$ in repeated measurement cycles (4-8 min) according to the equation:

$$\dot{\mathrm{M}}O_2 = V\left(\Delta\%\frac{sat}{t}\right)a.M_B \text{ , (4)}$$

where, V is the respirometry chamber minus fish volume, $\Delta\%\ sat/t$ is the change in oxygen saturation per unit time, α is the solubility coefficient of oxygen (mg O_2 %Sat^{-1} L^{-1}) in sea water (35 ppt), and M_B is the body mass of the fish in grams (Schurmann and Steffensen, 1997). The measurement cycles were interspersed by 1 min flushing periods in order to refresh the water within respirometers. In all estimates of metabolic rate only $\dot{M}O_2$ values with R^2 of >0.98 for the decline in oxygen per unit of time were used. The background oxygen consumption within chambers was assessed at the conclusion of thermal ramping and fish $\dot{M}O_2$ was adjusted following the same method outlined in Chapter 5.

4.2.4 Assessment of critical thermal maximum (CT_{max})

CT_{max} was measured under hyperoxia (200% air saturation) and normoxia (100% air saturation) in both species (N=9-10, *B. medius*: body mass=2.03g ± 0.24, length=56.6mm ± 2.1, *F. lapillum*: body mass=1.52g ± 0.12, length=55mm ± 1.2). Assessment of CT_{max} was carried out in a 60 L reservoir filled with either normoxic or hyperoxic seawater pre-heated to 21˚C. The reservoir accommodated 4 fish per run (~20 hours) and 2 fish of each species were included in each run. The fish were introduced to the reservoir and allowed to acclimate overnight (~16 hours) under conditions of normoxia or hyperoxia and stable temperature (~21˚C). Following overnight acclimation the temperature of the reservoir was raised at a rate of ~2˚C per hour until the fish lost equilibrium for a period of at least 10 s. The loss of equilibrium temperature was taken as the CT_{max} (Beitinger et al., 2000; Mora and Maya,

2006; Brijs et al., 2015; Sandblom et al., 2016). Temperature and water oxygen content were measured throughout the CT_{max} assessment using a FireSting O_2 meter (PyroScience, Aachen, Germany).

4.2.5 Assessment of ventilation frequency, ventilation amplitude and estimated ventilation volume

Ventilation frequency and amplitude were measured by video analysis in both species under normoxia or hyperoxia and thermal ramping (21, 24, 26, 28 and 29˚C) (N=8, *B. medius*: body mass=2.29g ± 0.35, length=50mm ± 1.9, *F. lapillum*: body mass=1.8g ± 0.15, length=49.4mm ± 1). There were no differences in body mass between treatment groups (ANOVA, DF=3, F=0.73, P=0.54). The measurements were carried out in an insulated 60 cm x 45 x 30 cm tank which was filled to a depth of 7 cm and continuously supplied with normoxic or hyperoxic seawater from a larger temperature controlled 60 L reservoir. The depth of the tank allowed video cameras to be mounted directly above pens made of cylindrical acrylic tubing which maintained the position of the fish throughout the measurement period. The experimental setup accommodated four fish and 2 of each species were used in each run. The fish were introduced to the pens and then allowed to recover overnight (~16 hour) under constant temperature (~21˚C) and oxygen conditions (normoxia or hyperoxia). Following overnight recovery a thermal ramping protocol was initiated where temperature was increased from 21 to 29˚C at a rate of ~2˚C per hour and videos were recorded at 21, 24, 26, 28 and 29˚C. A single video recording (~2 min) was taken at each temperature set point and thermal ramping continued immediately after the video had been taken.

Ventilation frequency and amplitude were quantified from videos using Image J imaging software (U. S. National Institutes of Health, Bethesda, Maryland, USA). Ventilation frequency was counted over 60 s in each video by using the Plot Z-axis function to measure the change in pixel intensity of an area adjacent to the operculum as it opened and closed. A 60 s time period has been used to estimate ventilation frequency in other fish species (Frisk et al., 2012). Ventilation amplitude was assessed by comparing the maximum width (mm) of the operculum when open to the width of the operculum when it was closed. The opening operculum was observed frame by frame during an opercula beat and width measured in the frame where the maximum separation between the right and left operculum was observed and

where the operculum were closed. Three randomly selected individual opercula beats were measured in each video and were converted to a percentage increase in operculum width. The mean of these three measurements was taken as the ventilation amplitude at each temperature. A plastic mesh grid of known dimensions attached to the base of the fish pens was used to calibrate the distance measurements in each video.

To estimate ventilation volume, the change in the volume of the head when the operculum fully opened and closed during an opercula beat was calculated. The shape of the head was treated as a half conical frustum and volume calculated from video frames according to the equation:

$$Volume = \frac{1}{3}\pi h(R_{2^2} + R_2 R_1 + R_{1^2})/2 \text{ , (5)}$$

where R_1 was equal to half the distance between the ventral tip of the opercula, R_2 was equal to half the distance between each side of the fish where the operculum pivoted during a ventilatory beat and h was the distance between R_1 and R_2 along the centre of the head of the fish (Fig. 4.3). The difference in the calculated head volume when the operculum were opened and closed was taken as the estimated stroke volume for an opercula beat and three opercula beats were measured in each video. The mean stroke volume of three opercula beats was multiplied by ventilation frequency to estimate ventilation volume as millilitres per minute per gram of body mass. A half conical frustum does not completely replicate the head shape of each species. As such, estimated ventilation volume is treated as a relative measure and only compared across experimental treatments within each species.

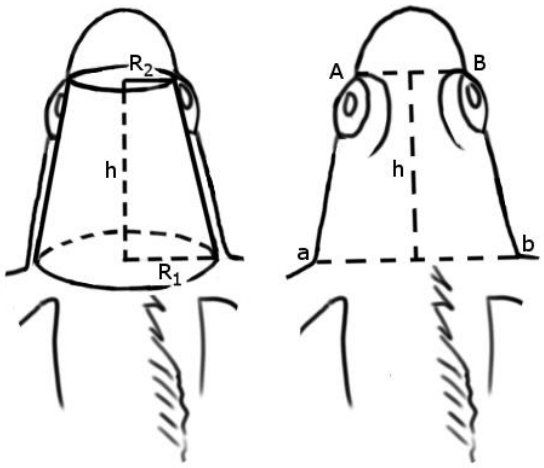

Figure 4.3 Parameters measured to estimate ventilation stroke volume. The head of the fish was treated as a half conical frustum to estimate change in volume when the opercula were opened and closed in a ventilatory beat. R_1 and R_2 were measured from video frames as half the distance between the left and right side of the fish (i.e. R_1= half the distance between a-b, R_2= half the distance between A-B). These points corresponded to the ventral tip of the opercula (a,b) and the point at which the opercula first pivoted in an ventilatory beat (A, B).

4.2.6 Statistics

All statistical analysis was carried out using the SPSS Statistics 24 or Sigma Plot 13.0 software packages. Two-way analysis of variance (ANOVA) with Holm-Sidak *post-hoc* comparisons was used to determine the effect of species and water oxygen content on SMR at 21˚C, MS at 21˚C, MS above routine $\dot{M}O_2$ at 29˚C and CT_{max}. For the analysis of MS above routine $\dot{M}O_2$ at 29˚C the data were log transformed. The impact of species, water oxygen content (hyperoxia vs normoxia) and thermal ramping temperature on routine $\dot{M}O_2$ were assessed using a three-way mixed ANOVA. For this analysis species and water oxygen content were set as between subjects factors and thermal ramping temperature as a within subjects factor. For the tests of within subjects effects a violation of sphericity was assumed and epsilon (Huynh-Feldt) adjusted *F* tests were run. For all *post-hoc* tests Holm-Sidak adjusted significance levels were used. Mixed three-way ANOVA was also used to assess the impact of species, water oxygen content and temperature on $\dot{M}O_{2,max}$. No three-way interactions were significant (P>0.05) and only the two-way interactions and main effects are reported in the results. Ventilation frequency, amplitude and estimated ventilation volume were compared within each species using repeated measures ANOVA with temperature set as a within subjects factor and water oxygen content set as a between subjects factor. For *F. lapillum* the natural logarithm of ventilation frequency was used in the analysis.

4.3 Results

4.3.1 The impact of hyperoxia on $\dot{M}O_2$

SMR was significantly higher in *B. medius* than in *F. lapillum* (ANOVA, DF=1, F=44.09, P<0.001) and in both species significantly higher under hyperoxia than normoxia (ANOVA, DF=1, F=9.03, P=0.005, Fig. 4.4A & 4.4B). The influence of hyperoxia on $\dot{M}O_2$ during thermal ramping was explained by an interaction between thermal ramping temperature and water oxygen content (ANOVA, DF=3.65, F=3.78, P=0.008). In both species $\dot{M}O_2$ was significantly higher under hyperoxia than normoxia but only at temperatures of 26, 28 and 29˚C (Fig. 4.4A & 4.4B). There was also an interaction between thermal ramping temperature and species (ANOVA, DF=3.65, F=12.65, P<0.001). While $\dot{M}O_2$ did not differ between the species at 21˚C, at temperatures of 24, 26, 28 and 29˚C *B. medius* had significantly higher $\dot{M}O_2$ than *F. lapillum.* Moreover, $\dot{M}O_2$ increased significantly from 21 to 24˚C and from 28 to 29˚C in *B. medius*, but there was no significant difference in $\dot{M}O_2$ over these temperature changes for *F. lapillum* (Fig. 4.4A & 4.4B).

The impact of species, temperature and water oxygen content on $\dot{M}O_{2,max}$ was explained by interactions between temperature and species (ANOVA, DF=1, F=4.15, P=0.049) and, temperature and water oxygen content (ANOVA, DF=1, F=22.4, P<0.001). Under all conditions $\dot{M}O_{2,max}$ was significantly higher in *B. medius* than in *F. lapillum* but the difference between the species was greater at 29°C (Fig. 4.4A & 4.4B). $\dot{M}O_{2,max}$ was significantly higher under hyperoxia than normoxia in all treatments, however the difference in $\dot{M}O_{2,max}$ between hyperoxia and normoxia was larger at 29˚C than at 21˚C (Fig. 4.4A & 4.4B). Interestingly, in both species $\dot{M}O_{2,max}$ increased significantly between 21 and 29˚C under hyperoxia whereas under normoxia there was no change in $\dot{M}O_{2,max}$ between these temperatures (Fig. 4.4A & 4.4B). At 21˚C MS was significantly greater under hyperoxia in both species (ANOVA, DF=1, F=13.12, P<0.001) and higher in *B. medius* than *F. lapillum* (ANOVA, DF=1, F=27.71, P<0.001) (Fig. 4.4A & 4.4B). Following thermal ramping to 29˚C MS above routine $\dot{M}O_2$ was significantly higher under hyperoxia than normoxia in both species (Fig. 4.4A & 4.4B). *B. medius* had significantly greater MS above routine $\dot{M}O_2$ than *F. lapillum* at 29˚C (ANOVA, DF=1, F=5.46, P=0.025).

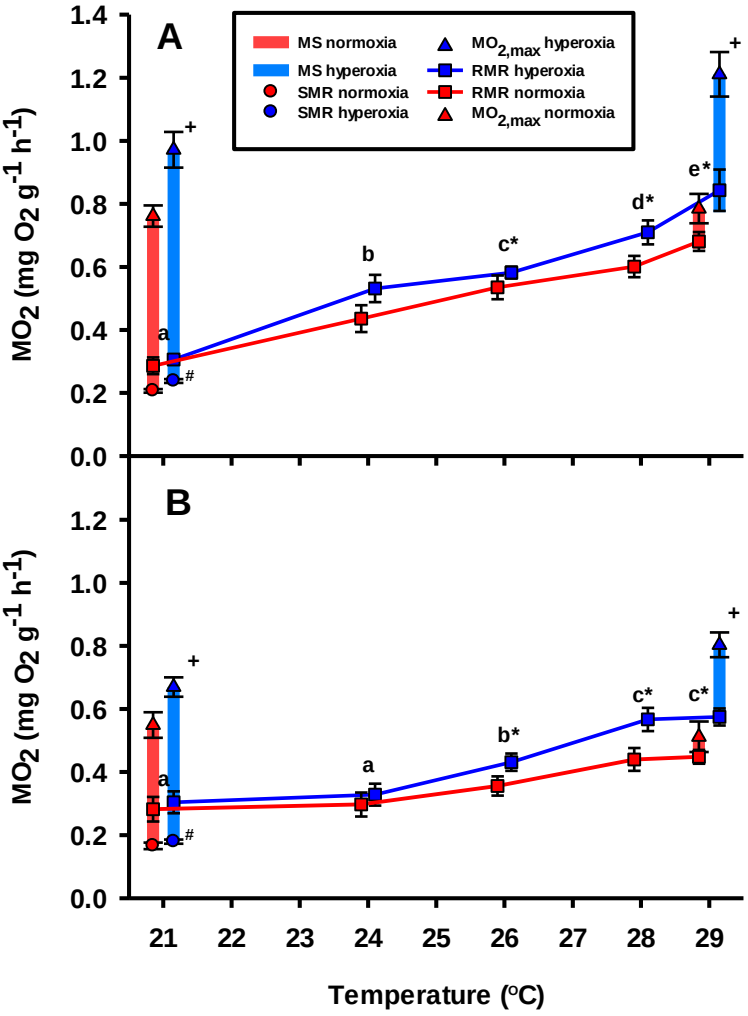

Figure 4.4 Metabolic rate ($\dot{M}O_2$) under normoxia and hyperoxia in *Bellapiscis medius* (A) and *Forsterygion lapillum* (B). All values are means ± s.e.m (N= 10). Red and blue symbols and lines show normoxia (100% air saturation) and hyperoxia (200% air saturation) respectively. Circles show standard metabolic rate (SMR) at 21˚C, triangles show maximum metabolic rate ($\dot{M}O_{2,MAX}$) at 21 and 29˚C and squares show routine $\dot{M}O_2$ at 21, 24, 26, 28 and 29˚C under thermal ramping (note symbols have been staggered for interpretation). The vertical red and blue shaded lines show metabolic scope at 21˚C and scope above routine $\dot{M}O_2$ at 29˚C. Hash symbols show a significant difference ($P<0.05$) in SMR between normoxia and hyperoxia at 21˚C. Cross symbols show significant differences ($P<0.05$) in $\dot{M}O_{2,max}$ and aerobic metabolic scope between normoxia and hyperoxia at 21 and 29˚C. Star symbols show a significant difference ($P<0.05$) in routine $\dot{M}O_2$ under thermal ramping between normoxia and hyperoxia. Lower case letters show significant differences ($P<0.05$) in routine $\dot{M}O_2$ between thermal ramping temperatures within each species.

4.3.2 Upper thermal tolerance limit (CT_{max})

There was a significant interaction between the effect of species and water oxygen content on CT_{max} (ANOVA, DF=1, F=4.19, P=0.048). Although the differences were small (<0.6˚C), the intertidal specialist *B. medius* had significantly higher CT_{max} than *F. lapillum* under both normoxia and hyperoxia (Fig. 4.5). There was no effect of hyperoxia on CT_{max} in *B. medius* but CT_{max} did increase (0.43˚C) under hyperoxic conditions in *F. lapillum* (Fig. 4.5).

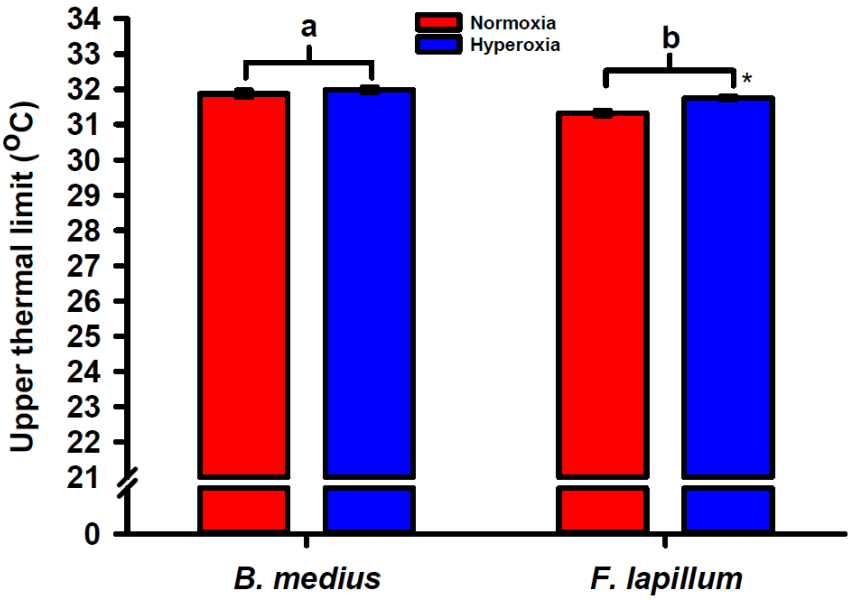

Figure 4.5 Upper thermal tolerance limit (CT_{max}) in normoxia and hyperoxia exposed *Bellapiscis medius* and *Forsterygion lapillum*. All values are means ± s.e.m (N= 9-10). Red and blue bars show normoxia (100% air saturation) and hyperoxia (200% air saturation) respectively. Stars show significant differences (P<0.05) between water oxygen content within species. Lower case letters show significant differences between species.

4.3.3 Ventilation frequency, ventilation amplitude and estimated ventilation volume

In *B. medius* and *F. lapillum* ventilation frequency during thermal ramping was significantly lower under hyperoxia than normoxia (*B. medius*: ANOVA, DF=1, F=13.74, P=0.002 and *F. lapillum*: ANOVA, DF=1, F=10.65, P=0.006, Fig. 4.6-1.1 & 4.6-1.2) but there was no effect of hyperoxia on ventilation amplitude (Fig. 4.6-2.1 & 4.6-2.2). Estimated ventilation volume during thermal ramping was also significantly lower under hyperoxia in

both species (*B. medius*: ANOVA, DF=1, F=44.1, P<0.001 and *F. lapillum*: ANOVA, DF=1, F=47.93, P<0.001, Fig. 4.6-3.1 & 4.6-3.2). During thermal ramping ventilation frequency increased significantly at higher temperatures in *B. medius* (ANOVA, DF=4, F=11.44, P<0.001, Fig. 4.6-1.1) but there was no effect of temperature in *F. lapillum* (ANOVA, DF=4, F=1.04, P=0.37, Fig. 4.6-1.2). Contrastingly, there was no change in ventilation amplitude across temperatures in *B. medius* (ANOVA, DF=4, F=0.96, P=0.43, Fig. 4.6-2.1) but, in *F. lapillum*, ventilation amplitude increased at higher temperatures (ANOVA, DF=4, F=28, P<0.001, Fig. 4.6-2.2). In both species, estimated ventilation volume increased with thermal ramping (*B. medius*: ANOVA, DF=4, F=21.98, P<0.001 and *F. lapillum*: ANOVA, DF=4, F=29.9, P<0.001, Fig. 4.6-3.1 & 4.6-3.2).

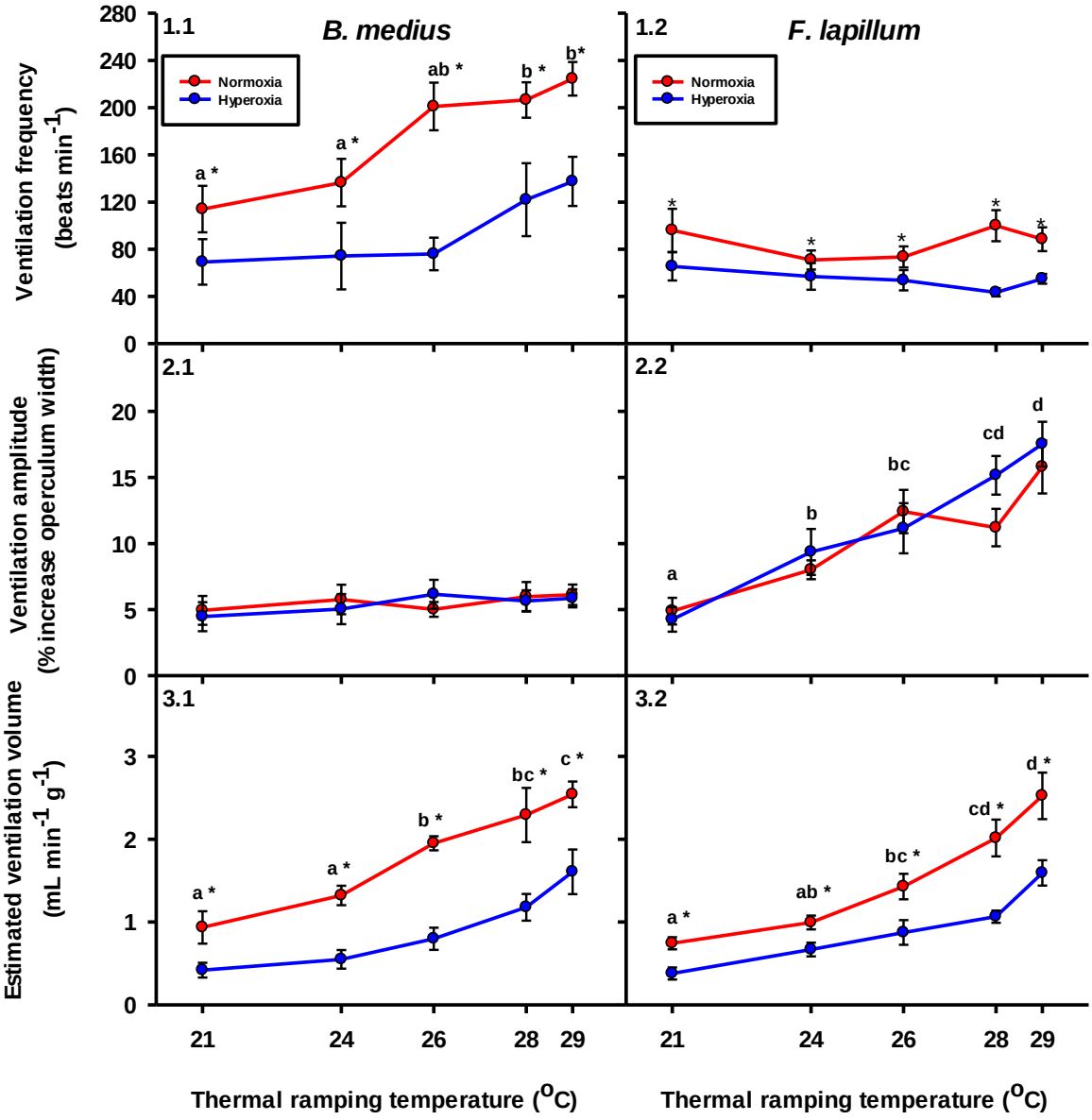

Figure 4.6 Ventilation frequency (1.1-1.2), ventilation amplitude (2.1-2.2) and estimated ventilation volume (3.1-3.2) during thermal ramping in normoxia and hyperoxia exposed *Bellapiscis medius* and *Forsterygion lapillum*. All values are means ± s.e.m (N= 8). Red and blue symbols and lines show normoxia (100% air saturation) and hyperoxia (200% air saturation) respectively. Lower case letters show significant differences (P<0.05) between thermal ramping temperatures and stars show significant differences (P<0.05) between normoxia and hyperoxia during thermal ramping.

4.4 Discussion

This study shows that hyperoxia increases $\dot{M}O_{2,max}$ and MS in intertidal triplefin fish. $\dot{M}O_{2,max}$ was higher with hyperoxia than normoxia at ambient temperature (21°C) and also after thermal ramping to ecologically relevant temperatures (29°C). Consequently, despite higher SMR and routine $\dot{M}O_2$, MS at 21°C and MS above routine $\dot{M}O_2$ at 29°C increased under hyperoxia. At the start of thermal ramping events in rock pools intertidal fish are first exposed to normoxia and ambient temperature but, as temperature increases, rock pools can also become super-saturated with oxygen (see Fig. 4.1B for an example). Therefore, comparisons of MS between ambient temperature normoxic conditions and, high temperature hyperoxic conditions are relevant. In hyperoxia, 79% and 64% of the MS available under normoxia and ambient temperature (21°C) was retained after thermal ramping to 29°C in *B. medius* and *F. lapillum* respectively. This contrasts with the near complete collapse of MS in both species at 29°C under normoxia or, more specifically without hyperoxia (Fig. 4.4). While hyperoxia did not provide a meaningful increase in the absolute thermal tolerance limits of either species, the observed retention of MS may allow intertidal fish to continue performing aerobically demanding activities, such as feeding and digestion, to higher temperatures than would otherwise be possible. These findings indicate that environmental hyperoxia may provide a 'metabolic refuge' for intertidal fish exposed to acute thermal stress during low tide ebbs if photosynthesis is active.

4.4.1 The influence of hyperoxia on aerobic metabolic scope

MS is thought to be a key determinant of fitness as it represents the boundaries within which aerobic activities can be performed (Claireaux and Lefrancois, 2007; Clark et al., 2013). Indeed, positive correlations between growth performance and MS have been demonstrated in several fish species (Jobling, 1981; Claireaux et al., 2000; Claireaux and Lefrancois, 2007; Khan et al., 2014b) including the triplefin *F. lapillum* (McArley et al., 2017). If increased availability of MS does indeed help to mitigate a prioritisation or constraint on simultaneous energetic processes (Lefrançois and Claireaux, 2003; Claireaux and Lefrancois, 2007; Clark et al., 2013) then an expansion of MS under hyperoxia may offset expected losses in performance during acute high temperature exposure. For example, if rock pools become hyperoxic and MS is expanded, feeding and digestion may be better

supported at high temperatures, despite increased basal costs. Interestingly, Hilton et al. (2008) showed that the intertidal triplefin *B. medius* was more abundant in rock pools with a high algal coverage suggesting a preference for rock pools likely to become hyperoxic during low tide. Greater MS under hyperoxia may also allow rock pool fish to avoid an anaerobic acidosis, which may develop during thermal ramping as anaerobic metabolism increases to meet ATP demands (e.g. Clark et al., 2008). Indeed, Devor et al. (2016) found hyperoxia mitigated the rise in plasma and muscle lactate levels in two Antarctic notothenioid fishes exposed to thermal ramping. So whether hyperoxia helps intertidal fish recover from heat stress by avoiding a metabolic acidosis or whether hyperoxia assists other aspects of performance (feeding, digestion, growth etc) during thermal ramping, would of course be interesting avenues for future research.

The exclusively intertidal triplefin *B. medius* has higher MS than *F. lapillum*, a species found more commonly in shallow sub-tidal habitats. *B. medius* is also found in rock pools higher in the intertidal zone than *F. lapillum* (T. McArley personal observation) and occupies habitat more prone to acute high temperature events and, possibly night time hypoxia too. Whether it is increased basal metabolic costs during thermal ramping or constrained maximum metabolic capacity during hypoxia, intertidal fish regularly face environmental conditions that limit MS. The high MS of *B. medius* may therefore reduce the occurrence of situations where limiting environmental conditions result in the prioritisation or constraint of aerobic activities (e.g. growth, reproduction, swimming etc).

4.4.2 Possible mechanisms by which aerobic metabolic scope increases under hyperoxia

In the current study SMR and routine $\dot{M}O_2$ both increased under hyperoxic conditions but not to the extent that $\dot{M}O_{2,max}$ increased. Thus, the observed increase in MS under hyperoxia was driven primarily by the response of $\dot{M}O_{2,max}$ and this is in line with the observations of Brijs et al. (2015) who saw the MS of European perch approximately double at 23°C under hyperoxia (~200% air saturation) as a result of expansion in $\dot{M}O_{2,max}$ This is not universal among all fish species, however, because hyperoxia did not increase $\dot{M}O_{2,max}$ or MS in either rainbow trout (Duthie and Hughes, 1987) or common sole (Lefrançois and Claireaux, 2003). Brijs et al. (2015) also suggested a higher $\dot{M}O_{2,max}$ under hyperoxia at temperatures approaching thermal limits (~34°C) but the results of the present study are the

first to directly demonstrate an increase in $\dot{M}O_{2,max}$ under hyperoxia following thermal ramping.

So, if MS expansion under hyperoxia results from increased $\dot{M}O_{2,max}$, how does this expansion occur? The oxygen consumption rate of an intact animal is the sum of aerobic metabolic demands of all tissues, which is mostly determined by the oxygen carrying capacity of blood and the rate at which the circulatory system delivers oxygen to respiring tissues. While hyperoxia likely increases $\dot{M}O_{2,max}$ by modulating these factors, the exact mechanisms by which this occurs remain unclear. European perch lacks a coronary circulation and, therefore, relies on the venous blood return to supply oxygen to the heart (Santer and Walker, 1980; Ekström et al., 2016). Although we do not know if triplefin fish also lack a coronary circulation it is not present in another blennioid fish species (*Blennius pholis*) (Santer and Walker, 1980). If triplefin fish do indeed lack a coronary circulation like European perch, one possibility is that cardiac output is improved under hyperoxia following exhaustive exercise due to an increased supply of oxygen to the heart via the venous blood return. Indeed, elevated partial pressure of oxygen in venous blood has been demonstrated in fish exposed to hyperoxia (Wilkes et al., 1981; Takeda, 1990; Ekström et al., 2016). This could also explain why there was no effect of hyperoxia on $\dot{M}O_{2,max}$ in rainbow trout (*Oncorhynchus mykiss*) (Duthie and Hughes, 1987) because salmonids possess a well-developed coronary circulation which supplies the heart directly with oxygenated blood (Santer and Walker, 1980; Ekström et al., 2017) and this could theoretically override the influence of hyperoxia on venous blood oxygen content, hence, cardiac output. An alternative suggestion is that cutaneous oxygen uptake is increased in hyperoxia (Wang et al., 2014; Brijs et al., 2015; Ekström et al., 2016). There is evidence that the contribution of the skin to gas exchange appears to be up-regulated following exercise in fish (Rummer et al., 2014) and cutaneous respiration provides a relatively greater contribution to the total $\dot{M}O_2$ of carp (*Cyprinus carpio*) under hyperoxia (Takeda, 1989).

While increased $\dot{M}O_{2,max}$ in hyperoxia may be beneficial for rock pool fish through enabling MS expansion, hyperoxia does appear to elevate basal metabolic costs. Here we hypothesised that basal metabolic costs decrease under hyperoxia, yet the SMR at ambient temperature (21°C) and routine $\dot{M}O_2$ at high temperatures (26-29°C) during thermal ramping were higher under hyperoxia than normoxia. This observation is somewhat paradoxical because, as expected, our measures showed that ventilation effort (and presumably the energetic cost of ventilation) was decreased with hyperoxia (Fig. 6). This cost pattern was

also apparent for the rainbow trout, which showed a transient increase in $\dot{M}O_2$ despite a 50% reduction in ventilatory flow rate under hyperoxia (Wood and Jackson, 1980), and spotted wolfish (*Anarhichas minor*) also showed higher routine $\dot{M}O_2$ under hyperoxia (Foss et al., 2003). A similar decrease in ventilation with hyperoxia has also been seen in carp (Takeda, 1990), the white sucker (*Catostomus commersonii*) (Wilkes et al., 1981) and an intertidal goby (*Gobius cobitis*) (Berschick et al., 1987) but in these species $\dot{M}O_2$ was unchanged, suggesting a relatively smaller increase in basal costs with hyperoxia. Taken together, these findings suggest the basal metabolic costs of fish seem to be raised under hyperoxia and these costs appear to override the reduced costs of ventilation. While higher basal metabolic costs under hyperoxia put a performance benefit of hyperoxia in question, in the current study they occurred alongside comparatively larger increases in $\dot{M}O_{2,max}$, such that MS was still expanded with hyperoxia. Clearly, further work is required to determine if intertidal fish facing thermal ramping are able to derive a performance benefit from expanded MS under hyperoxia.

4.4.3 The influence of hyperoxia on absolute upper thermal tolerance limits

Despite MS increasing at temperatures approaching critical limits, hyperoxia did not provide a biologically relevant increase in the absolute thermal limits of either triplefin species. This finding is in agreement with other recent studies, which found no or only a small increase in the critical thermal maximum of fish under hyperoxia (Healy and Schulte, 2012a; Brijs et al., 2015; Ekström et al., 2016; Devor et al., 2016). Furthermore, the U_{TL} of *B. medius* was only 0.55 and 0.23°C higher than *F. lapillum* under normoxia and hyperoxia respectively even though $\dot{M}O_{2,max}$ in *B. medius* was substantially higher (32-42% greater $\dot{M}O_{2,max}$) under all conditions. These findings therefore contribute to a growing body of literature suggesting that the absolute thermal limits of fish is not controlled by an inability of the cardiorespiratory system to deliver oxygen to tissues (Healy and Schulte, 2012a; Wang et al., 2014; Brijs et al., 2015; Devor et al., 2016; Ekström et al., 2016; Ern et al., 2016b). These findings also suggest that the constraints that set the absolute thermal limits of these species develop at a similar temperature under normoxia and hyperoxia. What these constraints are and when they develop in relation to loss of equilibrium is unknown, but beyond this temperature any expansion of MS under hyperoxia would probably be inconsequential as death will ensue rapidly.

The most extreme rock pool temperature observed over a 3-week period in summer was 30.1°C (Fig. 1A). While this temperature is below the CT_{max} of each triplefin fish species it is within 2°C of their absolute thermal limits even under hyperoxic conditions. Moreover, the rate of temperature increase used to determine CT_{max} (2°C per h) was faster than the rate at which peak temperatures observed in rock pools were reached (1.3°C per h). Therefore as U_{TL} is often reduced under slower rates of warming (Mora and Maya, 2006) the absolute thermal limits of these species may be even closer to observed maximum rock pool temperatures under fully replicated rock pool conditions. This presents a cause for concern because climate change is expected to result in more frequent and extreme transient heat waves (Perkins et al., 2012), which could push temperature maximums of many rock pools beyond the tolerance limits of temperate intertidal triplefin fishes. In this scenario, intertidal fishes may experience habitat loss and increased risk of overheating, unless intergenerational adaption can select for more thermally tolerant phenotypes.

A limitation of the current study was that both species were acclimated to constant environmental conditions prior to the assessment of CT_{max}. In the wild, rock pool fish are exposed to constantly fluctuating physico-chemical conditions which could potentially condition CT_{max} beyond the levels observed in the present investigation. The influence of prior exposure to fluctuating temperature and other relevant environmental conditions on CT_{max} in intertidal fishes should be considered in future studies.

4.4.4 Conclusions

In the intertidal triplefins *B. medius* and *F. lapillum* hyperoxia increased $\dot{M}O_{2,max}$ and MS both at ambient temperature and following acute thermal ramping to temperatures observed in rock pools. This finding suggests that algal mediated hyperoxia in rock pools mitigates the collapse of MS in intertidal fish which would otherwise occur under normal air saturated oxygen conditions and thermal ramping. Retention of MS under hyperoxia offers several potential benefits to intertidal fish facing acute high temperature exposure including less severe constraint of aerobically demanding activities and mitigation of anaerobic stress. However, since hyperoxia did not meaningfully increase the absolute thermal limits of either species, further studies are required to establish if there are benefits of hyperoxia for intertidal fish, and at what temperatures they occur relative to loss of equilbrium. The CT_{max} of both *B. medius* and *F. lapillum* were less than 2°C above maximal observed rock pool temperatures,

even with hyperoxia. This confirms that these species, particularly the exclusively intertidal *B. medius*, resides in habitat where they are periodically exposed to temperatures close to their thermal tolerance limits. With more extreme heat waves predicted with climate change, intertidal triplefins may face loss of habitat and increased risk of heat death as rock pool temperature maximums increase. With this understanding, a comprehensive analysis of the likely impact of climate change on the availability of thermally suitable rock pool habitats is warranted.

CHAPTER 5: THE EFFECTS OF CHRONIC WARM ACCLIMATION ON AEROBIC METABOLISM, GROWTH PERFORMANCE AND UPPER THERMAL TOLERANCE LIMITS IN THE NEW ZEALAND TRIPLEFIN FISH *FORSTERYGION LAPILLUM*

Chapter published as: McArley, T.J, Hickey, A.J, Herbert, N.A. (2017) Chronic warm exposure impairs growth performance and reduces thermal safety margins in the common triplefin fish (*Forsterygion lapillum*). Journal of Experimental Biology. 220: 3527-3535.

5.1 Introduction

Understanding the physiological responses of organisms to temperature change and the limits of thermal tolerance will play a key role in determining a species susceptibility or resilience to climate change (Somero, 2012). It has recently been suggested that studies investigating the effects of climate warming may underestimate the impacts on populations if physiological tolerances to environmental extremes are not included, and if only resting physiological functions are considered (Sandblom et al., 2016). The need to examine the impacts of warming on chronic and acute thermal tolerance collectively may also be particularly important in species that occupy habitats, such as those within marine intertidal zones, which are exposed to large acute temperature fluctuations (Nilsson and Lefevre, 2016). As the climate warms these species not only have to tolerate chronic increases in mean ambient sea temperature, but also tolerate greater extremes of acute temperature change due to the forecasted increase in the frequency and severity of transient heat waves (Meehl and Tebaldi, 2004; Perkins et al., 2012). Despite the universal occurrence of rock pools along coastlines, and that predicted changes in climate will likely impact in these environments, there is a surprising lack of research regarding both the chronic *and* acute thermal tolerance of intertidal biota such as fish.

A potential impact of climate change is that chronic warming will demand changes in aerobic metabolism and cause adjustments to essential life history traits such as growth, reproduction, foraging and locomotion, which lead to changes in fitness (Pörtner and Knust, 2007; Pörtner and Farrell, 2008). This theory, known as oxygen and capacity limited thermal tolerance (OCLTT), has emerged as a prominent hypothesis linking the thermal limits of metabolism to impacts of climate warming on multiple levels of biological organisation in

aquatic ectotherms (Pörtner, 2010; Clark et al., 2013; Nilsson and Lefevre, 2016). Under the OCLTT, the availability of aerobic metabolic scope (MS, the degree to which an organism can increase oxygen consumption above basal requirement) is the primary determinant and window of a species' thermal tolerance. This thermal window encompasses an optimum temperature where MS peaks (T_{optMS}), upper and lower pejus temperatures that mark the onset of MS decline, and more severe upper and lower critical temperatures where MS is abolished and survival time is limited (Pörtner and Farrell, 2008). In the case of warming, MS is thought to decline at supra-optimal temperatures, because the capacity of the cardiorespiratory system is not capable of increasing maximum oxygen uptake ($\dot{M}O_{2max}$) to a rate that keeps pace with increasing basal energetic requirements (Pörtner and Farrell, 2008; Pörtner, 2010). Crucially, it is commonly stated that MS and performance measures such as growth, reproduction and locomotion are causally linked (Pörtner and Knust, 2007; Claireaux and Lefrancois, 2007; Pörtner and Farrell, 2008; Pörtner, 2010; Clark et al., 2013; Schulte, 2015; Lefevre, 2016). Despite initial enthusiasm for the OCLTT hypothesis its general applicability in explaining thermal tolerance and its value in evaluating the potential impacts of climate change has become increasingly questioned (Clark et al., 2013; Nilsson and Lefevre, 2016). A recent meta-analysis (Lefevre, 2016) has demonstrated that not all marine ectotherms display the expected "increase-optimum-decrease" temperature response of MS that is a central pillar of the OCLTT hypothesis and that species of interest require assessment on a case by case basis to examine the temperature dependence of organismal performance and fitness (Nilsson and Lefevre, 2016). Other studies have also found the optimum temperature for performance and MS are not always matched (Grans et al., 2014; Norin et al., 2014) and, overall, there are actually few studies linking key performance metrics such as growth to MS *under* chronic thermal exposures (Clark et al., 2013).

The critical thermal maximum (CT_{max}) is the temperature at which an organism loses equilibrium during acute thermal ramping and is used as a measure of upper thermal tolerance in fish (Beitinger et al., 2000; Mora and Maya, 2006). Fish occupying rock pools may already experience acute exposure to temperatures exceeding critical limits, and climate change will likely increase temperature extremes further as heat waves are predicted to become more extreme (Perkins et al., 2012). In fish, warm acclimation can elevate upper thermal tolerance (Beitinger et al., 2000; Fangue et al., 2006; Drost et al., 2016); this indicates physiological plasticity may buffer a species' thermal limits as the climate warms. However, evidence suggests that this capacity to elevate the upper thermal tolerance point is

likely limited in the context of predicted temperature rises associated with climate change (Gunderson and Stillman, 2015). Moreover, the increase in CT_{max} with warm acclimation so far has always been less than the difference between two acclimation temperatures (Gunderson and Stillman, 2015). Consequently thermal safety margins (the difference between acclimation temperature and CT_{max}) are reduced with warming (Sandblom et al., 2016). Although rock pools are among the most likely habitats where the CT_{max} of fish is exceeded (and this risk increases with climate change) we are unaware of any study which specifically addresses the acclimation capacity of CT_{max} in intertidal fish in the context of climate change.

The upper critical temperature (T_{critMS}), not to be confused with CT_{max}, is incorporated into the OCLTT as the temperature at which MS becomes zero (Pörtner, 2010). Since warm acclimation increases the upper thermal tolerance of fish, this should also be associated with metabolic adjustments, which increase or maintain MS at high temperatures. Indeed, under the OCLTT, improved upper thermal tolerance in warm acclimated organisms is proposed to result from downregulation of mitochondrial function, which decreases resting oxygen demand and protects MS during warming (Pörtner, 2001). Conversely, cold acclimated organisms have higher mitochondrial densities to maintain capacity for MS and adenosine triphosphate (ATP) production under cold temperatures but this also increases oxygen demand during warming and decreases upper thermal tolerance (Pörtner, 2001; Fangue et al., 2009). Thus, at an equivalent elevated temperature, a thermally tolerant warm acclimated ectotherm would have a lower metabolic rate than that of a less tolerant individual acclimated to cooler temperatures (Fangue et al., 2009).

Forsterygion lapillum is a small (20-60 mm) benthic marine blennioid fish that occupies shallow subtidal reefs, harbours and intertidal rock pools of New Zealand. This species maintains MS across a thermal acclimation range of 15°C - 25°C and shows a surprisingly high T_{optMS} of 24°C (Khan et al., 2014a). Under the OCLTT framework, a T_{optMS} of 24°C would indicate that whole animal performance may be optimised at a temperature beyond the current day range, but this seems questionable since significant mortality (50%) occurs upon acclimation to 25°C, and skeletal muscle mitochondrial ATP production capacity shows signs of compromise at 24°C (Khan et al., 2014a). As an in-depth understanding of temperature-specific performance is not known for this species, the possibility of optimised performance at temperatures beyond current day conditions remains unclear.

As *F. lapillum* occupies intertidal rock pools *and* shallow subtidal habitats, this study set out to critically evaluate the chronic *and* acute thermal tolerance of *F. lapillum* after 12 weeks of thermal acclimation to 15°C, 18°C, 21°C and 24°C. To critically evaluate the growth performance and resilience of *F. lapillum* to climate change, the weight-specific growth rate was first measured at the 4 acclimation temperatures. It was hypothesised that the fitness performance of the 24°C group (i.e. fish chronically exposed to temperatures ~3°C above the current mean peak summer sea surface temperature (SST) in northern New Zealand) would be negatively impacted. As part of this testing, MS at the 4 temperatures was also measured and compared against growth to determine if growth performance and metabolic performance correlated across an extended timeframe as expected under the OCLTT framework. Combining archival sea surface temperature (SST) records with the temperature-growth data of *F. lapillum* then provided the opportunity to model the seasonal growth impact of future warming scenarios within the Northern geographical range of its distribution. Following the growth trial, the acute thermal tolerance (CT_{max}) of thermally-acclimated *F. lapillum* (15°C, 18°C, 21°C and 24°C) was then assessed to gauge whether plasticity in upper thermal tolerance exerts any influence over the ability of *F. lapillum* to withstand short term extremes in temperature during a thermal ramping test. We hypothesised that higher acclimation temperatures would increase upper thermal tolerance (Beitinger et al., 2000) and that this would associate with a lower metabolic rate on thermal ramping, as predicted by the OCLTT (Fangue et al., 2009).

5.2 Materials and Methods

5.2.1 Experimental animals

Wild *F. lapillum* were caught using bait catcher traps and transferred to the Leigh Marine Laboratory and housed in 30 L flow through ambient temperature seawater tanks (14.7°C ± 0.16, air saturated, 200 μm filtered, 35 ppt salinity) for a period of 14 days to allow laboratory acclimation. These fish had an initial total mean length of 55.3 mm (±0.55) and mass of 1.53 g (±0.03) and were sexually mature 1-2 year old fish based on length at age determinations for this species (Caiger, 2017). During this acclimation period they were fed daily to satiation on a mixture of crushed aquaculture pellets (Skretting, Australia) and

chopped pilchard. Animal ethics approval was granted from The University of Auckland Animal Ethics Committee (AEC approval 001801).

5.2.2 Growth trial

For the duration of the growth trial the fish were housed in 15 L flow through seawater tanks (air saturated, 200 μm filtered, 35 ppt salinity) maintained at the target experimental temperatures of either 15°C, 18°C, 21°C or 24°C (±0.3°C). The temperature was maintained in each tank by continually heating or cooling water to the required temperature with heat pumps. Each heat pump received seawater from insulated tanks which were continuously fed with fresh ambient temperature seawater (200 μm filtered, 35 ppt salinity) from the main laboratory supply. Three replicate tanks were each randomly assigned 8 fish at the four temperatures (N=24 fish per temperature). Each individual was tagged with visible subcutaneous elastomer (NMT INC Northwest Marine Technology, USA) and allowed to recover over 24 h at 15°C without feeding. The initial mass and length of each fish was then measured and the temperature of each tank was raised to the target experimental temperature at a rate of 3°C per 24 h^{-1}. Mass-specific growth rates (SGR % body mass day^{-1}) of individual fish were then assessed at 30 day intervals over a 12 week period according to:

$$SGR = \ln m_2 - \ln m_1 \,/\, t_2 - t_1 \times 100, \quad (6)$$

where, m_1 is the initial weight at the start of the growth period t_1 and m_2 is the final weight at the end of the growth period t_2. The overall growth rate of individual fish was taken as the mean SGR of the three 30 day growth periods. Throughout the growth trial each tank was fed daily to satiation on a mixture of crushed aquaculture feed (Skretting, Australia) and chopped pilchard. Ammonia, nitrate and nitrite were regularly measured in each tank and water quality remained high throughout the trial.

5.2.3 Respiromerty

At the conclusion of the growth trial, metabolic rate and acute thermal tolerance were then assessed in all individuals. Metabolic and thermal tolerance measurements were carried out in an automated respirometry set-up which could monitor four fish simultaneously in individual chambers (~20 h per run). All trials were completed over 35 days and, to avoid

confounding handling effects on metabolic measurements, growth measurements were not assessed during this period. The temperature treatment for each set of four fish was assessed in a cycled order so that the period between the conclusion of the 12 week growth trial and assessment of metabolic rate and upper thermal tolerance was as similar as possible across treatment groups.

The mass-specific O_2 consumption rate ($\dot{M}O_2$), reported as mg of oxygen consumed per g of body weight per hour (mg O_2 g^{-1} h^{-1}), was determined using automated intermittent-flow respirometry (Steffensen, 1989). Custom built respirometry chambers (42-96 mL and size matched to fish mass) were held within a 60 L reservoir filled with filtered (5 µm) UV-sterilised seawater, which was heated or chilled to the experimental temperature by continually pumping the seawater through a 40 L cooling tower containing an aluminium water cooled coil. The inlet of each chamber was connected to an automated Eheim compact 3000 submersible flush pump (EHEIM GmbH & Co. KG, Germany) which was switched on and off by a relay control unit (USB Power 8800 Pro, Aviosys International Inc, Taiwan) controlled by custom-coded software (Leigh Marine Laboratory). A magnetic stir bar was housed in a recess in the bottom of the respirometry chamber to ensure adequate water mixing and the oxygen concentration of water within the chamber was continuously measured using contactless sensor spots followed by FireSting O_2 meters (PyroScience, Aachen, Germany). The decline in O_2 concentration within a respirometry chamber was used to calculate $\dot{M}O_2$ in repeated measurement cycles (4-8 min) according to the equation:

$$\dot{\mathrm{M}}O_2 = V\left(\Delta\%\frac{sat}{t}\right)a.M_B, \quad (7)$$

where, V is the respirometry chamber minus fish volume, $\Delta\%\ sat/t$ is the change in oxygen saturation per unit time, α is the solubility coefficient of oxygen (mg O_2 %Sat^{-1} L^{-1}) in sea water (35 ppt), and M_B is the body mass of the fish in grams (Schurmann and Steffensen, 1997). The repeated measurement cycles were interspersed by automated flushing periods (1 min) so that the water within the chamber was refreshed and maintained at an O_2 concentration above 80% air saturation.

Metabolic measurements were completed in a sequence where $\dot{M}O_{2max}$ was assessed after exhaustive exercise and was then followed by measures of standard metabolic rate (SMR) during an overnight period of respirometry. $\dot{M}O_{2max}$ was determined following exhaustive exercise where fish were continually chased by hand for a period of 5 min. The chasing was carried out in a 30 L tank filled with air saturated sea water maintained at the

required experimental temperature. The fish were visibly exhausted after chasing and this method was used as it has been previously used to elicit maximal $\dot{M}O_2$ values in *F. lapillum* (Khan et al., 2014a) and is the most reliable method for obtaining $\dot{M}O_{2max}$ in benthic species that do not swim continuously in a flume (Clark et al., 2013; Norin and Clark, 2016). The fish was then transferred to a respirometry chamber within 30 s of the conclusion of chasing and repeating 4 min $\dot{M}O_2$ measurement cycles were initiated. $\dot{M}O_{2max}$ was taken as the highest $\dot{M}O_2$ value recorded in any measurement cycle, which in almost all cases was obtained from the first measurement cycle following exhaustive exercise. When the metabolic rate of fish was clearly declining, the measurement period was extended to 8 min and $\dot{M}O_2$ measured repeatedly for >16 h. During overnight $\dot{M}O_2$ measurements fish were left undisturbed in the respirometers and SMR was estimated from the mean of the lowest 10% of $\dot{M}O_2$ over this time (Khan et al., 2014a; Norin et al., 2014). MS was defined as the difference between the body mass corrected $\dot{M}O_{2max}$ and SMR (see below) for each individual. In all estimates of metabolic rate only $\dot{M}O_2$ values with R^2 of >0.95 for the decline in oxygen per unit of time were used.

5.2.4 Acute thermal tolerance and critical thermal maximum

Following SMR estimation, fish were subjected to thermal ramping to determine CT_{max}. In all cases thermal ramping for an individual fish began from the experimental temperature a particular fish had been acclimated to in the growth trial phase of the study. The temperature of the seawater reservoir was increased at a rate of 4°C h^{-1} and water temperature was continuously measured using a shielded temperature probe connected to a FireSting O_2 system (PyroScience, Aachen, Germany). After every 1°C increment in temperature the routine $\dot{M}O_2$ (RMR) of fish was measured over a measurement cycle length of 3-5 min. Thermal ramping and $\dot{M}O_2$ measurements continued until the point at which fish lost equilibrium for 10 s or more which was taken as the CT_{max} (Beitinger et al., 2000; Brijs et al., 2015; Sandblom et al., 2016). The fish was then removed from the respirometer and transferred to air saturated ambient temperature seawater (~21°C) for recovery. The maximum routine metabolic rate during thermal ramping (RMR_{maxTR}) was taken as the highest $\dot{M}O_2$ measurement obtained at any of the 1°C temperature increments prior to reaching the point of CT_{max}. The thermal safety margin for each fish was also calculated and

was defined as the temperature difference between CT_{max} and acclimation temperature (Sandblom et al., 2016).

Background oxygen consumption was measured immediately after thermal ramping. Pilot trials showed background oxygen consumption remained negligible throughout the overnight SMR measurement cycles but developed linearly during thermal ramping. A linear regression was therefore used to back calculate the background oxygen consumption values throughout thermal ramping and these were subtracted from fish $\dot{M}O_2$. The mean background oxygen consumption at 15°C, 18°C, 21°C and 24°C was 3.2 (±0.72), 3.4 (±0.5), 3.9 (±0.64) and 4.7 (±0.6) % of fish $\dot{M}O_2$ during thermal ramping respectively.

5.2.5 Scaling of metabolic measurements and statistics

To account for body mass differences the $\dot{M}O_2$ values were standardised to the mean body mass of all fish (2.5g) using the formula:

$$\dot{M}O_{2(2.5g)} = \dot{M}O_{2(meas)} \left(\frac{w}{w_{(2.5g)}}\right)^{(1-A)}, \quad (8)$$

where $\dot{M}O_{2(2.5g)}$ is the $\dot{M}O_2$ for a fish with the standardized (corrected) new weight of 2.5g, $\dot{M}O_{2(meas)}$ is the measured $\dot{M}O_2$, w is the weight of the fish, $w_{(2.5g)}$ is the standardized body mass of fish set to 2.5g and A is the weight exponent describing the relationship between metabolic rate and body weight (Schurmann and Steffensen, 1997). The mass exponent (A) describing the relationship between body mass and $\dot{M}O_2$ for SMR and $\dot{M}O_{2max}$ were 0.75 and 0.65 respectively and were derived from a dataset of triplefin $\dot{M}O_2$ measurements collected across multiple studies (T. McArley unpublished data). The values for these mass exponents are within the expected range for fish (Clarke and Johnston, 1999).

In all statistical tests, significance was accepted at $P < 0.05$. Mixed model analysis was used to perform a nested analysis of covariance and assess the effect of acclimation temperature on growth rate (SGR) following the model fitting procedure outlined by Zurr et al., (2009). The final model included initial fish mass as a covariate, temperature as a fixed main effect and, to control for possible tank effects, individual tanks were included as fixed random effects. The model assumptions of normality and homoscedasticity were satisfied and specific *post-hoc* comparisons were carried out using Tukey contrasts. The relationship

between SGR and temperature was also assessed with a second-order (non-linear) polynomial regression. To examine how climate warming may impact annual patterns of growth, the relationship between temperature and SGR derived from the laboratory based growth trial (Fig.1B) was used to calculate projected growth rates over an annual present day temperature regime and annual temperature regimes based on two warming scenarios (present day temperature +1.5°C and +3°C). The annual present day temperature regime was based on the 7 day temperature means from a 40 year SST record from the Leigh Marine Laboratory, which is in the North Eastern region of this species' geographical range and where the *F. lapillum* used in this study were sourced. SMR, $\dot{M}O_{2max}$, MS, CT_{max}, thermal safety margin and RMR_{maxTR} were each compared between acclimation temperatures using one way analysis of variance (ANOVA) with Holm-Sidak *post-hoc* comparisons and individual tanks treated as replicates (N=3 per temperature). The relationship between MS and temperature was also assessed with a second-order (non-linear) polynomial regression and linear regression was used to examine the relationship between SGR and MS across tank replicates. RMR during thermal ramping was compared between temperature acclimation treatments using two way repeated measures ANOVA with individual tanks set as subjects. Thermal ramping for each temperature acclimation treatment started at a different temperature so RMR was compared over a standardised temperature range of 24°C to 28°C. This temperature range was chosen as it was common to all four acclimation temperatures and did not include temperatures where any fish had obviously declining $\dot{M}O_2$ or had lost equilibrium. Finally, the relationship between RMR at 24°C and CT_{max} and RMR at 28°C and CT_{max} was examined using linear regression. All statistical tests were carried out using R (version 3.1.1) and Sigma Plot (Systat Software Inc, California, USA).

5.3 Results

5.3.1 Mortality

There were 9 mortalities during the 12 week growth trial but there were no significant differences in mortality rate between the temperature acclimation treatments (Chi-square= 2.33, df=3, P = 0.507). The surviving fish appeared in good health at all temperatures with no obvious signs of disease or abnormal behaviour indicative of a compromised health status.

5.3.2 Chronic thermal tolerance and the relationship between growth rate, metabolic rate and temperature

SMR was significantly different between the temperature acclimation treatments (ANOVA, df=3, F=20.2, P<0.001) and there was an overall trend of SMR to increase with higher temperature (Fig. 5.1A). *Post-hoc* tests showed SMR was significantly higher at 24°C than at 21°C (P=0.027), 18°C (P=0.002) and 15°C (P<0.001), and significantly higher at 21°C compared to 15°C (P=0.024). $\dot{M}O_{2max}$ appeared lowest in fish acclimated to 24°C but no significant differences (ANOVA, df=3, F=2.79, P=0.11) were found between temperature treatment groups (Fig. 5.1A). A parabolic relationship described the effect of acclimation temperature on MS, with MS peaking at 18.2°C (Fig. 5.1A). There was a significant main effect of temperature on MS (ANOVA, df=3, F=10.58, P=0.004) and *post-hoc* tests showed significantly lower MS at 24°C than at 15°C (P=0.019), 18°C (P=0.005) and 21°C (P=0.011). Fish held at all temperatures gained mass during the 12 week temperature acclimation period and there was a parabolic relationship between SGR and temperature with the highest growth rate seen at 18°C (0.68 % bm d^{-1} ± 0.041) but lower growth rates at 21°C (0.59 % bm d^{-1} ± 0.047), 15°C (0.51 % bm d^{-1} ± 0.034) and 24°C (0.39 % bm d^{-1} ± 0.048) (Fig. 5.1B). Mixed model analysis confirmed a significant main effect of temperature (df= 3, F= 5.28, P=0.027) on specific growth rate and *post-hoc* comparisons showed growth rate was significantly higher at both 18°C (P<0.001) and 21°C (P=0.041) compared to 24°C. The similar pattern in SGR and MS across the temperature acclimation treatments resulted in a significant linear relationship between these variables (R^2=0.48, P=0.01) with an overall trend for higher SGR to be associated with greater MS across the individual tank replicates (Fig. 5.1C).

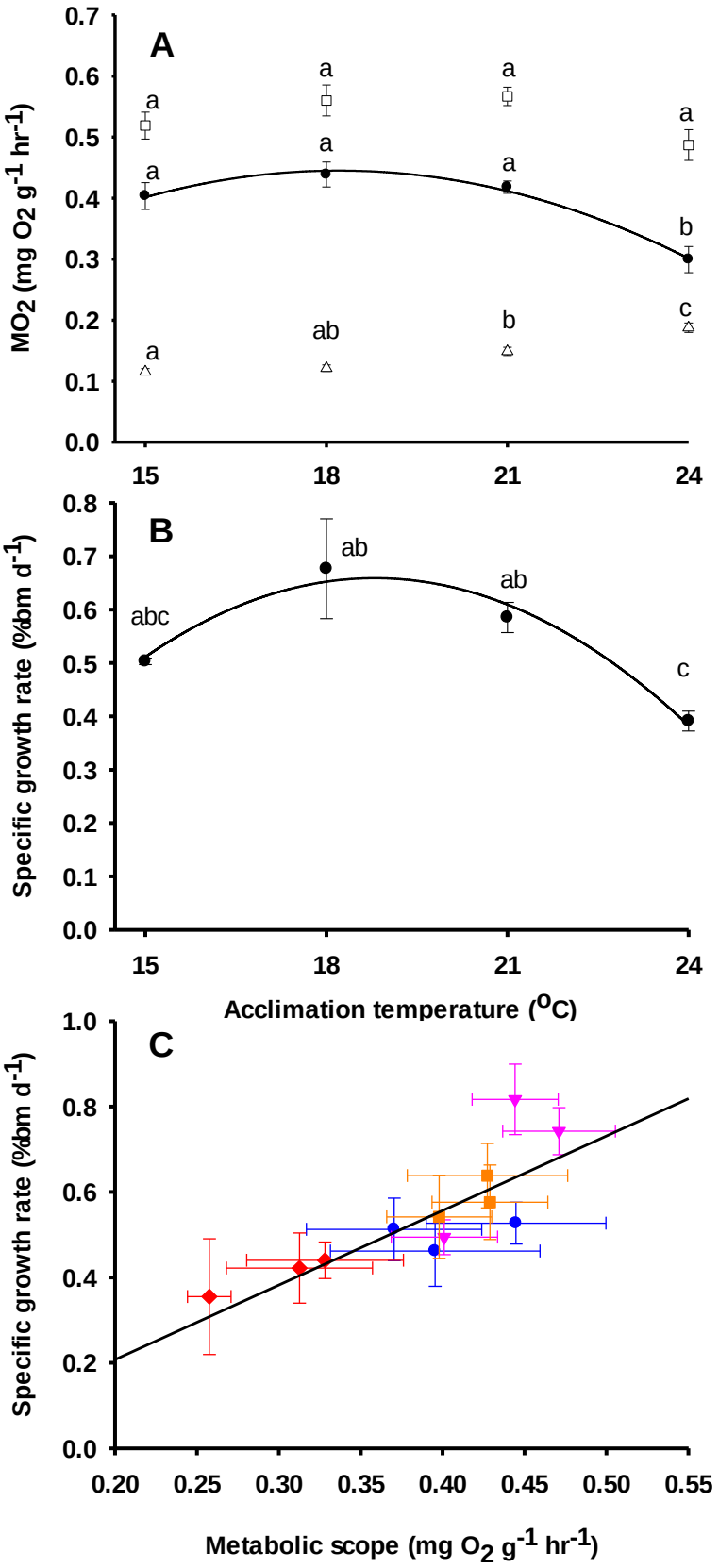

Figure 5.1 Metabolic rate and growth responses of *Forsterygion lapillum* acclimated to 15 °C, 18 °C, 21 °C and 24 °C. A: Mean (± s.e.m.) standard metabolic rate (open triangles), maximum metabolic rate (open squares) and aerobic metabolic scope (closed circles) (N=3 tank replicates for each temperature, N=18-23 fish per temperature). Significant differences (one-way ANOVA, $P < 0.05$) are shown for each of standard metabolic rate, maximum metabolic rate and aerobic metabolic scope by lower case letters which are not common between acclimation temperatures. The black line shows a polynomial (quadratic) regression ($y= -0.0043_x^2 + 0.1556_x -0.9709$, $R^2=0.79$, $P=0.0008$) between acclimation temperature and aerobic metabolic scope. B: Mean (± s.e.m.) specific growth rate (N=3 tank replicates for each temperature, N=20-23 fish per temperature). Significant differences ($P < 0.05$) are shown by lower case letters which are not common between temperatures. The black line shows a polynomial (quadratic) regression ($y -0.0102_x^2 + 0.3837_x -2.9479$, $R^2=0.67$, $P = 0.0069$) between temperature and specific growth rate. C: Mean (± s.e.m.) specific growth rate and aerobic metabolic scope of individual tank replicates (blue circles=15 °C, pink triangles=18°C, orange squares= 21°C, red diamonds=24°C, N=3 tank replicates for each temperature). The black line shows a linear regression between specific growth rate and aerobic metabolic scope ($R^2=0.69$, $P<0.001$).

5.3.3 Projected annual patterns of growth under present day and future (warming) temperature regimes

Under a present day annual temperature regime, optimum temperatures for growth in *F. lapillum* occur over the warmer summer and early autumn months between December and May but growth potential drops substantially over the cooler winter period between June and November (Fig. 5.2). Under a scenario where the temperature at all times of the year is 3°C warmer than present day, optimum temperatures for growth occur over late autumn, winter and spring (May to November) whereas growth potential declines substantially over the warm summer months (Fig. 5.2). Despite substantial changes in the projected annual pattern of growth under warming scenarios, only minor differences are projected in the mean annual growth rate of *F. lapillum* where a small but possibly insignificant increase exists compared to present day under +1.5°C and +3°C warming scenarios (Fig. 5.2).

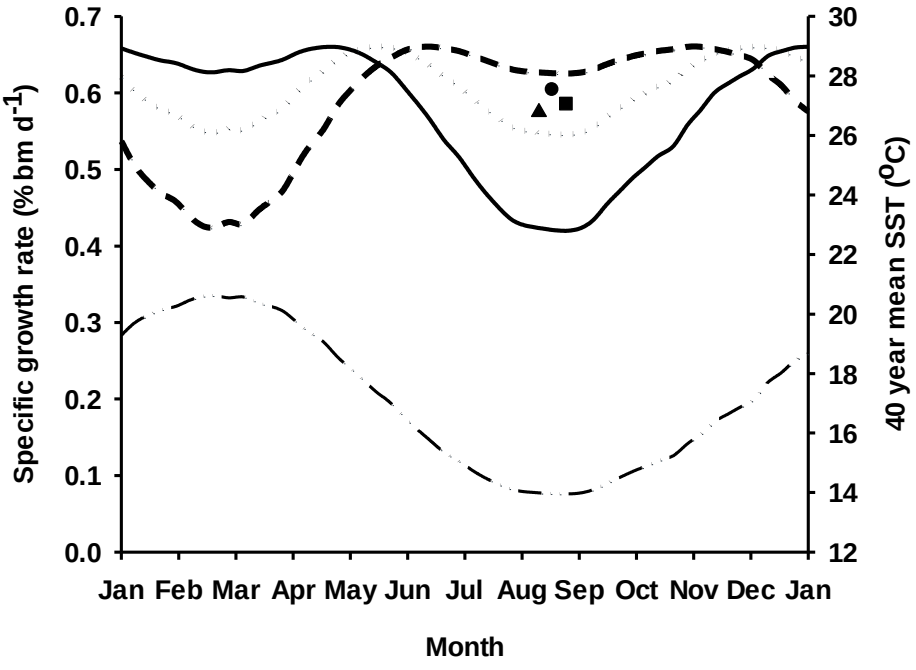

Figure 5.2 Projected annual cycle of growth performance in *Forsterygion lapillum* under present day and future (warming) temperature regimes. All values of projected specific growth rate (left axis) are calculated from a significant non-linear relationship between growth rate and temperature described by the equation $y=-0.0102_x^2 + 0.3837_x - 2.9479$ (Fig. 1B), where y is specific growth rate (% body mass per day) and x is temperature (°C). 40 year mean sea surface temperature (SST) from the Leigh Marine Laboratory site (dotted and dashed line, right axis) was used to calculate projected specific growth rate throughout the year for 40 year mean SST (solid line), 40 year mean SST + 1.5°C (dotted line), and 40 year mean SST + 3°C (dashed line). Symbols represent the mean annual projected growth rate for 40 year mean SST (triangle), 40 year mean SST + 1.5°C (circle), and 40 year mean SST + 3°C (square).

5.3.4 Acute thermal tolerance

Following thermal acclimation, CT_{max} was highest in fish acclimated to 24°C and lowest in those acclimated to 15°C and there was a significant main effect of acclimation temperature (ANOVA, df=3, F=32.39, P<0.001) (Fig. 5.3). *Post-hoc* tests showed fish acclimated to 24°C and 21°C had significantly higher CT_{max} than fish acclimated to 18°C (P=0.004, P=0.007) and 15°C (P<0.001, P<0.001) and fish acclimated to 18°C had significantly higher CT_{max} than fish acclimated to 15°C (P=0.015). The difference between acclimation temperature and CT_{max} (thermal safety margin) was progressively reduced with increasing acclimation temperature and there were significant differences in thermal safety margin between all temperature acclimation treatments (ANOVA, df=3, F=570.87, P<0.001) (Fig. 5.3).

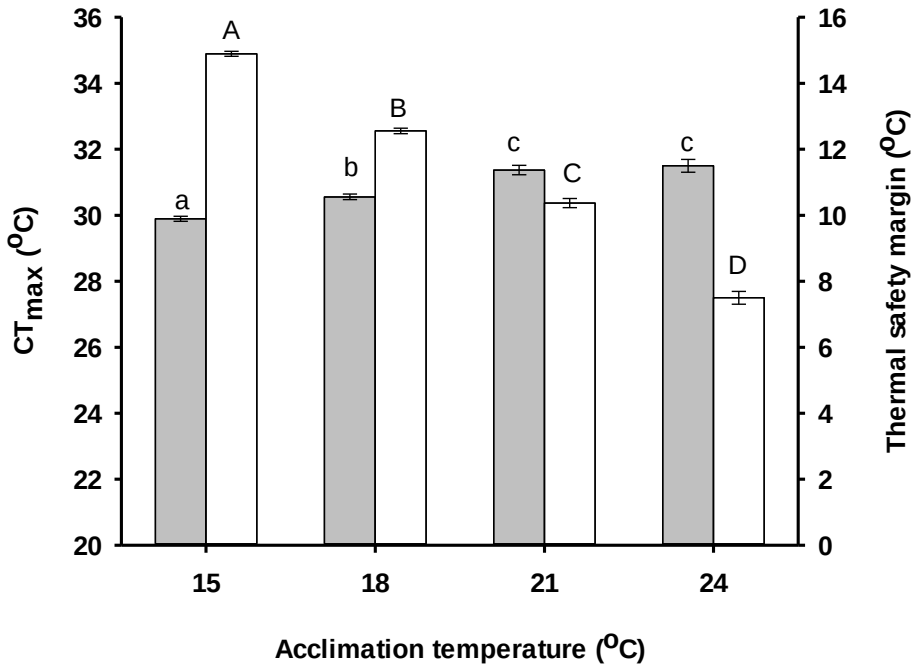

Figure 5.3 Upper thermal tolerance in *Forsterygion lapillum* acclimated to 15°C, 18°C, 21°C and 24°C. Grey and white bars show the mean (± s.e.m.) critical thermal maximum (CT_{max}, left-hand axis) and thermal safety margin (right-hand axis) respectively (N=3 tank replicates for each temperature, N=18-23 fish per temperature). The thermal safety margin is the difference between acclimation temperature and critical thermal maximum temperature. Dissimilar lower case and upper case letters above bars indicate significant differences (one-way ANOVA, p<0.05) between acclimation temperatures for CT_{max} and thermal safety margin respectively.

5.3.5 Metabolic rate during thermal ramping

During thermal ramping, the RMR of fish acclimated to all temperatures increased to a peak in an exponential manner and then declined suddenly (Fig. 5.4A). Across a common temperature range (24°C-28°C), there was a significant effect of acclimation temperature (ANOVA, df=3, F=17.13, P<0.001) and thermal ramping temperature (ANOVA, df=4, F=195.6, P<0.001) on RMR, and the interaction was significant (ANOVA, df=12, F=2.59, P=0.016). *Post-hoc* tests showed that RMR increased significantly (p<0.05) between 24°C and 28°C, but there were also significant differences between acclimation temperatures that were dependent on thermal ramping temperature. Between 24°C and 26°C, RMR was significantly higher in fish acclimated to 15°C and 18°C than in fish acclimated to 21°C and 24°C (Fig. 5.4A). However, at 27°C and 28°C, fish acclimated to 24°C had significantly lower RMR than those acclimated to 15°C, 18°C and 21°C, and those acclimated to 21°C had significantly lower RMR than 15°C acclimated fish (Fig. 5.4A). RMR attained a similar maximal $\dot{M}O_2$ value in the 15°C (0.58 mg O_2 g^{-1} hr^{-1} ± 0.028), 18°C (0.59 mg O_2 g^{-1} hr^{-1} ± 0.006) and 21°C (0.56 mg O_2 g^{-1} hr^{-1} ± 0.021) groups, but appeared to increase less in the 24°C group (0.49 mg O_2 g^{-1} hr^{-1} ± 0.015) (Fig. 5.4A & 5.4B). As a result, peak values of RMR during thermal ramping were significantly different between the temperature acclimation groups (ANOVA, df=3, F=4.98, P=0.031), and *post-hoc* tests confirmed that fish acclimated to 24°C had significantly lower RMR_{maxTR} than those acclimated to 18°C (P=0.043) (Fig. 5.4B). Interestingly, there was a significant inverse linear relationship between RMR and CT_{max} at 24°C (R^2=0.67, P=0.001) and also between RMR and CT_{max} at 28°C (R^2=0.48, P=0.013). As such, low RMR was consistently associated with a high CT_{max} and vice versa (Fig. 5.5).

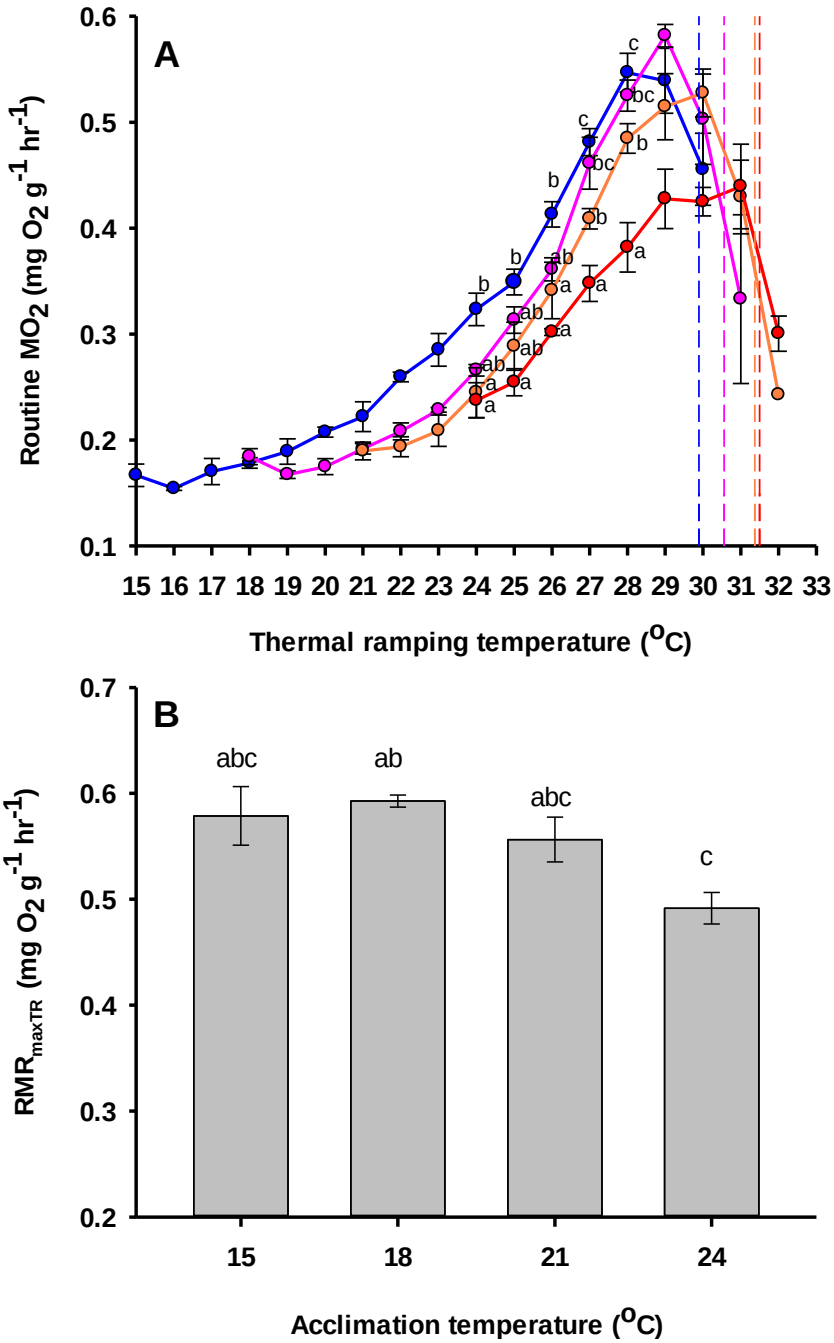

Figure 5.4 Routine metabolic rate during acute thermal ramping in *Forsterygion lapillum* acclimated to 15°C, 18°C, 21°C and 24°C. A: Routine metabolic rate ($\dot{M}O_2$) during thermal ramping. Data points represent the mean (± s.e.m.) of tank replicates (N=3 tank replicates for each acclimation temperature, N=18-23 fish per temperature). Blue=15°C, Pink=18°C, Orange=21°C, Red=24°C. Hatched vertical lines represents the mean critical thermal maximum for reference (i.e. data from Fig. 3). Lower case letters not shared between data points show significant differences ($P<0.05$) in routine metabolic rate among temperature acclimation treatments at thermal ramping temperatures between 24°C and 28°C. B: Maximum routine metabolic rate ($\dot{M}O_2$) during thermal ramping (RMR_{maxTR}). Data points represent the mean (± s.e.m.) of tank replicates. Lower case letters not shared between data points show significant differences (one-way ANOVA, $P<0.05$).

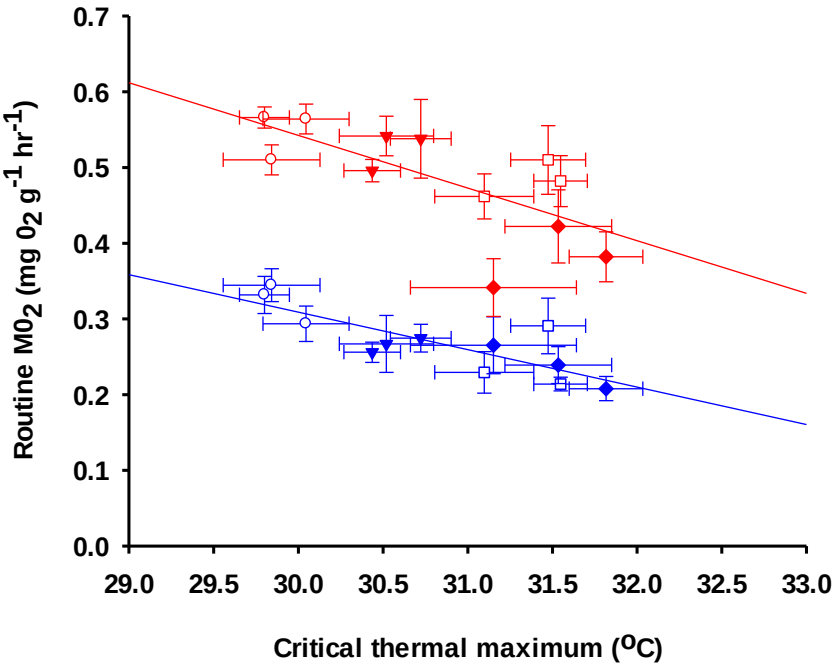

Figure 5.5 The relationship between routine metabolic rate during thermal ramping and critical thermal maximum in *Forsterygion lapillum* acclimated to 15°C, 18°C, 21°C and 24°C. Data points represent mean (± s.e.m.) routine $\dot{M}O_2$ at 24°C (blue symbols) and 28°C (red symbols), and mean critical thermal maximum (± s.e.m.) of each tank replicate (open circles=15°C, closed triangles=18°C, open squares= 21°C, closed diamonds=24°C, N=3 tank replicates for each temperature). The blue line shows a linear regression between routine $\dot{M}O_2$ at 24°C and critical thermal maximum (R^2=0.67, P<0.01) and the red line shows a linear regression between routine $\dot{M}O_2$ at 28°C and critical thermal maximum (R^2=0.48, P<0.05).

5.4 Discussion

To better understand how global warming may affect fish populations and their ecosystems, previous research has stimulated the need for more data on the response of respiratory variables, fitness related variables and upper thermal tolerance limits to warming in species of interest (Nilsson and Lefevre, 2016). In this respect the current research has adopted a novel approach in addressing growth and metabolic responses of the common triplefin fish to chronic *and* acute thermal changes. This provides insights to possible impacts of climate change for a highly abundant coastal species that resides in shallow subtidal and intertidal habitats. This work also allows an opportunity to assess the relevance of the OCLTT hypothesis in chronic and acute thermal tolerance.

5.4.1 Impact of chronic warm exposure on growth performance and metabolic scope

There are few studies which allow for the potential effects of thermal acclimation of metabolism on MS under chronic time scales (weeks to years) (Norin et al., 2014; Sandblom et al., 2014; Sandblom et al., 2016) and even fewer that include measurements of growth (Grans et al., 2014; Khan et al., 2014b; Donelson, 2015). In the warm northern geographical range of *F. lapillum*, mean SST peaks in February (20.6°C Leigh Laboratory SST record) and remains within 0.5°C of this value for a period of 11 weeks over the austral summer. Thus tolerance assessments at higher mean temperatures approximating future temperature predictions should be tested over a similar timeframe. This justifies our 12 week period of continuous temperature acclimation.

Chronic exposure to 24°C resulted in decreased growth rates and MS compared to lower temperatures (15°C, 18°C and 21°C). Our data therefore indicates that an average sea temperature rise to 24°C due to climate change will impair the whole animal and metabolic performance of this species during the hottest months of the year. Depressed MS at 24°C was driven by higher SMR, and a marginally decreased $\dot{M}O_{2max}$ in comparison to fish acclimated to lower temperatures. The metabolic response of *F. lapillum* to chronic warming therefore follows the response pattern outlined in the OCLTT, where maximum aerobic capacity is limited, and warming beyond an optimal temperature increases basal costs and reduces MS (Pörtner and Knust, 2007; Pörtner and Farrell, 2008; Pörtner, 2010). However, this pattern of restricted MS at higher temperature is not universal across species, as the MS of some fish increases continuously with temperature (i.e. it does not peak) (Lefevre, 2016), due to the expansion of $\dot{M}O_{2max}$ at higher temperatures. If MS theoretically sets a limit to the extent at which aerobically demanding processes can be performed beyond maintenance requirements (Clark et al., 2013), it is perhaps not surprising that the growth of *F. lapillum* fell at higher temperatures. MS was decreased and competing oxygen demanding processes were potentially partitioned or prioritised across a shrinking pool of aerobically derived energy. While the correlation between MS and growth found in this study is not unique (Jobling, 1981; Claireaux and Lefrancois, 2007; Khan et al., 2014b), there are limited studies linking key performance measures to MS under chronic temperature exposures (Clark et al., 2013). Other studies have found no association between temperature optimums for growth and MS in fish (Healy and Schulte, 2012a; Norin et al., 2014; Grans et al., 2014) and it is has become

apparent that, while the OCLTT may be applicable in some species, it is not universal (Nilsson and Lefevre, 2016; Lefevre, 2016).

Although it was not quantified, egg deposition was evident during the growth trial at 15˚C, 18˚C and 21˚C but not at 24˚C. This reproductive activity was unavoidable as wild *F. lapillum* spawn year round (Wellenreuther and Clements, 2007). Despite investment of energy in reproductive activity at 15˚C-21˚C these fish still grew faster than fish at 24˚C where no reproductive activity was observed. The absence of reproductive activity at 24˚C in this study could mean that spawning activity is constrained to cooler months if predicted warmer summertime conditions eventuate. Studies which specifically aim to address the influence of predicted future temperature regimes on the seasonality and performance of reproductive activity in this species would be of interest.

The pattern of SMR, $\dot{M}O_{2max}$ and MS in the current study contrasts with previous work for *F. lapillum* where, despite increasing SMR with higher temperature, a peak in $\dot{M}O_{2max}$ at 24°C resulted in higher MS at 24°C than at lower temperatures (Khan et al., 2014a). However, Khan et al. (2014a) acclimated fish for only 4 weeks (vs. 12 weeks in the current study), and fish may therefore have been in a different physiological state. The discrepancy in these findings highlights the importance of acclimation length in studies of this nature and future studies investigating the time course of physiological adjustments in this species would be valuable. The different pattern of MS may also result from methodological differences, as $\dot{M}O_{2max}$ was estimated over a substantially longer period by Khan et al. (2014a), and this may have underestimated the $\dot{M}O_{2max}$ at lower temperatures.

5.4.2 The effect of warming on an annual cycle of growth

Few studies assess climate change with respect to seasonal growth. Despite the forecast of impaired growth at projected higher summer temperatures, when considered on an annual cycle and taking into account seasonal temperature fluctuations, the annual growth of *F. lapillum* might be slightly improved with a universal 1.5°C and 3.0°C increase in temperature (Fig. 5.2). Warmer summer temperatures due to climate change are expected to result in chronic exposure to temperatures beyond current day conditions and compromised growth for *F. lapillum.* However, the influence of warming on growth during late autumn, winter, and early spring may actually improve overall growth performance as temperature

shifts closer to an optimal range (90% of maximum) (16.5°C-20.5°C Fig. 5.1). The geographical range of this species extends to the southern end of New Zealand where mean sea temperatures are cooler at all times of the year. Therefore, if southern populations have optimum temperature ranges for growth similar to the northern populations considered in this study, warming may serve to improve growth performance as temperatures are shifted closer to the optimum range for growth at all times of the year. The predicted growth rates, however, need to be interpreted with caution as they are based on average growth rates measured over an extended period (12 weeks) of continuous exposure to a single temperature. Wild populations face gradually rising and falling temperatures in spring and autumn and thus may be in a continual state of acclimation during these months which could alter the temperature growth relationship. It is also not known how gradual seasonal temperature changes between summer highs and winter lows, as occurs over 3 to 4 months in natural environments, impacts the temperature growth relationship during the relatively thermally stable winter and summer periods that follow. While seasonal temperature fluctuations undoubtedly play a role in determining when a species can grow fast, other factors such as food availability and competition, also likely play an important role (Jones, 1986; Metcalfe, 1986). Consideration of how factors such as these are impacted by warming is required to develop a more complete picture of the growth response to climate change. Furthermore, the relationship between temperature and growth rate under laboratory conditions may not be applicable to wild populations.

5.4.3 The effect of warming on acute thermal tolerance

Chronically exposing *F. lapillum* to higher temperatures over 12 weeks increased its upper thermal tolerance limit (CT_{max}) and this has been reported for other fish species (Beitinger et al., 2000; Healy and Schulte, 2012a; Akhtar et al., 2013; Zhang and Kieffer, 2014; Donelson, 2015; McDonnell and Chapman, 2015; Sandblom et al., 2016; Drost et al., 2016). However, thermal safety margins were reduced progressively because the increase in CT_{max} under acclimation to higher temperature was proportionately less than the increase in acclimation temperature (i.e. acclimation response ratios are less than 1). Low CT_{max} acclimation response ratios appear to be common among ectotherms and suggests that plasticity in upper thermal tolerance has limited potential to reduce overheating risk (Gunderson and Stillman, 2015). Indeed, CT_{max} only increased 0.12°C between the

acclimation temperatures of 21°C and 24°C in the current study (Fig. 3), suggesting that *F. lapillum* exposed to temperatures beyond the current day range do not have the acclimatory capacity to further increase CT_{max}. As a consequence the thermal safety margin was reduced to its lowest value upon acclimation to 24°C.

While subtidal populations of *F. lapillum* reside in more thermally stable environments this species also occupies intertidal rock pools where large temperature fluctuations occur. The significant reduction in thermal safety margin upon acclimation to a predicted future summertime temperature could therefore threaten survival or restrict available habitat for these intertidal populations. The one-off exposure to an acute temperature rise in the current study does not fully represent rock pool conditions and it is acknowledged that repeated acute temperature rises (as occurs over a tidal cycle) may further condition upper thermal tolerance. However, there was no evidence of repeated heat shock improving CT_{max} of the common killifish (*Fundulus heteroclitus*) (Healy and Schulte, 2012a). Acute temperature rises in rock pools may also coincide with algal mediated photosynthetic hyperoxia, but hyperoxia also appears to have no benefit to the CT_{max} of fish (Rutledge and Beitinger, 1989; Healy and Schulte, 2012a; Brijs et al., 2015). Intergenerational or adaptive genetic responses in the upper thermal tolerance of *F. lapillum* could therefore be essential for mitigating overheating risk in a warmer future, although these too appear limited for fish upper thermal tolerance (Sandblom et al., 2016).

5.4.4 $\dot{M}O_2$ and acute thermal tolerance

The OCLTT, with the differential availability of MS as the primary mechanism, seeks to unify explanations of thermal tolerance across the optimum to critical temperature range (Pörtner and Farrell, 2008). There is a difference, however, between how upper thermal tolerance is defined under the OCLTT and the majority of studies investigating upper thermal tolerance in fish which use CT_{max} (i.e. the temperature at which equilibrium is lost). Under the OCLTT the upper critical temperature (T_{crit}) is the point at which metabolic scope is nil (Pörtner and Farrell, 2008; Farrell, 2016) and how the CT_{max} fits within the OCLTT framework is unclear (Healy and Schulte, 2012a; Farrell,2016). Regardless, warm acclimation increases upper thermal tolerance in fish and aerobic metabolism in fish with higher CT_{max} should reflect either an extension or retention of MS at high temperatures.

Theoretically upper thermal tolerance could be increased through MS in two ways: (1) maximum aerobic capacity (i.e. $\dot{M}O_{2max}$) could be expanded at temperatures approaching critical limits, and/or (2) basal metabolic costs (i.e. SMR) could be reduced at temperatures approaching critical limits. In consideration of the first scenario, this study found no evidence of an expansion of maximum aerobic capacity with acclimation to warmer temperatures. In fact, despite having the highest CT_{max}, acclimation to the warmest temperature of 24°C restricted maximum aerobic capacity when measured at acclimation temperature (Fig. 5.1) and also limited maximum RMR at temperatures approaching critical limits (Fig. 5.4A and 4B). Although $\dot{M}O_{2max}$ was not directly measured at high temperatures, the RMR approaching critical limits should reflect maximum capacity, because under the OCLTT framework SMR and $\dot{M}O_{2max}$ are the same at the upper critical temperature. This study finds more support for decreases in basal metabolic costs supporting improved upper thermal tolerance. Acclimation to increasingly warmer temperatures resulted in a lower RMR at equivalent temperatures during thermal ramping (Fig. 5.4a) and a lower RMR at 24°C and 28°C was associated with higher CT_{max} (Fig. 5.5). These findings therefore fit the theory that physiological adjustments occur under warm acclimation to prevent the occurrence of high basal energetic costs (Pörtner, 2001; Seebacher et al., 2010; Sandblom et al., 2014; Norin et al., 2014). Lower metabolic rates in warm acclimated fish may result from down-regulation of mitochondrial function which prevents excessively high metabolic rate during warming (Pörtner, 2001; Fangue et al., 2009) and could preserve MS at higher temperatures. However, despite the observed correlations in Figure 5.5, it remains unclear if lower RMR during thermal ramping actually plays a functional role in setting upper thermal tolerance because a clear difference in the metabolic rate of fish from the 21°C and 24°C acclimation groups approaching their lethal thermal limit did not appear to dictate any difference in CT_{max}. Furthermore experimental manipulations of oxygen transport capacity through anaemia (Wang et al., 2014; Brijs et al., 2015), hypoxia (Ern et al., 2016b) and hyperoxia (Brijs et al., 2015) have little effect on upper thermal tolerance in fish.

5.4.5 Conclusions

In regard to chronic thermal tolerance, *F. lapillum* acclimated to 15°C, 18°C, 21°C and 24°C (over 12 weeks) experience a decline in MS and growth fitness at 24°C. This finding is in support of the OCLTT hypothesis due to the correlation between metabolic and

growth performance measures (Fig. 5.1). Unless genetic or transgenerational phenotypic acclimation can compensate, the main cause for concern would be that the metabolism and growth of *F. lapillum* would be sub-optimal with a 3°C temperature increase above current summertime SSTs. Few studies, however, assess climate change with respect to seasonal growth and our modelling approach suggests that projected climate change will shift optimal growth to alternative times of the year and would thus result in an annual growth rate that is not impacted negatively (Fig. 5.2). Chronic warm acclimation only modestly increased upper thermal tolerance and, consequently, thermal safety margins declined at high acclimation temperatures (Fig. 5.3). In line with expectations under the OCLTT hypothesis, the observed increase in upper thermal tolerance under warm acclimation was associated with reduced RMR on acute thermal ramping (Fig. 5.4A & 5.4B). In the context of global warming the acute thermal tolerance of *F. lapillum* is cause-for-concern because the ability of this species to withstand acute temperature change will decrease with every unit increase in peak chronic temperature. On that basis, the resilience of *F. lapillum* to future climate change will be reduced progressively as temperatures increase and the distribution of this common coastal species may become limited to thermally stable sub-tidal habitats or higher latitudes to cope.

CHAPTER 6: GENERAL DISCUSSION

This thesis examined aspects of the hypoxia (Chapter 2 and 3) and thermal tolerance (Chapter 4 and 5) in New Zealand triplefin fishes with a focus on intertidal species. The final chapter of this thesis describes the main findings of each chapter in the broader context of hypoxia and thermal tolerance in fish and identifies future directions where further work is needed. Rather than including distinct sections for limitations and future directions, I have chosen to integrate these throughout the general discussion.

6.1 The total hypoxia response of intertidal triplefin fish

In Chapter 2 of this thesis, I show that the exclusively intertidal triplefin fish *B. medius* has a lower P_{crit} than species that occupy both rock pools and shallow subtidal habitats (*F. lapillum*), and exclusively subtidal habitats (*F. varium* and *F. malcolmi*). This demonstrates that *B. medius* is able to maintain a stable rate of O_2 consumption under more severe levels of hypoxia - a finding that indicates *B. medius* is more hypoxia tolerant than its closely related subtidal counterparts. In addition to a low P_{crit}, the superior hypoxia tolerance of *B. medius* was suggested by an observed ability to maintain an upright position within respirometers at oxygen levels less than 10% of air saturation; whereas, the exclusively subtidal species (*F. varium* and *F. malcolmi*) frequently exhibited loss of equilibrium (LOE) at these severe levels of hypoxia. Since completing the experimental work for Chapter 2, further investigation (completed in conjunction with a fellow PhD student Jules Deveaux), has confirmed that *B. medius* tolerates severe hypoxia substantially better than the three *Forsterygion* species. In this additional work, we measured the time to loss of equilibrium (LOE), a proxy for hypoxia survival, in each species under exposure to a constant severe level of hypoxia (7% air saturation). The exclusively subtidal species (*F. varium* and *F. malcolmi*) first exhibited LOE at an O_2 level of 16% of air saturation, and the remaining individuals of these species could not maintain equilibrium below an O_2 level of 9% of air saturation. *Forsterygion lapillum*, an occupant of both shallow subtidal and rock pool habitats, fared better than the exclusively subtidal species surviving on average 20 min exposure at an O_2 level of 7% air saturation. The exclusively intertidal species *B. medius* was substantially better able to tolerate severe hypoxia than all three *Forsterygion* species, surviving on average 90 min exposure to an O_2 level of 7% air saturation. Thus, when

assessed as P_{crit} (Chapter 2) or LOE, it is evident that the intertidal specialist *B. medius* has superior hypoxia tolerance than its related exclusively subtidal counterparts (*F. varium* and *F. malcolmi*) and the occasional rock pool inhabitant *F. lapillum*. Notably, among the three more closely related *Forsterygion* species included in Chapter 2, *F. lapillum*, a species which is quite frequently observed in rock pools, was found to be more hypoxia tolerant than the two exclusively subtidal species *F. varium* and *F. malcolmi*. Among the closely related sculpins, intertidal species also have lower P_{crit} and the ability to survive severe hypoxia longer than subtidal species (Mandic et al., 2013). Thus, the commonality between triplefins (Perciformes) and sculpins (Scorpaeniformes) suggests that the high prevalence of hypoxia in rock pools has selected for hypoxia tolerant metabolic phenotypes in intertidal species from each of these distantly related orders (Mandic and Regan, 2018).

In Chapter 2 of this thesis key aspects of the total hypoxia response of intertidal triplefin fish were also identified. To cope with hypoxia, the intertidal specialist *B. medius*, initially relies on an enhanced capacity to sustain aerobic metabolism under low O_2 conditions. This ability in *B. medius* can be summarised as a low P_{crit}, which reflects an increased capacity to extract O_2 from low O_2 environments. The high extractive capacity for O_2 in *B. medius* was also reflected by higher maximal rates of O_2 consumption following exhaustive exercise, denoting possession of a cardiorespiratory system that, when pushed to its limits, can extract more O_2 at a given level of environmental O_2 availability. High $\dot{M}O_{2,max}$ affords *B. medius* with high aerobic MS, which may help avoid limitation of essential aerobic activities (e.g. feeding, digestion etc.) when facing hypoxia or increased temperature in rock pools. Indeed, *B. medius* not only maintains higher $\dot{M}O_{2,max}$ than subtidal species under normoxia as shown in chapter 2, but also under reduced O_2 levels of 80, 60 and 30% of air saturation (Stobie, 2017). At an O_2 level of 30% of air saturation, *B. medius* retains 44% of normoxic MS availability; whereas, *F. varium* retains only 24% of normoxic MS availability (Stobie, 2017).

The findings of Chapter 2 also revealed that intertidal triplefins have gills with a relatively large surface area and a high capacity for O_2 diffusional flux, and the intertidal species also had intrinsically high levels of Hb reflecting an elevated blood-O_2 carrying capacity; both of these physiological characteristics contribute to a high O_2 extractive capacity and low P_{crit}. Hilton et al. (2010) showed that *B. medius* has a higher ventricular mass to body mass ratio than *F. varium* and *F. malcolmi*. The larger heart of *B. medius* may enhance the capacity of the circulatory system to take up and deliver O_2 to tissues. Previous

studies have also shown that *B. medius* has a higher red blood cell O_2 binding affinity (lower P_{50}) (Innes and Wells, 1985) than *F. varium* and *F. malcolmi* (Brix et al., 1999). The lower red blood cell P_{50} of *B. medius* would allow the Hb of this species to saturate more with O_2 under hypoxic conditions and, undoubtedly, contributes to comparatively lower P_{crit} of this intertidal species. The cardiac mitochondria of *B. medius* have also been shown to have a higher rate of cytochrome c oxidase flux than the subtidal species *F. varium* and *F. malcolmi*, which may aid O_2 binding at low O_2 tensions within the mitochondria (Hilton et al., 2010b). This pattern of increased cytochrome c oxidase flux in *B. medius* compared to subtidal species has also been observed in brain mitochondria and skeletal muscle by a fellow PhD student (Jules Devaux unpublished data). Thus, from the level of the whole animal to the mitochondria, the physiology of *B. medius* reflects a hypoxia survival strategy geared towards continued reliance on aerobic metabolism for as long as possible under hypoxia exposure. The same physiological characteristics that differentiated *B. medius* from the three *Forsterygion* species (low P_{crit}, high $\dot{M}O_{2,max}$, high MS, high Hb, large gill SA, thin secondary lamellae) also differentiated *F. lapillum*, a species which is also known to inhabit rock pools, from two exclusively subtidal species (*F. varium* and *F. malcolmi*) placed within its own genus. Furthermore, many of the physiological characteristics of intertidal triplefins are shared by distantly related intertidal sculpin species suggesting "convergent evolution has played a role in shaping the hypoxic metabolic phenotype" (Mandic and Regan, 2018).

Presumably, one of the advantages of a low P_{crit} for an intertidal fish is that it lowers the hypoxic threshold which requires a transition to more costly anaerobic metabolism (Mandic et al., 2009). In many hypoxic episodes, a low P_{crit} may allow *B. medius* to completely avoid recruitment of anaerobic metabolism, particularly if it remains behaviourally inactive when O_2 availability deteriorates. Indeed, the observations of nocturnal hypoxia in rock pools inhabited by *B. medius* indicate that in many instances O_2 availability remains above the P_{crit} of this species, and the possession of a low P_{crit} may relegate a transition to anaerobic metabolism to only the most severe hypoxic episodes. A comparison of lactate accumulation in intertidal and subtidal triplefin species exposed to progressive hypoxia, however, is required to confirm if a low P_{crit} delays transition to anaerobic metabolism and at what point in relation to P_{crit} lactate accumulation first appears in each species. Moreover, a thorough examination of hypoxia in rock pools would be beneficial in determining how often intertidal triplefin fish face O_2 availability less than their P_{crit}.

In some instances O_2 availability in rock pools may become so depleted that a low P_{crit} alone is an insufficient coping mechanism and other strategies including a transition to anaerobic metabolism, metabolic rate depression and behavioural responses (e.g. aquatic surface respiration and aerial emergence) will be required in order to survive. Indeed, *B. medius* tolerates exposure to O_2 levels less than P_{crit} substantially better than subtidal species demonstrating an enhanced ability to balance energy supply and demand under severely limited O_2 availability. The findings of this thesis contributed less to understanding how *B. medius* manages to survive extreme hypoxia below P_{crit} for so much longer than the other species, but some insights have still been gained. While the benefit of a low P_{crit} to intertidal fish has been considered mostly in terms of avoiding or delaying a transition to anaerobic metabolism (Mandic et al., 2009; Richards, 2011; Speers-Roesch et al., 2013), it is also worth considering how a low P_{crit} influences the ability to maintain energy balance at O_2 levels below P_{crit}. One point, albeit a simple one, is that if two species, one with low P_{crit} and one with high P_{crit}, are exposed to a common level of severe hypoxia that is below the P_{crit} of each species, the species with low P_{crit} is able to meet a greater proportion of the O_2 demand associated with SMR at that level of hypoxia. For example, at an O_2 level of 7% of air saturation the $\dot{M}O_2$ of *B. medius* is equivalent to 79% of SMR, whereas the $\dot{M}O_2$ of *F. malcolmi* at the same O_2 level is equivalent to 34% of SMR. If a greater proportion of energy demand can be met aerobically, survival should be prolonged as glucose will be used more efficiently and respiratory acidosis will be less severe.

Beyond being able to extract more O_2 under severely hypoxic conditions, the capacity for anaerobic glycolytic flux and metabolic rate depression are proposed as the main factors contributing to an animal's ability to survive O_2 availability less than P_{crit} (Boutilier and St-Pierre, 2000; Bickler and Buck, 2007; Richards, 2011). In Chapter 2 I found *B. medius* had larger tissue glycogen stores than the *Forsterygion* species which may contribute to its superior ability to survive severe hypoxia. Simply put, large tissue glycogen stores may allow anaerobic glycolytic flux to be sustained for longer, thereby increasing the length of time which energy balance can be maintained under severe hypoxia. Indeed, the maintenance of large tissue glycogen stores is hypothesised to be a key feature of hypoxia tolerant fishes (Richards, 2011). It has also been hypothesised that the maximal activity of key glycolytic enzymes should be higher in hypoxia tolerant fish species, since this would theoretically contribute to a high capacity for anaerobic glycolytic flux (i.e. O_2 independent ATP production) (Mandic et al., 2013). There is limited information known about the activity of

glycolytic enzymes in triplefin fish, although Hickey and Clements (2003) did demonstrate (in caudal muscle tissue) a higher activity of the glycolytic enzyme phosphofructokinase (PFK) in *B. medius* compared to *F. lapillum* and *F. varium.* In the same study, however, there was no difference in the activity of two other glycolytic enzymes, lactate dehydrogenase (LDH) and pyruvate kinase (PK), between these species. Mandic et al. (2013) demonstrated among sculpins that hypoxia tolerance was associated with high anaerobic enzyme activity in brain tissue but not liver or muscle, so an assessment of anaerobic enzyme activity in triplefin brain tissue may reveal a more convincing association between hypoxia tolerance and the activity of glycolytic enzymes. A high tolerance of H^+ and lactate, which accumulates in tissues due to anaerobic ATP production, may also contribute to the ability of hypoxia tolerant fishes to survive for long durations of severe hypoxia and has been proposed to relate to the ability of metabolic enzymes to continue to function well at low pH (Scott et al., 2008). It has recently been demonstrated that the brain mitochondria of *B. medius* perform significantly better at low pH than the subtidal triplefin species, and that *B. medius* may even be able to couple respiratory acidosis to mitochondrial ATP production (Devaux et al., 2018).

Whether or not a metabolic rate depression below P_{crit} contributes to the superior hypoxia tolerance of *B. medius* is unknown. In some very hypoxia tolerant fish species P_{crit} is relatively high. This situation indicates that a controlled depression of metabolic rate in response to hypoxia exposure contributes to hypoxia tolerance through a reduction in energetic demand. An example is the Amazonian oscar (*Astronotus ocellatus*) which has a relatively high P_{crit} (6.1 kPa, 30% of air saturation) but, impressively, can survive complete anoxia up to 6 h at 28°C (Scott et al., 2008). There was no evidence of a similar strategy in *B. medius* as this species is clearly a strict oxygen regulator, and an early depression of metabolic rate during progressive hypoxia exposure is not part of the hypoxia survival strategy of this species. If metabolic rate depression does occur in intertidal triplefins, it must only be recruited at very severe levels of hypoxia exposure. Indeed, goldfish have recently been shown to recruit metabolic rate depression only under near anoxic conditions (Regan et al., 2017a), therefore it does remain a possibility that a species such as *B. medius* does the same. Since there are currently no reliable assessments of the ability of intertidal fish to perform metabolic rate depression, its role in the total hypoxia response of rock pool fish remains unknown.

Behavioural responses, which were not assessed in thesis, may also contribute to the superior hypoxia tolerance of intertidal triplefin fish, as these can provide access to more O_2

(e.g. via aquatic surface respiration or aerial emergence) or reduce energy expenditure through inactivity (Mandic et al., 2009). Aquatic surface respiration and aerial emergence have both been reported in *B. medius* subjected to hypoxia (Innes and Wells, 1985); however, my own observations of this species' behaviour during exposure to severe hypoxia (e.g. in the LOE trials conducted in Chapter 3) did not indicate any attempts to emerge, and aquatic surface respiration occurred only immediately prior to LOE. Innes and Wells (1985) report *B. medius* as regularly been observed "exposed to air under rocks" in the intertidal zone, however, in dozens of collection trips, I have only ever observed this species fully submerged in well-formed rock pools. Nevertheless, the contribution of aquatic surface respiration and aerial emergence to the hypoxia tolerance of intertidal triplefin fish warrants further investigation. Examining the activity levels of intertidal and subtidal species under progressive hypoxia would also be useful in determining whether a minimisation of energy expenditure through quiescent behaviour plays a role in hypoxia tolerance.

6.2 Ecological significance of the total hypoxia response of intertidal triplefin fish

The findings of this thesis are in agreement with the conclusion made by Mandic and Regan (2018) that the total hypoxia response of intertidal fishes is centred on the prioritisation of aerobic metabolism under all levels of hypoxia and supplemental ATP production through anaerobic metabolism at very severe levels of hypoxia. Mandic and Regan (2018) argue that the total hypoxia response (the mix of aerobic and anaerobic metabolism, and metabolic rate depression) observed in different fish is mostly related to the severity and duration of hypoxic episodes in a species' native habitat. Hypoxic events in rock pools can be severe, but they are only of short duration because, even in high intertidal pools, the incoming tide restores normoxia after less than 12h of emersion. Moreover, O_2 levels gradually decline throughout low tide so the most severe levels of hypoxia may develop for only a short duration towards the end of the emersion period. It also appears that, while some do become severely hypoxic during nocturnal low tides, many rock pools inhabited by intertidal triplefins retain enough O_2 for aerobic metabolism to be sustained and relied upon to survive. Metabolic rate depression, while an important strategy employed by some hypoxia tolerant fishes (Bickler and Buck, 2007; Vornanen et al., 2009), may not be suitable for intertidal fishes as the lethargic state it induces may elevate predation risk (Mandic and Regan, 2018). Furthermore, metabolic rate depression is associated with significant costs to

an organism such as oxidative stress (Carey et al., 2000), impaired cognitive and neurological function (Popov et al., 1992) and reduced sensory and motor activity (Choi et al., 1998). Therefore, if metabolic rate depression was continually recruited in response to hypoxia in rock pools these costs may accumulate and exceed any benefits gained from improved ability to match energy supply and demand (Regan et al., 2017a). For example, intertidal fish in a state of metabolic depression in response to hypoxia would have drastically reduced capacity to feed and digest food when normoxic conditions are returned by the incoming tide. The requirement for intertidal fish to station hold in strong tidal surges may also make a depressed metabolic state an inappropriate strategy for defence against hypoxia. Intertidal fish are also always in close proximity to air, where more O_2 can be accessed through aquatic surface respiration or emergence and, because hypoxia in rock pools tends to be nocturnal, the threat of aerial predation when performing these behaviours would be highly reduced. Thus, a total hypoxia response centred on continued utilisation of aerobic metabolism (i.e. a low P_{crit}) for as long as possible appears well suited to intertidal fish because: (1) severe hypoxia occurs for only a short duration, (2) adequate O_2 may often remain available during hypoxia, (3) behaviours that allow access to more O_2 (e.g. aquatic surface respiration) can be performed with low risk of aerial predation because hypoxia tends to be nocturnal, and (4) abiotic and biotic characteristics of rock pool habitats may act against the selection of metabolic rate depression.

In Chapter 3 of this thesis I found that *B. medius* tolerates on average 57 min exposure to an O_2 level of 7% air saturation at 21°C, a temperature representative of summertime nocturnal rock pool temperatures in the warm northern range of this species. This represents the duration across which energy balance can be maintained in this species when exposed to an O_2 level equivalent to approximately 50% of P_{crit}. Thus, while *B. medius* is certainly more hypoxia tolerant than subtidal triplefin species, it does not appear to have the anaerobic capacity to survive below P_{crit} for an extended or indefinite period of time, at least in its warm northern range during summer. In comparison, all be it at a lower temperature of 12°C, the tidepool sculpin survives 538 min exposure to an O_2 level equivalent to 24% of its P_{crit} (Mandic et al., 2013), and 720 min exposure to an O_2 level equivalent to 30% of its P_{crit} (Speers-Roesch et al., 2013). In Chapter 3 I also showed that the ability of *B. medius* to survive below P_{crit} is reduced following recovery from acute heat stress. This finding suggests that exposure to multiple sequential stressors, as can occur naturally in rock pools, renders *B. medius* less able to tolerate severe hypoxia. The findings of Chapter 3 indicate that *B. medius*,

despite possession of a low P_{crit}, may reside in rock pools where it is exposed to hypoxic conditions which are close to its maximum tolerance threshold during summer. Climate change will increase ambient sea temperatures and is also predicted to increase the frequency and severity of heat wave events (Perkins et al., 2012). With the understanding that *B. medius* may already reside in rock pools which are close to its maximum hypoxia tolerance threshold there is a strong need for further research examining the impact of predicted future temperatures on the hypoxia tolerance of this species. This is pertinent because hypoxia tolerance is impaired at higher temperatures in the vast majority of fish species examined so far (Rogers et al., 2016). There is also a need to combine knowledge of the hypoxia tolerance thresholds of intertidal fish with a better understanding of the heterogeneity in hypoxic conditions in different rock pools, which are highly varied in terms of shape, depth, elevation and inhabiting biota. This would allow predictions to be made about exactly which type of rock pools may become uninhabitable in the near future and how this will impact habitat availability for intertidal triplefin fish.

6.3 Tolerance to acute thermal stress in intertidal triplefin fish

Hypoxia is not the only environmental stressor that occurs in rock pools. When low tide aligns with the middle of the day temperatures in rock pools can increase rapidly above that of the surrounding ocean (Truchot and Duhamel-Jouve, 1980; Huggett and Griffiths, 1986). However, if there are photosynthetic organisms present (e.g. seaweeds, algae) acute temperature increases will co-occur with hyperoxia (Truchot and Duhamel-Jouve, 1980; Gunderson et al., 2016b). In Chapter 4 I showed that aerobic metabolic scope (MS) is not constrained to the same extent in intertidal fish facing acute heat stress under hyperoxic conditions, compared to fish facing acute heat stress under normoxia. This finding suggests environmental hyperoxia may provide a metabolic refuge for intertidal fish facing acute heat stress. Beyond improved aerobic performance, however, the results of Chapter 4 did not demonstrate any other benefits of hyperoxia. Thus, further studies are required to determine if intertidal fish really are advantaged when facing heat stress under hyperoxic conditions. Examinations of whether or not expanded MS under hyperoxia mitigates a requirement for anaerobic metabolism at high temperatures, or removes constraints on aerobic activities such as digestion at high temperatures, would be interesting avenues for future research.

In Chapter 4, despite the expansion of $\dot{M}O_{2,max}$ and MS at high temperature under hyperoxia, there was little difference in the absolute thermal tolerance limits of intertidal fish under hyperoxia compared to normoxia. This finding suggests that the lethal thermal tolerance limits of intertidal triplefin fish are not set by a capacity limitation of the cardiorespiratory system to supply O_2 to the working tissue, as hypothesised within the OCLTT framework (Pörtner, 2010). This is in agreement with several other studies which have found that experimental manipulations of O_2 supply capacity including hyperoxia (Healy and Schulte, 2012a; Brijs et al., 2015; Devor et al., 2016; Ekström et al., 2016), anaemia (Wang et al., 2014; Brijs et al., 2015) and even relatively severe hypoxia (Ern et al., 2016b; Ern et al., 2017) have little influence on the upper tolerance limits of fish facing acute thermal stress. Together these findings refute the notion that an inability to supply O_2 to tissues is the all-important factor in the upper thermal tolerance limits of fish. In Chapter 5 I measured the upper thermal tolerance limits of *F. lapillum* after 12 weeks acclimation to either 15, 18, 21 or 24°C. As expected the warm acclimated fish had higher upper thermal tolerance limits than cold acclimated fish. The improved upper thermal tolerance of warm acclimated fish, however, could not be reconciled with an enhanced capacity to supply O_2 to tissues as warm acclimated fish also had significantly lower rates of O_2 consumption at temperatures approaching critical limits. Potentially, lower rates of O_2 consumption at high temperatures allows warm acclimated fish, despite reduced maximal capacities, to retain MS and better manage energy supply and demand. However, despite clear differences in the metabolic rate of fish approaching thermal limits, there was no difference in upper thermal tolerance between fish acclimated to 21°C and 24°C. Thus, it remains questionable whether a reduction in metabolic costs associated with chronic warm acclimation actually plays a functional role in setting upper thermal tolerance limits.

The upper thermal tolerance limits determined for intertidal triplefin fish in Chapters 4 and 5 indicate that, under present day conditions, temperatures may approach lethal limits in at least some rock pools. This is particularly the case for the exclusively intertidal species *B. medius* as it occurs in high intertidal rock pools which are subject to the most extreme temperature events (Hilton et al., 2008). Chapter 4 also revealed that both *B. medius* and *F. lapillum* receive no additional capacity to tolerate thermal extremes under hyperoxia, which is prevalent in rock pools when high temperatures develop due to strong solar radiation and the photosynthetic activity of algae. In addition, Chapter 5 showed that chronic acclimation (12 weeks) to a temperature (24°C) approximately 3°C warmer than present day summer

conditions did not increase upper thermal tolerance limits in *F. lapillum.* This suggests phenotypic plasticity for upper thermal tolerance reaches its limits at current day peak summer temperatures and that thermal safety margins will be significantly reduced with predicted warming due to climate change. Plasticity in upper thermal tolerance limits was, however, only examined in *F. lapillum*, so whether or not other triplefin species have reduced thermal safety margins at predicted future temperatures should be examined. In the context of predicted increases in ambient sea temperatures and more frequent and severe heat wave events due to climate change (Perkins et al., 2012) the upper thermal tolerance limits of intertidal triplefin species, which appear relatively fixed, are a cause for concern. If temperature maximums increase in rock pools this may result in a loss of thermally suitable habitat and increased risk of heat death. In my observations, the exclusively intertidal *B. medius* will utilise even very small and shallow rock pools as habitat. Small rock pools are subject to the most extreme daytime temperatures as they have low thermal inertia, and it is this habitat that may become unsuitable in the near future. This may force a species like *B. medius* into only larger rock pools or those located lower on the shoreline and lead to significant declines in abundance. Intergenerational adaptive selection for more thermally tolerant phenotypes may provide additional resilience to the effects of warming, although these too appear limited for the upper thermal tolerance of fish (Sandblom et al., 2016).

To understand how climate change will influence habitat availability and over-heating risk in intertidal fish a better understanding of the thermal characteristics of rock pools is required. Rock pools are highly diverse in terms of shape, depth, volume, elevation, aspect and the amount of shading they receive from nearby substrate such as cliffs, boulders, overhanging rocks and crevices. All these physical characteristics influence the thermal profiles of rock pools during daytime low tide emersion and, undoubtedly, there is a diverse array of thermal conditions at any one time amongst the multitude of different pools located in the intertidal zone. Even within a single rock pool there may be refuges available to escape heat stress (e.g. in crack or recesses in rock pool walls). Identifying exactly which type of rock pools are most vulnerable to extreme heating events (especially those that exceed the thermal tolerance limits of intertidal fish), and what proportion of habitat these vulnerable pools comprise is an important step to predicting the effects of climate change on species such as *B. medius.*

6.4 Is there a link between chronic thermal tolerance and aerobic metabolic scope in triplefin fish?

One of the main threats of climate change to fish is that global warming will result in chronic exposure to temperatures beyond those experienced under current day conditions. Accordingly, there has been an explosion of research concerning the ability of fish to acclimate and adapt to predicted future temperatures (Nilsson et al., 2009; Munday et al., 2009; Donelson et al., 2012; Grans et al., 2014; Norin et al., 2014; Drost et al., 2016). A strong emerging theme has been that thermal physiology will play a major role in dictating tolerance to global change (Somero, 2012). A prominent hypothesis, the OCLTT, proposes that the ability of aquatic ectotherms to tolerate exposure to increased temperature is set by the availability of MS (Pörtner and Farrell, 2008). The OCLTT states that all aquatic ectotherms operate within a thermal window defined by MS availability and that, as temperature increases beyond an optimal range, MS collapses due to an inability of the cardiorespiratory to match O_2 supply and demand (Pörtner and Farrell, 2008). Moreover, within the OCLTT framework, MS availability is causally linked to whole organism fitness-related performance (e.g. growth, reproduction, swimming performance). Whether or not thermal tolerance in aquatic ectotherms is set by availability of MS has become a contentious issue among physiologists (Clark et al., 2013; Schulte, 2015; Jutfelt et al., 2018; Pörtner et al., 2018).

In Chapter 5 of this thesis I demonstrated a significant correlation between growth performance and MS in *F. lapillum* acclimated to 15, 18, 21, 24°C for 12 weeks. This finding is in support of the central prediction of the OCLTT that whole animal fitness-related performance is set by the availability of MS. However, in the latest version of the OCLTT hypothesis (Pörtner et al., 2017) MS is no longer defined as the difference between maximal rates of O_2 consumption and SMR as it was in its original form (Pörtner and Knust, 2007; Pörtner and Farrell, 2008; Pörtner, 2010). In particular the use of exhaustive chase protocols (as in Chapter 5) to define MS is now strongly discouraged by these authors, and they suggest only maximal O_2 consumption associated with steady-state routine activities should be used in any attempt to examine the applicability of this concept (Pörtner et al., 2017). Thus, in light of these recent changes to the OCLTT, which have come under strong criticism from many in the field (Jutfelt et al., 2018), it is actually unclear if the results of Chapter 5 can in fact be taken as evidence in support of this concept. Nevertheless, MS and growth performance were both reduced in *F. lapillum* at a temperature which may eventuate in the

warm high latitude range of this species in the near future. Indeed, the lower growth rates observed at high temperature may be linked to reduced MS due to a prioritisation of other aerobic activities, or a constraint on the ability to perform multiple aerobic activities simultaneously (Claireaux and Lefrancois, 2007; Clark et al., 2013). Much of the controversy surrounding the OCLTT is due to the fact that in many ectotherms $\dot{M}O_{2,max}$ does not plateau with increasing temperature (Lefevre, 2016), and some species maintain MS at temperatures approaching their lethal thermal limits (Norin et al., 2014; Grans et al., 2014; Claësson et al., 2016; Jensen et al., 2017). In these species the availability of MS is clearly not the main determinant of temperature dependent performance (e.g. Grans et al., 2014). Thus, the findings of Chapter 5 contribute to a growing acceptance that while the principles of the OCLTT may be applicable in some species (e.g. *F. lapillum*) it is by no means a unifying theory of thermal tolerance for all aquatic ectotherms.

The findings of Chapter 5 indicate that assessments of MS availability at relevant temperatures may be a useful tool in predicting how triplefin fish will be impacted by warming due to climate change. Indeed in Chapter 4, all be it under acute rather than chronic thermal exposure, *B. medius* and *F. lapillum* were observed to have constrained MS at high temperatures. This suggests the possibility that, as a group, triplefin fish may be vulnerable to a collapse in MS at high temperatures. As hyperoxia modified the response of MS to high temperature exposure, the presence of naturally occurring hyperoxic conditions in the habitat of a species would also need to be taken into account in any attempt to model how future warming trends might influence aerobic performance. Future studies should also include other relevant climate change related stressors (e.g. elevated CO_2, low pH) to better understand the impacts of global change. A limitation of using the availability of MS as a metric of temperature dependent performance is that O_2 consumption alone does not reflect the capacity for mitochondrial ATP production. The amount of ATP produced per unit of O_2 consumed (the P/O ratio or respiratory control ratio) is often reduced at high temperatures due to increased rates of proton leak across the inner mitochondrial membrane (Hilton et al., 2010b; Iftikar and Hickey, 2013). High leak rates reduce P/O ratios as leaked protons are not available to drive ATP synthesis (Brand, 2005). Variation in P/O ratio has been proposed as an important determinant of whole animal performance under different environmental conditions (Salin et al., 2015), and future studies investigating thermal tolerance in triplefins should incorporate assessments of the efficiency of mitochondrial ATP production alongside measurements whole animal O_2 consumption and performance (e.g. growth). While the

results of this thesis suggest MS is a helpful metric to assess tolerance to global warming in triplefin fishes, it should only be used alongside other measurements that, regardless of whether MS peaks or plummets above the pejus range, are informative of the fitness-related performance of the organism in question.

APPENDICES

Appendix 1. Characteristics of individual experimental runs in experiment 1 (chapter3). TR= thermal ramping heat shock.

Treatment group	Mean LOE time ± s.e.m. (min)	TR magnitude (°C)	TR time length (min)	TR heating rate (°C min^{-1})	Time to hypoxic exposure post TR (hh:mm)	Time to reach hypoxic set point (mm:ss)	O_2 saturation during hypoxic challenge (% air saturation)			Temperature during overnight recovery (°C)			Temperature during hypoxic challenge (°C)		
							Mean	*Min*	*Max*	*Mean*	*Min*	*Max*	*Mean*	*Min*	*Max*
Ambient no HS (N=12)															
Run 1 (*n*=6)	53.06 (10.16)	na	na	na	19:16	09:27	7.02	5.19	8.79	21.03	20.42	21.68	21.02	20.91	21.14
Run 2 (*n*=6)	57.47 (7.98)	na	na	na	19:18	10:19	6.88	5.47	8.37	20.72	20.21	21.52	21.07	20.97	21.19
+8°C HS (N=12)															
Run 1 (*n*=6)	40.86 (5.5)	8.04 (21.01-29.05)	301	0.027	19:15	09:37	6.98	5.66	8.3	20.88	20.18	21.59	21.06	20.96	21.23
Run 2 (*n*=6)	42.75 (6.67)	8.27 (20.79-29.06)	296	0.028	19:20	07:04	6.78	5.41	8.1	21.18	20.34	21.82	21.12	20.98	21.28
+10°C HS (N=12)															
Run 1 (*n*=6)	26.29 (5.24)	10 (21.05-31.05)	295	0.034	19:15	07:36	6.99	5.7	8.59	21.04	20.31	21.81	21.03	20.93	21.12
Run 2 (*n*=6)	27.8 (5.54)	10.08 (21.01-31.09)	299	0.034	19:21	09:06	6.85	5.46	8.13	21.05	20.44	21.75	21.04	20.92	21.15

Appendix 2. Characteristics of individual experimental runs in experiment 2 (Chapter 3). TR= thermal ramping heat shock.

Treatment group	TR magnitude (°C)	TR time length (min)	TR heating rate (°C min^{-1})	Post TR time period to progressive hypoxia exposure (h)	Total time length of progressive hypoxia exposure (min)	Temperature during SMR assessment (°C)			Temperature during P_{crit} assessment (°C)		
						Mean	*Min*	*Max*	*Mean*	*Min*	*Max*
Ambient no HS (N=10)											
Run 1 (*n=4*)	na	na	na	18.33	292	20.58	20.17	21.12	21.12	20.92	21.3
Run 2 (*n=4*)	na	na	na	18.76	271	21.36	21.06	21.55	21.19	20.99	21.37
Run 3 (*n=2*)	na	na	na	18.56	267	20.95	20.86	21.13	21.12	21.01	21.33
+8°C HS (N=10)											
Run 1 (*n=1*)	7.83 (21.2-29.03)	300	0.027	18.85	274	20.6	20.42	21.03	20.99	20.89	21.04
Run 2 (*n=3*)	8.1 (20.97-29.07)	313	0.026	18.1	261	21.5	21.17	21.76	21.09	21.05	21.22
Run 3 (*n=4*)	7.99 (20.97-28.96)	310	0.026	18.33	270	20.7	20.56	21.11	21.24	20.96	21.35
Run 4 (*n=2*)	8 (21.02-29.02)	282	0.029	19.5	267	20.8	20.73	21.07	21.07	20.95	21.27
+10°C HS (N=8)											
Run 1 *(n=3*)	10.16 (20.84-31)	284	0.036	19	273	20.98	20.91	21.23	21.2	21.12	21.24
Run 2 (*n=2*)	10.21 (20.87-31.08)	289	0.036	19.1	266	21.09	20.99	21.48	21.22	21.11	21.29
Run 3 (*n=1*)	10.01 (21.1-31.11)	309	0.033	18.5	272	20.97	20.86	21.15	20.99	20.89	21.07
Run 4 (*n=2*)	10.02 (21.03-31.05)	286	0.036	19.4	291	20.89	20.69	21.15	20.99	20.94	21.04

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