PHENOTYPIC DRIVERS OF HYPOXIA TOLERANCE IN A TROPICAL, DIADROMOUS FISH (*Lates calcarifer*)

Thesis submitted by

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Frontispiece: Barramundi performing aquatic surface respiration

STATEMENT OF CONTRIBUTION OF OTHERS

The data chapters in this thesis are a collection of collaborative work with my supervisors Dr Guy Carton and Dr Timothy Clark, with further contributions from Dr Jodie Rummer and Professor Dean Jerry. Experimental design, data collection, data analysis and interpretation in context were primarily conducted by myself. Further contributions in the form of intellectual guidance and financial support, as well as essential feedback on experimental design, statistical analysis and editing of manuscript drafts were provided by co-authors.

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Reports

Jerry, D.R., Smith-Keune, C., Hodgsone, L., Pirozzi, I., Carton, A.G., Hutson, K.S., Brazenor, A.K., Gonzalez, A.T., Gamble, S., **Collins, G.M.** and VanDerWal, J. (**2013**) Vulnerability of an iconic Australian finfish (barramundi – *Lates calcarifer*) and aligned industries to climate change across tropical Australia. Townsville, QLD, Australia. Fisheries Research Development Corporation and James Cook University. Project No. 2010/521. 222 p.

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Collins, G.M., Clark, T.D., Rummer, J.L. and Carton, A.G., 2013, Resting metabolism and hypoxia tolerance are conserved across genetically distinct sub-populations of an iconic, tropical Australian teleost (*Lates calcarifer*). Society for Experimental Biology Annual Conference, València, Spain.

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Collins, G.M., Clark, T.D. and Carton, A.G., 2015, Physiological plasticity v. inter-population variability: understanding drivers of hypoxia tolerance in a tropical, estuarine fish. Australian Society for Fish Biology Annual Conference, Sydney, Australia.

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The image (painting) of a barramundi on the front cover of this thesis is an original artwork by Tom Montgomery (https://tommontgomery.wordpress.com/home/fish2/).

GENERAL ABSTRACT

Increasing coastal eutrophication and rising global temperatures are placing substantial pressure on wild fish populations. In the tropics, routinely high temperatures close to the equator have the combined effects of reducing the solubility of O_2 in water, increasing metabolic rates and enhancing thermal stratification. Such conditions may be particularly pronounced in lentic freshwater environments, and have led to a suite of adaptations by tropical fish species. Inter-specific diversity in hypoxia tolerance is widely acknowledged, however, many species exist not as a single, continuous population, but rather as multiple populations that may be distributed over broad latitudinal gradients, potentially spanning thousands of kilometres. In the absence of horizontal migration and genetic mixing between populations, and with differences in prevailing environmental conditions (such as temperature and dissolved O_2), such populations may become phenotypically and even genotypically divergent over time.

Performance of fish populations have been extensively investigated in the context of temperature, particularly with regard to projected temperature increases from climate change models. Despite the intrinsic relationship between temperature, O_2 and metabolism in fish, population differences in hypoxia tolerance have received little research attention to date. To address this knowledge gap, the hypoxia tolerance of geographically and genetically divergent populations of juvenile barramundi from across the distribution in

northern Australia was assessed (*Chapter 2*). Juvenile barramundi were collected from five hatcheries across northern Australia, and were assessed for their resting O_2 consumption rate ($\dot{M}O_2$) and critical O_2 level (O_{2CRIT}) using intermittent-flow respirometry. Measurements for all five populations were made at temperatures considered benign for this species across its distribution in Australia (26°C) and at temperatures that may be encountered during the pre-wet season (36°C). *A posteriori* comparisons revealed significant temperature effects for both resting $\dot{M}O_2$ and O_{2CRIT}, but no conclusive evidence for population differences in either measure. The results from this study indicate a similar capacity for barramundi populations to regulate metabolism in response to hypoxia at typical and warm temperatures. The magnitude of temperature effects on O_{2CRIT} for barramundi was lower than for many other tropical and temperate fish species, indicating that barramundi retain a high capacity to regulate metabolism in hypoxic environments at high temperatures.

Over long time scales (tens to hundreds of thousands of years), populations that are genetically divergent may become either locally adapted to a specified range of conditions (if conditions are constrained within a narrow range), or they may retain a large degree of physiological plasticity (if the environments they inhabit are highly variable). Further, dissolved O_2 may display high spatial and temporal variability in coastal freshwater and estuarine systems that is often overlooked in empirical studies. To assess the contribution of population-of-origin or physiological plasticity to hypoxia tolerance, juvenile barramundi (one tropical and one sub-tropical population) were exposed to daily fluctuations in dissolved O_2 (>85% to <10% saturation)

for 0 (control), 8 or 16 d (*Chapter 3*). Fish (separate cohorts) were then assessed for either resting $\dot{M}O_2$ and O_{2CRIT}, or haematological parameters. No changes in any parameters were detected after 8 d, however after 16 d a reduction in O_{2CRIT}, and increases in both haematocrit and haemoglobin were observed. No population differences were detected for any measured parameter. This study demonstrates that barramundi populations are capable of acclimating to diel-cycling hypoxic conditions following repeated exposure, and that such changes are accompanied by improvements to blood-O_2 carrying capacity.

Inter-specific diversity in hypoxia tolerance of teleost fish is widely acknowledged, however the extent of intra-specific diversity in hypoxia tolerance is less well understood, due in part to the logistical and temporal constraints of measuring performance across a large number of individuals. Further, the temporal repeatability and hence the reliability of hypoxia tolerance measures have received virtually no attention in the broader scientific literature. To address these knowledge gaps, ~800 juvenile barramundi were first separated into hypoxia tolerance categories (*Chapter 4*) based on time to loss of equilibrium (LOE) tests: sensitive, intermediate and tolerant. Following a recovery period, fish were then assessed for growth performance, metabolic regulation under hypoxia (O_{2CRIT}) and repeatability of time to LOE. Further, the relationship between $\dot{M}O_2$ and DO during O_{2CRIT} tests was assessed using non-linear regression and broken-stick regression techniques, which represents a novel approach to handling such a large empirical data set. Fish were reliably separated into different hypoxia tolerance categories, yet there were no significant category effects for any of

the subsequently measured variables: growth rate, feed conversion ratio, standard metabolic rate or O_{2CRIT}. Non-linear regression was more robust in describing the relationship between $\dot{M}O_2$ and DO than the more commonly used broken-stick regression method. Surprisingly, there was no significant relationship between two independent measures of hypoxia tolerance: time to LOE and O_{2CRIT}. The time to LOE test was broadly repeatable after ~100 d. This study highlights the extent of intra-specific diversity in hypoxia tolerance that can exist within fish populations.

All previous assessments of hypoxia tolerance were necessarily conducted on unfed individuals to eliminate the influence of specific dynamic action (SDA) on metabolic responses. In the wild, and on commercial fish farms, metabolism of fish may be elevated above resting levels due to feeding behaviour. Digestive responses are potentially influenced by dissolved O_2 conditions, however, few studies have assessed this possibility in tropical fish. Further, no studies have assessed the effect of hypoxia tolerance phenotype on digestive metabolic responses. Therefore, barramundi were separated into hypoxia tolerance phenotypes (sensitive or tolerant) based on time to LOE tests (*Chapter 5*). Fish were then fed a restricted ration (2.5% of their body-mass), before being assessed for $\dot{M}O_2$ under normoxic (>85% saturation) and chronic hypoxic (~35% saturation) conditions. There was no effect of hypoxia tolerance phenotype for any measured parameter, and negligible differences in a range of digestive responses (SDA magnitude, SDA duration, SDA coefficient) under normoxic or hypoxic conditions. The results from this study suggest that barramundi are capable of maintaining maximal digestive capacity even when O_2 drops to 35% saturation.

The results from this thesis demonstrate that barramundi are extremely resilient to bouts of environmental hypoxia, and retain a strong tolerance to hypoxic conditions at extremely warm temperatures. The lack of population differences in hypoxia tolerance may be explained by the retention of a high degree of physiological plasticity as a strategy for responding to environmental hypoxia. The extent of phenotypic diversity in hypoxia tolerance is impressive, and the influence of this diversity on performance is still poorly understood. The homogeneity of performance between hypoxia tolerance phenotypes across two experiments and a number of measured parameters suggests that hypoxia tolerance is unrelated to several other performance metrics under either normoxic or hypoxic conditions. Future research should be directed at further exploring the relationship between phenotypic diversity in hypoxia tolerance and fitness, with an objective to more clearly elucidate the ecological consequences of trait variability.

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LIST OF ABBREVIATIONS

AGR Absolute Growth Rate

AIC Akaike's Information Criterion

ASR Aquatic Surface Respiration

BOD Biochemical O_2 Demand

BSR Broken-stick Regression

BW Body-mass

CTV Critical Trigger Value

DE Digestible Energy

DO Dissolved O_2

FC Food Consumption

FCE Food Conversion Efficiency

FCR Food Conversion Ratio

FR Feeding Rate

Hb Haemoglobin

Hct Haematocrit

HCT Hypoxia Challenge Test

HRA Hypoxia Resilience Analysis

INT Intermediate

K Condition Factor

LOE Loss of Equilibrium

LT_{50} Median Lethal Time

MCHC Mean Cellular Haemoglobin Concentration

$\dot{M}O_2$ O_2 Consumption Rate

NH_3/NH_4^+ Ammonia

NLR Non-linear Regression

NO_2^- Nitrite

NO_3^- Nitrate

NT Northern Territory

O_2 Oxygen

$[O_2]_{CRIT}$ Critical O_2 Saturation

O_{2CRIT} Critical O_2 Level

pH Decimal Cologarithm of Hydrogen

P_{CRIT} Critical O_2 Tension

PO_2 O_2 Partial Pressure

QLD Queensland

SDA Specific Dynamic Action

SENS Sensitive

SGR Specific Growth Rate

SMR Standard Metabolic Rate

TOL Tolerant

WA Western Australia

CHAPTER 1. GENERAL INTRODUCTION

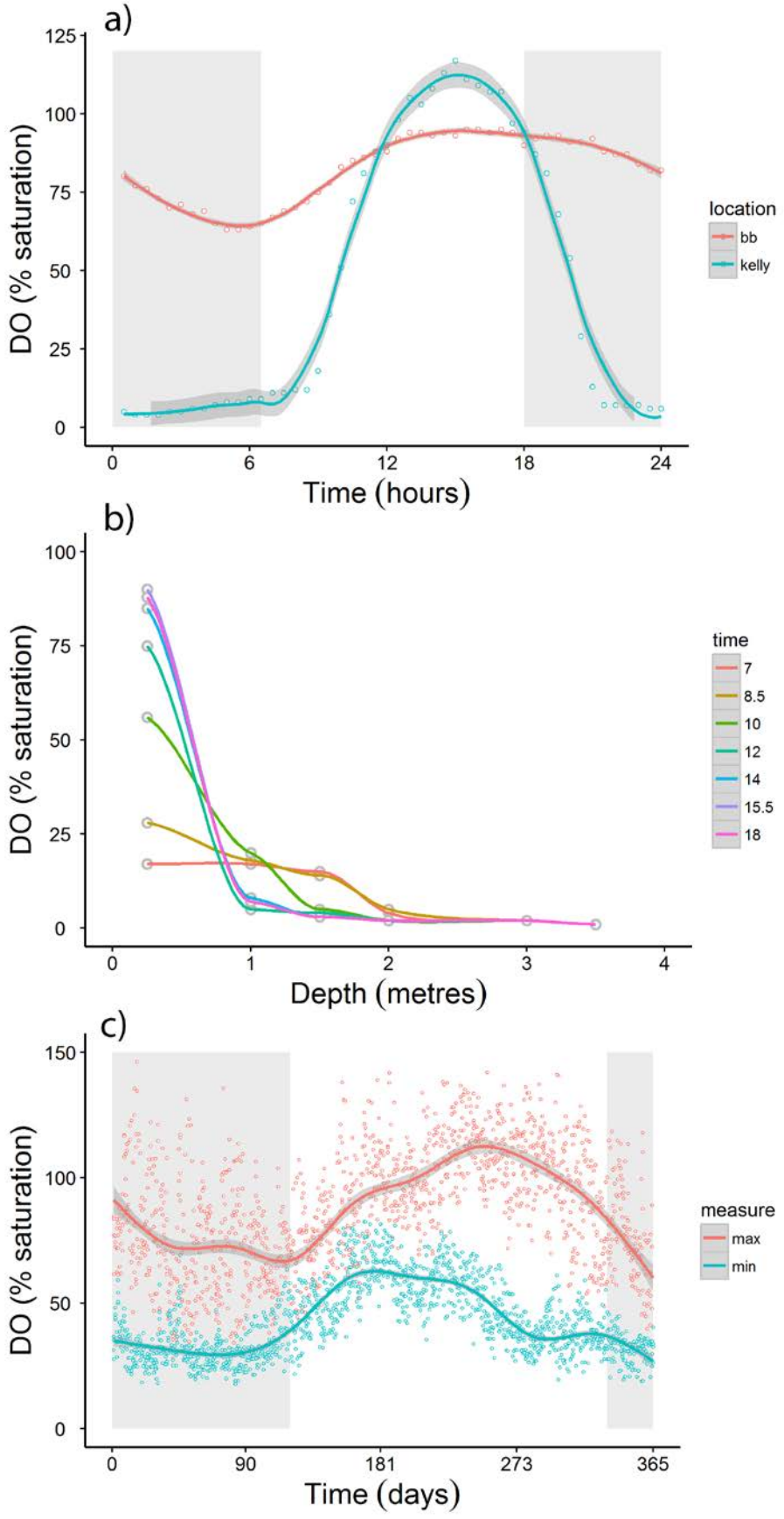
Environmental hypoxia

Low dissolved oxygen (DO), known as hypoxia, was identified by aquatic ecologists as being detrimental to fish as early as the 1920s (Díaz and Rosenberg, 2011; Fry and Hart, 1948; Jones, 1952), and is widely regarded as being an important and ubiquitous stressor for aquatic life (Butler and Burrows, 2007). Critical hypoxia is defined in broad terms as the partial pressure of oxygen (O_2) when physiological function becomes compromised (Farrell and Richards, 2009). Environmental hypoxia may be either a seasonal (Arend et al., 2011; Díaz and Rosenberg, 2011; Townsend and Edwards, 2003) or daily (Butler and Burrows, 2007; Nilsson et al., 2004; Val et al., 2005) phenomenon that occurs in natural, undisturbed waters due to three primary mechanisms: 1, thermal stratification, 2, high biochemical O_2 demand (BOD) and 3, diel cycling due to fluctuations in O_2 production (photosynthesis) and respiration.

Thermal stratification is particularly prevalent in tropical lakes and wetlands (Val et al., 2005) and occurs due to differences in water density (Figure 1). Under such conditions the cooler, denser waters (hypolimnion) become separated from the air-water interface (epilimnion) and, in the absence of submerged macrophytes, much or all of the O_2 may be consumed, leading to severe hypoxia or anoxia. In shallow waters (freshwater and estuarine

systems), the epilimnion may cool to such an extent during the night that complete mixing occurs, however, in deeper waters a distinct thermocline can develop and may persist until a major disturbance causes a turnover (Felsing and Glencross, 2004). In some instances, the bottom waters of deep lakes (Verburg et al., 2003) and continental shelf slopes (Helly and Levin, 2004; Reichart et al., 1998) may become permanently anoxic. Such variability in water quality constrains activity and habitat use by aquatic fauna (Borsuk et al., 2002; Chabot and Claireaux, 2008; Kramer, 1987; discussed further below).

Biochemical O_2 demand (BOD) is the quantity of O_2 required by heterotrophic microbes to break down organic material in water (Brenniman, 1999), whereas biological O_2 demand is the total demand for O_2 of all biological organisms in water (Pearson et al., 2003). An increase in the quantity of organic material present in water, such as accumulation in benthic sediments or a sudden increase throughout the water column from pre-flush rain events (Waltham et al., 2013), may increase BOD to the point where DO becomes life-threatening to obligate aerobes. Such conditions can result in large-scale fish kills (Bishop, 1980; Small et al., 2014; Thronson and Quigg, 2008; Townsend et al., 1992), which can have long-lasting ecological impacts.


a)
DO (% saturation)
125
100
75
50
25
0
0
6
12
18
24
Time (hours)
location
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b)
DO (% saturation)
100
75
50
25
0
0
1
2
3
4
Depth (metres)
time
7
8.5
10
12
14
15.5
18
c)
DO (% saturation)
150
100
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0
90
181
273
365
Time (days)
measure
max
min

Figure 1 - Spatial and temporal variability in dissolved O_2 from selected locations in tropical north-east Queensland, Australia. Diel fluctuations in O_2 recorded 300 mm below the surface from two locations in the Burdekin River (panel a): open river, ~350km from the river delta (bb; red) and a floodplain wetland covered by native floating plants, ~30km from the river delta (kelly; blue), adapted from Loong et al. (2005). Shaded (grey) regions indicate an absence of sunlight. Panel b: spatial and temporal variability in dissolved O_2 from a floodplain wetland on the Herbert River, adapted from Butler (2008). Time of day is indicated by the different colours and numbers in the figure legend: 7 = 0700, 8.5 = 0830, 10 = 1000, 12 = 1200, 14 = 1400, 15.5 = 1530, 18 = 1800. Panel c: seasonal variability in maximum and minimum surface dissolved O_2 (red and blue, respectively), recorded on a commercial fish (barramundi) farm ~100km south of Cairns, Australia (Chung, C. and Hayward, S., personal comm.). Shaded (grey) regions indicate the wet (monsoon) season (December – April) in the Australian wet tropics.

Diel-cycling in DO is common in many waterways, but may be particularly prevalent in shallow, lentic systems with high plant or algal biomass (Almeida-Val et al., 2005; Butler, 2008). O_2 production due to photosynthesis occurs only during the daylight hours and such systems may become O_2 deficient overnight (Figure 1). The amplitude of diel cycling may be further influenced by natural weather events (e.g. cloud cover) which may restrict O_2 production. Such fluctuations may result in daily physiological and behavioural adjustments to fish and other aquatic fauna (Tyler and Targett, 2007; discussed further below).

Anthropogenic influence on O_2 in water

“Globally, human activities have led to large-scale modification of landscapes at the expense of ecosystem function and services” (Díaz, 2010). Hypoxia has increased substantially over the past 50 years in many coastal and

estuarine systems adjacent large-scale human development (Figure 2), with notable examples including the Baltic Sea (Conley et al., 2011), Gulf of México (Rabalais et al., 2007) and Chesapeake Bay (Rabalais et al., 2010). The primary influence of human activity on water quality has been through the alteration of nutrient cycles, in particular nitrogen and phosphorous from the agricultural industry, and effluent from municipal wastewater (Díaz, 2010). Such changes can result in large-scale increases in primary production that may lead to eutrophication (an increase in the rate of supply of organic matter to an ecosystem; Boesch, 2002; Nixon, 1995), and hence a concomitant increase in BOD and decrease in DO to critically low levels. Anthropogenic influences on O_2 in water may be further exacerbated in the future due to the rapid average increase in global temperatures, attributed to a rise in carbon dioxide (CO_2) and methane gas in the atmosphere from the burning of fossil fuels (BoM and CSIRO, 2014; IPCC, 2013; Rabalais et al., 2010). Such increases in temperature are predicted to affect aquatic life through elevated metabolic demand for O_2, lower solubility of O_2 in water and by exacerbating thermal stratification (Altieri and Gedan, 2015; Diaz and Breitburg, 2009; Harley et al., 2006; Vaquer-Sunyer and Duarte, 2008; Vaquer-Sunyer and Duarte, 2011; Figure 4).

Hypoxia may also be an issue in artificial environments created by people for intensive aquaculture (ponds, tanks, raceways and cages), where stocking densities and nutrient inputs are unnaturally high. Indeed, low O_2 and concomitant high CO_2 as a result of high stocking densities and nutrient inputs are widely recognised as imposing a limit on the productivity of such systems, and considerable effort is often invested in alleviating such

problems in order to maximise system productivity (Colt and Orwicz, 1991; Cruz-Neto and Steffensen, 1997; Summerfelt et al., 2000). In addition to being a problem inside intensive aquaculture systems, the wastewater from aquaculture can deliver large quantities of nutrients to river and estuarine systems and, if not properly treated, may further contribute to freshwater and coastal eutrophication (Cai et al., 2013; Lin et al., 2002), exacerbating the pressure on wild fish populations.

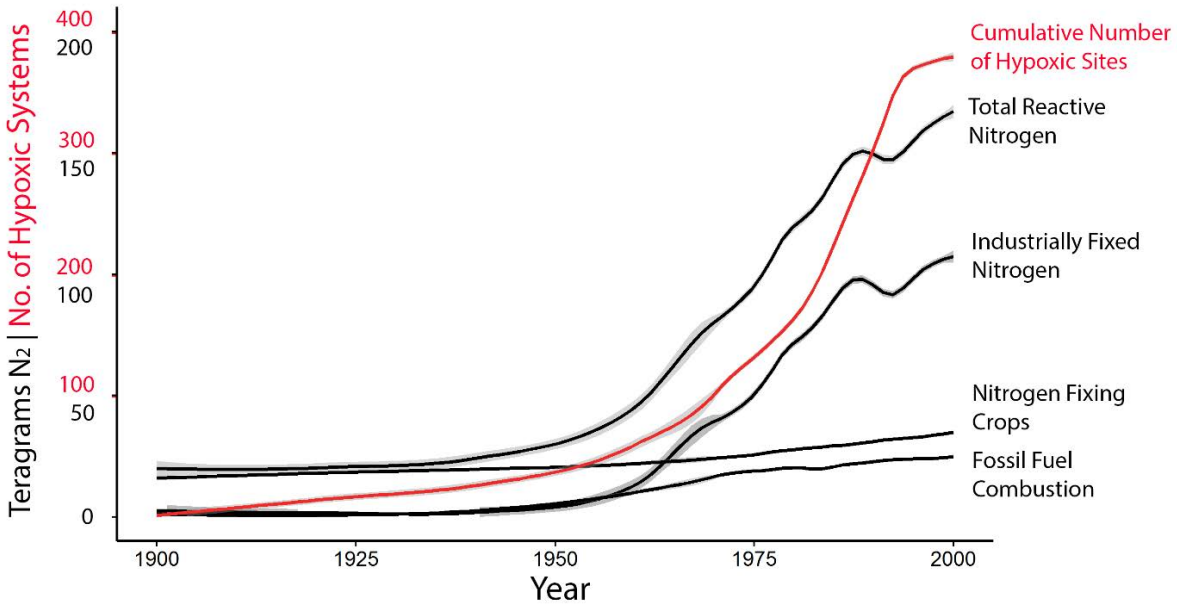

Figure 2 - Increase in nitrogen entering coastal waterways from the years 1900 to 2000 (adapted from Diaz and Breitburg, 2009). The red line displays the cumulative number of hypoxic sites in coastal waterways over the same period.

Behavioural and Physiological Responses by Fish to Hypoxia

Mobile aquatic vertebrates such as fish exhibit a range of behavioural, morphological and physiological changes accompanying hypoxia exposure

that vary depending on the severity and magnitude of the hypoxic event (reviewed by Almeida-Val et al., 2005; Bickler and Buck, 2007; Claireaux and Chabot, 2016; Hochachka and Lutz, 2001; Pollock et al., 2007; Richards, 2009; Wu, 2002). Responses to environmental changes, such as hypoxia can be broadly classified as short-term, plastic responses or longer term, evolutionary adaptations. Plastic responses can be further categorised as reversible (e.g. seasonal changes or acclimation; Fu et al., 2011; Love and Rees, 2002) or irreversible (developmental plasticity; West-Eberhard, 2005).

Avoidance behaviour and aquatic surface respiration

Probably the most common behavioural response employed by fish is avoidance of hypoxic water (Burleson et al., 2001; Kramer, 1987), however, some fish actively make forays into hypoxic water of varying durations for either foraging (Behrens et al., 2012; Plambech et al., 2013), or to seek refuge from predation (Chapman et al., 1995; Schofield and Chapman, 2000). If hypoxia is severe and inescapable, fish may resort to further behavioural adjustments, including skimming the O_2 -rich surface layer of the water (commonly known as aquatic surface respiration (ASR); Abdallah et al., 2015; Almeida-Val et al., 2005), or for some species, air-breathing (Clark et al., 2007; Fritsche et al., 1993).

Changes at the gills and heart

One of the simplest and most common responses by fish to sub-lethal hypoxia is to maintain O_2 uptake by increasing the amplitude and frequency

of gill ventilation (Flint et al., 2015; Perry et al., 2009). Such responses serve to maximise the blood/water partial pressure gradient, and hence to minimise the reduction in arterial partial pressure of O_2 and maintain a consistent supply of O_2 to tissues (Perry et al., 2009). Oxygen uptake may be further assisted by increasing the surface area of the gills (Chapman et al., 2000; Fu et al., 2011), although this response is not ubiquitous and in some cases a decrease in gill surface area can occur (Borowiec et al., 2015). Acute hypoxic episodes result in bradycardia for many fish species and under these conditions, cardiac output may be compensated for by an increase in stroke volume (Gamperl and Driedzic, 2009).

Changes in the blood

A suite of changes occur within the blood during hypoxia to compensate for a decline in environmental O_2, with alternative changes occurring during functional hypoxia (rigorous exercise) (Wells, 2009a). A fishes' ability to tolerate environmental hypoxia is correlated, in large part, to O_2 carrying capacity of the blood (Figure 3), and the O_2 binding affinity of haemoglobin (Hb; Mandic et al., 2009; Richards, 2011). Fish that are better adapted to environmental hypoxia tend to have Hb that exhibits a high affinity for O_2 and blood that is relatively insensitive to changes in pH (i.e. a less pronounced Bohr effect), while the reverse seems to be more typical of fish that have an athletic lifestyle (Wells, 2009a). It is worth mentioning here that many fish often contain more than one form of Hb in blood (Wells et al., 1997), some of which have entirely different characteristics (Weber, 1996). In addition to the properties of Hb mentioned above, the O_2 carrying capacity of fish blood is modulated by the quantity of red blood cells, the quantity of Hb present within

the blood and changes to Hb function by phosphates (ATP and GTP) present within erythrocytes (Rasmussen et al., 2009; Wells, 2009a). When environmental O_2 declines to low levels, large changes in O_2 carrying capacity of the blood may be experienced over a relatively small range in DO (Figure 3), adversely affecting the capacity for aerobic metabolism.

Changes to oxygen consumption

The availability of O_2 was described by Fry (1971) as a limiting factor for metabolism by aquatic life. The functional role of O_2 in metabolism is as a terminal electron acceptor in the oxidation of organic substrates which, through a series of biochemical reactions in the mitochondria, results in the generation of ATP (Hochachka and Somero, 2002). As O_2 declines fish may regulate the rate of O_2 consumption ($\dot{M}O_2$) to sustain aerobic metabolism, but below the critical O_2 level (O_{2CRIT}; also referred to in the literature as the critical O_2 tension (P_{CRIT})) a fish's $\dot{M}O_2$ transitions from being independent to being dependent upon O_2 in the environment (Claireaux and Chabot, 2016; Richards, 2009; Figure 3). At such critically low levels (typically between 10 and 40% of air saturation in teleost fishes (Rogers et al., 2016)), glycolysis and/or phosphagen mobilisation become the primary sources of ATP generation (Boutilier and St-Pierre, 2000; Hochachka and Somero, 2002; Richards, 2009). The decline in $\dot{M}O_2$ below O_{2CRIT} is often precipitous (Figure 3) and is an ubiquitous response by fish species during severe hypoxia. The O_{2CRIT} is therefore a common measurement in ecological physiology and has been utilised extensively for broad inter-species comparisons of hypoxia tolerance (Fu et al., 2014; Mandic et al., 2009; Richards, 2011; Rogers et al., 2016).

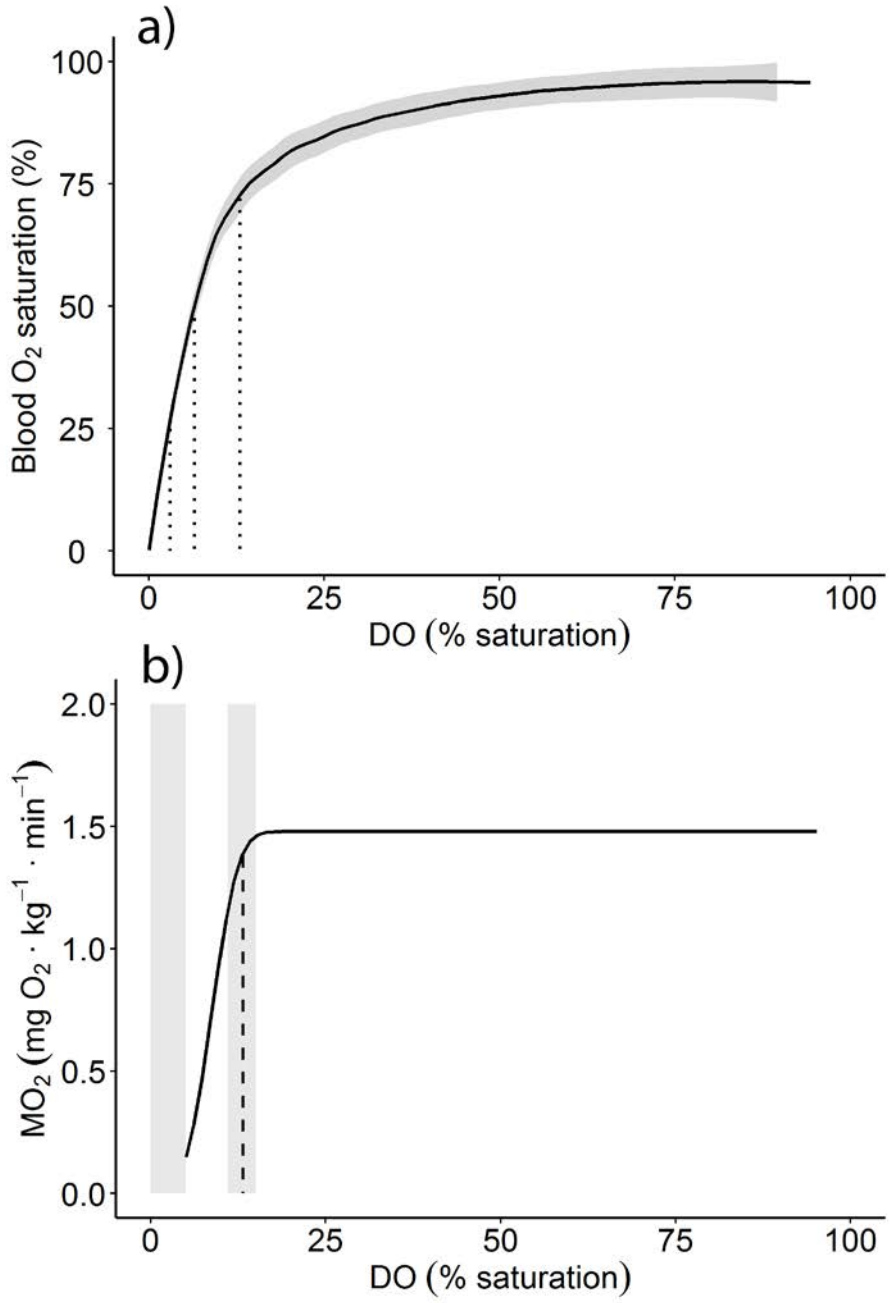

Figure 3 - Relationships between blood-O_2 saturation (panel a) and metabolic rate (panel b) and dissolved O_2 in fish. The blood-O_2 saturation curve (panel a) has been modified from Grigg (1969), and is from channel catfish (*Ictalurus punctatus*) blood at 24°C. The vertical lines at 13%, 6.5% and 3% water dissolved O_2 saturation indicate where blood-O_2 saturation is equal to 75%, 50% and 25%, respectively. The relationship between $\dot{M}O_2$ and DO is from data collected for this thesis using barramundi (*Lates calcarifer*) held at 30°C. The vertical dashed line at ~13% saturation indicates the critical O_2 level (O_{2CRT}), and the shaded grey region around the O_{2CRIT} represents the region of transition from oxy-regulation to oxy-conformation. The shaded grey region ≤ 5% saturation indicates the region where fish are likely to rapidly lose equilibrium.

Intra-specific diversity in hypoxia tolerance

While it is generally accepted that species display a broad array of responses to hypoxia (Fu et al., 2014; Mandic et al., 2009), less well understood is the influence of hypoxia as a selective evolutionary pressure on localised fish populations. Recent evidence has indicated that populations of fish inhabiting different environments (e.g. well-oxygenated rivers vs. shallow, aperiodically hypoxic wetlands) may elicit differences in physiology and morphology (Crispo and Chapman, 2010; Martínez et al., 2009; Sneddon and Yerbury, 2004; Timmerman and Chapman, 2004). The quantity of O_2 in the water and metabolic rates of aquatic fauna are proportional to water temperature (Figure 4). Persistent high temperatures are characteristic of tropical environments (Almeida-Val et al., 2005) and while great effort has been expended in the characterisation of fish performance at elevated temperatures across latitudinal gradients (Dhillon and Schulte, 2011; Donelson and Munday, 2012; Grabowski et al., 2009; Righton et al., 2010; Whitehead et al., 2011), population-level responses to hypoxia are less-well understood.

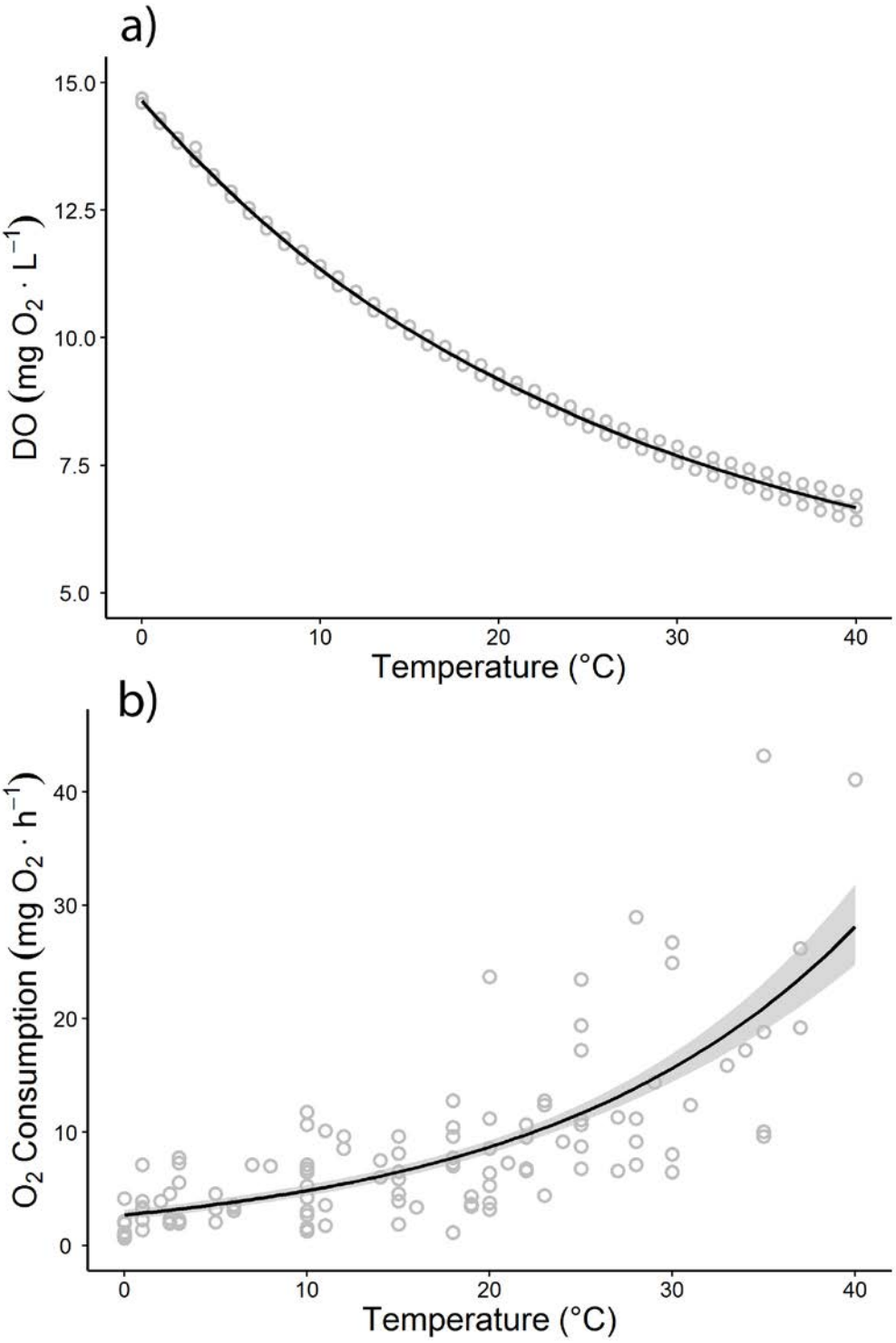

Figure 4 - Relationships between the solubility of O_2 in freshwater (Boutilier et al., 1984; Davis, 1975; FAO, 1987; panel a) and resting O_2 consumption rates of teleost fish (modified from Clarke and Johnston, 1999; panel b) and water temperature. The relationship between O_2 solubility in freshwater and temperature is defined by the equation: $f(x) = -0.00005x^3 + 0.0069x^2 - 0.3906x + 14.616$, where x is equal to water temperature

Study Species (Lates calcarifer)

The species of teleost fish used for this thesis was the barramundi (also known as Asian Sea Bass; Jerry, 2014; Figure 5). Barramundi (*Lates calcarifer*) occur throughout the tropical and sub-tropical Indo-Pacific and are of substantial economic, ecological and cultural importance in the region. Barramundi are diadromous and make regular forays into both fresh and salt water environments. A critical period of the life history for this species is spawning in estuaries (salinities >25 psu are required for hatching and egg development; Thépot and Jerry, 2015), which typically occurs during the monsoon season. Following spawning, juveniles disperse into local mangrove and wetland habitats before migrating into the upper reaches of rivers, where they may remain for several years (Milton et al., 2005; Milton and Chenery, 2005). While some juveniles and adults may migrate into adjacent river systems, tagging studies indicate that movement between river systems is relatively low in Australia (Davis, 1986; Russell and Garrett, 1988). The localised nature of distribution, together with historical barriers to dispersal (Keenan, 2000) has led to the formation of localised population structure in Australia (Figure 6). Recent evidence has indicated that differences between populations may exist for thermal tolerance (Edmunds et al., 2012; Edmunds et al., 2010; Newton et al., 2010; Newton et al., 2013) and other performance measures (Rodgers and Bloomfield, 1993).

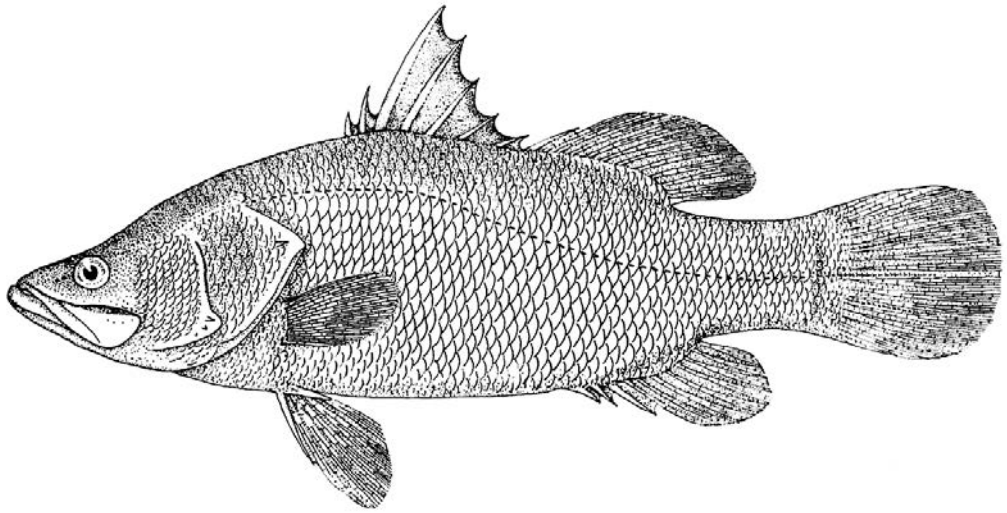

Figure 5 - *Lates calcarifer* juvenile sketch, Katayama and Taki (1984)

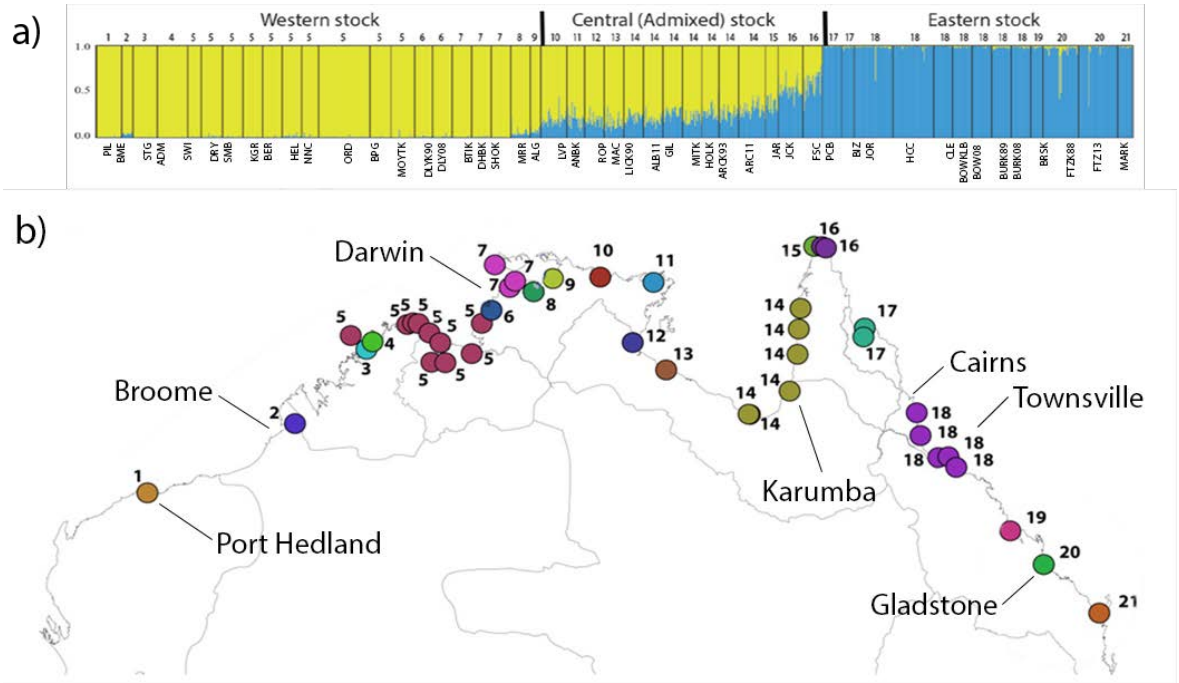

Figure 6 - Map of the northern Australian coastline (panel b; modified from Jerry et al., 2013) with major freshwater bioregions indicated as light grey lines on the figure (Unmack, 2001). Major population centres across northern Australia are indicated by the text and diagonal lines. Genetically distinguishable sub-populations of barramundi are indicated on the map as coloured circles and corresponding numbers (1 – 21). The co-ancestry plot immediately above the map (panel a) (and corresponding numbers) indicates the degree of relatedness between the two major stocks, as identified using 16 microsatellite loci and pairwise F_{ST} comparisons: Western (yellow) and Eastern (blue).

Research aims and thesis objectives

The primary aims of this thesis were to use barramundi as a model to examine intra-specific variability in hypoxia tolerance in tropical fish populations, and to understand the biological consequences of variability in hypoxia tolerance both within and between fish populations. All of the data chapters presented herein are broadly related to these aims, yet there is a clear dichotomy in this thesis with data chapters 2 and 3 focussing on variability in hypoxia tolerance between populations, and chapters 4 and 5 focussing on variability within populations (Figure 7). As such, the data chapters presented in this thesis have been written as stand-alone manuscripts for publication, yet they complement each other to form a comprehensive examination of the primary aims.

Barramundi was selected as an appropriate species for this research due to their: 1, broad distribution and availability across a large latitudinal gradient, 2, distinct and known genetic population structure, 3, propensity to inhabit coastal wetlands that are subject to large variability in temperature and dissolved O_2, and 4, substantial ecological, economic and cultural importance across the natural distribution. Increasing thermal stratification due to rising temperatures, and eutrophication from higher population pressure in coastal, tropical waterways mean that fish species such as barramundi are likely to encounter a higher incidence of hypoxic stress in the future.

The specific objectives of this thesis were to:

1. Quantify the metabolic responses of barramundi populations to the combined pressures of high temperature and low O_2
2. Assess the capacity for acclimation to cyclic bouts of low O_2 in barramundi populations
3. Determine the extent of intra-specific variability in hypoxia tolerance of barramundi populations
4. Assess intra-specific diversity in performance of barramundi hypoxia tolerance phenotypes
5. Quantify the metabolic responses of barramundi hypoxia tolerance phenotypes to the combined challenges of digestion and hypoxia

Chapter 2 investigates the resting metabolic rate and critical O_2 level (O_{2CRIT}) of barramundi at benign and warm temperatures, and was essential for characterising metabolic responses to hypoxia in barramundi, as well as for broad population-level comparisons. In *Chapter 3* the effects of daily hypoxia exposure on metabolic and haematological responses of a sub-tropical and tropical population were assessed. *Chapter 4* builds upon the results in the preceding two chapters by assessing the diversity of hypoxia tolerance phenotypes that exist within sub-tropical and tropical populations. Growth performance and metabolism of hypoxia tolerance phenotypes are then assessed, as well as the repeatability of hypoxia tolerance tests in order to assess the biological consequences of variability in hypoxia tolerance within fish populations, and to determine the efficacy of spot measurements of hypoxia tolerance in physiological studies. *Chapter 5* investigates the

metabolic responses to digestion in hypoxia tolerance phenotypes under normoxia and chronically low O_2 conditions. The main findings from these four data chapters are discussed in *Chapter 6*.

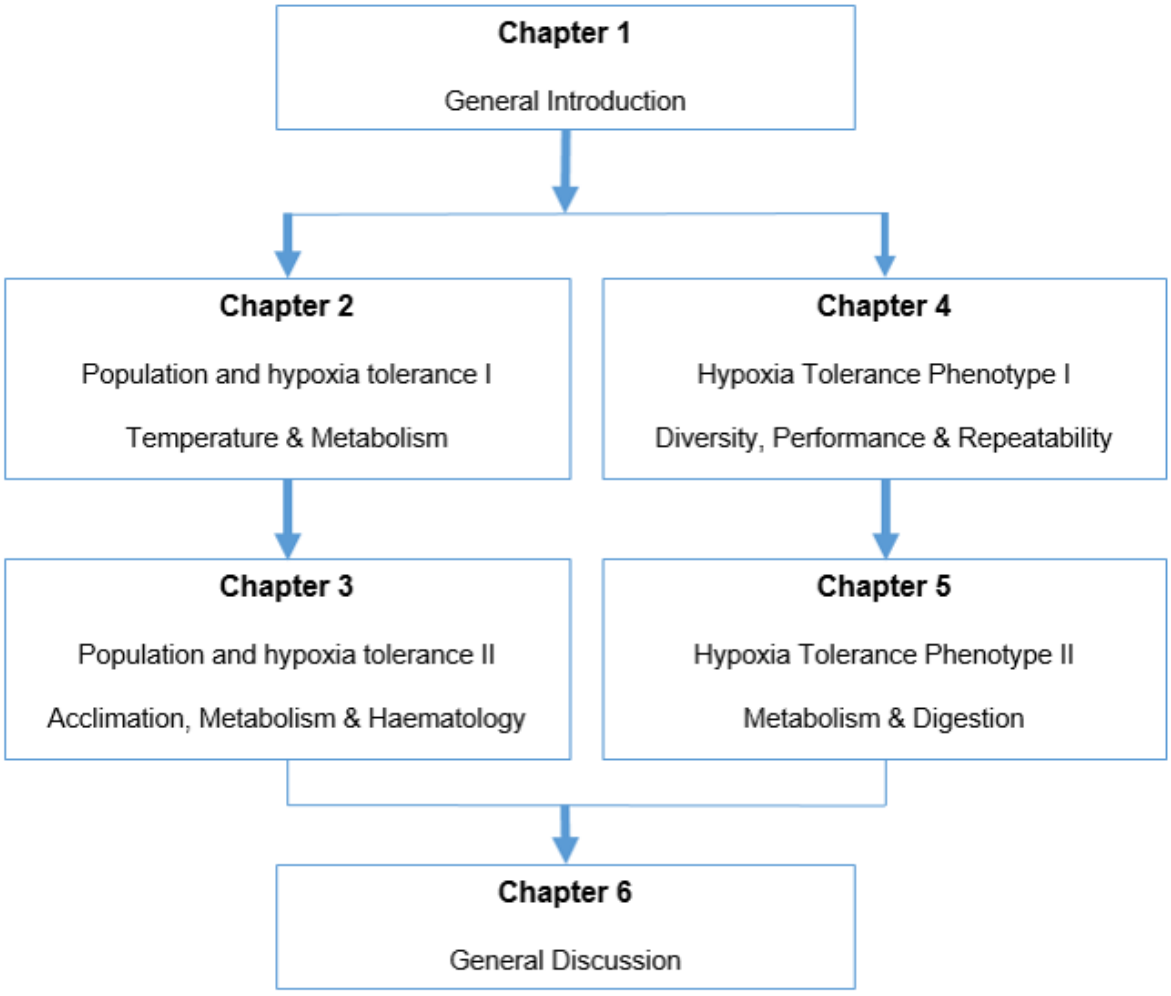

Figure 7 - Chapter structure of thesis

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CHAPTER 2. HYPOXIA TOLERANCE IS CONSERVED ACROSS GENETICALLY DISTINCT POPULATIONS OF AN ICONIC, TROPICAL AUSTRALIAN TELEOST (*LATES CALCARIFER*)

Abstract

Tropical coastal systems are particularly prone to periods of environmental hypoxia, which can result from organismal respiration as well as thermal stratification, and may be further exacerbated by anthropogenic disturbances. In this study I used five genetically distinct populations of Australian barramundi (*Lates calcarifer*) to examine the extent of intraspecific variability in hypoxia tolerance. Fish were maintained at two temperatures (26°C or 36°C), representing the seasonal thermal range for this species across its tropical distribution in Australia. All fish maintained a constant O_2 consumption rate ($\dot{M}O_2$) as air saturation of the water decreased from 100% down to a critical O_2 level (O_{2CRIT}) of 15.44 ± 3.20% (mean ± SD) and 21.07 ± 3.92% at 26°C and 36°C, respectively. Mean O_{2CRIT} did not differ between populations. No differences were found for resting $\dot{M}O_2$ between populations at 26°C, however modest differences were detected between two populations at 36°C. Resting $\dot{M}O_2$ was lower for populations at 26°C than at 36°C. I conclude that both hypoxia tolerance and resting $\dot{M}O_2$ are conserved across the distribution of barramundi in Australia, which reflects the capacity of this species to cope in environments with large fluctuations in both temperature and dissolved O_2.

Introduction

The availability of dissolved O_2 (DO) in aquatic systems is critical in defining the abundance and distribution of biological communities (Pearson et al., 2003). In freshwater and estuarine systems, O_2 can fluctuate between normoxia and severe hypoxia due to respiration of organisms inhabiting such systems and thermal stratification (Brauner and Val, 2005; Pearson et al., 2003). In tropical regions, initial rains at the onset of the monsoon season can carry high loads of organic material into freshwater and estuarine systems causing a rapid depletion of O_2, which can be fatal for resident animal populations (Bishop, 1980; Erskine et al., 2005; Townsend et al., 1992). Furthermore, natural occurrences of hypoxia may be exacerbated by anthropogenic disturbances such as agriculture and urbanisation (Pollock et al., 2007).

Altered weather patterns resulting from climate change have the potential to exacerbate periods of environmental hypoxia. Higher water temperatures reduce the solubility of O_2 in water while concurrently increasing the metabolic demand for O_2 in aquatic ectotherms (Diaz and Breitburg, 2009; Weiss, 1970). As such, the unprecedented rate of temperature rise predicted under current climate change scenarios may push many aquatic species close to, or above, their upper thermal thresholds (Huey et al., 2012; Morrongiello et al., 2011; Tewksbury et al., 2008). This may be particularly pronounced at very low latitudes near the equator, where species may be adapted to mean temperatures that may span only 1-2°C annually (Tewksbury et al., 2008). Thus, it is likely that the elevated

temperatures predicted to occur in the future will progressively increase the prevalence and severity of hypoxia in aquatic systems, with flow-on effects to larger scale processes such as population growth, fitness and ecosystem dynamics (Pollock et al., 2007).

Fish can physiologically and behaviourally respond to fluctuating DO, although such responses are highly species- and context-dependent. For example, fish that encounter hypoxic conditions may actively avoid areas of low DO (Herbert and Steffensen, 2005; Poulsen et al., 2011), increase gill ventilation volume (Fernandes and Rantin, 1989; Jesse et al., 1967), increase the number of red blood cells and haemoglobin concentration (Wells et al., 1989), and/or induce bradycardia (Randall and Smith, 1967). Exposure to acute or chronic hypoxia can have sub-lethal effects such as altered behaviour and reduced growth and reproduction, which may be further exacerbated by increasing water temperatures (Vaquer-Sunyer and Duarte, 2008).

The physiological responses to hypoxia are regarded to be dependent on the frequency and severity of the hypoxic event (Farrell and Richards, 2009). Below a certain DO, most fish are unable to regulate their O_2 consumption rate ($\dot{M}O_2$) independently of ambient O_2 levels and consequently enter a state of O_2 conformity (Fernandes et al., 1995; Prosser and Brown, 1961; Schurmann and Steffensen, 1997). The level of DO at which this conformity occurs is referred to as the critical O_2 level (O_{2CRIT}) and is commonly used as a measure of the hypoxia tolerance of a species (Prosser and Brown, 1961; Schurmann and Steffensen, 1997).

The prevalence of intraspecific local adaptation (or interdemic variation) may influence the capacity for sub-populations to respond to environmental changes (Grabowski et al., 2009; Tobler et al., 2011; Whitehead et al., 2011). It has previously been proposed that selection pressure for hypoxia tolerance may lead to variation among populations for species with broad habitat ranges (Timmerman and Chapman, 2004). A number of studies to date have found evidence for local thermal adaptation in metabolic traits between populations of species including killifish (*Fundulus heteroclitus*; Dhillon and Schulte 2011) and Atlantic cod (*Gadus morhua*; Sylvestre *et al*. 2007; Grabowski *et al*. 2009). Local adaptation to hypoxia between populations of fish species across broad spatial scales, however, is less well understood and no study has investigated this for a higher-level predator.

To help address this knowledge gap, I investigated O_{2CRIT} and resting $\dot{M}O_2$ in five different populations of Australian barramundi (*Lates calcarifer*) spanning ~12° of latitude (Figure 8). Experiments were performed at two temperatures chosen to represent typical (26°C) and warm (36°C) summer conditions. The main aims of this study were to; 1) investigate whether populations differ in their O_{2CRIT} and/or resting $\dot{M}O_2$, 2) quantify how different populations respond to the combined challenges of temperature and hypoxia, and 3) determine whether hypoxia tolerance is related to latitudinal position. I expected barramundi populations from lower latitudes to be more hypoxia tolerant than their higher latitude counterparts due to the inverse relationship between water temperature and O_2 solubility. I also expected barramundi (irrespective of population) to exhibit higher metabolic rates at warmer

temperatures, and to consequently be less hypoxia tolerant due to the elevated metabolic demand for O_2.

Materials and Methods

Experimental Animals and Holding Conditions

Barramundi juveniles were obtained from five commercial hatcheries located at Broome (Broome Aquaculture Centre), Darwin (Darwin Aquaculture Centre), Karumba (Barramundi Discovery Centre), Townsville (Mainstream Aquaculture) and Gladstone (Gladstone Water Board Barramundi Hatchery) (Figure 8). All five hatcheries use broodstock sourced from local rivers that are separated by a minimum of 700 km (Fig. 8). Wild populations of Australian barramundi differ genetically (Doupé et al., 1999; Keenan, 1994) and broodstock maintained at these locations have been identified as genetically distinct (Smith-Keune et al, unpub. data). Prior to experimental treatments, fish were on-grown to a size of ~200 g over approximately 10 months in tanks containing fresh water connected to a recirculating system at the Marine and Aquaculture Research Facility Unit of James Cook University, Townsville, Australia. All fish were fed ~1% body mass per day using a commercial pelleted feed (Ridley Aquafeed, Narangba, Australia), and maintained at 26 ± 1°C under a 12:12 hour photoperiod. Dissolved O_2 (DO) was maintained >75% saturation in the holding tanks and water quality was monitored daily. Individual fish were weighed and measured prior to experiments and following respirometry. Mean (± SD) body

mass and condition factor of fish after respirometry were 194.2 ± 22.6 g and 1.2 ± 0.1, respectively.

$$K = \frac{10^5 \times \text{mass}}{\text{length}^3}$$

Equation 1 - Condition factor (Froese, 2006): mass (g) and length (mm)

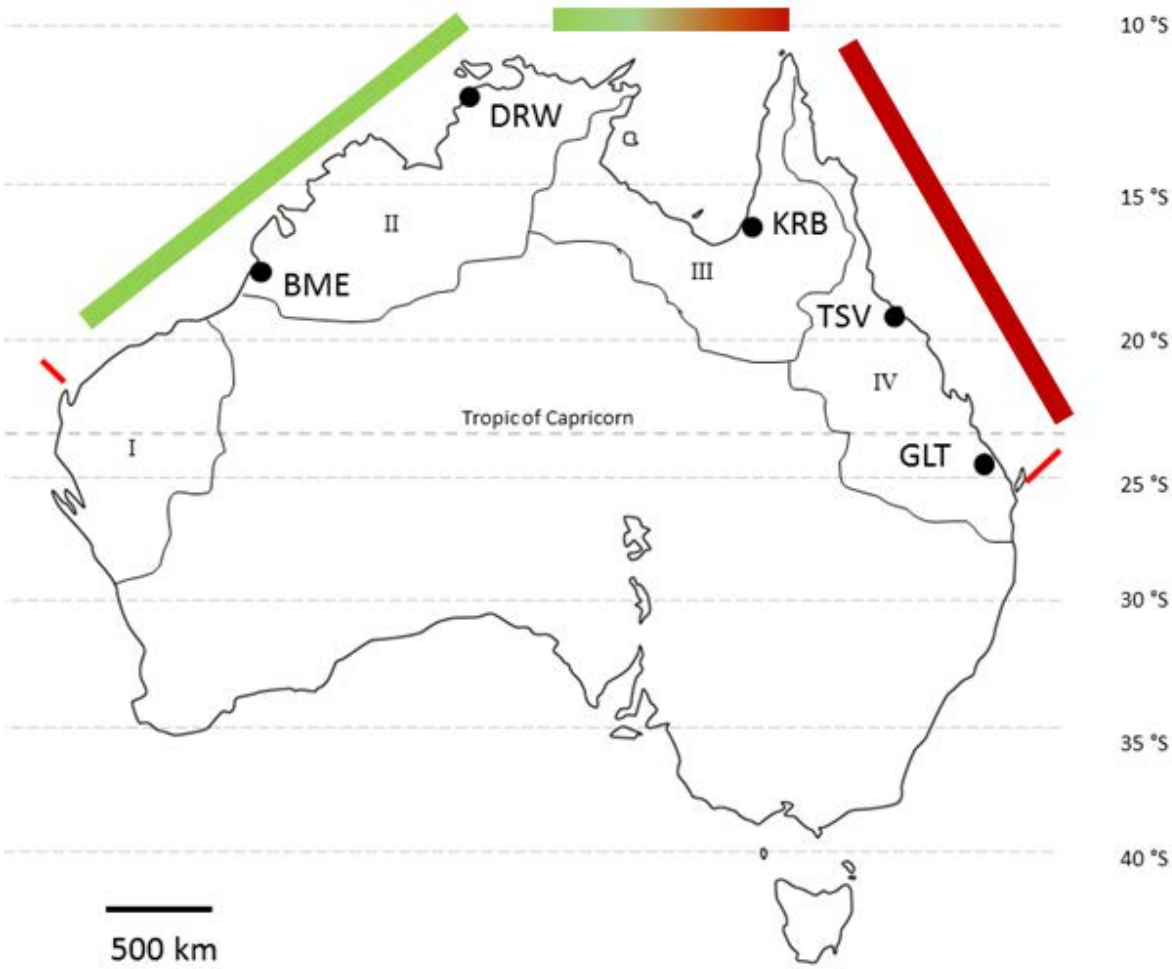

Figure 8 - Map of Australia showing where individual populations of barramundi were collected, as follows: BME, Broome; DRW, Darwin; GLT, Gladstone; KRB, Karumba; and TSV, Townsville. Major river drainage divisions of northern Australia have been redrawn from (Hutchinson and Dowling, 1991) and are indicated as follows: I, Indian Ocean; II, Kimberley and Arafura Sea; III, Gulf of Carpentaria; and IV, North East Coast. Small, red lines adjacent to the coast indicate the southerly limits of the species' distribution. The thicker green and red lines indicate the major stocks as per Figure 6.

Experimental Design

Temperature treatments were selected based on a review of atmospheric and sea-surface temperatures obtained from Australian government databases (AIMS, 2012; BOM, 2012) and from a comprehensive review of river temperatures from previously published data (Pusey et al., 1998; Stuart and Berghuis, 2002; Webster et al., 2005). Based on this information, the two temperature treatments were; 1) a 'typical' temperature (26°C), which is representative of the annual mean across the species distribution in Australia and the temperature at which the fish had been held long-term, and 2) a 'warm' temperature (36°C), which is regarded to be representative of the upper limit that wild populations experience (Russell and Garrett, 1983), but still within the known tolerance limits for the species (Bermudes et al., 2010). Prior to experiments, fish were removed from their holding tanks and acclimated to one of two temperatures in a separate system where water temperature could be closely controlled. While fish in the 26°C treatment did not undergo a temperature change relative to prior holding conditions, fish used in the 36°C treatment were acclimated by increasing the temperature by 1°C per day and then held at 36°C for a minimum of 7 days prior to experimentation. All fish were fed as stated above, but food was withheld for 24 h prior to conducting respirometry to minimise the effect of specific dynamic action on O_2 consumption measurements.

Respirometry

All measurements were performed using intermittent flow-through respirometry following best practices outlined in Clark *et al*. (2013b). Respirometers (volume = 10.3 L) were fitted with a small Perspex window to allow fish to be observed during respirometry trials. Each respirometer was connected to two pumps; a single recirculating pump to keep water within the chamber mixed, and a flush pump to supply the chamber with aerated water between $\dot{M}O_2$ measurements. Respirometers and flush pumps were submerged in a shallow 1000 L tank with vigorous aeration to provide both stable temperature conditions during experiments (26.2 ± 0.5 °C and 36.4 ± 0.2 °C) and to supply the chambers with ~100% saturated water during the flush cycle.

Temperature-compensated O_2 concentration ($mg \cdot L^{-1}$) of the water within each chamber was continuously recorded (0.5 Hz) using O_2 sensitive REDFLASH® dye on contactless spots (2mm) adhered to the inside of each chamber and linked to a Firesting Optical O_2 Meter (Pyro Science e. K, Aachen, Germany) *via* fibre-optic cables. Oxygen sensing equipment was recalibrated daily using a one-point 100% saturation calibration and an electrical (factory-calibrated) zero. Following initial measurements of background respiration in the respirometers, individual fish that had been fasted for 24 h were placed in respirometers in the evening and allowed to acclimate to the respirometers for 16 h.

During the acclimation period, the flush pumps attached to each respirometer were set to a 30:15 min on:off cycle and $\dot{M}O_2$ (mg $O_2 \cdot kg^{-1} \cdot min^{-1}$) was measured from the decline in O_2 in each respirometer during each 15 min off cycle. Resting $\dot{M}O_2$* was calculated for each fish as the mean of the lowest three measurements recorded during the acclimation period.

Following the chamber acclimation period and resting $\dot{M}O_2$ measurements, each flush pump was turned off and fish were permitted to deplete the O_2 within their respective respirometers down to 5% air saturation. $\dot{M}O_2$ was calculated for each consecutive 5 min period during the decline in O_2 (~1.5 - 4 hours depending on temperature). Upon reaching 5% air saturation, each flush pump was turned on to restore O_2 levels to 100% saturation. Preliminary trials conducted using similar sized fish indicated that depletion to 5% saturation was sufficient to calculate O_{2CRIT} but still above the O_2 levels that induce loss of equilibrium. Two values were identified from each O_{2CRIT} experiment; 1) pre-hypoxia $\dot{M}O_2$, which was calculated from the mean of the lowest three 5-min measurements recorded between 100% and 75% saturation after flush pumps had been turned off, and 2) O_{2CRIT}, which was determined using previously established methods (Corkum and Gamperl, 2009; Nilsson et al., 2010). Briefly, O_{2CRIT} was determined by fitting two linear regression lines to the measurements (one line based on the calculated pre-hypoxia $\dot{M}O_2$, and another line based on the decrease in $\dot{M}O_2$ observed during the later stages of the O_{2CRIT} test) and calculating the intersection point of the two lines (Nilsson et al., 2004; Figure 9).

* The terminology "resting MO_2" (Chapters 2 and 3) and standard metabolic rate (SMR; Chapters 4 and 5) are used to describe the oxygen consumption rate of a fish at rest. While these terms relate to different measurements in the broader literature, they can be considered synonymous in the context of this thesis, as the methodology to measure each was highly similar between studies

After the completion of each trial, additional background respiration measurements were obtained for each chamber in the absence of fish. Any change in background respiration between the start and end of experiments was assumed to be linear when correcting fish $\dot{M}O_2$.

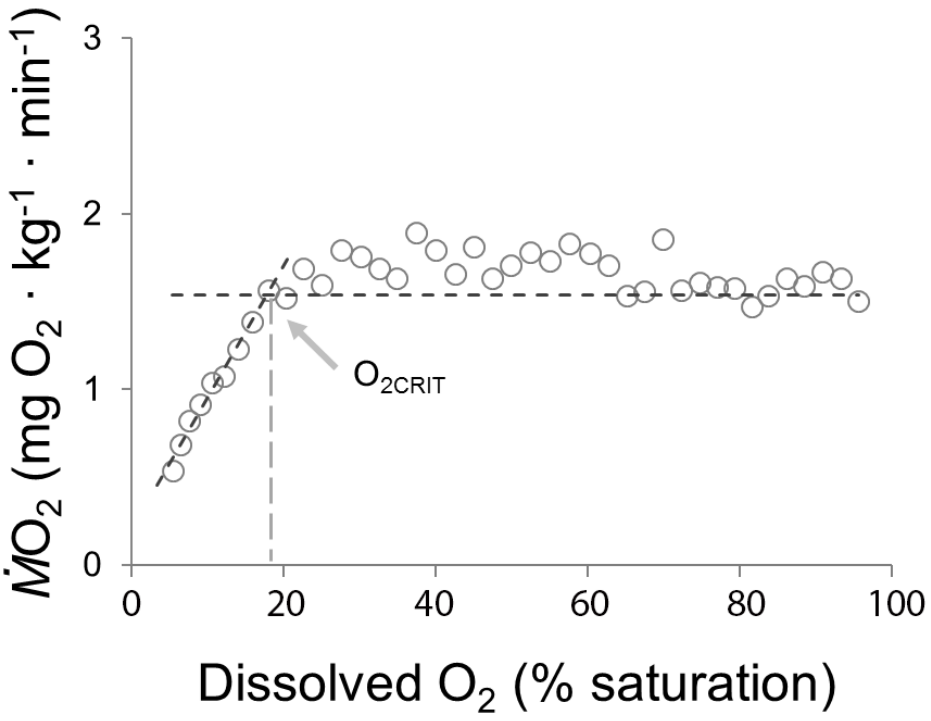

Figure 9 - Plot of O_2 consumption rate ($\dot{M}O_2$) against dissolved O_2 for one fish, illustrating how the critical O_2 level (O_{2CRIT}) was calculated as the intersection of two lines: the horizontal line is calculated as the mean of the lowest 3 $\dot{M}O_2$ measurements ≥ 75% saturation, and the line with positive slope at low DO was fit using least-squares regression.

Data Analyses and Statistics

All O_2 consumption rate measurements (mg O_2 · kg^{-1} · min^{-1}) were calculated using commercial software (LabChart v. 7, ADInstruments, Sydney, Australia) from the slope of the decline in O_2 concentration according to the formula:

$$\dot{M}O_2 = \frac{\Delta O_2}{\Delta T} \times V \times \frac{1}{M}$$

Equation 2 - Oxygen consumption rate ($\dot{M}O_2$ mg $O_2 \cdot kg^{-1} \cdot min^{-1}$), where ΔO_2 = the change in water DO (mg $\cdot L^{-1}$), ΔT = the change in time (s), V = volume of the respirometer (L) and M = the mass of the fish (g).

Resting $\dot{M}O_2$, pre-hypoxia $\dot{M}O_2$, and O_{2CRIT} measurements were obtained for 114 barramundi from the five geographically distinct sub-populations, with individual fish considered as an individual replicate for population and temperature treatments. Individual replicates where O_{2CRIT} was not calculated (N = 7) were excluded from further analyses. The temperature coefficient (Q_{10}) was calculated as:

$$Q_{10} = \frac{R_2}{R_1}^{\left(\frac{10}{T_2 - T_1}\right)}$$

Equation 3 - Temperature coefficient (Q_{10}), where R is resting $\dot{M}O_2$, T is temperature, 1 represents values at 26^{o}C, and 2 represents values at 36^{o}C.

Statistical analyses were performed using SPSS v. 20 (IBM, Chicago, IL, USA). A general linear model was used to assess the effect of the two main factors (population and temperature) on resting $\dot{M}O_2$, pre-hypoxia $\dot{M}O_2$ and O_{2CRIT}. These analyses were followed by one-way ANOVA with Student-Newman Keuls *post hoc* tests for O_{2CRIT} and the non-parametric Kruskal-Wallis test with Dunn's *post hoc* comparison (where the assumption of normality was not met) for resting $\dot{M}O_2$ and pre-hypoxia $\dot{M}O_2$ to assess population differences at each temperature (Zar, 2010). Homogeneity of variance and normality were assessed using Levene's test and normal

quantile-quantile (Q-Q) plot respectively. Data are presented as means ± standard deviation and results were considered statistically significant at $P < 0.05$.

Results

Irrespective of population, resting $\dot{M}O_2$ of fish acclimated to 26°C was significantly lower than resting $\dot{M}O_2$ of fish acclimated to 36°C (1.47 ± 0.24 mg $O_2 \cdot kg^{-1} \cdot min^{-1}$ vs. 3.10 ± 0.43 mg $O_2 \cdot kg^{-1} \cdot min^{-1}$ for all populations combined), ($F_{1, 110} = 563.57$, $P < 0.001$; Figure 10). There was a significant interaction between population and temperature for resting $\dot{M}O_2$ ($P = 0.025$). No differences were found for resting $\dot{M}O_2$ between populations at 26°C ($H_5 = 1.446$, $P = 0.84$). At 36°C, resting $\dot{M}O_2$ differed from that at 26°C ($H_5 = 11.25$, $P = 0.0239$; Figure 10), although Dunn's *post hoc* comparison did not identify differences between individual populations. Pre-hypoxia $\dot{M}O_2$ was higher for the Townsville population than for Darwin at 26°C ($H_5 = 11.90$, $P = 0.018$) and was higher for the Gladstone population than for Broome or Karumba at 36°C ($H_5 = 17.02$, $P = 0.002$). There was no significant difference between resting $\dot{M}O_2$ and pre-hypoxia $\dot{M}O_2$ at 26°C ($F_{1, 92} = 0.38$, $P = 0.540$) or 36°C ($F_{1, 116} = 0.00$, $P = 1$) (Table 1). Average Q_{10} for resting $\dot{M}O_2$ and pre-hypoxia $\dot{M}O_2$ across all populations was 2.12 ± 0.30 and 2.12 ± 0.36.

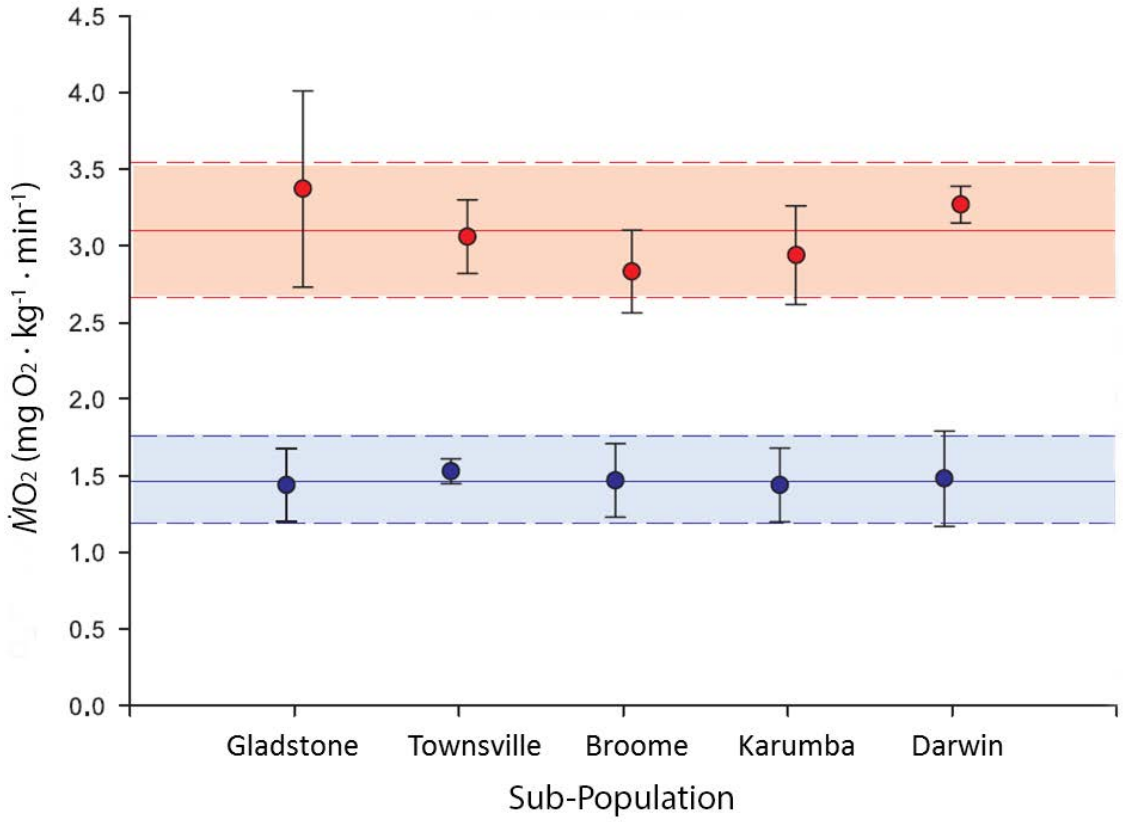

Figure 10 - Resting O_2 consumption rates ($\dot{M}O_2$; means ± standard deviation) for five barramundi populations at 26°C (blue; N = 48) and 36°C (red; N = 59). The continuous and dashed horizontal lines represent the mean and standard deviation for all populations at each temperature.

Barramundi exhibited a common and clear trend in response to decreasing O_2 with fish maintaining a relatively constant $\dot{M}O_2$ above the O_{2CRIT}, followed by a steep decline (Figure 11). There was no significant interaction between population and temperature for O_{2CRIT} (P = 0.486). Temperature had a significant effect on mean O_{2CRIT} across all populations, with lower O_{2CRIT} at 26°C than at 36°C ($F_{1, 114}$ = 69.97; P < 0.001; Fig. 11). No significant differences were observed for mean O_{2CRIT} between four of the five populations tested at both 26°C and 36°C (Figure 11). The exception was that mean O_{2CRIT} was highest for fish from Darwin at both 26°C and

36°C (18.91 ± 2.97% and 23.84 ± 3.49%, respectively). O_{2CRIT} measurements varied between individuals, with minimum and maximum values spanning 8.63 to 23.02% saturation at 26°C (mean = 15.44 ± 3.20% saturation) and 13.47 to 31.17% saturation at 36°C (mean = 21.07 ± 3.92% saturation). Fish displayed signs of distress such as erratic movements and pale body colour below O_{2CRIT}, however all fish recovered fully upon returning to 100% saturated conditions.

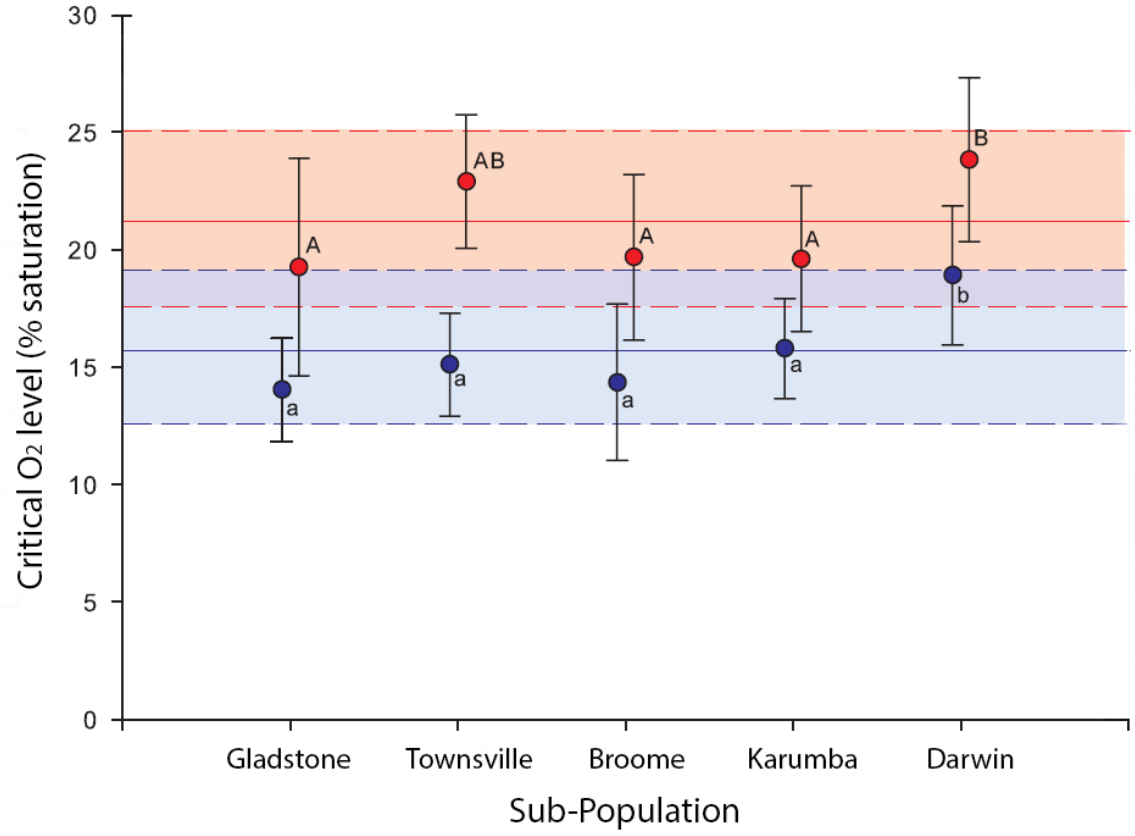

Figure 11 - Mean critical O_2 level (O_{2CRIT}; mean ± standard deviation) for five populations of barramundi at 26°C (blue; *N* = 50) and 36°C (red; *N* = 63). The continuous and dashed horizontal lines represent mean and standard deviation, respectively, of O_{2CRIT} for all populations at each temperature. Letters indicate significant differences ($P < 0.05$; a < b and A < B).

Table 1- Mean ± standard deviation of results from respirometry. Superscript letters indicate significant differences.

	Resting $\dot{M}O_2$	Pre-Hypoxia $\dot{M}O_2$	O_{2CRIT}
		26°C	
Gladstone	1.45 ± 0.24 ^{a}	1.34 ± 0.22 ^{ab}	14.05 ± 2.20 ^{a}
Townsville	1.63 ± 0.22 ^{a}	1.72 ± 0.22 ^{b}	15.12 ± 2.18 ^{a}
Broome	1.47 ± 0.24 ^{a}	1.56 ± 0.24 ^{ab}	14.35 ± 3.33 ^{a}
Karumba	1.44 ± 0.24 ^{a}	1.48 ± 0.28 ^{ab}	15.81 ± 2.13 ^{a}
Darwin	1.48 ± 0.31 ^{a}	1.25 ± 0.33 ^{a}	18.91 ± 2.97 ^{b}
		36°C	
Gladstone	3.37 ± 0.64 ^{c}	3.48 ± 0.68 ^{d}	19.27 ± 4.63 ^{b}
Townsville	3.06 ± 0.24 ^{bc}	3.14 ± 0.21 ^{cd}	22.92 ± 2.84 ^{bc}
Broome	2.83 ± 0.27 ^{b}	2.84 ± 0.26 ^{c}	19.69 ± 3.53 ^{b}
Karumba	2.93 ± 0.32 ^{bc}	2.89 ± 0.48 ^{c}	19.62 ± 3.11 ^{b}
Darwin	3.27 ± 0.37 ^{bc}	3.02 ± 0.28 ^{cd}	23.84 ± 3.49 ^{c}

Discussion

Species inhabiting thermally stable environments (e.g. equatorial or polar) are thought to be physiologically constrained within a narrow range of environmental thresholds, suggesting that they may be particularly susceptible to increasing temperatures and increasing variability in mean temperatures (Tewksbury et al., 2008). Due to the inverse relationship between temperature and O_2 solubility in water (Diaz and Breitburg, 2009), I expected barramundi from lower latitudes to be more hypoxia tolerant than their higher latitude counterparts. Contrary to expectations, Darwin fish (low latitude) displayed a slightly higher average O_{2CRIT} (less hypoxia tolerant) than fish from the other four populations tested at both control and warm temperatures. However, on the whole, I was unable to find consistent and conclusive evidence for local adaptation in hypoxia tolerance between

Australian barramundi populations separated by large longitudinal and latitudinal distances.

Interspecific variation in hypoxia tolerance, as derived from O_{2CRIT} measurements, exists for a number of temperate and tropical fish species at temperatures that are routine or typical (Table 2). Species such as Nile tilapia (*Oreochromis niloticus*) can regulate $\dot{M}O_2$ down to 11 ± 3% saturation, whereas other species such as Atlantic salmon (*Salmo salar*) can regulate $\dot{M}O_2$ to only 36 ± 3% saturation (Table 2). Schofield and Chapman (2000) reported a O_{2CRIT} of 15% saturation for juvenile Nile perch (*Lates niloticus*) at 20°C, and also noted the tendency of Nile perch to congregate at the surface layer of water during periods of aquatic hypoxia in an attempt to enhance O_2 uptake from the more aerated surface waters (commonly referred to as aquatic surface respiration (ASR)). It has been suggested, however, that Nile perch are inefficient at using this technique to obtain O_2 (Schofield and Chapman, 2000). Anecdotal reports exist of farmed barramundi aggregating at the surface during periods of low DO, however the capacity for barramundi to utilise ASR remains unclear. No attempt was made to measure ASR in this study, however future experiments using respirometers with an air pocket at the surface (see Lefevre et al., 2016) would help to elucidate the potential for ASR in barramundi and therefore their capacity to exist for extended periods in hypoxic waters below O_{2CRIT}.

Barramundi feature in mass mortality events in northern Australia (Townsend et al., 1992), and previous reports indicate that barramundi succumb rapidly to hypoxia under severe conditions. Pearson *et al.* (2003) reported a 'lethal DO concentration' of ~ 15% saturation for barramundi living

in temperatures ranging 28 – 30°C, however details of the fish and experimental design were unclear, making the results difficult to interpret in the context of the present study. In contrast, Wu (1990) reported no mortality for 150 – 250 g barramundi held in saltwater after 8 h of exposure to ~ 15% saturation at 25°C, but exposure to ~ 8% saturation resulted in 50% mortality after 6.5 h. Butler *et al*. (2007) reported an 'acute asphyxiation concentration' of 4% saturation for 200 g barramundi in freshwater at 28°C. Fish were visibly distressed under severe acute hypoxia in the present study, however there was only one mortality during the O_{2CRIT} tests despite the fact that each individual fish was exposed to DO of ~ 4-5% saturation in order to obtain precise O_{2CRIT} measurements.

As well as measuring 'acute asphyxiation', Butler *et al.* (2007) investigated the relationship between gill ventilation volume and frequency in response to declining DO for barramundi at 28°C. Butler *et al.* (2007) demonstrated that this species increases ventilation rates down to approximately 15-20% saturation, followed by a steep decline in ventilation rate as DO continues to decline. The maximum ventilation rate observed by Butler *et al*. (2007) corresponds closely with the observed O_{2CRIT} measurements presented here. This suggests that increasingly elevated ventilatory requirements may be needed to maintain a state of O_2 regulation by the fish under acute hypoxic conditions. Furthermore, a sharp decline in both ventilation rate and $\dot{M}O_2$ may be inevitable once DO drops below O_{2CRIT}.

There is varying evidence in the literature for local adaptation of performance traits in populations of Australian barramundi. Cairns (latitude 16°S) and Burrum River (26°S) barramundi have been found to exhibit no

differences in specific growth rate, critical thermal minima or critical thermal maxima between populations (Burke, 1994; Keenan, 2000; Rodgers and Bloomfield, 1993). Recent research, however, has documented differences in performance traits at high temperatures for Australian barramundi from lower (northern) latitudes compared with those from higher (southern) latitudes through the measurement of critical swimming speed (Edmunds et al., 2010) and time to loss of swimming equilibrium when challenged with acute water heating (Newton et al., 2010). Edmunds et al (2012) found elevated transcript abundance of the glycolytic enzyme lactate dehydrogenase-B (*ldh-b*) in southern (Gladstone) populations of Australian barramundi, compared with northern (Darwin) populations and suggested that southern populations may be better adapted to cooler temperatures. Differences were observed for pre-hypoxia $\dot{M}O_2$ in this study, however such differences were not consistent with resting $\dot{M}O_2$, neither was there conclusive evidence for a latitudinal trend. The results from this study indicate that both resting $\dot{M}O_2$ and hypoxia tolerance are conserved across populations of Australian barramundi.

Higher temperatures consistently result in elevated $\dot{M}O_2$ for teleost fish, irrespective of species or life history, reflecting increased metabolic requirements at higher temperatures (Brett and Groves, 1979; Clark et al., 2011b; Fry and Hart, 1948). Barramundi elicit a two-fold increase in resting $\dot{M}O_2$ (Q_{10} = 2.12) over a 10°C increase in temperature. Higher $\dot{M}O_2$ at warmer temperatures might be expected to induce a decrease in hypoxia tolerance in all fish species due to the increased O_2 demands of the fish. Results from previous studies on species such as Nile tilapia (Fernandes and Rantin, 1989; Mamun *et al*., 2013), rainbow trout (*Oncorhynchus mykiss*; Ott

et al., 1980) and common carp (*Cyprinus carpio*; Ott *et al*., 1980) indicate that O_{2CRIT} appears to be less temperature-sensitive than what is typically found for resting $\dot{M}O_2$, however this is not consistent across all teleosts. Species such as Atlantic salmon (Barnes *et al*., 2011), Doederlein's cardinal fish (*Ostorhinchus doederleini*; Nilsson *et al*., 2010) and Atlantic cod (Schurmann and Steffensen, 1997) elicit a large increase in O_{2CRIT} with increasing temperature, particularly towards upper thermal tolerance limits. My results indicate that O_{2CRIT} displays only a small increase for barramundi from typical (26°C) to high (36°C) temperatures, demonstrating the resilient nature of this species to the synergistic effects of temperature and environmental hypoxia.

Table 2 - Critical O_2 level (O_{2CRIT}; expressed as % saturation) measurements for a range of temperate and tropical fish species

Species	O_{2CRIT} (% saturation)	Temperature (°C)	Fish Mass (g)	Authors
Salmo salar	36 ± 3	18	151 ± 41	Barnes *et al*., 2011 [1]
	45 ± 4	22		
Lates niloticus	15 ± 2	20	4 - 28	Schofield and Chapman (2000) [2]
Prochilodus scrofa	14	25	244 ± 76	Fernandes *et al*., 1995 [2]
	28	35	295 ± 49	
Anguilla anguilla	16	25	(yellow phase)	Cruz-Neto and Steffensen (1997) [2]
Oreochromis niloticus	11 ± 3	25	301 ± 46	Fernandes and Rantin (1989) [2]
	19 ± 0	35		
Lates calcarifer	15 ± 3	26	191 ± 23	Present Study
	21 ± 4	36		

[1] O_{2CRIT} converted to % saturation from mg · L^{-1} (100% saturation = 9.4 mg · L^{-1} at 18 °C and 8.7 mg · L^{-1} at 22 °C)

[2] O_{2CRIT} converted to % saturation from mmHg (100% saturation = 159 mmHg)

Aside from temperature, a number of other environmental parameters can impact the hypoxia tolerance of fish. The ability to adjust to hypoxic conditions through a lowering of O_{2CRIT} following pre-exposure has been reported for the epaulette shark (*Hemiscyllium ocellatum*; Routley *et al*., 2002) and goldfish (*Carassius auratus*; Fu *et al*., 2011), although Cook *et al*. (2011) found no differences in O_{2CRIT} between naïve and hypoxia-conditioned silver sea bream (*Pagrus auratus*). Henriksson *et al.* (2008) demonstrated that alterations in salinity can increase the O_{2CRIT} by up to 30% in prickly sculpin (*Cottus asper*) acclimated to freshwater, compared with fish adapted to seawater, however the same trend was not observed in the closely related Pacific staghorn sculpin (*Leptocottus armatus*). Barramundi inhabit environments that are prone to acute and chronic hypoxia, and such environments also experience broad fluctuations in salinity and temperature. Currently there is no understanding of the effects of exposure to repetitive (short term) or chronic (long term) hypoxia on the physiology and consequently performance of barramundi. However, this topic warrants future research to elucidate more fully the capacity of fishes to tolerate extreme and variable environments.

Elevated temperatures due to climate change are predicted to increase the frequency and severity of hypoxic events through lower O_2 solubility, increased animal respiration rates and enhanced stratification (Diaz and Breitburg, 2009; Justic et al., 2001). Such conditions have the potential to increase habitat availability at higher latitudes while leading to the deterioration of habitats at lower latitudes (Graham and Harrod, 2009). Nutrient inputs to aquatic coastal systems from human waste and agriculture,

combined with habitat degradation through increased land use, have the potential to further exacerbate the effects of environmental hypoxia on physiological systems. The present study has shown for the first time that a tropical euryhaline fish, barramundi does not display obvious local adaptation in resting $\dot{M}O_2$ and hypoxia tolerance. This strongly suggests that all populations of this species in northern Australia can cope equally well in environments with large fluctuations in both temperature and dissolved O_2.

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CHAPTER 3. PHYSIOLOGICAL PLASTICITY VERSUS INTER-POPULATION VARIABILITY: UNDERSTANDING DRIVERS OF HYPOXIA TOLERANCE IN A TROPICAL ESTUARINE FISH

Abstract. Physiological plasticity and inter-population variability (e.g., local adaptation) are two key drivers in determining the capacity for species to cope with environmental change, yet the relative contribution of each parameter has received little attention. Here, I investigate the acclimation potential of two geographically-distinct populations of the barramundi (*Lates calcarifer*) to diel hypoxia. Fish were exposed to a daily hypoxia challenge of 6 h below 62% saturation, down to a minimum of 10 ± 5% saturation, followed by a return to normoxia. Respiratory and haematological variables were assessed after 8 and 16 d of daily hypoxia exposure. Hypoxia tolerance (measured as the critical O_2 level; O_{2CRIT}) was not different between populations and not different from control fish after 8 d O_{2CRIT} = 20.7 ± 2.8% saturation), but improved similarly in both populations after 16 d (O_{2CRIT} = 16.5 ± 3.1% saturation). This improvement corresponded with increases in haematocrit and haemoglobin, but not an increase in the mean cell haemoglobin concentration. Given the similarity of the response between these two geographically-distinct populations, I conclude that hypoxia tolerance for barramundi may be more dependent on physiological plasticity than inherent variability between populations.

Introduction

Environmental hypoxia occurs naturally in many aquatic environments due to high biological O_2 demand and stratification (Diaz and Breitburg, 2009). However, many aquatic systems, particularly in coastal regions, are predicted to undergo more frequent and severe episodes of hypoxia as anthropogenic pressure continues to increase and the climate continues to change (Diaz and Breitburg, 2009; Gillanders et al., 2011). In the tropics, environmental hypoxia may be particularly pronounced as water temperatures and metabolic rates of ectothermic animals are elevated in parallel (Almeida-Val et al., 2005), and the solubility of O_2 in water decreases with increasing temperature (Davis, 1975). Understanding the acclimation potential of aquatic organisms to hypoxia will be critical to predict the impact of increasing eutrophication and hypoxia on tropical aquatic systems.

Many vertebrates with broad geographical distributions exist not as a single continuous population, but rather as multiple populations that may occur in partial or complete reproductive isolation. Prevailing environmental conditions, including temperature and dissolved O_2, are expected to drive selection pressure for locally adapted genotypes and/or phenotypes (Crispo and Chapman, 2008; Tewksbury et al., 2008). Tolerance to acute environmental change such as hypoxia may be achieved in the short term through plasticity in physiological traits (Crispo and Chapman, 2010; Via et al., 1995). Such responses may subsequently come under genotypic control through genetic accommodation, resulting in locally adapted populations

(West-Eberhard, 2005). Determining the relative importance of physiological plasticity and inter-population variability in conferring hypoxia tolerance is important for understanding how fish populations will respond to an increasing prevalence of environmental hypoxia (Pollock et al., 2007; Vaquer-Sunyer and Duarte, 2008).

Across the tropical and sub-tropical Indo-Pacific, the Asian sea-bass or barramundi (*Lates calcarifer*) exists as multiple genetically-distinct populations (Chenoweth et al., 1998). This species experiences substantial variability in temperature and dissolved O_2 across spatial scales and exhibits localised habitat affinity (Moore and Reynolds, 1982; Russell and Garrett, 1988). These attributes make it an excellent candidate species to investigate the roles of physiological plasticity and inter-population variability in determining hypoxia tolerance. Two populations of barramundi were selected for the present study; one from a low latitude, tropical location (Broome, Western Australia [WA], 17° 57') and one from a higher latitude, sub-tropical location (Gladstone, Queensland [QLD], 23° 50'; Figure 8). Average annual sea-surface (28.7 ± 2.1°C vs. 23.9 ± 2.4°C), river-surface (28.0 ± 1.8°C vs. 19.8 ± 3.8°C) and air (26.7 ± 3.2°C vs. 22.6 ± 3.5°C) temperatures differ substantially between the tropical (WA) and sub-tropical (QLD) locations, respectively (AIMS, 2012; BOM, 2012; Burford et al., 2011; Kay et al., 1999; Pusey et al., 2004; Stuart and Berghuis, 2002). Given that temperatures are higher at low latitudes and daily fluctuations in dissolved O_2 due to photosynthesis and respiration are severe (Val et al., 2005), I hypothesised that barramundi living at lower latitudes would be better adapted and show greater physiological plasticity to environmental hypoxia than their

conspecifics living at higher latitudes. To test this hypothesis, I exposed the two geographically-distinct populations of barramundi to diel cycling hypoxia and quantified the responses in haematology and respiration at distinct time points over 16 d of exposure.

Materials and methods

Animals and holding conditions

Barramundi juveniles were obtained from commercial hatcheries at a low-latitude, tropical site (Broome, WA) and a high-latitude, sub-tropical site (Gladstone, QLD) and air-freighted to Townsville, QLD. All fish were approximately 3 months of age when collected. Both hatcheries use locally-sourced broodstock and are separated by > 6,000 km (coastal distance). Additionally, environmental conditions at the two hatcheries were very similar during juvenile rearing, with dissolved O_2 similar at both locations (>75% air saturation) and mean rearing temperature slightly higher for the tropical location (28.0 ± 1.1°C) than for the sub-tropical location (26.4 ± 1.4°C) (Hayes, T., pers. comm., Aris, A., pers. comm.). The origin of fish used in this study was verified using fin-clip samples for DNA extraction and microsatellite sequencing, in comparison with known samples (Smith-Keune, unpub. data; for full details of methods, see Jerry et al. (2013)). Fish were held in 2,500 L tanks in a freshwater recirculating system and grown to a mass of ~ 300 g at 26 ± 1°C over 24 months. This period of time helped to ensure that the two populations were acclimated to the same conditions such that inter-population differences could be investigated without the

confounding effects of different acclimation histories. Dissolved O_2 was maintained >80% air saturation and photoperiod was maintained at 12:12 h (L:D) during the holding period. All fish were moved to the experimental tank system 11 d prior to the commencement of experiments to allow them to settle. Temperature in the experimental system was increased from 26°C to 30°C at the rate of 1°C · d^{-1} and the new temperature (30°C) was maintained for 7 d prior to commencing the experimental protocol.

Experimental design

An experiment was conducted to test the effects of repeated diel hypoxia exposure on the aerobic metabolism and haematology of the fish, with population (WA or QLD) and number of consecutive days exposed to hypoxia (0, 8 or 16 d) as the main factors. Fish were randomly allocated to receive one of two experimental sampling procedures: haematology or respirometry. Throughout the experiment, individuals were used for either respirometry or haematology, but never for both. Further, individuals were used only once for sampling at a single time point and thus all measurements collected are considered independent. Experimental procedures were replicated over two consecutive months (December, 2013 and January, 2014) to achieve desired sample sizes. Four fish (mean mass = 314 ± 27 g; Fulton's condition factor (K; Froese (2006)) = 1.23 ± 0.03) were placed into each of 50 × 100 L tanks (25 tanks in each of December and January).

Tanks were considered sampling units (N = 4, haematology; N = 3, respirometry) and fish within tanks (N = 4) were considered sub-samples.

Time = 0 d acted as the initial control group (*N* = 16 fish per population for haematology, *N* = 12 fish per population for respirometry). Control measurements (i.e., constant normoxia) were additionally taken for a sub-set of fish (*N* = 4 per population) on each of days 8 and 16. These control measurements were statistically similar to the initial control group (Dunnett's *post hoc* test; see below), and so all control measurements were subsequently pooled to create one single control group (*N* = 24 per population) against which the treatment groups were compared (see *Data analysis*). Treatment allocation for tanks was randomised between all 50 tanks and across the two months.

Hypoxia acclimation

Environmental hypoxia is defined by Farrell and Richards (2009) as the O_2 partial pressure where physiological function is first compromised. Farrell and Richards (2009) also state that this definition of hypoxia is plastic, depending on the physiological system (species) being examined. Butler and Burrows (2007) identified a critical trigger value (CTV) of 62.5% saturation for barramundi (below this dissolved O_2 (DO) the fish began increasing their ventilation rate to maintain homeostasis). Therefore, for the purposes of defining a hypoxia treatment protocol in the present study, DO below 62% saturation was considered hypoxic.

The hypoxia treatment protocol simulated natural fluctuations in dissolved O_2 within northern Australian rivers (Butler and Burrows, 2007). Hypoxia in experimental tanks was achieved through the addition of nitrogen gas to a

common ‘sump’ tank connected to the recirculating system using a custom-built gas-diffusion column. Dissolved O_2 was controlled in each tank by adjusting rates of water flow and aeration. The hypoxia treatment consisted of a decline in dissolved O_2 from >75% saturation down to 10.0 ± 4.6% saturation, followed by a return to normoxia (Figure 12). Dissolved O_2 was maintained below 62% saturation for 6.0 ± 0.8 h each day. Fish were fed a commercial pelleted food at a rate of 2% body-mass · d^{-1}, 2 h following the daily return to normoxia. All fish were fasted for 48 h prior to respirometry or blood sampling to minimise the effect of digestive processes on the measurements. Water temperature was maintained at 30.6 ± 0.2°C in experimental tanks. The experimental system was exposed to natural daylight and hence fish experienced a natural photoperiod (13:11 h, L:D) for the duration of experiments.

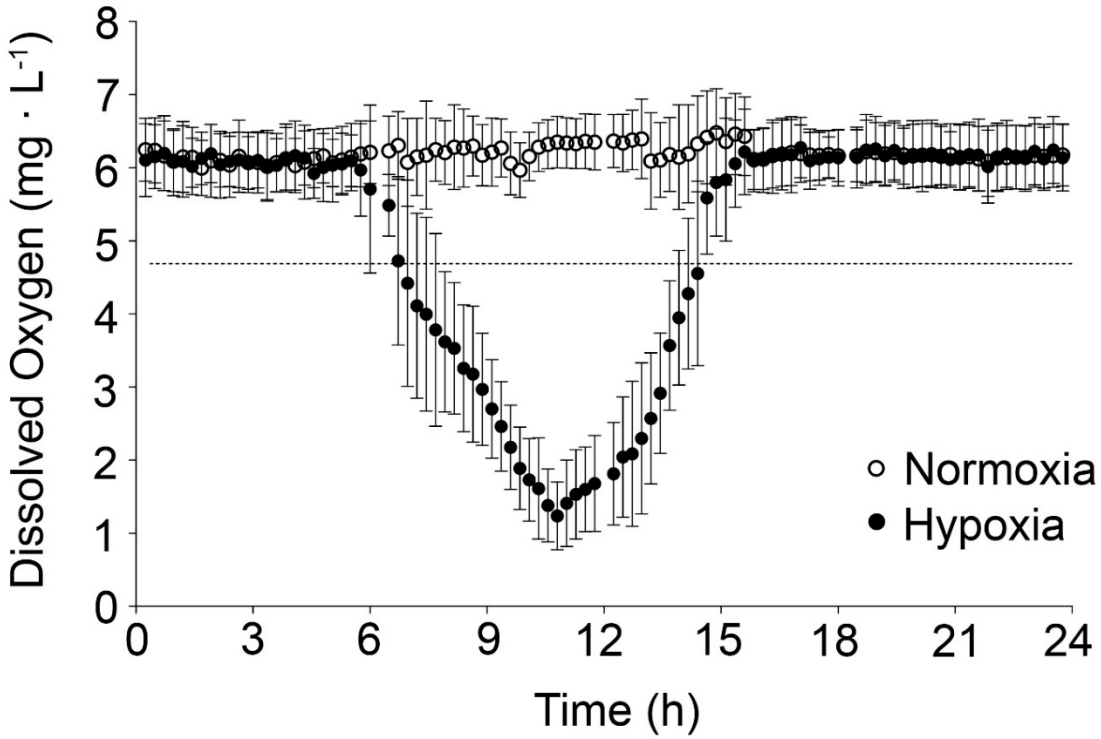

Figure 12 - Average dissolved O_2 (DO: mg · L^{-1}) measurements (N = 32 per data point) recorded during the experimental period. Each data point represents the mean ± standard deviation for 15-min intervals. Open circles indicate DO within normoxic tanks, and closed circles indicate DO in tanks undergoing daily hypoxia. The dashed horizontal line indicates the DO considered hypoxic for this species (62% saturation: equal to 4.86 mg · L^{-1} at 30°C).

Respirometry

Oxygen consumption rates ($\dot{M}O_2$) were measured using intermittent flow-through respirometry in subsets of fish on days 0, 8 and 16 using techniques described in Collins et al. (2013) and following best practises (Clark et al., 2013a). Briefly, fish (*N* = 4 per day; *N* = 84, total) from one experimental tank were transferred from the experimental system to respirometers (3.3 L; 1 fish per respirometer). All respirometers were submerged in a 1,000 L tank that provided fully air-saturated water during the flush cycle of respirometry. Oxygen concentration within each chamber was recorded using a Firesting

Optical O_2 Meter (Pyro Science, Aachen, Germany). Fish were acclimated to the chamber for 19 h (Figure 13), with $\dot{M}O_2$ being recorded every 15 min between flush cycles. Resting $\dot{M}O_2$ was calculated as the mean of the lowest 5% of $\dot{M}O_2$ measurements over the 19 h period.

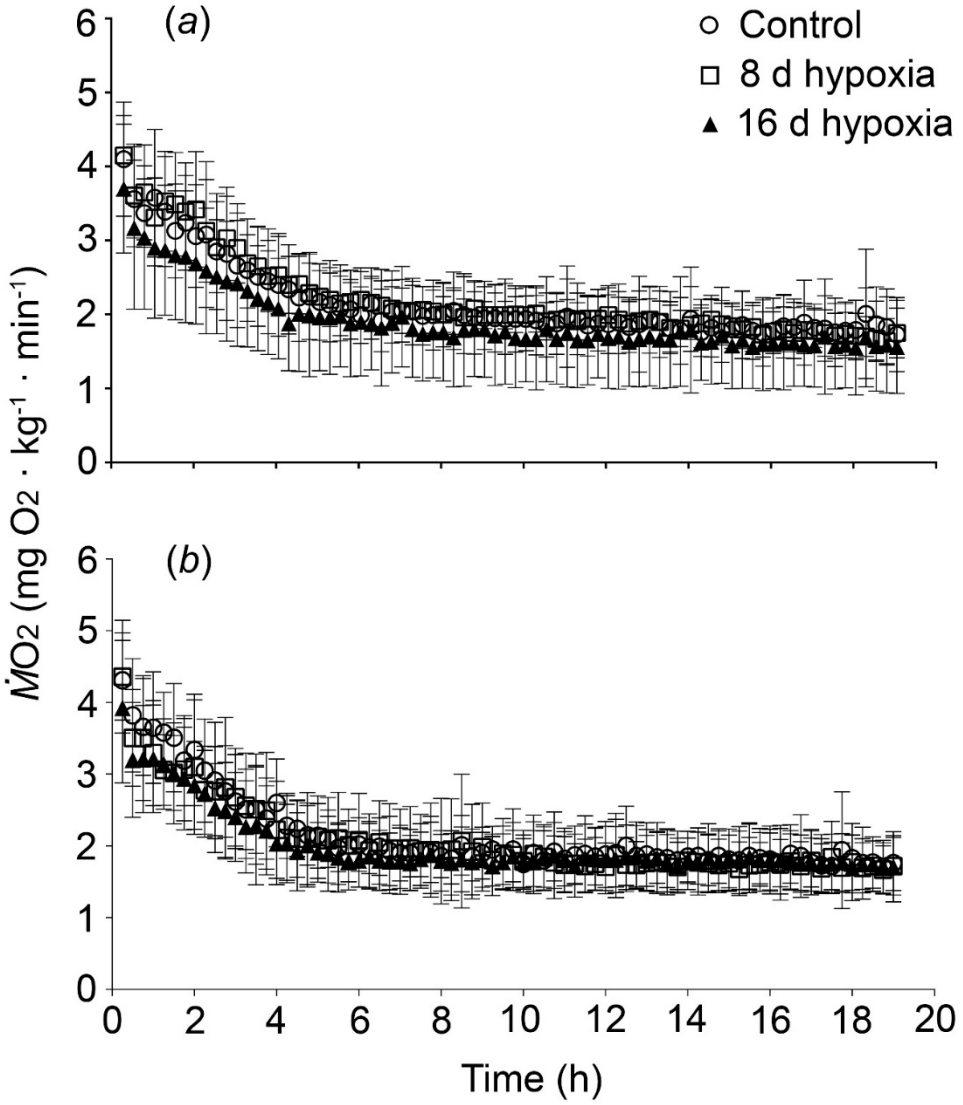

Figure 13 - Oxygen consumption rates ($\dot{M}O_2$: mg O_2 · kg^{-1} · min^{-1}) recorded every 15 min during the chamber acclimation period of respirometry for tropical (a) and sub-tropical (b) populations of barramundi (30°C). Each data point represents the mean ± standard deviation.

Following the acclimation period, the respirometer was sealed until the fish depleted the O_2 within the chambers to 5% saturation, after which the flush pump was turned on again to restore O_2 levels to 100% saturation. The $\dot{M}O_2$ was calculated for each consecutive 3 min period during the decline in O_2 and the procedure lasted approximately 1.5 h. Two linear regressions were fitted to the $\dot{M}O_2$ measurements and the O_{2CRIT} was calculated as the intersection point of the two slopes as per Nilsson et al. (2010), (Figure 14). Individual fish where O_{2CRIT} was not able to be calculated (N = 3) were excluded from further analyses. Water temperature was maintained at 29.5 ± 0.3°C during respirometry, and fish were exposed to a 12:12 (L:D) photoperiod.

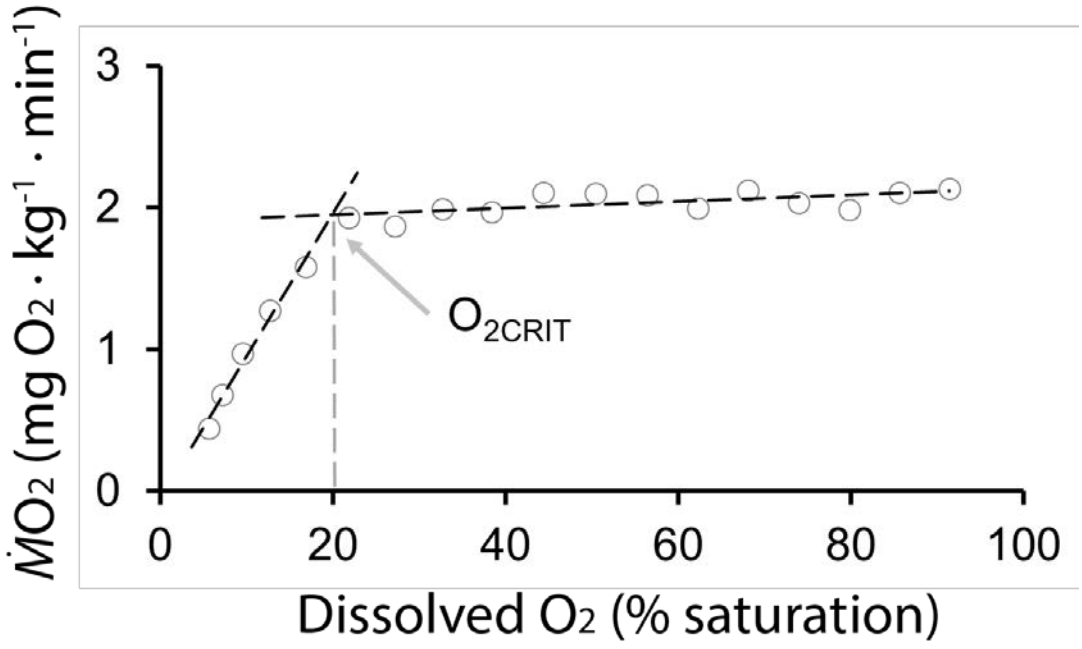

Figure 14 - Plot of O_2 consumption rate ($\dot{M}O_2$) against dissolved O_2 for one fish, illustrating how the critical O_2 level (O_{2CRIT}) was calculated in this study as the intersection of two lines, fit using least-squares regression.

Blood sampling

Following the final period of hypoxia exposure, fish were held under normoxic conditions overnight before blood sampling the next morning, during the time period of 09:00 to 12:00 h. Normoxic control fish were sampled at the same time as fish exposed to hypoxia. Fish were removed individually from experimental tanks and blood was collected from the caudal vasculature using heparinised syringes (Li^+ heparin, Merck Millipore, Victoria, Australia; 21G needles). Only one tank was disturbed at any one time and all blood sampling was completed within 6 min of initial tank disturbance. Average time to blood collection following removal from the tank was not different between tropical (67.4 ± 5.8 s) and sub-tropical (66.9 ± 13.4 s) populations, respectively, or between sampling cohorts (see Clark et al. (2011a) for discussion of blood sampling efficacy). Glucose and lactate were measured from whole blood using commercial test strips (Accutrend®; Roche Diagnostics, Indiana, USA) (Wells and Pankhurst, 1999). Haematocrit (Hct) was measured using heparinised micro-capillary tubes following centrifugation at 10,000 *g* for 3 min. Packed red cell volume was measured to the nearest mm using callipers. Haemoglobin (Hb) was measured using a hand-held analyser including a correction for fish blood (Hemocue, Ängelholm, Sweden; see Clark et al. (2008)).

$$\mathrm{MCHC} = \frac{\mathrm{Hb}}{\mathrm{Hct} \times 0.01}$$

Equation 4 - Mean cellular haemoglobin concentration (MCHC): haemoglobin ($g \cdot dL^{-1}$)

Mass (g) and total length (mm) were recorded for every fish following either respirometry or blood sampling. Fish mass (312.8 ± 24.5 g and 314.2 ± 32.8

g) and condition factor (K: 1.25 ± 0.02 and 1.22 ± 0.04) were not different between respirometry or blood sampling cohorts, respectively.

Data analysis

Data analysis was performed using GraphPad Prism v. 6 (La Jolla, California, USA). Treatment means for haematology or respirometry were compared using a general linear model (GLM) with experimental treatment (control or exposed to hypoxia for 8 or 16 d) and population origin (tropical or sub-tropical) as fixed factors. Normality and homoscedasticity were assessed using Kolmogorov-Smirnov test and Bartlett's test, respectively. Control measurements (i.e., constant normoxia) taken from each population at 0 d (N = 16 per population for haematology or N = 12 per population for respirometry), 8 d (N = 4 per population) and 16 d (N = 4 per population) were compared using Dunnett's *post hoc* test to determine if initial measurements were significantly different from those collected from the same population of fish held in normoxia throughout the experiment. No differences were detected between controls across days ($P > 0.05$ for all tests) and consequently data were re-analysed using a combined mean for all control measurements within each population. Differences between treatments were assessed and quantified using Tukey's *post hoc* test. Results were considered statistically significant when $P \leq 0.05$. All data, with the exception of boxplots (10^{th} to 90^{th} percentile), are presented as means ± standard deviation.

Results *

Respirometry

There were no differences in resting $\dot{M}O_2$ between populations or treatment groups (Overall mean resting $\dot{M}O_2$ for controls = 1.54 ± 0.24 and 1.52 ± 0.34 mg $O_2 \cdot kg^{-1} \cdot min^{-1}$ for tropical and sub-tropical populations, respectively). Fish of both populations and treatments exhibited a common trend in response to decreasing DO that was characterised by a relatively stable $\dot{M}O_2$ above the O_{2CRIT}, followed by a steep decline (Figure 14). The O_{2CRIT} did not differ between populations on day 0, yet the GLM revealed that duration of hypoxia exposure had an overall significant effect on the O_{2CRIT} of both populations ($F_{2, 79} = 15.87$, $P < 0.0001$). After 8 d of hypoxia treatment, the O_{2CRIT} was not different from controls in either population, however after 16 d O_{2CRIT} was significantly lower in both tropical (17.0 ± 2.2% saturation) and sub-tropical (16.0 ± 3.7% saturation) populations (Figure 15). No differences existed in O_{2CRIT} between populations, and there was no interaction between population and hypoxia treatment (population, $F_{1, 79} = 2.33$, $P = 0.1308$ | population*hypoxia treatment, $F_{2, 79} = 0.006$, $P = 0.9943$).

* The results presented herein for Chapter 3 are identical to those presented in Collins et al, 2016, Mar FW Res, 67(10), 1575-1582. The data have since been re-analysed due to improvements in analytical methodology during the writing of this thesis. The results of the additional analyses are presented in Appendix D of this thesis (see Figures 57 to 65 and Tables 11 to 16).

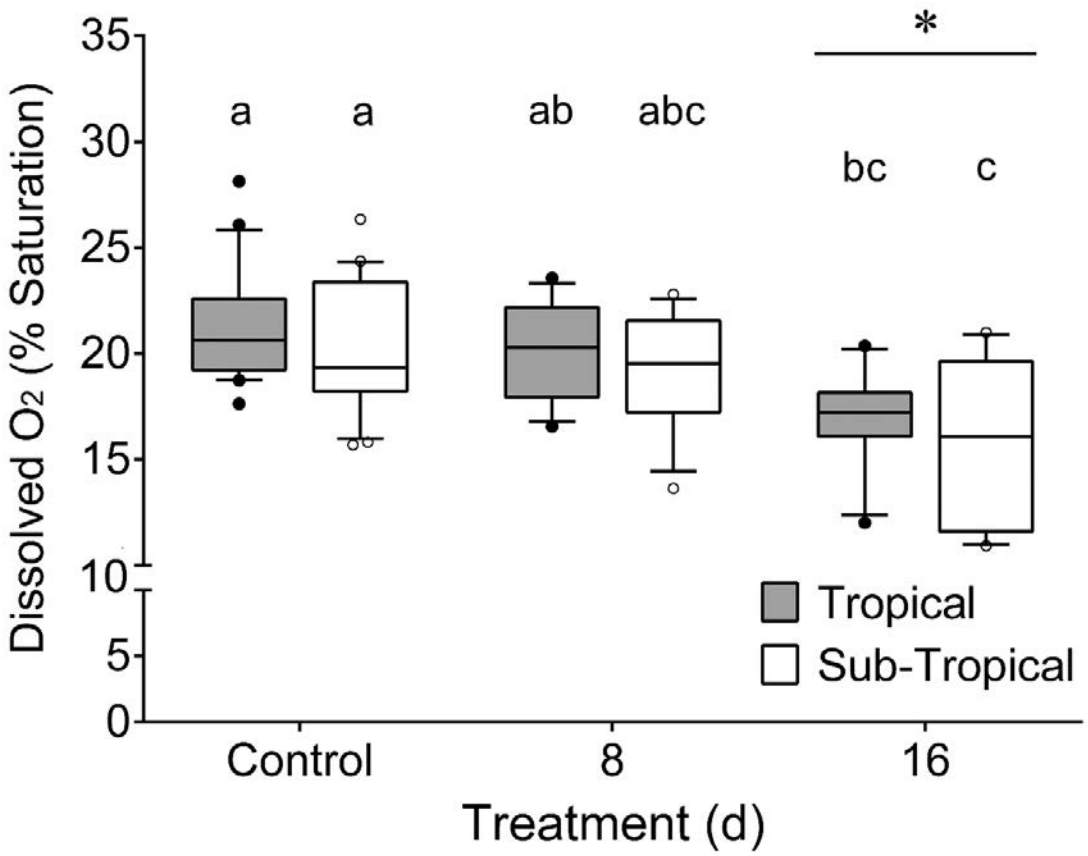

Figure 15 - Critical O_2 level (O_{2CRIT}) boxplots (10^{th} to 90^{th} percentile) for tropical (grey) and sub-tropical (white) populations of barramundi. Fish were exposed to normoxia (control) or a daily hypoxia challenge for 8 or 16 days. Each box within the plot represents the average of three replicate tanks (*N* = 12 fish) for treatments (8 and 16 days) or five replicate tanks (*N* = 20 fish) for controls (sampled on Days 0, 8 and 16). Statistically significant differences (*P* < 0.05) between times are denoted by an asterisk. Statistically similar values between populations and time are denoted by similar letters above boxes, whereas dissimilar letters indicate differences (*P* < 0.05; a > b > c).

Haematology

Exposure of barramundi to diel hypoxia had a significant effect on Hct ($F_{2, 94}$ = 7.933, P = 0.0007) and Hb ($F_{2, 98}$ = 6.361, P = 0.0025) that only emerged after 16 d (Figure 16, Table 3). At 16 d, Hct and Hb were higher than controls for the sub-tropical population but not for the tropical population. Both Hct and Hb were significantly higher in sub-tropical fish than for tropical fish (Hct, $F_{1, 94}$ = 4.075, P = 0.0464; Hb, $F_{1, 98}$ = 6.187, P = 0.0146). There were no effects of either population or hypoxia exposure time on MCHC (Figure 16), and there was no interaction between population and hypoxia treatment for Hct ($F_{2, 94}$ = 1.096, P = 0.3385), Hb ($F_{2\ 98}$ = 1.146, P = 0.3222) or MCHC ($F_{2, 94}$ = 0.984, P = 0.3776). Blood glucose (1.11 ± 0.40 mmol · L^{-1} and 1.24 ± 0.54 mmol · L^{-1}) and lactate (0.74 ± 0.16 mmol · L^{-1} and 0.89 ± 0.25 mmol · L^{-1}) were not different between hypoxia treatments for tropical and sub-tropical populations, respectively (Table 3), however glucose was higher for the sub-tropical population in general (glucose: $F_{2, 95}$ = 2.384, P = 0.0977; $F_{1, 95}$ = 8.972, P = 0.0035 | lactate: $F_{2, 98}$ = 1.51, P = 0.2262; $F_{1, 98}$ = 2.11, P = 0.1491).

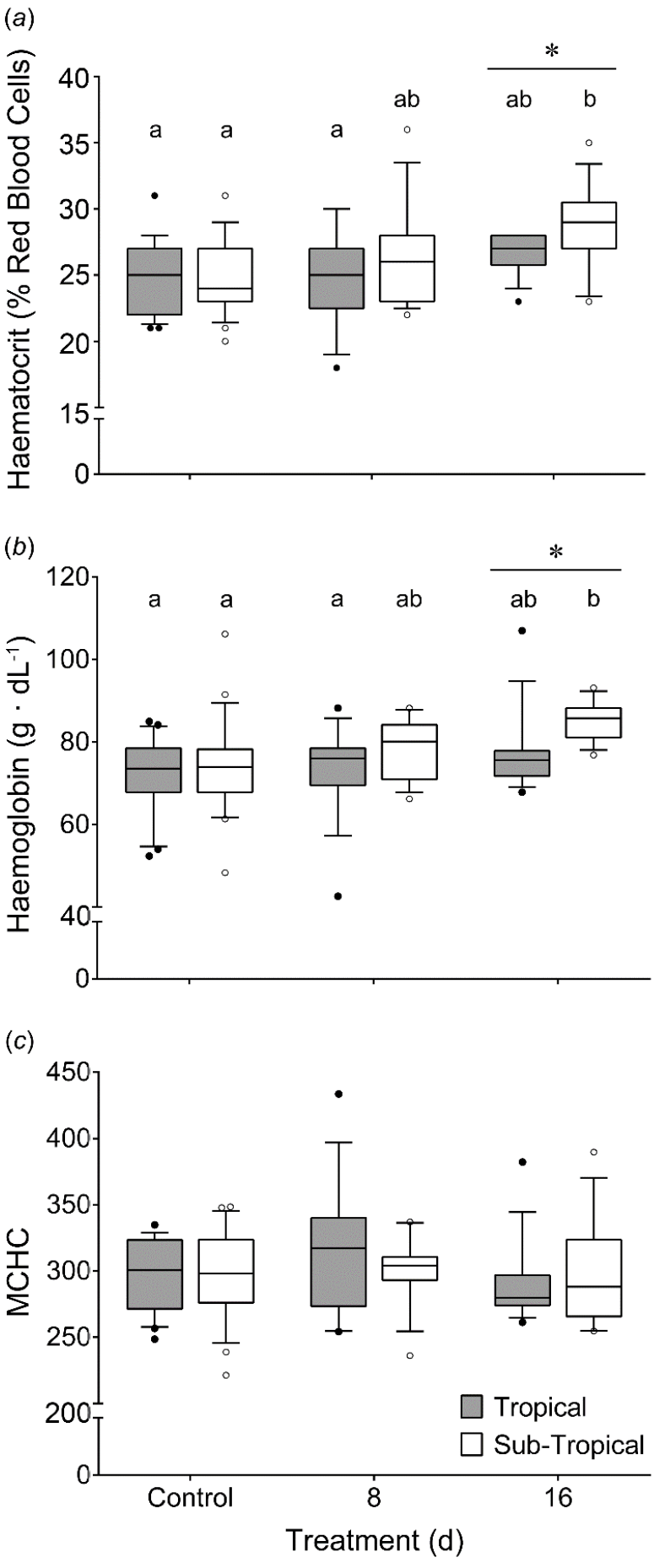
(a)
Haematocrit (% Red Blood Cells)
40
35
30
25
20
15
0
a
a
a
ab
ab
b
*
(b)
Haemoglobin (g · dL-1)
120
100
80
60
40
0
a
a
a
ab
ab
b
*
(c)
MCHC
450
400
350
300
250
200
0
Tropical
Sub-Tropical
Control
8
16
Treatment (d)

Figure 16 - Mean haematocrit (a, upper panel), haemoglobin (b, middle panel) and mean cellular haemoglobin concentration (MCHC; c, lower panel) in two populations of Australian barramundi: tropical (Broome, WA; grey) and sub-tropical (Gladstone, Qld; white). Fish were exposed to normoxia (control) or a daily hypoxia challenge for 8 or 16 days. Each box within the plot represents the average of four replicate tanks (N = 16 fish) for treatments (8 and 16 days) or six replicate tanks (N = 24 fish) for controls (sampled on Days 0, 8 and 16). Statistically significant differences ($P < 0.05$) between times are denoted by an asterisk. Statistically similar values between populations and time are denoted by similar letters above boxes, whereas dissimilar letters indicate differences ($P < 0.05$; b > a).

Discussion

The present study demonstrates for the two populations of diadromous barramundi examined in this study that short-term acclimation to diel cycling hypoxia is driven primarily by physiological plasticity rather than population of origin. Natural selection is expected to favour the preservation of physiological plasticity in populations that experience large environmental fluctuations (Chapman et al., 2000). This concept has been examined extensively in the context of temperature (Righton et al., 2010; Seebacher et al., 2015), however the role that population of origin or physiological plasticity play in hypoxia tolerance has received less attention. Improved hypoxia tolerance following diel hypoxia exposure, as observed in this study (20% decrease in O_{2CRIT} after 16 d), has been demonstrated in geographically and phylogenetically distant species, including southern catfish (*Silurus meridionalis*; 22% decrease), qingbo (*Spinibarbus sinensis*; 29% decrease) and the epaulette shark (*Hemiscyllium ocellatum*; 29% decrease) (Dan et al., 2014; Routley et al., 2002; Yang et al., 2013). No differences were observed in resting $\dot{M}O_2$ between populations or treatments which is consistent with previous studies (Dan et al., 2014; Yang et al., 2013). Despite the variability

in metabolic acclimation between species, the capacity and magnitude of hypoxia acclimation appear to be conserved across a broad range of fishes and this may reflect the importance of physiological plasticity as a survival strategy in environments prone to hypoxia.

The O_{2CRIT} is a common measurement for determining hypoxia tolerance and represents the transition of an animals' $\dot{M}O_2$ from being independent to being dependent on environmental O_2 (Almeida-Val et al., 2005; Nilsson and Ostlund-Nilsson, 2004). A decrease in O_{2CRIT}, as observed in this and other studies, indicates an increased capacity for O_2 regulation during conditions of hypoxia and thus an improved ability to utilise aerobic metabolism, which is energetically more efficient than anaerobic metabolism. Diel-cycling O_2 levels occur year-round in some tropical freshwater systems, but may become more frequent and severe as water recedes from main river channels and becomes concentrated in isolated water bodies (Almeida-Val et al., 2005; Brauner and Val, 2005; Waltham et al., 2013). Such increases in diel-cycling of O_2 are strongly influenced by the quantities of micro-algae and plants present in these systems (Waltham et al., 2013). In the present study, acclimation to daily hypoxia (lower O_{2CRIT}) occurred between 8 and 16 d in both populations. These results indicate that hypoxia tolerance improves following a number of consecutive days of hypoxia exposure. This functional response likely improves survival in the natural environment as waters recede and conditions of diel-cycling dissolved O_2 become more severe. Whether a further decrease in O_{2CRIT} is possible after more than 16 d of daily hypoxia is not known, but based on the studies outlined above it appears

likely that a 20-30% reduction in O_{2CRIT} might represent a limit to most species.

Haematological parameters increased for the two barramundi populations following hypoxia exposure, however the magnitude of the increase was greater for the sub-tropical population. Both Hct and Hb were higher for the sub-tropical population, however this did not translate into enhanced hypoxia tolerance (lower O_{2CRIT}) when compared with the tropical population. Blood is the primary transport medium for O_2 and it is expected that acclimation to hypoxia would be accompanied by changes to the O_2 transport capacity of fish blood (Kind et al., 2002; Tetens and Lykkeboe, 1981; Wells, 2009a). This hypothesis is supported by observations in goldfish (*Carassius auratus*), where chronic hypoxia exposure elicits a reduction in O_{2CRIT} that correlates with increased Hb and lamellar surface area (Fu et al., 2011). Contrary to my findings, killifish (*Fundulus heteroclitus*) increase Hb and Hct following exposure to chronic hypoxia but not diel-cycling hypoxia, with O_{2CRIT} decreasing by a similar magnitude under both chronic and diel-cycling hypoxia (Borowiec et al., 2015). Increases in circulating Hct and Hb were observed in both Atlantic cod (*Gadus morhua*) and snapper (*Pagrus auratus*) following hypoxia exposure, however this was not accompanied by any change in hypoxia tolerance (Cook et al., 2013; Petersen and Gamperl, 2011). Such increases in circulating Hb may be accompanied by improved blood- O_2 affinity and a reduction in erythrocyte organic phosphates, as observed in rainbow trout and European eel (*Anguilla anguilla*) (Bushnell et al., 1984; Wood and Johansen, 1972). While many of these aspects remain to be investigated in barramundi, the observed increases in both Hct and Hb

would likely increase O_2 carrying capacity of the blood and enable improved O_2 uptake during daily hypoxic episodes. Changes to blood parameters under hypoxia appear to be species-specific and may also be related to life-history, as well as being influenced by the nature of the hypoxic episode.

Table 3 - Mean ± standard deviation of results from the haematology component of the experiment.

		parameter				
Treatment	Population	Hct	Hb	MCHC	Glucose	Lactate
Control	Tropical	0.25 ± 0.02	72.50 ± 3.62	297.28 ± 10.17	1.02 ± 0.26	0.73 ± 0.07
	Sub-Tropical	0.25 ± 0.01	74.00 ± 4.47	292.94 ± 22.50	1.31 ± 0.39	0.76 ± 0.08
8	Tropical	0.25 ± 0.01	73.75 ± 4.35	304.75 ± 3.76	0.81 ± 0.22	0.82 ± 0.16
	Sub-Tropical	0.26 ± 0.02	78.00 ± 5.29	300.20 ± 17.78	1.25 ± 0.44	0.78 ± 0.17
16	Tropical	0.27 ± 0.02	77.50 ± 4.36	288.35 ± 10.60	1.23 ± 0.43	0.75 ± 0.10
	Sub-Tropical	0.29 ± 0.03	85.50 ± 3.79	304.50 ± 34.06	1.33 ± 0.40	0.92 ± 0.20

The observation of limited inter-population differences is consistent with previous findings for populations of hatchery-reared barramundi exposed to short-term hypoxia (Collins et al., 2013), although it must be acknowledged that the use of only two populations in this study imposes some limitations as regards inferences for adaptive responses for the species as a whole (Garland and Adolph, 1994). Previous studies with barramundi populations have reported that some traits may display differences associated with temperature (Edmunds et al., 2012; Edmunds et al., 2010; Newton et al., 2010), however acute hypoxia tolerance appears conserved across the distribution irrespective of acclimation temperature (Collins et al., 2013). The routine metabolic rate and aerobic scope of barramundi is reported to be

similar across different populations and temperatures (Gamble et al., 2014; Jerry et al., 2013) and may indicate a similar potential for hypoxia acclimation across the distribution of this species in Australia, however this remains to be demonstrated empirically. Both dissolved O_2 and temperature display large spatio-temporal variation in riverine and estuarine systems of northern Australia (Butler, 2008; Butler and Burrows, 2007), and these results indicate that hypoxia may be less important than temperature in driving population differences for large estuarine fish species. While every effort was made in the present study to control environmental conditions, I was not able to control for any developmental plasticity or maternal effects that may have occurred in the hatcheries prior to fish collection. Further studies across the distribution of this species in the tropical and sub-tropical Indo-Pacific may help to decipher any role of developmental plasticity or maternal effects contributing to hypoxia tolerance, as well as helping to further elucidate the role of hypoxia in driving local adaptation and physiological plasticity in fishes.

The necessity for hypoxia acclimation may be mitigated where refugia with conditions of less severe dissolved O_2 exist (Burleson et al., 2001; Kramer, 1987; McNeil and Closs, 2007). Avoidance behaviour was not assessed in the present study, as hypoxia in the treatments was inescapable, however dissolved O_2 in northern Australian rivers may vary greatly within the same body of water (Butler, 2008; Butler and Burrows, 2007; Waltham et al., 2013). Fish such as barramundi may rely on less severely affected areas for persistence during times of severe hypoxia, however where aquatic systems have been degraded by urbanisation and agriculture (Pearson et al., 2013),

refugia may not exist and physiological plasticity, as observed in the present study, may be the only mechanism for survival. Fish species such as weakfish (*Cynoscion regalis*) of north-east coastal USA have been observed to undertake diel horizontal migrations associated with declining O_2 levels. Laboratory testing with weakfish has demonstrated that hypoxia tolerance correlates strongly with abundances observed in the natural environment, indicating that avoidance is a critical survival strategy during severe environmental hypoxia (Brady et al., 2009; Stierhoff et al., 2009; Tyler and Targett, 2007). Hypoxia avoidance studies have not been conducted with barramundi, and despite an increasing understanding of hypoxia tolerance and respiration in this species (Butler et al., 2007; Collins et al., 2013; Flint et al., 2015; Jerry et al., 2013; Norin et al., 2014), further research is required to fill this knowledge gap.

The capacity for barramundi and other warm-water species to acclimate to hypoxia over timescales relevant to climate change remains uncertain. Nevertheless, my results suggest that physiological plasticity is a strong driver of hypoxia acclimation over short timescales in barramundi and may be similarly important across the species' distribution, given that I found no differences in plasticity for hypoxia tolerance between two genetically-distinct and geographically distant populations. It would be prudent for future studies to assess physiological responses of three or more populations, as such an approach would enable a greater elucidation of adaptive responses to hypoxia in this and other species.

CHAPTER 4. INTRASPECIFIC DIVERSITY IN HYPOXIA TOLERANCE OF A TROPICAL FISH IS HIGHLY PLASTIC AND NOT DEPENDENT ON METABOLIC PHENOTYPE

Abstract

Occurrences of hypoxia are increasing in freshwater and coastal aquatic systems, amplifying the selection pressure on individual phenotypes within fish populations. Substantial intraspecific variability in hypoxia tolerance has been documented in some species, yet the temporal repeatability of this tolerance and the underlying mechanisms remain poorly understood. Here, I address these knowledge gaps by first using loss of equilibrium (LOE) tests to assign ~800 barramundi (*Lates calcarifer*) into three hypoxia tolerance groups (sensitive, intermediate and tolerant). I then assessed individuals for their standard metabolic rate (SMR), critical O_2 level (O_{2CRIT}), growth performance, and temporal repeatability of time to LOE. Surprisingly, I found no relationship between the median time to LOE (LT_{50}) and O_{2CRIT} across the tolerance groups, nor between O_{2CRIT} and SMR at an individual level. Indeed, O_{2CRIT} means and variability were similar across all three hypoxia tolerance groups. The group-level time to LOE was not correlated with growth rate but was weakly, yet significantly, repeatable after 105 days. These findings highlight a disconnect between the interpretations derived from LOE vs. O_{2CRIT} measurements, and reveal a dramatic individual-level plasticity in hypoxia tolerance that is not driven by baseline metabolic demands.

Introduction

The capacity for any species to tolerate rapid environmental change is largely dependent on the diversity of phenotypes within a population. Dissolved O_2 displays large spatial and temporal variation in coastal and freshwater aquatic systems, however the frequency and severity of environmental hypoxia has increased in the past century due to nutrient enrichment from anthropogenic sources (Díaz and Rosenberg, 2011). Hypoxia affects a range of ecological and physiological processes (Abdallah et al., 2015; Almeida-Val et al., 2005; Chapman and McKenzie, 2009; Tyler and Targett, 2007) and acts as a significant pressure on many fish populations in concert with other natural and anthropogenic influences such as temperature and pH (Diaz and Breitburg, 2009; Peña et al., 2010). Phenotypic diversity in hypoxia tolerance has been documented for a small range of temperate fish species (Fu et al., 2014; Scott et al., 2015; Shimps et al., 2005; Speers-Roesch et al., 2013), yet comparatively little is known of tropical species despite a high prevalence of hypoxia in warm environments (Val et al., 2005). While there have been some recent advances in our understanding of hypoxia tolerance strategies in fish (Dhillon et al., 2013; Mandic et al., 2009; Richards, 2011), both the temporal repeatability and underlying drivers of intraspecific variability in hypoxia tolerance remain poorly understood.

The emphasis throughout many historical studies on hypoxia tolerance and other physiological traits has been on identifying trends between group means (Borowiec et al., 2015; Collins et al., 2013; Healy and Schulte, 2012),

rather than exploring the inherent inter-individual or intra-specific variability (Joyce et al., 2016; Marras et al., 2010; Norin et al., 2016). Such an approach may elucidate general, population-level patterns in environmental tolerance, but provides little information on the broader ecological consequences of trait variability and the inherent capacity of individuals and populations to tolerate change (Bennett, 1987; Killen et al., 2016). Despite an increasing focus on hypoxia as an environmental parameter of concern for many fish, intra-specific variability in hypoxia tolerance and its influence on metabolic regulation, growth and survival have received scant attention (Roze et al., 2013). Closer examinations of trait variability will enable a better understanding of the sensitivity of populations and species to environmental change, as well as helping to decipher the functional advantages of different phenotypes.

Understanding phenotypic diversity in traits such as hypoxia tolerance relies on robust and repeatable approaches that enable comparisons across studies and species. There are three common, non-lethal experimental techniques used to assess hypoxia tolerance in obligate water-breathing fish: the critical O_2 level (O_{2CRIT}), the DO at loss of equilibrium (LOE), and the time to LOE following hypoxia exposure (Rogers et al., 2016). These three metrics are often measured in isolation without cross-validation (Collins et al., 2016; Shimps et al., 2005), and while it is generally acknowledged that the mechanisms underlying each measure differ (McBryan et al., 2016) and that substantial diversity in responses below O_{2CRIT} exist between species (Fu et al., 2014; Shimps et al., 2005), it is intuitive and often implied that they should be highly correlated (Claireaux and Chabot, 2016; Collins et al., 2016; Dan et

al., 2014; Farrell and Richards, 2009). Further, while parameters such as O_{2CRIT} and time to LOE may inform intra-specific variation in hypoxia tolerance at a given time point, they may lack temporal repeatability and thus may not be representative of future performance (Dohm, 2002; Killen et al., 2016; Norin and Malte, 2011). Accordingly, forecasting the performance of fish in response to environmental hypoxia requires prior information on both the underlying variability, and the repeatability of hypoxia tolerance measures.

Here, many of these knowledge gaps are addressed using the barramundi (*Lates calcarifer*), a euryhaline fish that routinely encounters hypoxic conditions in its native range throughout the sub-tropical and tropical coastal Indo-Pacific. Intra-specific variability in hypoxia tolerance in sub-tropical and tropical populations is assessed to test the hypothesis that hypoxia tolerance shows broad intra-specific diversity and correlates with other performance metrics at the population level. I investigate whether individual- and population-level assessments of hypoxia tolerance are repeatable over time and robust across different methods. I also examine whether any relationship exists between hypoxia tolerance and growth performance. Specifically, fish are first separated into groups based on the time to LOE under hypoxic conditions, and then assessed for their standard metabolic rate (SMR), O_{2CRIT}, growth performance and temporal repeatability of time to LOE. I hypothesise that (1) time to LOE is repeatable over time, (2) time to LOE and O_{2CRIT} correlate strongly and thus provide synonymous assessments of

hypoxia tolerance, and (3) hypoxia tolerance can be used at an individual- or population-level to provide insight into whole-animal performance and fitness.

Materials and Methods

Animals and Holding Conditions

Barramundi were obtained from two commercial hatcheries in sub-tropical (Gladstone; 23°S) and tropical (Innisfail; 17°S) regions of Queensland (QLD), Australia. At the time of arrival fish were of comparable age (3 months) and size (~40 mm). Fish were held in a freshwater recirculating system (29°C) for three months prior to experimentation and fed a commercial diet *ad libitum*. All fish were exposed to a natural photoperiod (13 h light: 11 h dark) throughout the study.

Initial Hypoxia Challenge Test (HCT)

Hypoxia challenge tests (HCTs) at 29.1 ± 0.3˚C (means ± SD herein) were conducted on randomly selected replicate groups (24 tanks; 100 L; $N \approx 33$ fish · tank^{-1}) over a period of six days ($N = 788$ fish in total). Average fish mass was 18.7 ± 4.0 g and stocking density within HCT tanks was 6.2 ± 1.2 kg · m^{-3}. The HCT consisted of a decrease in DO from normoxia (>80% of air saturation) to severe hypoxia (10% saturation) over 76 ± 20 min, after which DO was maintained at 5.6 ± 0.9% saturation until fish lost equilibrium (Figure 43). DO was controlled in tanks by altering rates of water flow, aeration and the addition of nitrogen gas through ceramic diffuser stones, and was logged every 5 min using luminescent DO probes (Hach, Loveland, CO, USA). Fish

were not denied access to the surface and were observed performing aquatic surface respiration (ASR; i.e., gulping surface water) throughout the experiment. LOE was defined as an inability to maintain an upright position in the water column and failure to regain equilibrium within 5 s of being gently nudged by the experimenter using a plastic rod (Roze et al., 2013; Zambonino-Infante et al., 2013). When LOE was reached, fish were removed from the experimental tanks and the mass, length and time to LOE were recorded. The time to LOE was calculated for each fish as the time taken to lose equilibrium following the depletion of DO below 10% saturation.

Fish were separated into five labelled recovery tanks containing air saturated water, based on the time to LOE. The lower and upper quartiles were labelled hypoxia sensitive (referred to hereafter as ‘sensitive’) and hypoxia tolerant (tolerant), respectively. Fish in the semi-interquartile range were labelled hypoxia intermediate (intermediate; control), and the remaining fish in the interquartile ranges were removed and not used thereafter. LOE tests were terminated after 410 ± 21 min, and any fish that had not lost equilibrium (~3% of individuals) were removed and placed into the tolerant recovery tank. Fish were given three weeks to recover following the HCT prior to further tests. Sub sets of these fish were used for either growth or respirometry experiments, as outlined below.

Growth Trial

Following the initial HCTs, subsets of barramundi (N = 60 fish per population, per hypoxia category; 3 replicate tanks per treatment (N = 360 in total)) were assigned randomly to 18 × 100 L tanks and fed a commercial pelleted feed (EWOS, Bergen, Norway) to satiation, twice daily for 45 d (30.4 ± 0.7 °C). Uneaten pellets were removed from tanks 20 min following feeding and counted. Specific growth rate, feed consumption and feed conversion ratio were calculated as per (Yang et al., 2013).

Oxygen consumption rates ($\dot{M}O_2$)

Oxygen consumption rates ($\dot{M}O_2$) were measured on subsets of fish ($N \approx 36$ per population, per hypoxia category; N = 211 in total) using intermittent flow-through respirometry over a period of 40 days as described in Collins et al. (2013) and following best practices (Clark et al., 2013a; Svendsen et al., 2015). Briefly, respirometers (1.5 L) were submerged in a temperature-controlled (29.1 ± 0.3°C) tank that provided fully saturated water during the flush cycle. DO was recorded using Firesting optical O_2 sensors (Pyroscience; Aachen, Germany). Mass-standardised $\dot{M}O_2$ was calculated between each flush cycle for each fish as per equation 2. The SMR for each fish was calculated using the quantile method, as per Chabot et al. (2016b). O_{2CRIT} tests were conducted on individuals using closed-respirometry, as per Collins et al. (2016). Fish involved in respirometry trials were not used for any further tests.

HCT Repeatability

Fish (*N* = 354) were fed *ad libitum* once daily for 5 weeks after the completion of the growth trial, after which HCTs were conducted using the methodology described above to assess the temporal repeatability of LOE measurements (i.e., ~105 days following the first HCT). HCTs were conducted over 5 consecutive days (*N* ≈ 20 fish per HCT tank). Average fish mass was 130.2 ± 26.7 g and stocking density within experimental LOE tanks was 25.66 ± 2.11 kg m^{-3}. DO was maintained at 5.1 ± 0.9% saturation until LOE was reached, and then each individual was measured and handled as outlined above. LOE tests were terminated after 428 ± 9 min, and any fish that had not lost equilibrium (~3% of individuals) were removed and placed into a recovery tank.

Data Analysis

Data analyses were performed using R v.3.2.1 (R Core Team, 2012). Results were considered statistically significant at $P \leq 0.05$ and all data are presented as mean ± standard deviation unless otherwise stated.

Time to LOE data were compared using survival analysis procedures (Roze et al., 2013; Zambonino-Infante et al., 2013). As LOE and not mortality was assessed, the survival analysis is referred to herein as the 'hypoxia resilience analysis' (HRA) to avoid confusion. Fish that had not lost equilibrium at the completion of HCTs were right-censored. The HRA procedure consisted of fitting a Cox proportional hazard's model to assess differences between population, hypoxia category and HCT, with fish mass included in the model

as a covariate. The proportional hazard's assumption was assessed *via* examination of Schoenfield residuals and the accompanying chi-square test-statistic for each population, hypoxia category and HCT.

The O_{2CRIT} was calculated for each fish using broken-stick regression (BSR) (Muggeo, 2008), non-linear regression (NLR; Weibull function) (Marshall et al., 2013) and least-squares regression accompanying SMR (Claireaux and Chabot, 2016), (full details provided in Appendix B; see also Figures 28, 34). The Weibull function and first derivative were defined as:

$$f(\mathrm{x}) = \mathrm{a} \times \left(1 - \mathrm{e}^{-\left(\frac{\mathrm{x}}{\mathrm{b}}\right)^{\mathrm{c}}}\right)$$

Equation 5 - Weibull function: x = DO, a = asymptote, b = scale, c = shape

$$f'(\mathrm{x}) = \mathrm{a} \times \left(\frac{\mathrm{c}}{\mathrm{b}}\right) \times \left(\frac{\mathrm{x}}{\mathrm{b}}\right)^{(\mathrm{c}-1)} \times \mathrm{e}^{-\left(\frac{\mathrm{x}}{\mathrm{b}}\right)^{\mathrm{c}}}$$

Equation 6 - Weibull function (first derivative): x = DO, a = asymptote, b = scale, c = shape

With parameters ***a***, ***b*** and ***c***, and where x is equal to DO. The O_{2CRIT} was calculated for each fish where $f'(x) = 0.029$.

It was desirable to perform goodness of fit tests to select a model that best described the relationship between $\dot{M}O_2$ and DO. Least-squares regression accompanying SMR did not meet this criterion and thus was excluded from this procedure. Model fit to $\dot{M}O_2$ data (BSR or NLR) during the O_{2CRIT} tests were compared using Akaike weights (w_i(AIC)) as per (Wagenmakers and Farrell, 2004). NLR proved most robust (median w_i(AIC) = 0.96; 1st and 3rd

quartiles = 0.38 and 1.0, respectively; Figure 29) and thus all O_{2CRIT} estimates and comparisons reported herein are from NLR calculations, unless otherwise stated (further details can be found in *Appendices A* and *B*).

Comparisons between $\dot{M}O_2$ or growth data across groups were conducted using mixed model ANOVA (Bates et al., 2016; Pinheiro et al., 2014), with the main factors (population and hypoxia category) included as fixed effects and experimental day ($\dot{M}O_2$ data) or tank (growth data) included as random effects (Thorarensen et al., 2015). Multiple comparisons of means were performed using Tukey's Honest Significant Difference. Correlations within experiments and the repeatability of the median time to LOE (LT_{50}) were assessed using Pearson's product-moment correlation coefficient (r).

Results

Population Effects

Significant differences existed between tropical and sub-tropical populations for all growth parameters (Figure 42) and for time to LOE in the first HCT (Figures 18a, 44; Table 4), but not for SMR, O_{2CRIT} (Figure 45) or time to LOE in the second HCT (Figure 44). No significant differences were detected between populations in the global Cox Proportional Hazard's Model (further details in *Appendix B*).

Initial hypoxia challenge test (HCT)

Barramundi displayed substantial variability in hypoxia tolerance and were separated into distinct groups based on the time to LOE (Figure 17). Sensitive (LT_{50} = 77 min), intermediate (LT_{50} = 144 min) and tolerant (LT_{50} = 320 min) fish displayed significant differences in time to LOE (populations pooled: likelihood ratio test: LRT = 1 418, df = 4, $P < 0.0001$), (Figures 18a, 44a; Table 4). Time to LOE varied from 2 to 430 min for sub-tropical fish and from 38 to 425 min for tropical fish (LRT = 1418, df = 4, $P < 0.0001$). A further 26 fish (N = 18, sub-tropical; N = 8, tropical) did not lose equilibrium. There was a significant positive correlation between fish mass and time to LOE in the initial HCT (r = 0.409, df = 786, $P < 0.0001$; Figure 22).

*Standard metabolic rate and O_{2CRIT} **

Surprisingly, hypoxia category had no significant effect on O_{2CRIT} ($F_{2, 22}$ = 0.659, P = 0.527; Figure 18c, d). Moreover, there was no significant relationship between the time to LOE and O_{2CRIT} (r = 0.389, df = 4, P = 0.446), (Figure 18a). SMR was similarly not different between the hypoxia categories ($F_{2, 22}$ = 1.845, P = 0.182), and the overall relationship between SMR and O_{2CRIT} was not significant (r = -0.077, df = 209, P = 0.267), (Figure 18b). In contrast to the assessment of hypoxia tolerance based on time to LOE, larger fish appeared less hypoxia tolerant based on O_{2CRIT} measurements (Figure 36; r = 0.408, df = 209, P < 0.0001). However, this was probably a consequence of the mass-dependent rate of O_2 depletion within respirometers (Figure 36).

* A *post hoc* power analysis was conducted on the data for O_{2CRIT} and SMR and is presented in Appendix B (see Figures 49 and 50). The model formulation and output for O_{2CRIT} and SMR is presented in Tables 7 and 9, respectively. The confidence intervals for model parameters for O_{2CRIT} and SMR are presented in Tables 8 and 10, respectively.

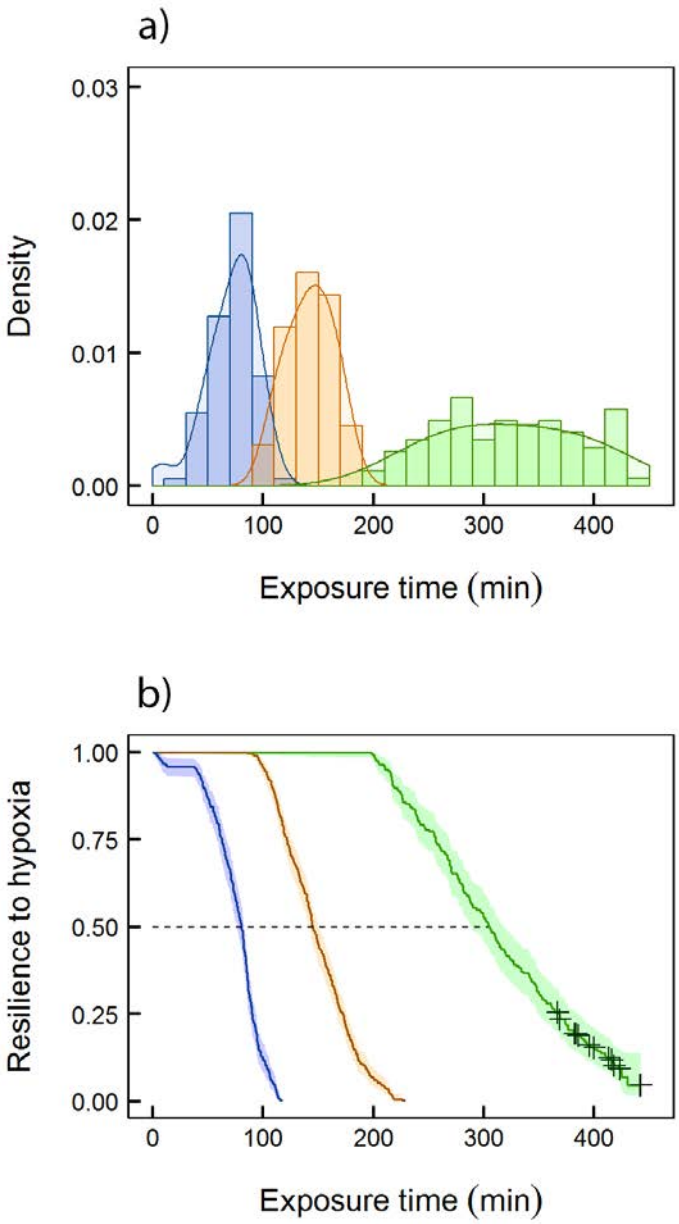

Figure 17 - Density distribution of time to loss of equilibrium in the first HCT (panel a), where blue, orange and green bars and density lines represent sensitive, intermediate and tolerant fish, respectively. Sample sizes are: sensitive (*N* = 200); intermediate (*N* = 209); tolerant (*N* = 173). Right-censored data are not included in histograms or density functions. Panel b illustrates survival functions (solid line ± shaded 95% confidence limits) where blue represents sensitive fish, orange represents intermediate fish, and green represents tolerant fish.

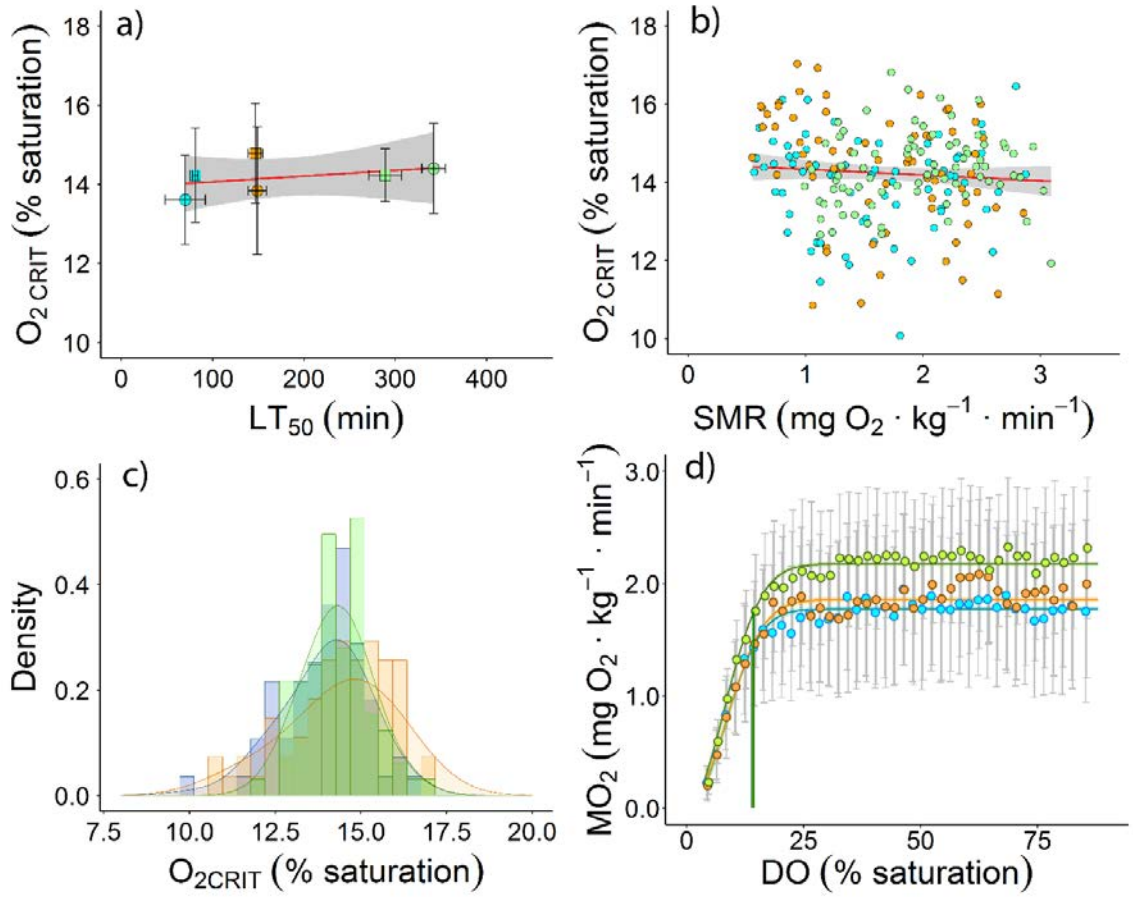

Figure 18 - Panel a: the relationship between O_{2CRIT} of barramundi (*N* = 211) plotted against the mean of the LT_{50} (*N* = 788) for each population and hypoxia category (see Table 5 for mean of LT_{50} results). Error bars represent standard deviation. Circles and squares represent sub-tropical and tropical populations, respectively. Blue, orange and green symbols represent hypoxia-sensitive, -intermediate and -tolerant fish, respectively. The red line ± 95% confidence limits (shaded grey area) is a fitted linear regression. Panel b: O_{2CRIT} for all fish plotted against SMR, along with a fitted linear regression (solid red line; data pooled for sub-tropical and tropical populations). Panel c: density histogram and density distribution (Gaussian kernel density estimates) of O_{2CRIT}, where blue, orange and green shading represent sensitive (*N* = 67), intermediate (*N* = 66), and tolerant (*N* = 78) fish, respectively. Panel d: mean ± standard deviation for each 2% decline in DO and fitted Weibull functions for sensitive (blue), intermediate (orange) and tolerant (green) fish.

Growth Trial

There was a significant effect of hypoxia category on absolute growth rate (AGR; $F_{2, 12} = 5.406$, $P = 0.021$), but not on any of specific growth rate (SGR), mean daily food consumption, feed conversion efficiency (FCE) or feed conversion ratio (FCR) (Figure 19; $P > 0.05$ for all non-significant results). Full details of the results from the growth trial are presented in *Appendix B* and illustrated in Figures 19 and 42.

Hypoxia Challenge Test (HCT) Repeatability

Consistent with the first HCT, I observed substantial variability in time to LOE (Figure 20): 16 to 382 min for sub-tropical fish, and 19 to 427 min for tropical fish. A further 13 fish ($N = 9$, sub-tropical, $N = 4$, tropical) did not lose equilibrium. There was no significant difference in LT_{50} between fish initially assigned to the sensitive group (LT_{50} = 93 min) and those assigned to the intermediate group (LT_{50} = 110 min; $P = 0.119$); however, fish initially assigned to the tolerant group (LT_{50} = 147 min) displayed significantly longer time to LOE (LRT = 145, df = 4, $P < 0.0001$). This observation was further supported by the shape of the non-linear relationship between the residual LT_{50} (Figure 21a). As in the initial HCT, fish mass and time to LOE remained positively correlated despite the fish being ~7-times larger in the second trial (r = 0.522, df = 352, $P < 0.0001$), (Figure 22).

There was a significant difference in LT_{50} between the first and second HCT, with fish in the first HCT (LT_{50} = 131 min) exhibiting a longer time to LOE than fish in the second HCT (LT_{50} = 114 min), (LRT = 968, df = 5, $P <$

0.0001), (Figure 21 b, c). The group-level repeatability of the HCT after 105 d was statistically significant (r = 0.968, t = 7.756, df = 4, P = 0.001).

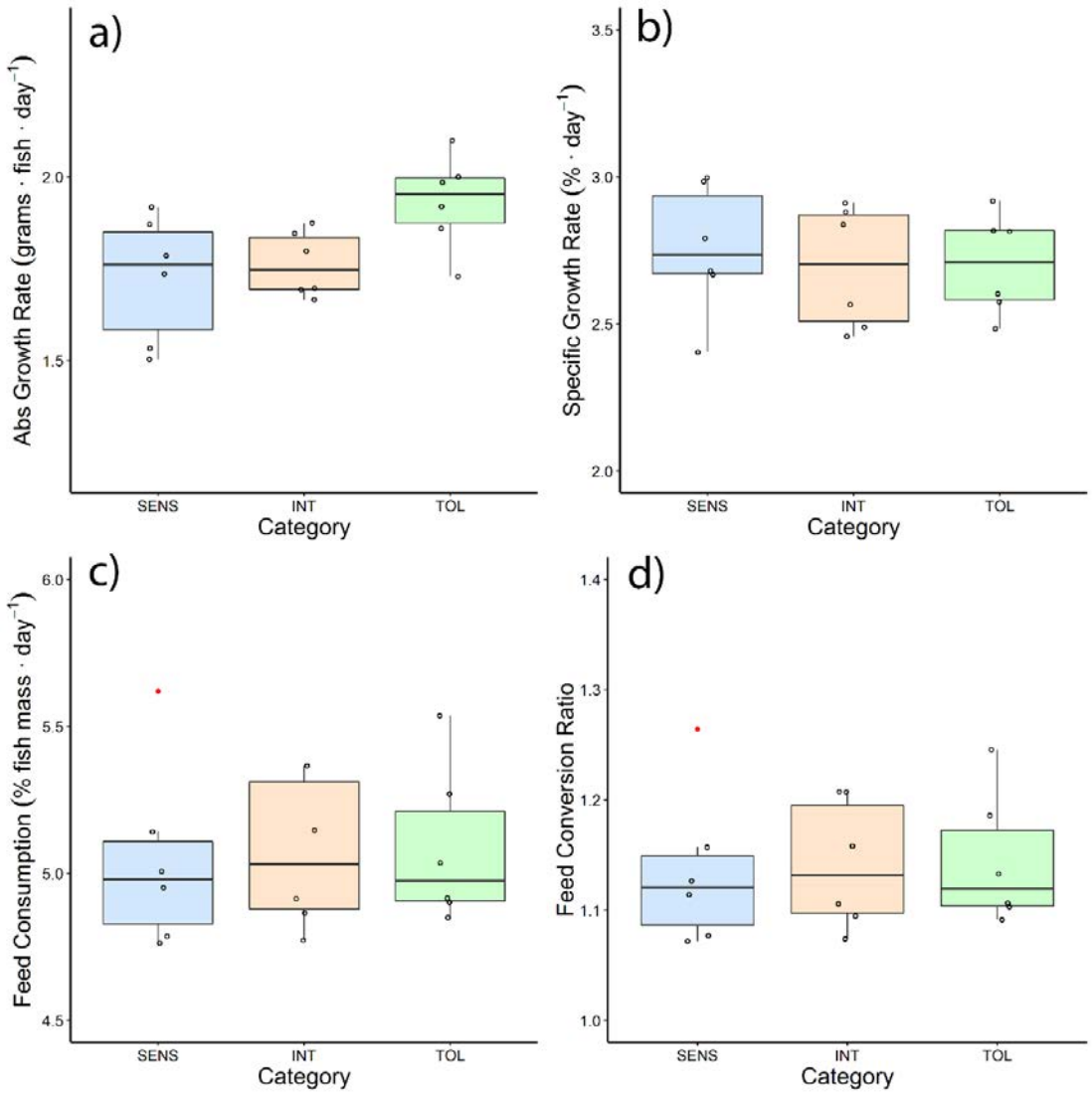

Figure 19 - Boxplots displaying growth parameters: absolute growth rate (panel a), specific growth rate (panel b), feed consumption rate (panel c) and feed conversion ratio (panel d). Raw data are overlayed as points.

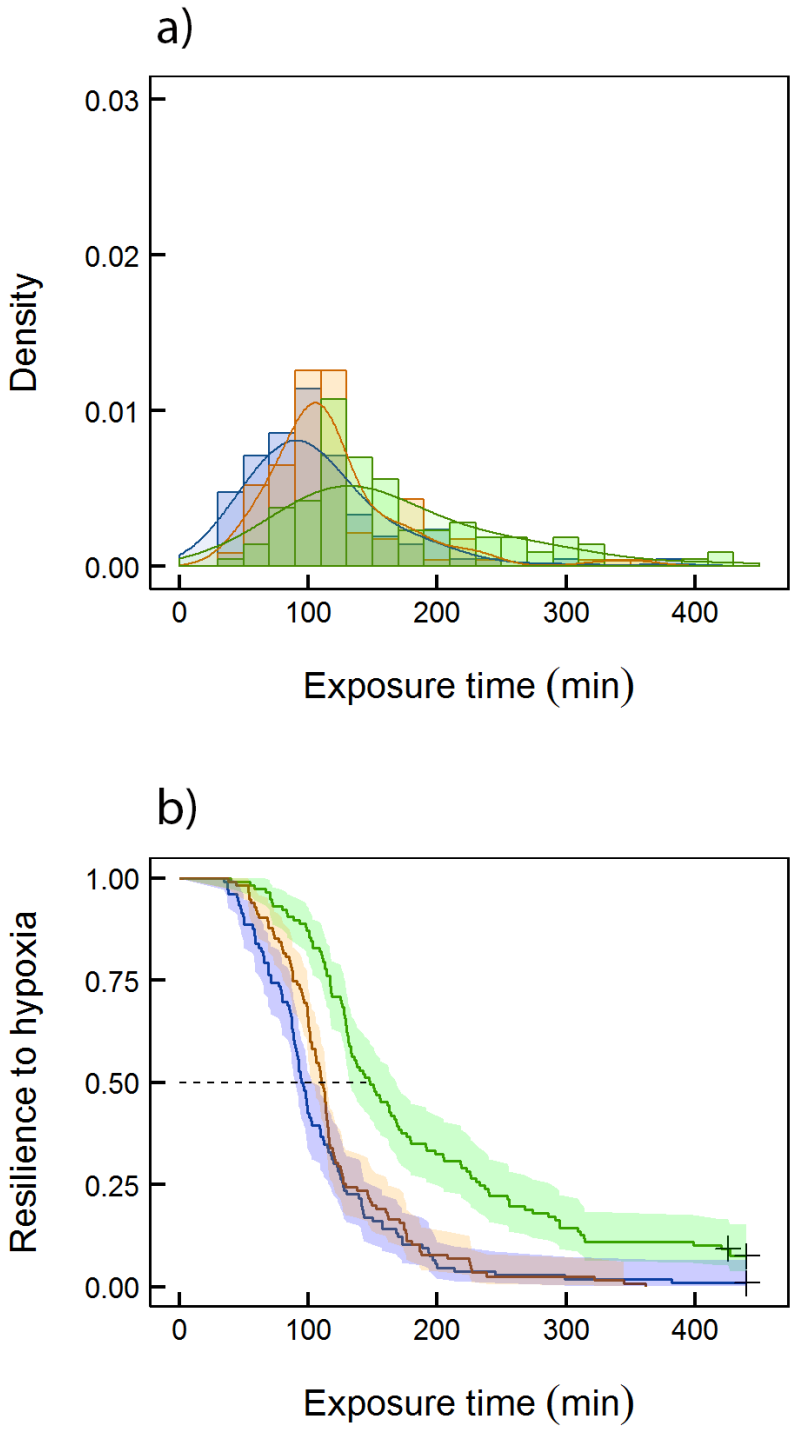

Figure 20 - Density distribution of time to loss of equilibrium in the second HCT (panel a), where blue, orange and green bars and density lines represent sensitive, intermediate and tolerant fish, respectively. Sample sizes are: sensitive (*N* = 115); intermediate (*N* = 120); tolerant (*N* = 119). Right-censored data are not included in histograms or density functions. Panel b illustrates survival functions (solid line ± shaded 95% confidence limits) where blue represents sensitive fish, orange represents intermediate fish, and green represents tolerant fish.

Discussion

Hypoxia tolerance and repeatability

This study shows that barramundi have a broad array of hypoxia tolerance phenotypes that are at least weakly repeatable across hypoxia categories (groups) and the two populations assessed over a period of ~105 days. Indeed, while the sensitive and intermediate groups were not clearly distinguishable in the second HCT, the tolerant group demonstrated clear separation throughout the study as illustrated by an approximately log-normal distribution of the density functions (Figure 21). Intriguingly, the tolerance of individuals within categories was highly plastic, indicating that individual rankings of hypoxia tolerance within a population can shift dramatically even when environmental conditions remain benign. As far as I am aware, this is the first study to demonstrate the extent of temporal plasticity of hypoxia tolerance across large cohorts of individuals within fish populations. Notably, the substantial diversity in hypoxia tolerance (time to LOE) between groups did not translate to differences in growth rates or SMR, suggesting that hypoxia tolerance does not share common mechanisms with these traits.

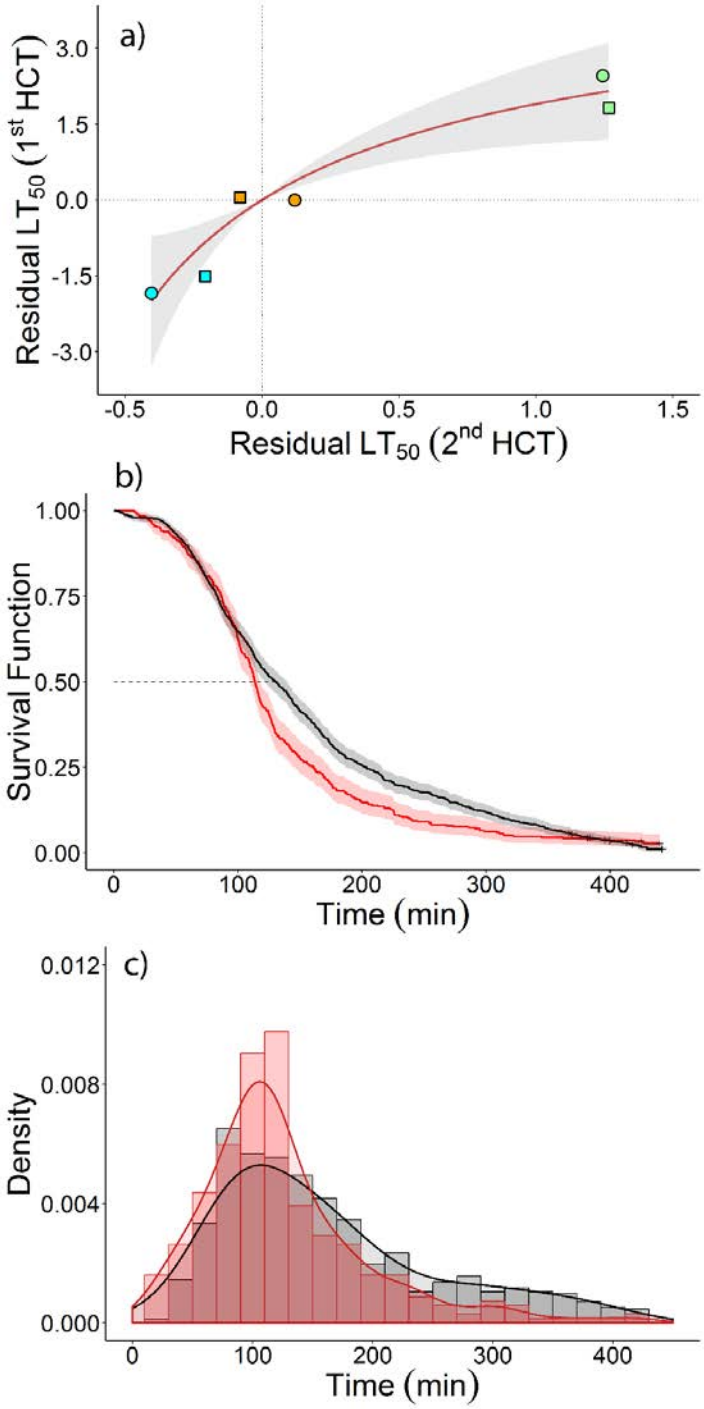

Figure 21 - Repeatability of residual LT_{50} in the HCT (panel a) for sub-tropical (squares) and tropical (circles) populations of barramundi. Blue, orange and green symbols represent sensitive, intermediate and tolerant hypoxia categories, respectively. The red line is a fitted hyperbolic equation. Panel b: Survival functions (solid line ± shaded 95% confidence limits) for the first (black; *N* = 788) and second (red; *N* = 354) HCT (data pooled for all hypoxia categories and populations). Panel c: Density distribution of time to LOE in the first (grey bars; *N* = 788) and second (red bars; *N* = 354) HCT. Black and red lines overlayed represent fitted density functions for the first (black) and second (red) HCT.

Hypoxia category and O_{2CRIT}

Unexpectedly, I found no relationship between time to LOE and the capacity of fish to regulate metabolism in response to hypoxia (O_{2CRIT}; Figure 18). Given that O_2 changes from a limiting to a lethal factor as DO falls below O_{2CRIT} (Claireaux and Chabot, 2016), it is generally implied that O_{2CRIT} may serve as an indicator of survival for species when DO becomes critically low. Despite this, my study adds to a growing literature indicating that O_{2CRIT} does not provide a universal metric of hypoxia tolerance (Dhillon et al., 2013; Fu et al., 2014; Scott et al., 2015; Speers-Roesch et al., 2013). This raises questions regarding what constitutes hypoxia tolerance, how it should be measured, and what isolated measurements reveal about long-term, ecologically-relevant levels of hypoxia tolerance in individuals. While the answers to these questions must await further research, the present study takes a significant step towards highlighting the knowledge gaps that remain to be filled. Importantly, I have shown that there exists a clear disconnect between the mechanisms responsible for endurance to severe hypoxia (time to LOE) and those responsible for the regulation of metabolism in the face of declining environmental O_2 (O_{2CRIT}). Such mechanistic responses await elucidation through future research, but may include changes to the quantity of ATP produced per O_2 atom consumed (Salin et al., 2015) or increased expression of myoglobin (Wells, 2009b) when O_2 declines below the O_{2CRIT}.

Influence of mass on hypoxia tolerance

Consistent with previous observations from a tropical fish (Almeida-Val et al., 2000), I observed a positive relationship between mass and time to LOE in both the first and second HCTs (Figure 22). Additionally, the mass co-variate was highly significant in the HRA, providing further support for a strong role of mass in time to LOE. These findings are consistent with previous observations of DO at LOE for barramundi (Butler et al., 2007) and rainbow trout (Roze et al., 2013), indicating that smaller fish are faster to lose equilibrium and therefore at greater risk of mortality than larger fish during severe hypoxia. Despite this, hypoxia tolerance was not a simple function of mass, as LT_{50} in the second HCT was actually lower than in the first HCT, despite the fish being nearly 7-times larger. Only 17% and 27% of the variation in time to LOE could be explained by fish mass in the first and second HCTs, respectively. Moreover, it was not necessarily the largest individuals that completed the 7.25-h HCT without losing equilibrium (black circles in Figure 22). The observed differences between individuals may be attributed to factors such as the capacity for metabolic suppression during hypoxia (Hochachka, 1990; Van den Thillart et al., 1994), the efficacy of aquatic surface respiration (Chapman and McKenzie, 2009), and the utilisation of anaerobic metabolism (Hochachka and Somero, 2002). While the role that mass plays in determining hypoxia tolerance for individuals or populations remains speculative (Nilsson and Östlund-Nilsson, 2008; Sloman et al., 2006), the present study demonstrates that this relationship can be masked when hypoxia tolerance is quantified using measures of O_{2CRIT} rather than time to LOE.

Hypoxia category and growth performance

The relationship between metabolic rate and growth performance of fish has been investigated extensively for a range of temperate fish species, particularly in relation to social status, behaviour and feeding rate (Auer et al., 2015; Burton et al., 2011; Gifford et al., 2014; Metcalfe et al., 1995; Nakano, 1995; Yamamoto et al., 1998), where fish that exhibit a higher metabolic rate tend to exert greater dominance behaviour and often grow faster. The capacity for metabolic regulation (O_{2CRIT}) and metabolic rate (SMR) are predicted to be positively correlated (although no such relationship was observed in this study; see Figure 18a), and it may therefore have been expected that growth performance would correlate inversely with hypoxia tolerance. Stated another way, fish that have a higher SMR tend to be faster growing and conventional theory suggests that fish with a higher SMR also have a higher O_{2CRIT} and are therefore less tolerant of hypoxia. I observed a positive relationship between fish mass and time to LOE, such that increased weight gain for individuals through the growth trial correlated with in an increase in time to LOE. An alternative hypothesis is therefore that hypoxia tolerance will correlate positively with growth rate, where fish that exhibit higher growth rates will increase in mass faster and will be more tolerant of hypoxia. I found no evidence for a relationship between hypoxia tolerance and any parameter associated with growth in this study (Figure 19), however a marked difference was observed in growth responses between the two populations examined (Figure 42) with sub-tropical fish exhibiting a lower feed consumption, higher SGR and lower FCR. The two populations of

barramundi examined in this study originate from the east coast of Queensland and hence do not display large genetic differentiation (Loughnan et al., 2015), however, previously observed differences in performance attributes such as swimming speed (Edmunds et al., 2010) and thermal tolerance (Newton et al., 2010) between sub-tropical and tropical populations are consistent with my observation of differences in growth performance between high and low latitude populations. While it is difficult to infer any adaptive responses between sub-tropical and tropical populations on the basis of a two population study (Garland and Adolph, 1994) the phenotypic responses observed may be attributed to differences in prevailing environmental conditions between locations coupled with genetic drift (BOM, 2012; West-Eberhard, 2005), but could also be attributed to genotypic and phenotypic differences between family lines (Anttila et al., 2013) or year-classes (Scott et al., 2015) of hatchery populations.

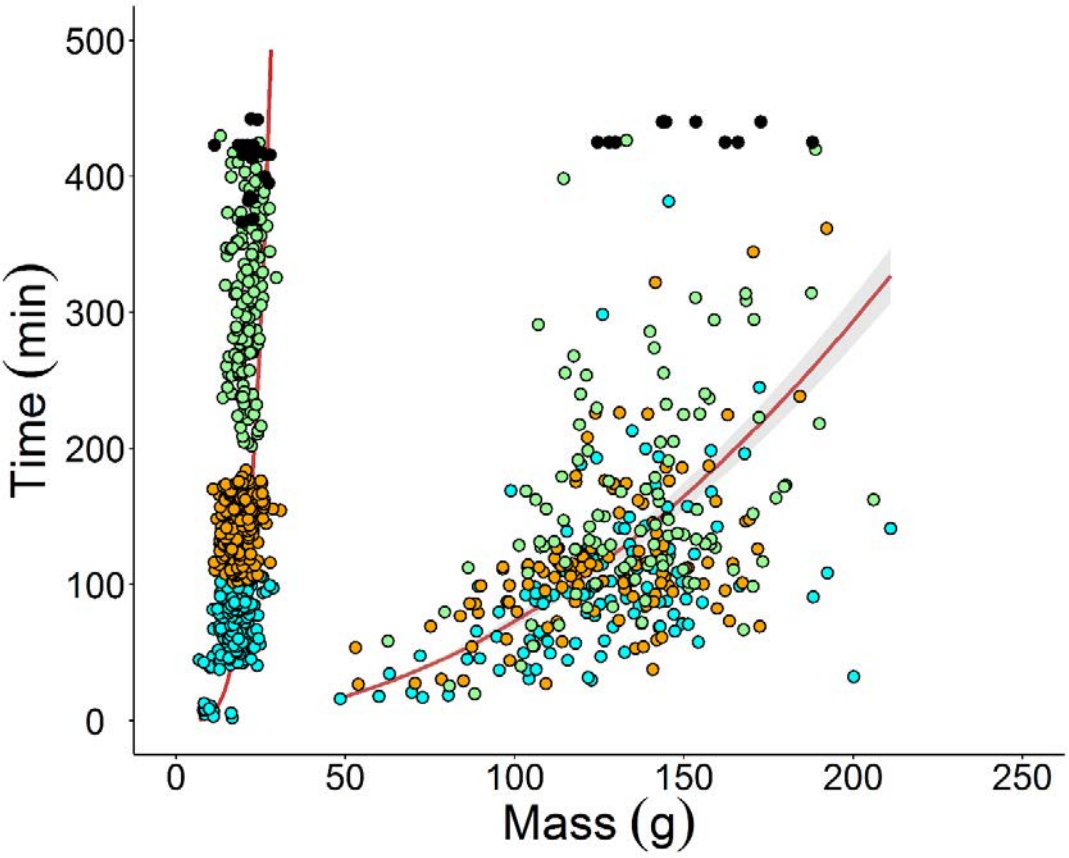

Figure 22 - Fish mass plotted against time to LOE for the first and second HCT. Fish in the first HCT were < 40 g, and fish in the second HCT were > 40 g. Blue, orange and green circles represent hypoxia sensitive, intermediate and tolerant fish, respectively. Black circles represent fish that did not lose equilibrium throughout the ~7 hour trial. The red lines represent fitted non-linear regressions.

Conclusion

This study demonstrates for the first time that hypoxia tolerance (time to LOE) exhibits substantial intra-specific variability yet it is only loosely repeatable at a group level. Indeed, the bounds of inherent hypoxia tolerance are set at the level of the population, as represented by a normal distribution, but individual fish can dramatically shift their position in the distribution over relatively short temporal scales (<105 days). Moreover, I have shown that time to LOE is not correlated with O_{2CRIT}, which overturns existing belief that

the two metrics of hypoxia tolerance are synonymous and/or provide qualitatively similar results. This finding calls for a re-appraisal of the ecological significance of O_{2CRIT}, and a deeper understanding of the ecological relevance of the different approaches used to measure hypoxia tolerance. A range of physiological adjustments can improve hypoxia tolerance of fishes following long-term or repeated exposure to hypoxic conditions, yet the improvements are often minor and there appears to be a rather rigid lower limit (O_{2CRIT} ~ 10-15% air saturation) that is largely conserved across species (Borowiec et al., 2015; Collins et al., 2016; Dan et al., 2014). Thus, while many fish species including barramundi are adapted to withstand natural bouts of environmental hypoxia, individuals are bound to relatively inflexible limits of hypoxia tolerance that will be increasingly challenged as anthropogenically-induced hypoxic bouts become more severe and frequent.

This chapter is currently in preparation for submission to the journal Ecology of Freshwater Fish

CHAPTER 5. METABOLIC RESPONSES TO DIGESTION ARE INDEPENDENT OF ENVIRONMENTAL OXYGEN AND HYPOXIA TOLERANCE PHENOTYPE IN A TROPICAL FISH

Abstract

Environmental hypoxia is increasing in tropical aquatic systems due to elevated nutrient levels from anthropogenic sources, amplifying the selection pressure on fish populations. Increasing hypoxia will likely exacerbate sub-lethal effects on fish populations and may impact ecosystem productivity through individual responses such as poor digestion and slower growth. Here, barramundi juveniles were separated into hypoxia tolerance phenotypes (sensitive and tolerant) using hypoxia challenge tests. Fish were then fed a restricted ration (2.5% body-mass) and their specific dynamic action (SDA) was assessed during digestion using intermittent-flow respirometry under normoxic (~95% saturation) or hypoxic (~35% saturation) conditions. There was no effect of hypoxia on the magnitude or duration of the SDA response, however the SDA coefficient was marginally, yet significantly lower under hypoxic conditions. Hypoxia phenotype had no effect on the SDA response, nor on the capacity for metabolic regulation when faced with declining environmental O_2 (O_{2CRIT}). These results suggest that barramundi are capable of maintaining maximal digestive capacity under chronic, moderate hypoxia, and that such responses are not influenced by hypoxia phenotype.

Introduction

Environmental hypoxia is a naturally occurring phenomenon in many freshwater, estuarine and marine systems that can occur due to thermal stratification, depth-dependent limits in photosynthesis, high biochemical O_2 demand, and/or pre-flush rain events at the onset of the monsoon season (Butler and Burrows, 2007; Diaz and Breitburg, 2009; Val et al., 2005; Waltham et al., 2013). A variety of anthropogenic pressures are increasing the severity of hypoxia in freshwater systems, including nutrients from agriculture and urbanisation (Diaz and Breitburg, 2009) and the spread of exotic plants (Perna and Burrows, 2005). Hypoxia is also common in aquaculture, where high stocking densities can lead to O_2 depletion following feeding, or through an accumulation of organic waste in benthic sediments (Avnimelech and Ritvo, 2003). Prevailing high water temperatures make tropical systems particularly susceptible to hypoxic events, due to elevated organismal metabolic demands, lower solubility of O_2 in water and thermal stratification (Val et al., 2005). Increasing human development, combined with rising average global temperatures and extreme weather events are predicted to exacerbate the frequency and severity of hypoxia in tropical systems (Diaz and Breitburg, 2009), placing further pressure on fish populations.

Fish experience a range of energetic demands that elevate metabolism above resting levels, and of paramount importance for growth and

development is the efficient processing of food, commonly referred to as specific dynamic action (SDA) (Secor, 2009; Wang et al., 2009). In broad terms, SDA is defined as the quantity of energy expended on ingestion, digestion, absorption and assimilation of a meal (Secor, 2009). Environmental hypoxia may compromise SDA processes by imposing an upper limit on aerobic metabolic capacity during digestion (Fry, 1971; Jourdan-Pineau et al., 2010; Wang et al., 2009). The effects of hypoxia on feeding and SDA have been characterised for several temperate fish species, and include reduced appetite and growth rates (Chabot and Dutil, 1999) as well as prolongation of digestion (Dupont-Prinet et al., 2013; Jordan and Steffensen, 2007). The effects of hypoxia on metabolic responses may be particularly severe in the tropics due to high metabolic demands and a concomitant reduced solubility of O_2 in water, and may become more severe in the future as the climate continues to warm (Aust Gov - BoM, 2015) yet aside from recent research on a facultative, air-breathing catfish (Nelson et al., 2007; Zhang et al., 2010), baseline information on SDA responses in tropical species remains scarce.

While it is broadly recognised that substantial diversity in hypoxia tolerance exists between fish species (Chapman et al., 1995; Fu et al., 2014; Richards, 2011), the impressive diversity in hypoxia tolerance within species (inter-individual variability) is often overlooked in empirical physiological studies (Scott et al., 2015). Accordingly, while the average responses for performance traits associated with hypoxia are increasingly being elucidated (Collins et al., 2016; Fu et al., 2014; Mandic et al., 2013), the underlying drivers and biological consequences of individual-level variability remain

poorly understood. Specifically, it is not known whether an increased ability to tolerate hypoxia translates into other advantages or improved performance, such as faster growth or an improved capacity to process meals during hypoxic conditions.

Aims and Hypotheses

Here I examine these knowledge gaps using the Asian sea-bass / barramundi (*Lates calcarifer*), a euryhaline fish of substantial ecological and economic importance throughout the tropical and sub-tropical Indo-Pacific. I assess intra-specific variability in metabolism following digestion during normoxia and chronic, moderate hypoxia to test the idea that hypoxia compromises SDA responses. The moderate hypoxic treatment was selected based on prior information of dissolved O_2 conditions commonly encountered by wild populations (Loong et al., 2005) and in aquaculture (Bade, T., personal comm.). I first separate fish into hypoxia tolerance phenotypes based on the time to LOE under hypoxic conditions (hypoxia challenge tests; HCTs), and then assess O_2 consumption rates ($\dot{M}O_2$) following feeding. I hypothesise that (1) moderate hypoxia will increase the duration and magnitude of the SDA response, and (2) hypoxia tolerant phenotypes will display less disruption of the SDA response during hypoxia compared with sensitive phenotypes, and (3) fish that are more hypoxia tolerant, as assessed *via* HCTs, will have a concomitant lower O_{2CRIT}.

Materials and Methods

Animals and Holding Conditions

Juvenile barramundi (*N* = 120) were obtained from a commercial hatchery (GFB Fisheries, Townsville (19°S), Queensland (QLD), Australia) and road-freighted to an outdoor, covered aquarium research facility at James Cook University, Townsville, QLD. At the time of arrival, fish were approximately 3 months old and 40-50 mm total length. Fish were held in 18 × 100 L tanks connected to a freshwater recirculating system (total system volume = 15 kL) at 30.1 ± 0.3°C for one month prior to experimentation, and fed a restricted ration (1% of body mass) every second day with a commercial pelleted feed (EWOS, Bergen, Norway: 42 $g \cdot kg^{-1}$ crude protein, 12 $g \cdot kg^{-1}$ crude lipid). Fish were exposed to a natural photoperiod (approximately 13 h light:11 h dark) throughout the study.

Hypoxia challenge test (HCT)

Hypoxia challenge tests (HCTs) were conducted on fish in four replicate tanks (100 L; *N* = 30 fish per tank; 30.5 ± 0.1°C), as per the methodology outlined in *Chapter 4*. Briefly, fish were transferred to experimental tanks 12 h prior to HCTs and feed was withheld from fish for 48 h prior to HCTs to minimise the effects of handling and digestion on hypoxic responses. The HCT consisted of a decrease in dissolved O_2 (DO) from normoxia (>80% saturation) to severe hypoxia (<10% saturation) (Figure 51). DO was lowered in tanks by reducing water flow and aeration and by bubbling nitrogen gas through ceramic diffuser stones. DO was recorded every 5 min using

luminescent/optical dissolved O_2 meters (Hach, Loveland, CO, USA). The time taken to reach severe hypoxia in each tank was 105 ± 11 min (Figure 51). Once severe hypoxia was achieved in the experimental tanks, DO was maintained at 6.7 ± 0.8 % saturation until fish lost equilibrium. Fish were not denied access to the surface and were observed performing aquatic surface respiration (i.e., gulping surface water) throughout the experiment.

Loss of equilibrium (LOE) was defined as an inability to maintain position in the water column and a failure to regain equilibrium within 5 s of being gently tapped by the experimenter with a short length of polyethylene tubing (Roze et al., 2013). When LOE was reached, fish were removed from the experimental tanks, and mass and length were recorded, along with the time of LOE. Fish were then placed into one of three labelled recovery tanks containing air saturated water. The first 20% of fish to lose equilibrium were placed into a tank labelled hypoxia sensitive (HS). The intermediate 60% of fish were placed into a tank labelled hypoxia intermediate (HI). LOE tests were terminated after 4 h and 15 min, and all fish that had not lost equilibrium (the final 20%) were placed into a tank labelled hypoxia tolerant (HT). Fish were maintained in these groups for all subsequent tests. A subset of HI fish were used for standard metabolic rate (SMR) measurements, whereas subsets of the HS and HT fish were used for measures of specific dynamic action (SDA) and critical O_2 level (O_{2CRIT}; see below). Fish were given four weeks of recovery following the HCT in normoxic water prior to further tests.

Determination of SMR and handling stress

SMR was determined for a subset of unfed, HI fish, for the purpose of accounting for handling stress associated with respirometry. The DO during SMR measurements was adjusted according to one of the two experimental treatments: normoxia (>90% saturation; *N* = 16 fish) or hypoxia (~35% saturation; *N* = 16 fish) (Figure 52). Oxygen consumption rates ($\dot{M}O_2$) were measured using intermittent flow-through respirometry as described in Collins et al. (2013) and following best practices (Clark et al., 2013a; Svendsen et al., 2015). Briefly, 8 fish per day were netted and transferred individually into respirometers (1.5 L). Care was taken to ensure that the time from netting to transferral into the respirometers was ≤ 15 s for all fish. The respirometers were submerged in a temperature-controlled (29.4 ± 0.2 °C) 1,000 L reservoir tank that provided new water to chambers during the flush cycle of respirometry. DO was controlled in the tank by manipulating rates of aeration, and was >90% saturation at the time of introduction to respirometers for all fish. The hypoxic treatment was achieved through the addition of nitrogen gas *via* ceramic diffuser stones. Fish in the hypoxic treatment were subject to a gradual reduction in DO over ~3 h, immediately following the introduction of fish to chambers. Submersible pumps (Eheim, Deizisau, Germany) were used to ensure that DO in the reservoir tank was homogeneous. Oxygen concentration was recorded in each individual respirometer using a Firesting Optical O_2 meter (Pyroscience; Aachen, Germany). Fish were acclimated to respirometers for 22.0 ± 1.1 h, and $\dot{M}O_2$ was recorded every 12 min between flush cycles, resulting in ~ 110 $\dot{M}O_2$ measurements for each individual fish (Figure 52). Mass-standardised $\dot{M}O_2$ measurements were calculated using

LabChart® software (ADI Instruments; Sydney, NSW, Australia) and according to equation 2. The O_2 data during the closed-chamber period of respirometry were assessed visually for linearity throughout the analysis to confirm the reliability of measurements. Background microbial respiration was measured three times following the removal of fish from respirometers. The average background respiration rate was then subtracted from the total respiration rate for each respirometer throughout $\dot{M}O_2$ calculations. All respirometers, including connecting fittings and tubing, were thoroughly cleaned with hot water before the introduction of new fish to avoid microbial accumulation. Following the ~ 22 h recovery period, fish were removed from the respirometers and their mass and length recorded.

Determination of SDA

Fish from each of the HS and HT groups were used for SDA measurements (N = 12 fish per hypoxia phenotype, per DO treatment; N = 48 fish total). Fish were first separated into feeding tanks (N = 4 fish per 100 L tank) 24 h prior to the measurement of $\dot{M}O_2$ for SDA. Each fish was weighed and measured before introduction to the tanks. The biomass of fish in each tank was then used to calculate the feeding ration (2.5% body mass) for each tank. All feeding throughout the experiment was conducted in normoxic conditions (DO > 85% saturation). After ~24 h of settling time in the tanks, feed was distributed slowly and evenly throughout the tank, and feeding behaviour was observed to ensure that all fish received an approximately equal portion of allocated feed. Fish were transferred individually into respirometers 1 h after

feeding. The time between netting and placement in respirometers was <15 s to minimise handling stress on the fish.

DO was >90% saturation at the time of introduction to respirometers for all fish. Fish allocated to the hypoxia treatment were subjected to a gradual reduction in DO to 35% saturation over ~3 h immediately following their introduction to chambers (Figure 55). Fish were left in the respirometers for ~53 h for the collection of $\dot{M}O_2$ measurements during digestion. $\dot{M}O_2$ was recorded every 12 min between flush cycles, resulting in ~ 270 $\dot{M}O_2$ measurements for each individual fish. At the completion of each three-day SDA trial a minimum of half the system volume (i.e. ≥ 500 L) was replaced with new, filtered water before the introduction of new fish.

Determination of O_{2CRIT}

Following the ~53 h digestion period, O_{2CRIT} tests were conducted on each individual to complement *Chapter 4* and further assess the hypothesis that time to LOE and O_{2CRIT} provide synonymous assessments of hypoxia tolerance. To achieve this, respirometers were manually sealed *via* solenoid valves and fish depleted DO in the respirometers to ~ 5% air saturation, after which the flush pump was turned on to restore DO to treatment levels (>90% saturation or ~35% saturation for normoxic and hypoxic treatments, respectively). Fish were removed from respirometers 1 h after the completion of O_{2CRIT} tests. $\dot{M}O_2$ was calculated for each consecutive 100 s period during the decline in DO, and the DO point where $\dot{M}O_2$ began dropping rapidly (i.e., O_{2CRIT}) was calculated as described below.

Data Analysis

All data analyses were performed using R v. 3.2.1 (R Core Team, 2012). Results were considered statistically significant at $P < 0.05$ and all data are presented as mean ± standard deviation unless otherwise stated.

Accounting for handling stress

The SMR for each fish was calculated using the quantile method (Chabot et al., 2016b), with *P* (probability) set to 0.1. A local polynomial regression was fit to the $\dot{M}O_2$ data for unfed, hypoxia intermediate fish ($N = 32$), with α (the parameter that controls the degree of smoothing) fixed at 0.4. The average time taken to return to SMR after introduction to chambers was calculated where the fitted regression dropped within a fixed percentage of the SMR according to equation 7:

$$f(\mathrm{x}) = 0.003x^3 + 0.013x^2 - 0.13x + 0.28$$

Equation 7 - Percentage of the SMR (x), used to determine that SDA was complete for each fish

The use of a third-order polynomial to determine the completion of handling stress was deemed appropriate due to the approximately 3-fold difference between the lowest and highest SMR between individual fish (lowest and highest SMR during the handling stress experiment were 0.92 and 2.79 mg $O_2 \cdot kg^{-1} \cdot min^{-1}$, respectively). The return to SMR took 207 ± 84 min and recovery from handling stress was thus deemed complete at approximately 300 min after introduction to chambers, which is consistent with previous

observation for this species (Collins et al., 2016; Rodgers et al., 2016; see Figures 13, 52 of this thesis). There was no significant relationship between the time to completion of handling stress and SMR (r = 0.11, df = 30, *P* = 0.545), and there was no significant difference in either time to completion of handling stress (*t* = -0.126, df = 21.73, *P* = 0.901) or SMR (*t* = -0.839, df = 28.77, *P* = 0.408) between fish held in normoxia or hypoxia.

An exponential decay function was then fitted to the $\dot{M}O_2$ data to describe the return to SMR following handling (Figure 53), according to the equation:

$$N(t) = N_0 \times e^{-\lambda \times t}$$

Equation 8 - Exponential decay function: t = time (min), λ was fixed at 0.135

The $\dot{M}O_2$ corrected for handling stress was similar to the method employed by Green et al. (2006) and was calculated as:

$$\dot{\mathrm{M}}\mathrm{O}_{2\ \mathrm{CORRECTED}}(i) = \dot{\mathrm{M}}\mathrm{O}_{2\ \mathrm{PREDICTED}}(i) - \alpha$$

Equation 9 - $\dot{M}O_2$ corrected: $\dot{M}O_{2\ \mathrm{PREDICTED}}$ = predicted values from *N*(t), α = SMR

This same approach to correct for handling stress was applied to the fed fish, where an exponential decay function was fit to the first 300 min of $\dot{M}O_2$ data, and α was calculated using the quantile method from the first 300 min of $\dot{M}O_2$ data from that individual.

SDA calculations

SDA calculations were similar to the additive quantile regression smoothing (rqss) method used by Chabot et al. (2016a), however I chose to fit local polynomial regressions (loess curves with α fixed at 0.4) to the $\dot{M}O_2$ data as these provided a visually better fit (Figures 23 and 54). SDA was deemed complete when the loess curve dropped to within a fixed percentage of the SMR, according to equation 7. The SDA magnitude was calculated for each individual fish by integrating the area under the loess curve (AUC):

$$\text{SDA Magnitude} = (\text{AUC}_1 + \text{AUC}_2 - \text{AUC}_{\text{SMR}}) \times 13.86 \times 0.001$$

Equation 10 - SDA Magnitude: AUC_1, AUC_2 and AUC_3 are defined in equations 11, 12 and 13. 13.86 = the conversion factor from mg O_2 to joules (Gessman and Nagy, 1988).

$$\text{AUC}_1 = \int_{\text{SDA}_{\text{START}}}^{\text{SDA}_{\text{END}}} \text{L}\, dt$$

Equation 11 - Area under the loess curve (L): SDA_{END} and SDA_{START} = the last and first predicted values of $\dot{M}O_2$ along the loess curve

$$\text{AUC}_2 = \int_{0}^{\text{SDA}_{\text{START}}} \text{LM}\, dt$$

Equation 12 - Area under the linear model (LM): SDA_{START} = the first predicted value of $\dot{M}O_2$ along the loess curve

$$\text{AUC}_{\text{SMR}} = \int_{0}^{\text{SDA}_{\text{END}}} \text{SMR}\, dt$$

Equation 13 - Area under the SMR curve: SDA_{END} = the last predicted value of $\dot{M}O_2$ along the loess curve

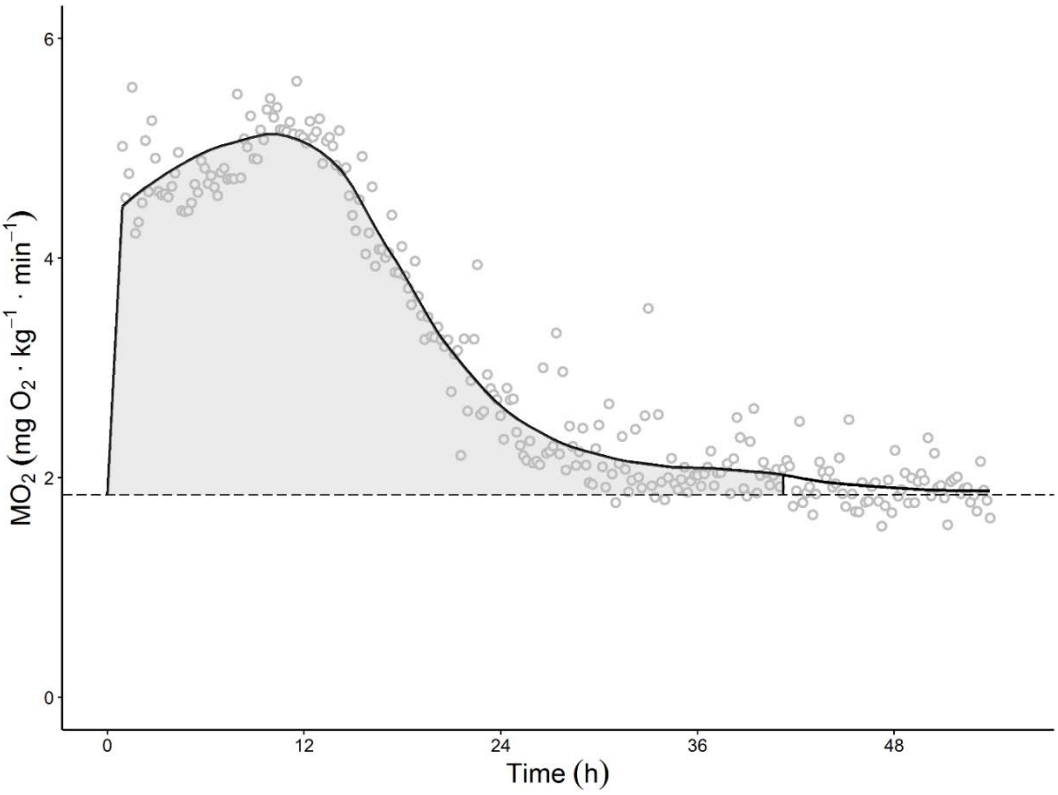

Figure 23 - Oxygen consumption rate ($\dot{M}O_2$) as a function of time (h) for a representative individual. A local polynomial regression was fitted to the $\dot{M}O_2$ data for each fish. The horizontal (dashed) line indicates the standard metabolic rate. The shaded area beneath the curve and above the SMR indicates the SDA response. The return to SMR after feeding is indicated by the solid, vertical line at ~42 h.

The duration of the peak period during the SDA process was determined from binned $\dot{M}O_2$, with bin widths set to 1 h (5 measurements per h). The completion of the SDA peak was calculated when $\dot{M}O_2$ fell below 0.8 × factorial scope, where factorial scope was calculated for each individual as $\dot{M}O_2$ peak / SMR.

The SDA coefficient (SDA_{COEF}) was calculated as:

$$\mathrm{SDA_{COEF}} = \frac{\mathrm{SDA\ Magnitude\ \times Fish\ Mass}}{\mathrm{Feed\ Consumed \times DE}}$$

Equation 14 - SDA Coefficient: SDA Magnitude (kJ · kg^{-1}), fish mass (kg), feed consumed (g), digestible energy (DE: kJ · g^{-1})

O_{2CRIT} calculations

The critical O_2 level (O_{2CRIT}) was calculated using each of three methods: broken stick regression (BSR; Muggeo, 2008; R Core Team, 2012), piecewise regression accompanying SMR (LSR_{SMR}; Claireaux and Chabot, 2016) and non-linear regression (NLR; Weibull function) as per Marshall et al. (2013), where the O_{2CRIT} is calculated from the first derivative of standardised $\dot{M}O_2$ measurements: $f'(x) = 0.029$. All O_{2CRIT} results presented are from non-linear regression, unless otherwise stated. Further details of O_{2CRIT} calculations can be found in *Appendix A*.

Statistical comparisons

Experiments were conducted using a 2 × 2 factorial design, with hypoxia phenotype (hypoxia sensitive or hypoxia tolerant) and DO treatment (normoxia or hypoxia) as the two main factors. Comparisons of means between groups were conducted using mixed model ANOVA (Pinheiro et al., 2014), with hypoxia phenotype and DO treatment included as fixed effects and respirometer number included as a random effect in the model. Normality of data was assessed by a visual examination of a normal Q-Q

plot, frequency histogram and Shapiro-Wilk test. Homogeneity of variance was assessed using Bartlett's test. Multiple comparisons of means were performed using Tukey's Honest Significant Difference test.

Results

SDA

There were no significant differences between hypoxia tolerance phenotypes for any of the parameters measured, and there were no significant interactions between hypoxia phenotype and DO treatment. Fish mass was similar for hypoxia phenotype ($F_{1, 45}$ = 0.058, P = 0.811) and DO treatment ($F_{1, 45}$ = 1.776, P = 0.189). Feed consumption as a percentage of body mass was similar for hypoxia phenotype ($F_{1, 45}$ = 0.199, P = 0.658) and DO treatment ($F_{1, 45}$ = 0.01, P = 0.969). Gross feed consumption was marginally higher for the normoxia treatment (0.94 ± 0.06 and 0.91 ± 0.02 g for the normoxia and hypoxia treatments, respectively; $F_{1, 44}$ = 5.955, P = 0.019), likely driven by small yet non-significant differences in body-mass (39.2 ± 4.6 and 37.6 ± 3.5 g for the normoxia and hypoxia treatments, respectively).

There was no significant effect of DO treatment on SDA duration ($F_{1, 37}$ = 0.159, P = 0.692; mean = 38.9 ± 6.3 h) or SDA magnitude ($F_{1, 37}$ = 3.314, P = 0.077; mean = 46.3 ± 11.2 kJ · kg^{-1}), however the SDA coefficient was marginally, yet significantly lower in the hypoxia treatment ($F_{1, 37}$ = 4.478, P = 0.041; SDA_{COEF} = 14.7 ± 3.3% and 13.0 ± 2.9% for normoxia and hypoxia, respectively), (Figures 24, 25). There was a strong, positive relationship

between SDA magnitude and SDA_{COEF} in fish, irrespective of DO treatment or hypoxia phenotype (Figure 56). Time to completion of the $\dot{M}O_2$ peak was not affected by DO treatment and averaged 16.2 ± 3.4 h. $\dot{M}O_2$ peak and standard metabolic rates were not different between DO treatments and averaged 4.25 ± 0.92 and 1.60 ± 0.42 mg $O_2 \cdot kg^{-1} \cdot min^{-1}$, respectively. Factorial scope in $\dot{M}O_2$ during digestion was 2.71 ± 0.29. There was no difference in SMR between unfed fish from the handling stress trial and those that had recently completed the SDA response ($F_{1, 64}$ = 0.548, P = 0.462), indicating that digestion was complete after ~53 h.

O_{2CRIT}

The O_{2CRIT} was significantly lower in the hypoxia treatment (11.49 ± 0.90 % saturation), compared with the normoxia treatment (12.39 ± 1.33 % saturation), ($F_{1, 37}$ = 7.146, P = 0.011; Figure 26). This may have resulted because the time from closing the chambers to achieving O_{2CRIT} was significantly shorter in hypoxia (31 ± 5 min) compared with normoxia (85 ± 12 min) due to the hypoxia treatment starting at a DO of ~35% rather than >90% saturation ($F_{1, 46}$ = 424.93, P < 0.0001). There was a marginal, yet significant difference in the mean O_{2CRIT} calculated using the different methods (both DO treatment groups combined), with NLR (11.94 ± 1.21 % saturation) producing marginally higher O_{2CRIT} than BSR (11.05 ± 1.34 % saturation; $F_{2, 141}$ = 5.050, P = 0.008). There was no difference between LSR_{SMR} and either BSR or NLR (11.64 ± 1.62 % saturation). Further details of O_{2CRIT} comparisons can be found in *Appendix A* (see Figure 31).

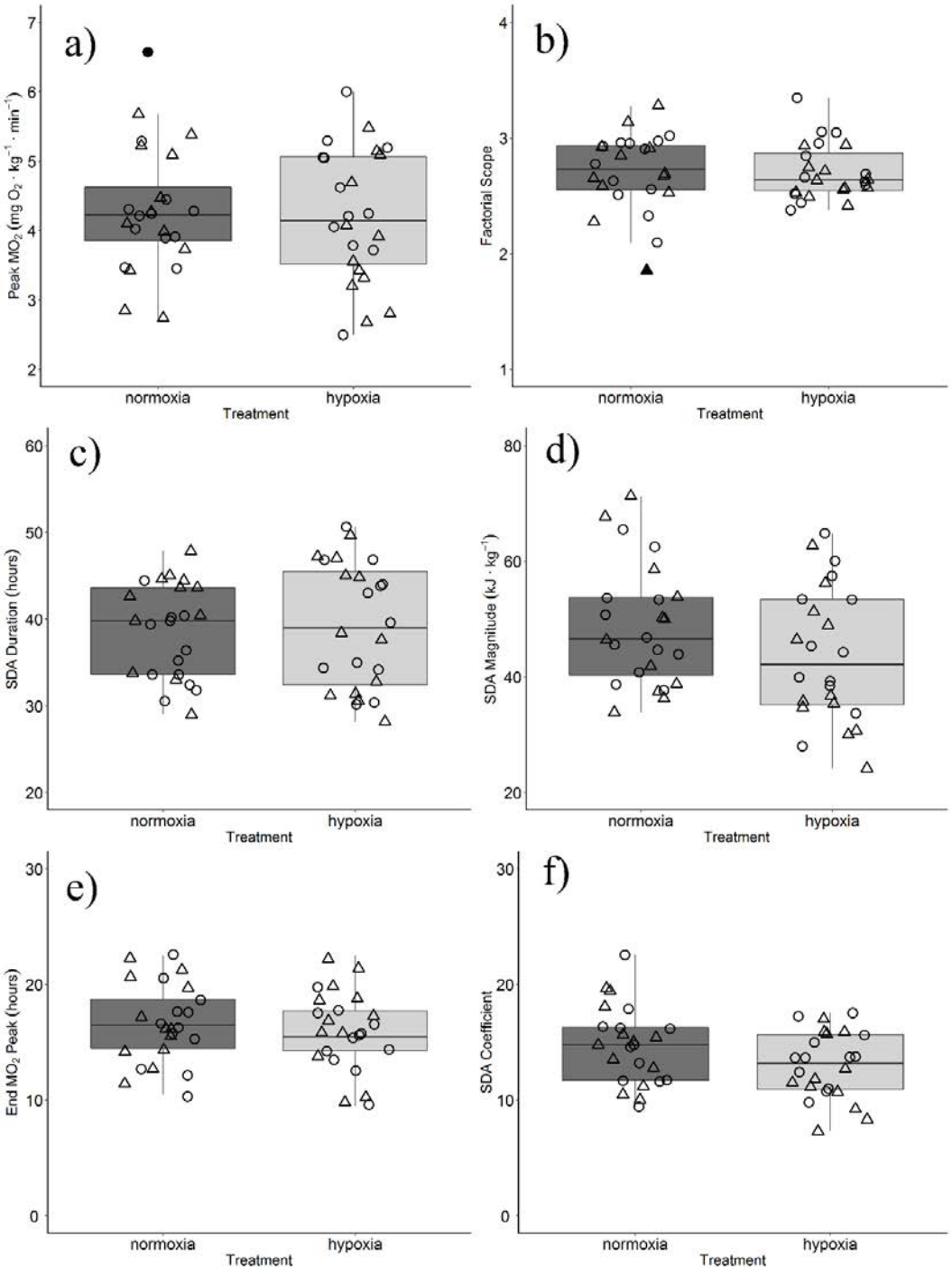

Figure 24 - Boxplots with raw data overlayed for $\dot{M}O_2$ peak (panel a), factorial scope (panel b), SDA duration (panel c), SDA magnitude (panel d), end of the $\dot{M}O_2$ peak (panel e) and SDA coefficient (panel f). Dark and light grey boxes indicate fish maintained in the normoxia and hypoxia treatments, respectively. Open circles and triangles represent hypoxia-sensitive and –tolerant fish, respectively. Outliers are presented as either filled (black) circles or triangles for –sensitive or –tolerant fish, respectively.

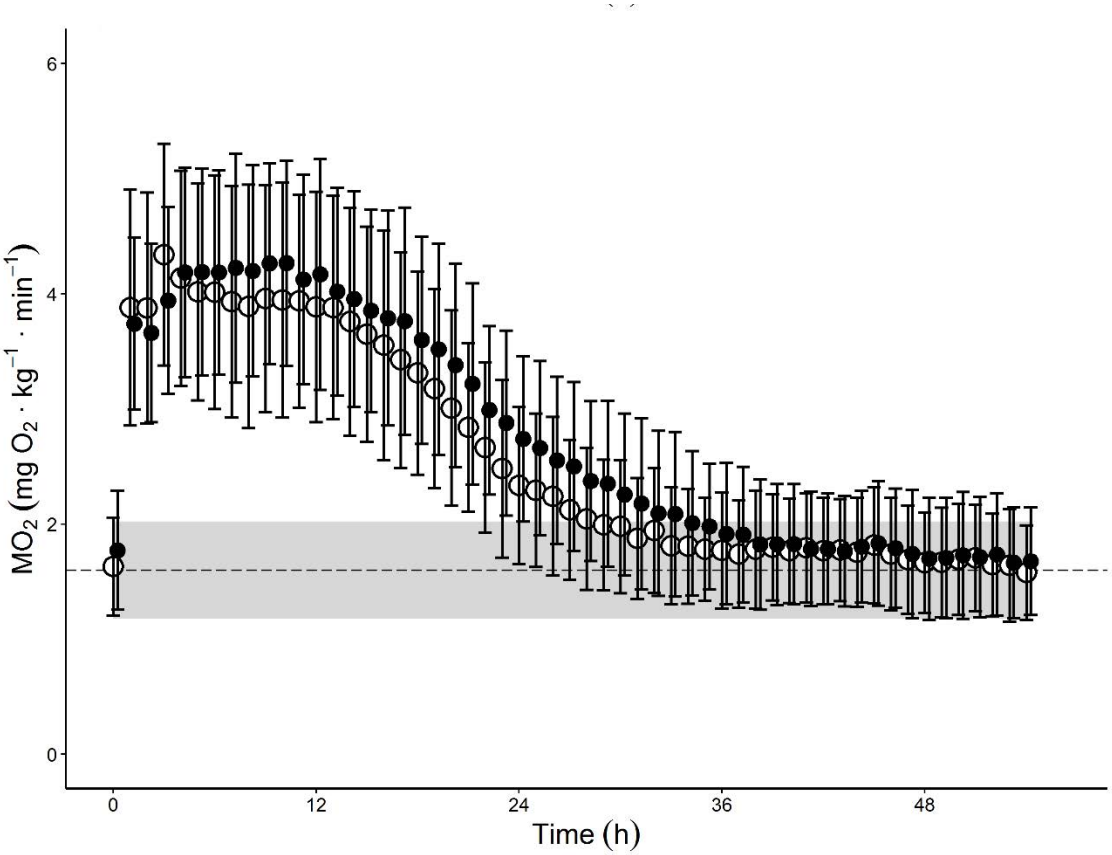

Figure 25 - Oxygen consumption rate ($\dot{M}O_2$) as a function of time (h) for all fish used in this study. The horizontal (dashed) line and shaded area indicates the mean standard metabolic rate ± standard deviation for all fish. Each point indicates the mean of 24 fish for each hour of data collected, and error bars indicate ± standard deviation. The open and closed circles at time = 0 indicate the SMR calculated for unfed fish prior to SDA trials (i.e. those fish used for the determination of handling stress). Open and black circles indicate fish maintained in the hypoxia and normoxia treatments, respectively.

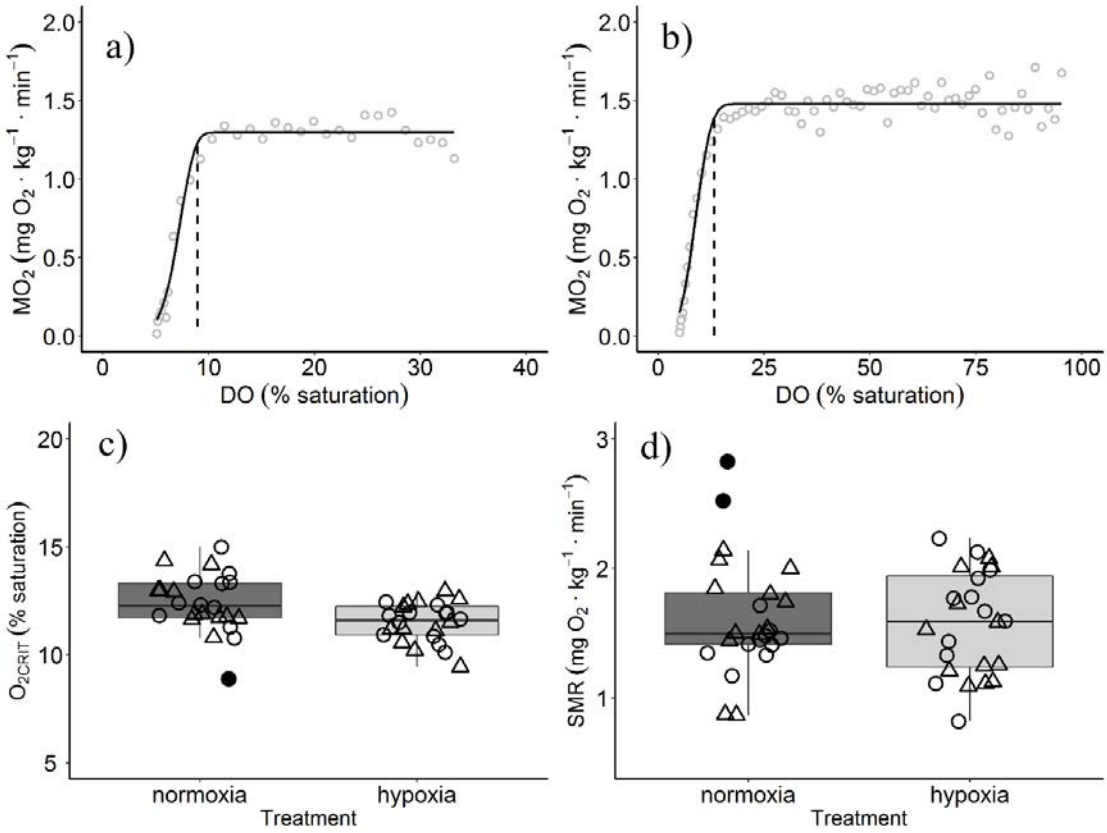

Figure 26 - Oxygen consumption rate ($\dot{M}O_2$) plotted against dissolved O_2 (% saturation) for one fish maintained in the hypoxia treatment (panel a) and one fish maintained in the normoxia treatment (panel b). A Weibull function was fitted to the $\dot{M}O_2$ data for each fish (panels a and b), and the critical O_2 level is indicated as the dashed, vertical line. Boxplots with raw data overlayed (panels c and d) for the O_{2CRIT} and SMR, respectively. Dark and light grey boxes indicate fish maintained in the normoxia and hypoxia treatments, respectively. Open circles and triangles represent hypoxia-sensitive and –tolerant fish, respectively. Outliers are presented as either filled (black) circles or triangles for –sensitive or –tolerant fish, respectively.

Discussion

Barramundi exhibit metabolic responses to digestion typical of most fish species, with a characteristic elevation of metabolic rate after feeding, followed by a steady decline back to resting levels. All SDA parameters measured were within ranges expected, based on studies with other teleost fish (Jobling, 1981; Jordan and Steffensen, 2007; Lefevre et al., 2012; Wang et al., 2012). Aside from a small difference in SDA_{COEF} between DO treatments, exposure to chronic, moderate hypoxia did not substantially affect SDA responses in resting, juvenile barramundi. These results indicate that, in spite of a likely reduction in aerobic scope under hypoxic conditions (Chabot and Claireaux, 2008; Norin et al., 2016), barramundi are capable of maintaining maximal digestive capacity even when DO drops to 35% saturation. Such responses to digestion are broadly consistent with those observed for southern catfish (*Silurus meridionalis*) and common sole (*Solea solea*), where SDA responses are unaffected at DO >48% and >30% saturation, respectively (Chabot and Claireaux, 2008; Zhang et al., 2010). More severe hypoxia, as commonly encountered by barramundi and other tropical species in the natural environment (Butler and Burrows, 2007), may lead to increases in each of SDA magnitude, duration and coefficient, as observed for Greenland halibut (Dupont-Prinet et al., 2013; 21% saturation), Atlantic cod (Jordan and Steffensen, 2007; 30% saturation) and southern catfish (Zhang et al., 2010; 24% saturation). Acclimation to diel-cycling or chronic DO may further influence SDA responses (Dan et al., 2014), however the magnitude of any potential improvements await elucidation through further research.

At an individual level, SDA_{COEF} correlated strongly with SDA magnitude, irrespective of hypoxia phenotype or DO treatment (Figure 56e). The lower SDA_{COEF} during hypoxia indicates that digestion may be more efficient under hypoxic conditions, or perhaps more likely, that more nutrients pass through the digestive tract without being assimilated (i.e. a decrease in digestive efficacy or efficiency). Studies with Atlantic cod and silver bream indicate that feed conversion efficiency (FCE) may decline with decreasing DO to ≈ 45% and 24% saturation, respectively (Chabot and Dutil, 1999; Wang et al., 2009). Sea-bass, turbot and spotted wolf-fish, however, all display no change to FCE compared with normoxia at 40%, 45% and 37% saturation, respectively (Foss et al., 2002; Pichavant et al., 2001), reflecting a degree of inter-specific variability that may potentially be attributed to differences in lifestyle and environment. Much of the changes to digestion and assimilation under hypoxia are attributed to reduced feed intake (Brett and Groves, 1979; Wang et al., 2009), however, all feeding was conducted in normoxia in this study and thus feed intake was similar between DO treatments.

The lack of differences between hypoxia tolerance phenotypes indicates that the capacity to tolerate near-lethal hypoxia (time to LOE) is not related to metabolic responses for this species, either during digestion (SDA responses) or under hypoxic stress for metabolic regulation (O_{2CRIT}). These findings are broadly consistent with previous research on this species (*Chapter 4*), in which group-level hypoxia tolerance phenotypes displayed no differences in growth performance (in normoxia) or O_{2CRIT}. Thus, while hypoxia tolerance phenotypes may differ in their capacity for anaerobic metabolism, aerobic capacity (e.g. when O_2 is ≥ O_{2CRIT}) appears to be

homogeneous between phenotypes. Further research that directly assesses aerobic scope under O_2 limitation (Jordan and Steffensen, 2007), capacity for metabolic suppression (Hochachka, 1990) and alterations to biochemical pathways (Wade et al., 2014) across hypoxia tolerance phenotypes will assist to further elucidate the functional basis of trait variability in this and other fish species.

Under natural conditions, barramundi and other fish likely encounter energetic demands that elevate metabolism above resting levels during digestion, including horizontal migration, predator avoidance and prey-capture (Brett and Groves, 1979). While the results from this study demonstrate no effects of hypoxia on digestion in resting barramundi, the combined effects of exercise and hypoxia were not explored. Studies on several temperate fish species have demonstrated that reductions to aerobic scope under hypoxic conditions during digestion (Chabot and Claireaux, 2008; Jordan and Steffensen, 2007) are related to a deference of SDA responses under exercise (Dupont-Prinet et al., 2009; Jourdan-Pineau et al., 2010). Such responses may lead to a longer SDA duration through longer gastrointestinal feed retention, and altered gastrointestinal blood flow (Dupont-Prinet et al., 2009; Eliason and Farrell, 2014). Further alterations to SDA responses, such as increased SDA duration through elevated CO_2 (Tirsgaard et al., 2015) or changes to the SDA magnitude through elevated temperature (Guinea and Fernández, 1991; Guinea and Fernández, 1997; McLeod and Clark, 2016) may further affect fish populations as atmospheric CO_2 continues to increase and the climate continues to warm (Aust Gov - BoM, 2015).

Conclusions

This study provides the first assessment of respiratory metabolic responses during digestion for barramundi. The results presented demonstrate that SDA is largely unaffected in barramundi when DO declines to ~35% saturation. Hypoxic conditions are common both in the natural environment and in aquaculture and the results presented in this chapter highlight the resilience of barramundi to bouts of environmental hypoxia. While this study shows negligible effects of moderate hypoxia on SDA responses, future studies should examine sub-lethal effects of hypoxia on factors like growth rate and feed conversion efficiency to determine whether they may be responsible for the slight reduction in SDA_{COEF} documented here.

CHAPTER 6. GENERAL DISCUSSION

At the inception of this thesis, there was very little empirical information on the capacity for tropical fish populations to tolerate hypoxia across broad latitudinal gradients. Further, the extent of intra-specific variability in hypoxia tolerance and the biological consequences of this variability had received virtually no research attention. The work in this thesis utilised a range of techniques (experimental and analytical) to assess the effects of environmental hypoxia on population-level and individual-level metabolic responses in juvenile barramundi. The biological consequences of variability in hypoxia tolerance were assessed through a range of performance measures, including haematology, growth, metabolic regulation and digestion of populations and hypoxia tolerance phenotypes. This research has addressed several critical knowledge gaps and provides a solid foundation for future research into the functional basis of hypoxia tolerance in fish.

The discussion below addresses the implications of results presented in the preceding four data chapters. The selection of ecologically relevant dissolved O_2 treatments is discussed first, followed by a brief discussion of population-level responses to hypoxia in tropical fish. The ecological consequences of intra-specific variability in hypoxia tolerance are then discussed, followed by a brief analysis of the relationship between O_2 consumption rates and dissolved O_2 in fish. Opportunities for future research are presented, along with some concluding remarks about the results presented in this thesis.

Dissolved O_2 treatments

Dissolved O_2 is highly dynamic across spatial and temporal scales in tropical systems (Figure 1) and one of the challenges in this thesis was to devise hypoxic treatments that approximate conditions encountered by barramundi, both in the natural environment and in aquaculture. The daily variability in O_2 used in *Chapter 3* is common in environments that barramundi inhabit across tropical Australia (Loong et al., 2005). The acute hypoxia treatment used during time to LOE tests in *Chapter 4* is likely similar to conditions that result in fish kills (Townsend and Edwards, 2003), as >95% of fish had lost equilibrium (a response that closely precedes mortality) at the end of these tests. The more moderate, chronic hypoxia treatment in *Chapter 5* is routinely encountered on commercial fish farms with no mortality (Hayes, T., personal comm.) and also in the natural environment (Loong et al., 2005). Responses by fish to hypoxia vary depending on the duration and intensity of the hypoxic event (Borowiec et al., 2015; Dan et al., 2014), and while it is impractical to assess every environmental scenario the hypoxic treatments used in this thesis (acute, chronic and diel-cycling hypoxia) encompass the range of conditions encountered by this species, both in its natural environment and in aquaculture.

Population-level responses to hypoxia

One of the primary objectives of this thesis was to investigate population-level hypoxia tolerance in barramundi. No conclusive evidence for population differences in hypoxia tolerance was found in any of *Chapters 2*, *3* and *4* for either O_{2CRIT}, or time to LOE. All populations examined in this thesis appear similarly able to regulate $\dot{M}O_2$ at high temperatures. Further, sub-tropical and tropical populations appear equally able to acclimate to hypoxia following repeated, daily exposure, albeit with some observed differences in haematological parameters. These results strongly indicate that whole-animal hypoxia tolerance is conserved across the distribution of this species in Australia, however the limitations in inferring adaptive responses across the species' distribution on the basis of two-population studies must be acknowledged (Garland and Adolph, 1994). Incorporation of three or morw populations with greater genetic divergence and from lower latitudes (i.e. from Indonesia, Vietnam and Malaysia) in future studies would assist in further resolving whether population of origin plays any significant role in underpinning hypoxia tolerance above and beyond short-term phenotypic plasticity.

Global temperatures have been increasing recently, and projected temperature increases of 1.3 to 2.6°C are predicted across tropical northern Australia by 2100 under an intermediate emissions scenario (Aust Gov - BoM, 2015). The relationship between temperature and dissolved O_2 in water, together with high metabolic demands at elevated temperatures (Figure 4) was expected to lead to a reduction of hypoxia tolerance, and thus

impaired performance of fish under warmer conditions, irrespective of population. It was hypothesised in *Chapter 2* that fish which routinely experience warmer temperatures (i.e. those from low latitudes) may be better adapted to the combined challenges of elevated temperature and lower O_2. A general reduction in hypoxia tolerance was observed at 36°C, however O_{2CRIT} tests conducted in warmer conditions took considerably less time than at cooler temperatures because of a more rapid rate of O_2 utilisation in the respirometers. It is therefore difficult to separate the effects of temperature on O_{2CRIT} from the speed of the decline in O_2 within chambers (see Figure 36b). Regardless of this caveat, the magnitude of the reduction in O_{2CRIT} did not differ substantially between the populations examined. These results provide further evidence that local adaptation for hypoxia tolerance is unlikely for this species.

The similar capacity for hypoxia acclimation between the populations in *Chapter 3* demonstrates that physiological plasticity is an important strategy for this species' response to environmental hypoxia. In *Chapter 3* it was hypothesised that fish from lower latitudes may experience greater daily fluctuations in O_2 due to consistently higher temperatures, and that those populations may therefore display greater physiological plasticity. No such differences between the populations were found, potentially due to the highly variable nature of O_2 in the environment across the distribution of this species. Reversible, plastic responses to hypoxia are not atypical for tropical fish species that frequently encounter environmental hypoxia (Almeida-Val et al., 2005; Chapman et al., 1999) and the lack of population differences

observed in *Chapter 3* demonstrates that physiological plasticity is an important strategy for coping with environmental hypoxia.

Intra-specific diversity in hypoxia tolerance

Almost 30 years ago, Albert F. Bennett (1987) wrote about the “tyranny of the golden mean” and of the classical experimental approaches employed by researchers in ecological physiology, including those used in *Chapters 2* and *3* of this thesis. Bennett (1987) posits that there is a great diversity of information contained within populations that is often disregarded by researchers in their search for the average response of a performance trait. The two main questions that may be addressed by assessing variability in performance within populations are: 1, what is the functional basis of trait (performance) variability, and 2, what are ecological or biological consequences of trait variability (Bennett, 1987). The latter of these two questions was addressed in *Chapters 4* and *5* through the comparison of performance traits in individuals or groups of individuals within populations (Joyce et al., 2016; Ozolina et al., 2016; Roze et al., 2013), after finding little evidence for differences in hypoxia tolerance between populations in *Chapters 2* and *3*.

The separation of fish into groups based on their tolerance to hypoxia and subsequent performance tests revealed little difference in metabolism or growth performance between hypoxia tolerance phenotypes. These results indicate that performance in normoxia may not be related to hypoxia tolerance. Further, metabolic responses to digestion were largely unaffected

between phenotypes under either normoxic or hypoxic conditions. At present there is very little information on the underlying drivers of individual-level variability in hypoxia tolerance within fish populations. Previous research on southern catfish (*Silurus meridionalis*) has demonstrated that, at the population-level, growth rates decline following daily exposure to diel-cycling hypoxia, along with a concomitant improvement in hypoxia tolerance (Yang et al., 2013). It is unclear, however, from studies such as that conducted by Yang et al., (2013) whether those individuals that displayed the greatest improvement in hypoxia tolerance elicit any change from the average response in growth. Complementary assessments of aerobic scope and growth parameters across a range of dissolved O_2 conditions with a focus on individual-level responses would assist in shedding further light on the performance of hypoxia tolerance phenotypes, and hence the ecological consequences of variability in hypoxia tolerance.

The large sample sizes used in *Chapter 4* provided a unique opportunity to undertake some detailed analyses of performance traits. The lack of group (hypoxia category) differences in O_{2CRIT} observed in this thesis (*Chapter 4*), together with the absence of any relationship between O_{2CRIT} and time to LOE provide strong evidence that these two measures of hypoxia tolerance are not correlated. These findings are somewhat surprising, as the implication is often made that fish with a higher O_{2CRIT} are more sensitive to environmental hypoxia, will require a greater reliance on anaerobic metabolism during acute hypoxia and will therefore be at greater risk of losing equilibrium faster (Claireaux and Chabot, 2016). Stated another way, fish that take longer to lose equilibrium would be expected to have a lower

O_{2CRIT}. As mentioned in the discussion section of *Chapter 4*, these results contribute to a growing body of literature which indicates that time to LOE and O_{2CRIT} measures of hypoxia tolerance are unrelated across a broad range of fish species.

The temporal repeatability of hypoxia tolerance has received very little attention in contemporary ecological physiology. It may be reasonable to expect some traits to demonstrate changes over time that may be associated with ontogeny (Almeida-Val et al., 2000) or seasonal differences (Arend et al., 2011; Love and Rees, 2002). Nevertheless, over shorter temporal scales (weeks to months) and with small overall changes in body-mass, metabolic traits are expected to be temporally repeatable in individuals or groups of individuals (Maciak and Konarzewski, 2010; Marras et al., 2010; Norin and Malte, 2011; Norin et al., 2016). The results presented in *Chapter 4* demonstrate that hypoxia tolerance is loosely repeatable at a group level over ~100 d. Similar responses have also been observed for European Sea-Bass, in which hypoxia tolerance was broadly repeatable over an 18 month period (Joyce et al., 2016), however there is considerable scope for future research investigating the repeatability of hypoxia tolerance measures. One caveat of conducting repeated measures on individuals is that experiments used to assess hypoxia tolerance (HCTs) may be inherently biased: that is, individuals that are more tolerant of hypoxia will also, by default, receive a greater duration of hypoxia exposure than more sensitive fish during HCTs. Care must therefore be taken to ensure that sub-lethal effects are minimised through adequate time periods between measurements, combined with behavioural observations of individuals throughout such studies.

Aerobic scope and hypoxia

Aerobic scope was not directly assessed in this thesis, however, some indirect comparisons with other studies involving barramundi are possible. Reductions in aerobic scope (10 – 50%) are observed during moderate hypoxia (45% saturation, 29°C, 35 ppt; Norin et al., 2016), however digestive responses remain unaffected (35% saturation, 30°C, 0 ppt; present study), with factorial scope (as measured during digestion) ≈ 2.7. Norin et al. (2014) estimate that factorial scope (as measured during exercise) is ≈ 4 (29°C, 35 ppt). At the critical O_2 level (~12 - 18% saturation at 30°C, 0 ppt), aerobic scope is predicted to be equal to zero (Claireaux and Chabot, 2016). It can thus be speculated that a rapid decline in aerobic capacity occurs between ~15% and ~35% saturation (Figure 27), and that activity becomes constrained by aerobic scope at these O_2 levels. A conceptual model for the relationship between the maximum metabolic rate (MMR; Norin and Clark, 2016) and DO for barramundi is presented here (Figure 27), however, validation of this model with empirical data requires further research.

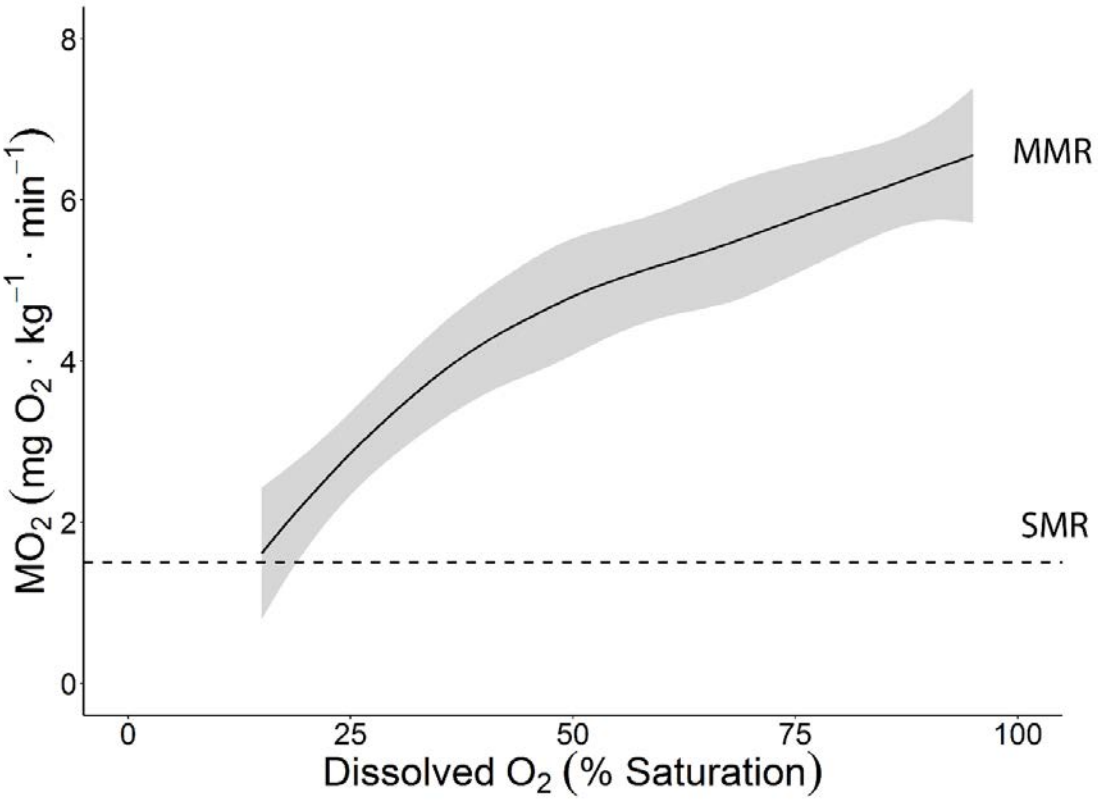

Figure 27 - Conceptual model of the relationship between maximum metabolic rate (MMR) and dissolved O_2 for barramundi at 30°C, adapted from Jordan and Steffensen (2007), with data specific for barramundi from this thesis, as well as (Norin et al., 2014; Norin et al., 2016).

Relationship between $\dot{M}O_2$ and DO

Further to the broad assessments of hypoxia tolerance measures and the repeatability of hypoxia tolerance tests in *Chapter 4*, the large sample sizes used provided an opportunity to re-assess the relationship between $\dot{M}O_2$ and DO. While such analyses are hardly new (Hudson, 1966; Mueller and Seymour, 2011; Nickerson et al., 1989; Tang, 1933; Yeager and Ultsch, 1989), this analysis is particularly timely as there is no single method used by researchers to estimate the O_{2CRIT} of fish species (Rogers et al., 2016). Despite two excellent recent reviews on the subject (Claireaux and Chabot,

2016; Rogers et al., 2016) and much commentary, there has been no detailed analysis of this relationship conducted using a large, empirical data set from a single species or study (to the best of my knowledge). The analysis presented in *Appendix A* demonstrates that non-linear regression provides an overall better fit to $\dot{M}O_2$ data than the more favoured broken-stick regression methods for the large data set from *Chapter 4*, as predicted by Marshall et al. (2013). Surprisingly, the opposite was true for $\dot{M}O_2$ data from *Chapter 5* (Table 4).

The method presented by Claireaux and Chabot (2016) (least-squares regression accompanying SMR (LSR_{SMR}), used in *Chapter 2* of this thesis) is based on the metabolic theory presented in Fry (1971), but does not lend itself to model comparison, as the SMR portion of the curve is estimated using a quantile or other method (Chabot et al., 2016b) rather than least squares or log likelihood (Muggeo, 2008). Nevertheless O_{2CRIT} estimates obtained from LSR_{SMR} should be similar to those from broken-stick regression (Yeager and Ultsch, 1989; used in Chapter 3), and this was indeed observed in data from *Chapters 4* and *5* (Figures 28, 31; Table 4).

Claireaux and Chabot (2016) argue that traditional methods ignore a large amount of information collected before the O_{2CRIT} test (i.e. the $\dot{M}O_2$ data used to estimate SMR), yet the counter argument is equally strong, perhaps even more so: that all information above O_{2CRIT} (between ~20% and ~85% saturation) is virtually disregarded in LSR_{SMR}. Accordingly, LSR_{SMR} enables an estimation of the point of transition (O_{2CRIT}), but makes no attempt to describe the relationship between $\dot{M}O_2$ and DO. Given recent advances in

computing power and the relative simplicity with which non-linear regression may be fit to physiological data, it would seem pertinent for future studies to adopt a similar approach to that used in *Chapters 4* and *5* of this thesis (and similar to the analysis presented by Stoffels, 2015), where multiple models are fitted to the data and compared. The results presented in this thesis strongly indicate that NLR provides the best fit to describe the relationship between $\dot{M}O_2$ and DO. However, from such conclusions arise further questions, such as what is the relevance of a critical point along a continuous function? Further analytical methods such as the 'regulation index' proposed by Mueller and Seymour (2011) have the potential to provide further insight into the capacity for fish to regulate metabolism during hypoxia, outside of the more traditional paradigm.

Conclusions and opportunities for future research

Increasing global temperatures will likely exacerbate the effects of environmental hypoxia through increasing metabolic rates, and reducing the solubility of O_2 in water (Diaz and Breitburg, 2009). Further, daily fluctuations in dissolved O_2 in natural systems may be accompanied by changes in pH, associated with fluctuations in CO_2 (Butler and Burrows, 2007), which may be further exacerbated by global temperature changes and increasing greenhouse gas emissions (Aust Gov - BoM, 2015). Even without temperature changes, hypoxia is expected to become more frequent and severe in many freshwater and coastal aquatic systems due to increasing nutrient inputs and further eutrophication. There is increasing interest in

assessing the synergistic effects of multiple environmental stressors on performance of fish populations and to examine if such traits are correlated (Burnett, 1997; Cruz-Neto and Steffensen, 1997; McBryan et al., 2013; McKenzie et al., 2003; Melzner et al., 2012; Pörtner and Knust, 2007; Roze et al., 2013). Many of the findings presented in this field are currently lacking in broad consensus. For instance, acclimation to warm conditions may result in improvements to hypoxia tolerance in Atlantic killifish (McBryan et al., 2016) and common sole (Zambonino-Infante et al., 2013). Norin et al. (2016), however, found that thermal tolerance may be inversely related to hypoxia tolerance in barramundi and similarly, Roze et al. (2013) demonstrated an inverse relationship between thermal tolerance and hypoxia tolerance in rainbow trout. Nevertheless, with expected changes to environmental conditions mentioned above and with considerable uncertainty about the capacity for fish to acclimate or adapt to such conditions, investigating the synergistic effects of temperature, hypoxia and CO_2 on performance in fish remains an important area for future research.

The results presented in this thesis focussed primarily on whole-animal metabolic responses to hypoxia, however it is evident from the broader literature and the results presented here that there remains considerable scope to investigate the first question posed by Bennett (1987): what is the functional basis of trait variability? In particular, while broad inter-species comparisons may reveal evolutionary adaptations to hypoxia (Mandic et al., 2009; Richards, 2011), it is becoming increasingly apparent that there is an enormous intra-specific diversity of traits such as hypoxia tolerance that is often not explained under current frameworks. More specifically, the

mechanisms (molecular, physiological, morphological and behavioural) that enable ~10% of individuals to tolerate severe hypoxia for >5 h, while ~10% of individuals can only tolerate hypoxia for <1 h (*Chapter 4*) remain to be elucidated. Further research in this area would benefit from a focus on drivers of inter-individual variability, and in particular on performance differences in metabolic phenotypes (Auer et al., 2015; Metcalfe et al., 2016; Norin et al., 2016)

The repeatability of hypoxia tolerance measures has received very little research attention, and any studies that do exist have focussed on loss of equilibrium measures (Joyce et al., 2016). Given the importance attributed to O_{2CRIT} in the broader literature, it is perhaps surprising that not a single study (to the best of my knowledge) has assessed the temporal repeatability of this measure. Nevertheless, the results from *Chapter 4* (that hypoxia tolerance is only loosely repeatable at a group-level) suggest that further research is required to examine the temporal repeatability of time to LOE measures over longer time scales, and to assess the heritability of hypoxia tolerance.

It would seem prescient to repeat studies (where logistically feasible) to obtain further confidence in results. Recent studies with Atlantic salmon (Anttila et al., 2013) and rainbow trout (Scott et al., 2015), together with findings from the research presented in this thesis, have demonstrated that enormous variability in hypoxia tolerance measures may be found within different family lines, across year-classes of hatchery populations, and between different hatcheries. Such results highlight a distinct problem currently encountered within ecological physiology and more broadly across

many disciplines of science: that replication within studies does not necessarily equal repeatability or reliability of results (Alberts et al., 2015; Baker, 2016; Clark et al., 2016; Halsey et al., 2015; Nosek et al., 2015; Parker et al., 2016). This may be particularly problematic for studies using hatchery populations (where genetic diversity is low), yet hatchery populations of fish contain enormous potential for examining questions of interest in contemporary ecological physiology due to: 1, the capacity to obtain large numbers of individuals at low cost, 2, consistency and reliability of supply and 3, the ability to obtain fish that are accustomed to the presence of people, thereby reducing the potential confounding effects of handling stress on measurements. Regardless of caveats such as these, the results from this thesis provide a solid foundation for future researchers to assess and more fully elucidate the functional basis of hypoxia tolerance in fish populations.

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APPENDIX A - RELATIONSHIP BETWEEN $\dot{M}O_2$ AND DO

During the course of this thesis two key papers emerged (Claireaux and Chabot, 2016; Marshall et al., 2013) that presented new opportunities for the analysis of O_{2CRIT} data. Consequently, the O_{2CRIT} data collected for *Chapters 4* and *5* were analysed using not one but three methods: non-linear regression (NLR; Marshall et al., 2013), broken-stick regression (BSR; Muggeo, 2008; Yeager and Ultsch, 1989) and least-squares regression accompanying SMR (LSR_{SMR}; Claireaux and Chabot, 2016). These methods were then compared (where appropriate) using Akaike's Information Criterion (AIC) as a means of assessing the relationship between $\dot{M}O_2$ and DO. The large sample sizes used in *Chapter 4* presented a unique opportunity to assess the relationship between $\dot{M}O_2$ and dissolved O_2 using a large, empirical data set, in the first such analysis of its kind (to the best of my knowledge).

BSR and LSR_{SMR} were by far the most commonly used methods for analysing O_{2CRIT} data at the time of writing this thesis. NLR has been used very little to assess hypoxia tolerance, perhaps due to the absence of a distinct "break-point" at the intersection of two lines. Both BSR and NLR are fit to the data through minimising the negative log-likelihood (BSR) or the residual sums of squares (NLR), and thus provide a basis for model comparison using AIC. No such method may be applied to LSR_{SMR}, and this technique was therefore excluded from this component of the analysis. Due to slight differences in methodology between the two chapters (*4* and *5*), it

was not considered appropriate to analyse both data sets collectively and they have therefore been kept separate.

NLR provided an overall better fit to $\dot{M}O_2$ data than BSR in *Chapter 4*, and may therefore be a better descriptor of the relationship between $\dot{M}O_2$ and DO (Figure 29; Table 4). This was predicted by Marshall et al. (2013), however the analysis presented here benefited from the use of empirical, rather than theoretical data, and thus was not reliant on assumptions used to generate simulated data sets. Somewhat surprisingly, the reverse was true for data from *Chapter 5*: BSR provided a better fit to $\dot{M}O_2$ data than NLR (Figure 32; Table 4).

Regardless of the method used to describe the relationship between $\dot{M}O_2$ and DO, all O_{2CRIT} estimates were generally within the range expected for this species (~10 – 20% saturation). Parameter estimates for the NLR (Weibull) function are presented here (Table 4), together with goodness-of-fit profiles (Figure 30).

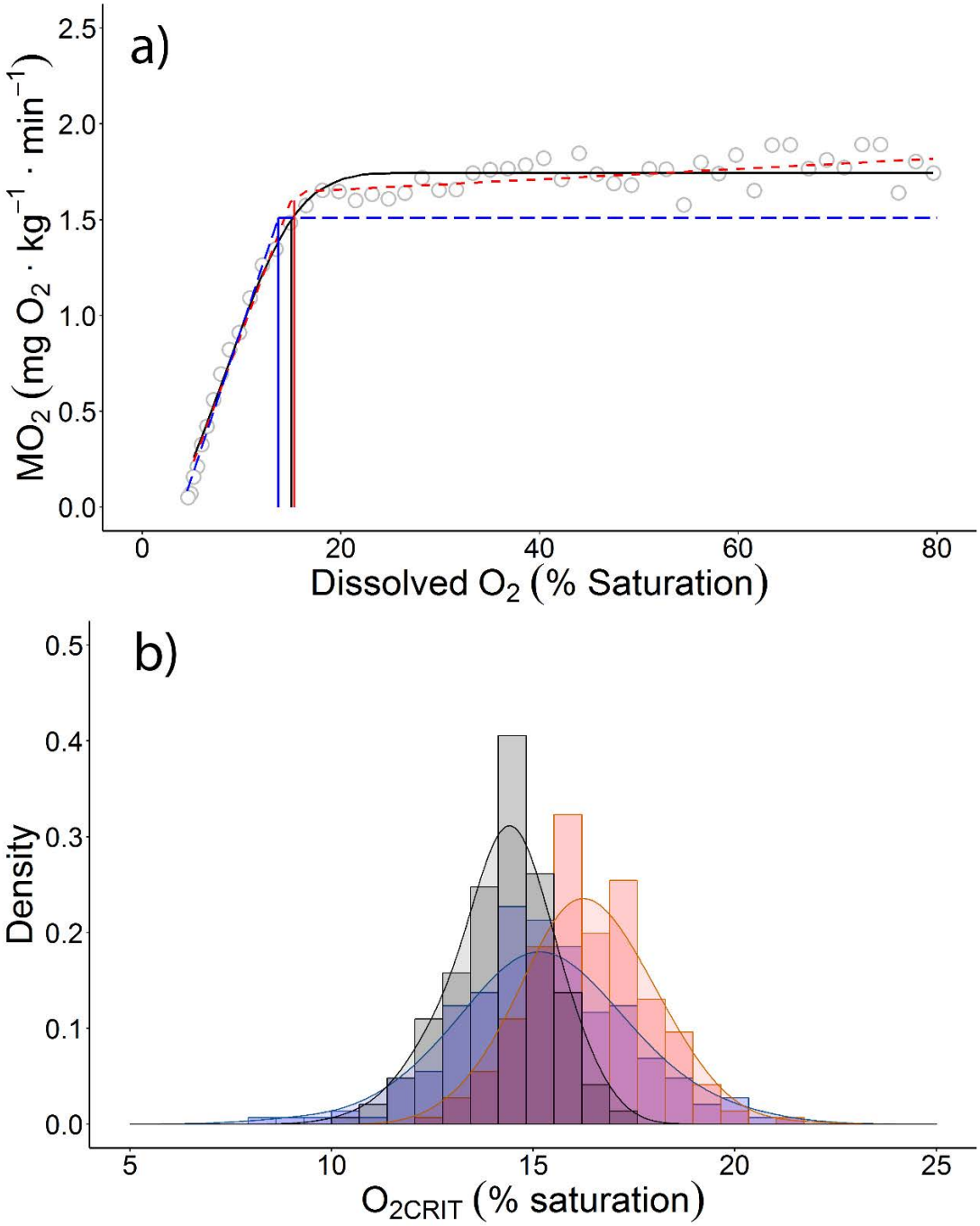

Figure 28 - Relationship between $\dot{M}O_2$ and DO, for one individual barramundi (panel a), with three different models fit to the data: non-linear regression (Weibull function; black line), segmented (broken-stick) regression (red line) and least-squares regression accompanying SMR (blue line). The vertical line for each function indicates the O_{2CRIT} estimate. Panel b is a density histogram with density functions (Gaussian) overlayed that represents the O_{2CRIT} estimated for 211 fish (*Chapter 4*) using three methods: non-linear regression (grey bars, black line), segmented regression (red bars and line) and least-squares regression accompanying SMR (blue bars and line).

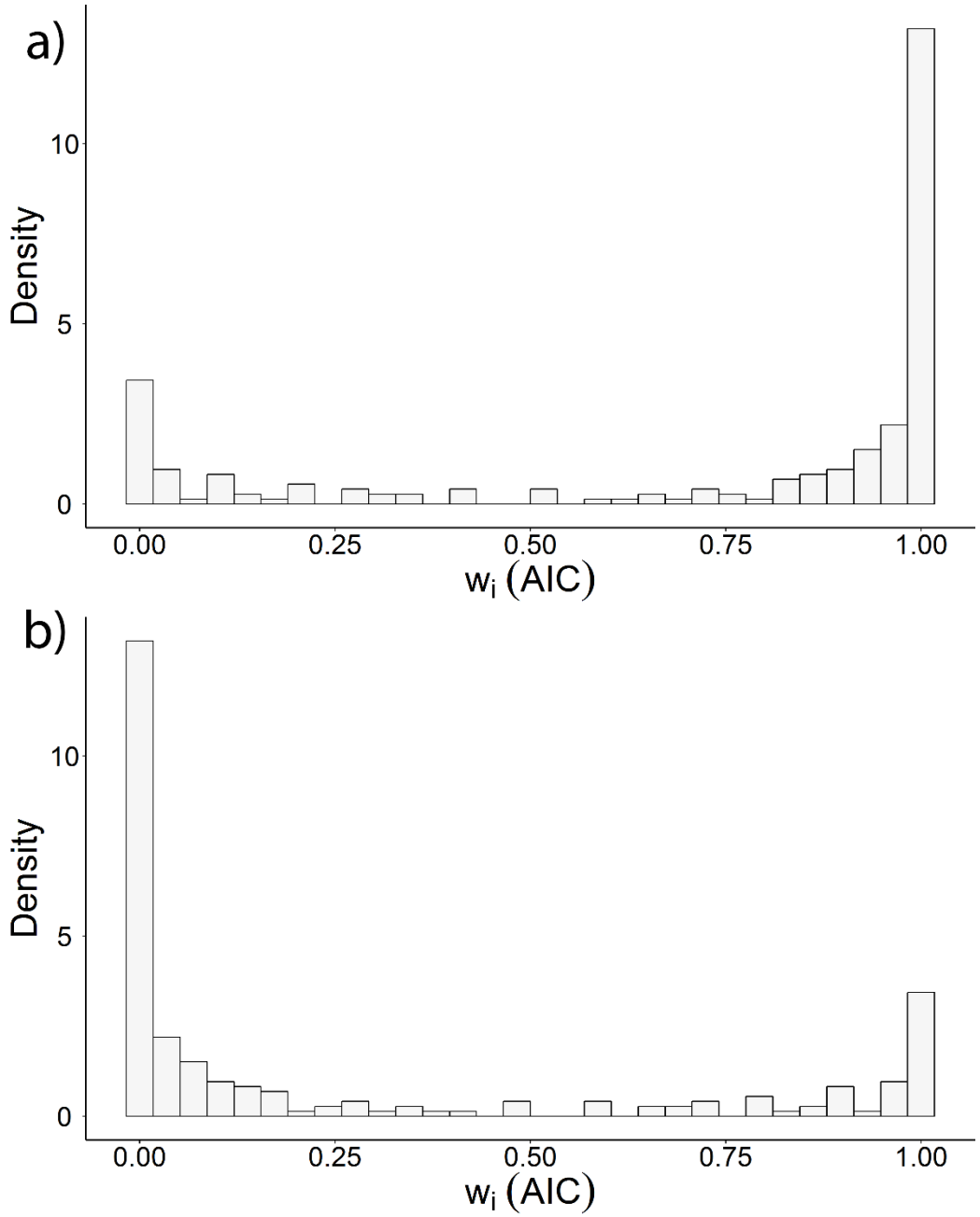

Figure 29 - Density histograms of Akaike weights for non-linear regression (a) and broken-stick regression (b) for 211 fish (*Chapter 4*).

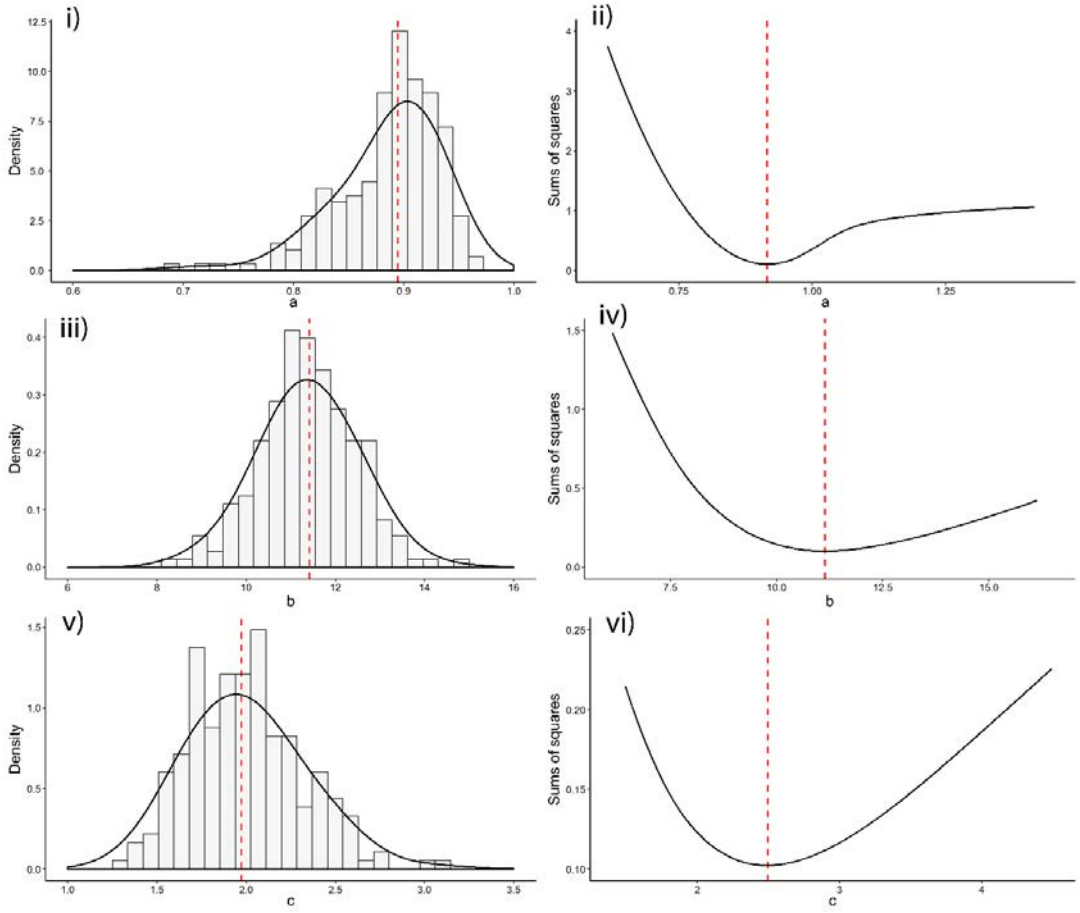

Figure 30 - Parameter estimates for all fish (i, iii and v) and goodness of fit profile plots for parameters from one individual fish (ii, iv and vi) for the Weibull function (*Chapter 4*). Parameters a, b and c represent the asymptote, scale and shape of the Weibull function. The vertical red line on histograms indicates the median value. The vertical red line on goodness of fit plots indicates the minimum of the sums of squares for each parameter for that fish.

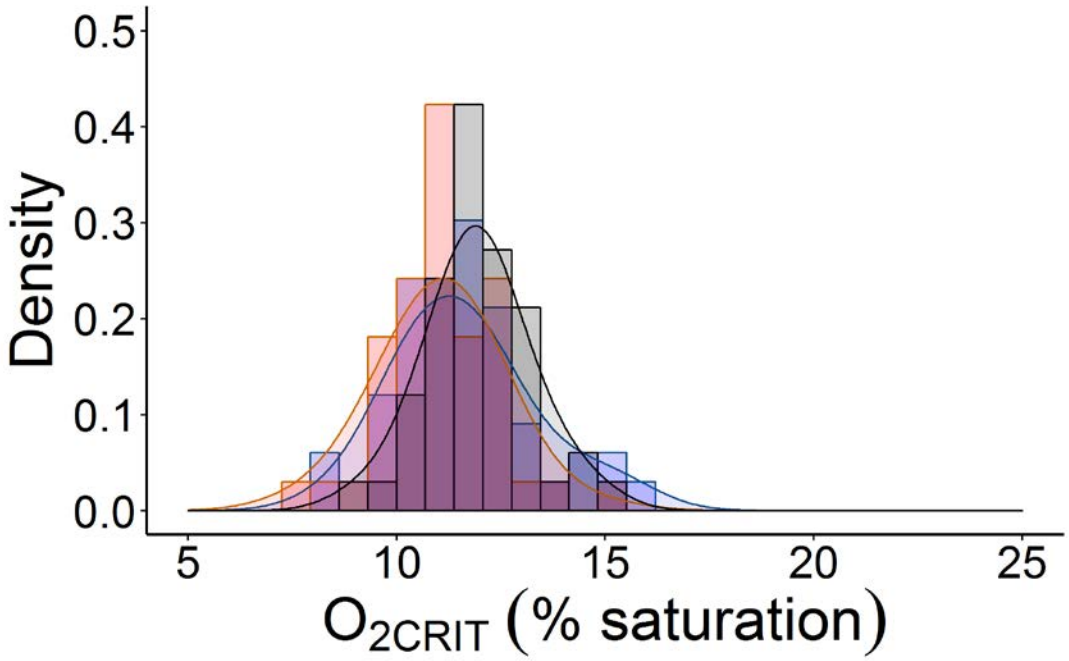

Figure 31 - Density histogram with density functions (Gaussian) overlayed that represents the O_{2CRIT} estimated for 48 fish (*Chapter 5*) using three methods: non-linear regression (grey bars, black line), segmented regression (red bars and line) and least-squares regression accompanying SMR (blue bars and line).

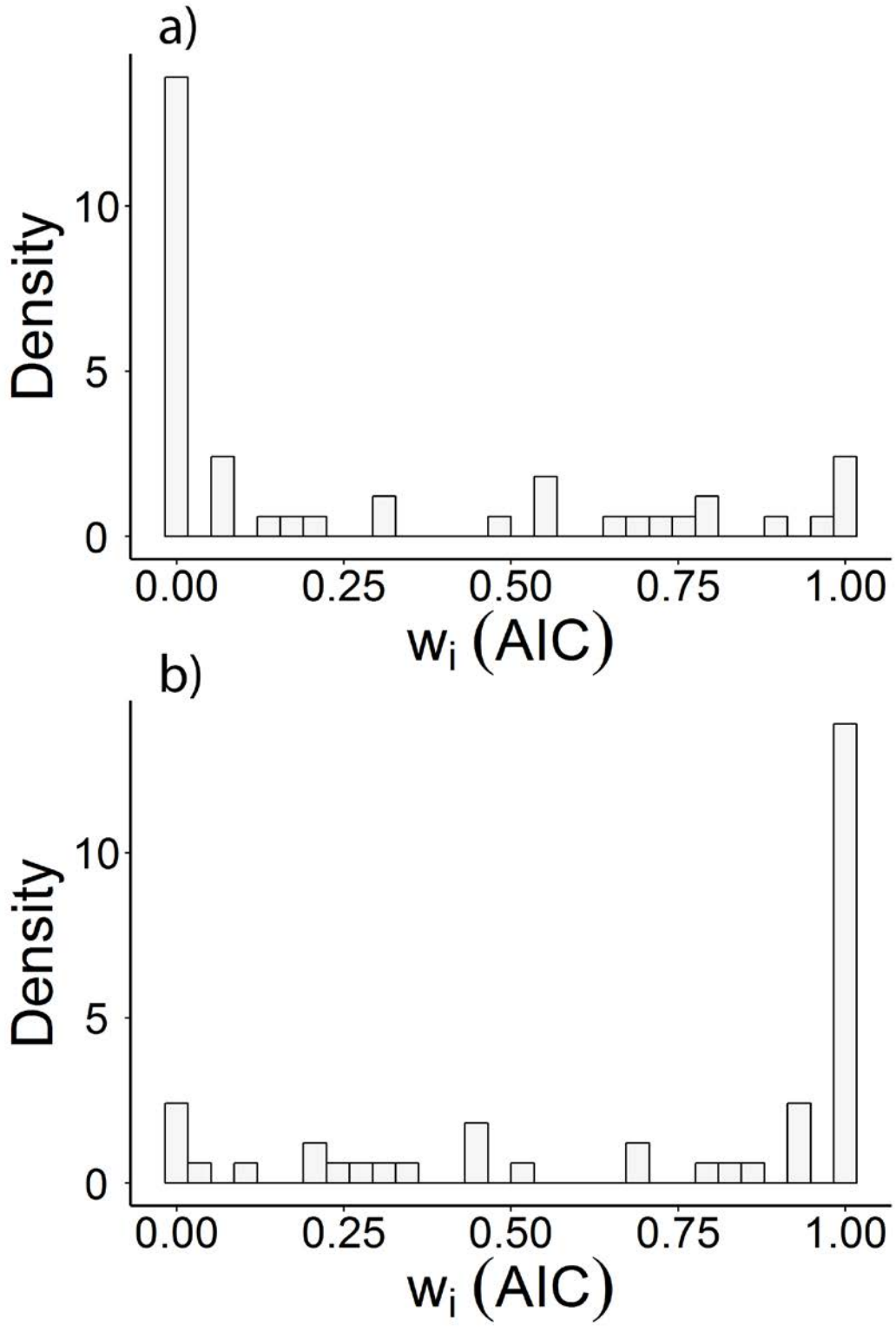

Figure 32 - Density histograms of Akaike weights for non-linear regression (a) and broken-stick regression (b) (*Chapter 5*).

Table 4 - Results for O_{2CRIT} estimates from *Chapters 4* and *5*

	Mean ± SD	Median (Q_1 - Q_3)	Range (min - max)
Chapter 4			
O_{2CRIT}			
NLR	14.23 ± 1.22 [a]	14.32 (13.55 - 15.00)	10.07 - 17.03
BSR	16.39 ± 1.47 [c]	16.25 (15.45 - 17.39)	12.73 - 21.56
LSR_{SMR}	15.22 ± 2.09 [b]	15.10 (13.95 - 16.45)	8.50 - 21.20
Aggregated	15.28 ± 1.86	15.20 (14.14 - 16.40)	8.50 - 21.56
w_i(AIC)			
NLR	-	0.96 (0.38 - 1.00)	-
BSR	-	0.04 (0.00 - 0.62)	-
Parameter estimates			
a	0.89 ± 0.05	0.89 (0.86 - 0.92)	0.67 - 0.96
b	11.39 ± 1.06	11.41 (10.76 - 12.14)	8.32 - 14.74
c	2.00 ± 0.32	1.97 (1.75 - 2.19)	1.30 - 3.08
SMR	1.72 ± 0.63	1.72 (1.18 - 2.24)	0.54 - 3.09
Chapter 5			
O_{2CRIT}			
NLR	11.94 ± 1.21 [b]	11.89 (11.21 - 12.50)	8.89 - 14.99
BSR	11.05 ± 1.34 [a]	11.12 (10.09 - 11.90)	7.53 - 14.90
LSR_{SMR}	11.64 ± 1.62 [ab]	11.65 (10.50 - 12.23)	8.50 - 15.60
Aggregated	11.54 ± 1.44	11.55 (10.64 - 12.25)	7.53 - 15.60
w_i(AIC)			
NLR	-	0.08 (0.0004 - 0.59)	-
BSR	-	0.92 (0.41 - 1.00)	-
Parameter estimates			
a	0.87 ± 0.07	0.89 (0.85 - 0.91)	0.70 - 0.97
b	8.88 ± 0.92	8.67 (8.33 - 9.34)	7.44 - 11.23
c	3.19 ± 1.06	3.23 (2.53 - 3.86)	1.14 - 6.36
SMR	1.60 ± 0.42	1.52 (1.33 - 1.87)	0.82 - 2.82

Data are presented as mean ± standard deviation, median (1st and 3rd quartiles) and range (minimum and maximum). Super-script letters next to mean O_{2CRIT} estimates indicate significant differences: a < b < c. NLR = non-linear regression; BSR = broken-stick regression; LSR_{SMR} = least-squares regression accompanying SMR. w_i(AIC) = Akaike weights. Parameter estimates (a, b and c) are the asymptote, scale and shape parameters, respectively, for the Weibull function.

APPENDIX B – SUPPORTING INFORMATION FOR CHAPTER 4

Supplementary Materials and Methods

$\dot{M}O_2$ Measurements

Oxygen consumption rates ($\dot{M}O_2$) were measured on sub-sets of fish ($N \approx 40$ per population, per hypoxia tolerance category: $N = 233$ in total) using intermittent flow-through respirometry as described in Collins et al. (2013) and following best practices (Clark et al., 2013a; Svendsen et al., 2015). Briefly, 8 fish per day were transferred from the experimental system and placed individually in respirometers (1.5 L). The respirometers were submerged in a temperature-controlled (29.10 ± 0.29˚C) 1,000 L tank that provided fully saturated water during the flush cycle of respirometry. Oxygen concentration was recorded in each individual respirometer using a Firesting Optical O_2 Meter (Pyroscience; Aachen, Germany). Fish were acclimated to respirometers for 17.8 ± 1.2 h, and $\dot{M}O_2$ was recorded every 12 min between flush cycles, resulting in ~ 90 $\dot{M}O_2$ measurements for each individual fish (Figure 33). There was no relationship between acclimation time (ranging 15.2 – 20.8 h) and SMR ($r = 0.089$, df = 209, $P = 0.199$) or O_{2CRIT} ($r = 0.106$, df = 209, $P = 0.123$). $\dot{M}O_2$ was calculated using LabChart® software (ADI Instruments; Sydney, NSW, Australia) and according to Equation 2. The O_2 data during the sealed cycles of respirometry were assessed visually for

linearity throughout the analysis to confirm the absence of spurious measurements. Background microbial respiration was measured prior to the fishes' introduction to the respirometer, and again following the fishes' removal from the respirometer. The average of three background $\dot{M}O_2$ readings was subtracted from the total $\dot{M}O_2$ to account for microbial respiration in $\dot{M}O_2$ calculations. Respirometers were cleaned regularly to avoid microbial accumulation, and background microbial respiration was generally negligible throughout the experiment.

Following the ~ 18 h acclimation period, the respirometers were manually sealed *via* a polyethylene valve and fish depleted DO in the respirometers to ~ 5% air saturation, after which the flush pump was turned on to restore DO levels to 100% saturation. $\dot{M}O_2$ was calculated for each consecutive 100 s period during the decline in DO and the procedure lasted 96 ± 20 min (dependent on fish size; see Figure 36). $\dot{M}O_2$ measurements were used to calculate the critical O_2 level (O_{2CRIT}) using each of three methods: broken-stick regression (BSR) using the 'segmented' package in R v. 3.2.1 (Muggeo, 2008; R Core Team, 2012), non-linear regression (NLR; Weibull function) as per Marshall et al. (2013), where the O_{2CRIT} is calculated from the first derivative ($f'(x) = 0.029$), and least-squares regression accompanying SMR (LSR_{SMR}; Claireaux and Chabot, 2016). Representative examples of the O_{2CRIT} estimate methods are presented in Figures 28a and 34, and $\dot{M}O_2$ data from all O_{2CRIT} tests are presented in Figure 28b. The NLR approach provided an overall better fit to $\dot{M}O_2$ measurements than the BSR approach (median w(AIC) for BSR = 0.04: 1st and 3rd quartiles = 0.00 and 0.62,

respectively) (Figure 29). The distribution and density functions for each of the three O_{2CRIT} methods are presented in Figures 28 and 35.

Growth Trial

Barramundi from the three hypoxia tolerance groups (*N* = 60 in each group; 3 replicate tanks per group with 20 fish·tank^{-1}) and populations (*N* = 2) were assigned randomly to 18 × 100 L tanks (*N* = 360 in total). Random tank allocation was achieved using pseudo-random number generation in R v. 3.2.1.

Fish were fed a commercial pelleted feed (EWOS, Bergen, Norway) to satiation, twice daily for 45 d (first feed at 08:30 and second feed at 15:30). Uneaten pellets were removed from the tanks 20 min following feeding. These were then counted for each tank and at each feeding period for subsequent calculations of feeding rate and feed conversion ratio (detailed below). Uneaten feed was calculated for each tank as the number of pellets remaining after feeding × average pellet mass (0.0488 ± 0.0015 g). DO was 73 ± 9% saturation during the experiment and was maintained by increasing rates of aeration and water flow as required. Temperature of the water was maintained at 30.4 ± 0.7 °C. Stocking density within the tanks increased from 6.8 ± 0.6 kg · m^{-3} at the beginning of the growth trial, to 22.8 ± 1.7 kg · m^{-3} at the completion. Water quality parameters (ammonia, nitrites and pH) were monitored daily with test kits (Aqua One, Ingleburn, NSW, Australia) and were maintained within acceptable levels (NH_3/NH_4^+ = 0.2 ± 0.4 ppm; NO_2^- =

1.8 ± 1.5 ppm; pH = 6.8 ± 0.3) by replacing 15% of the system volume daily with fresh, dechlorinated and carbon-filtered water.

Fish were weighed and measured at the beginning of the study and again after 7, 25 and 45 d. Accordingly, the first, second and third growth periods were of 6, 18 and 20 d, respectively. The following equations were used to determine absolute growth rate (AGR), specific growth rate (SGR), feeding rate (FR), feed conversion efficiency (FCE) and feed conversion ratio (FCR), as per Yang et al. (2013).

$$\mathrm{AGR} = \frac{\Delta \mathrm{W}}{\Delta \mathrm{T}}$$

Equation 15 - Absolute growth rate, where W = mass (g) and T = time (days)

$$\mathrm{SGR} = 100 \times \frac{[\mathrm{Ln}(\mathrm{W_T}) - \mathrm{Ln}(\mathrm{W_0})]}{\Delta \mathrm{T}}$$

Equation 16 - Specific growth rate, where Ln = natural logarithm, W_T = mass (g) at time = T, W_0 = mass at time = 0, T = time (days)

$$\mathrm{FR} = \mathrm{F_C} \times \frac{2}{[(\mathrm{W_T} + \mathrm{W_0}) \times \Delta \mathrm{T}]} \times 100$$

Equation 17 - Feeding rate, where F_C = feed consumption (g), W_T = mass (g) at time = T, W_0 = mass at time = 0, T = time (days)

$$\mathrm{FCE} = \frac{\Delta \mathrm{W}}{\mathrm{F_C}} \times 100$$

Equation 18 - Feed conversion efficiency, where W = mass (g) and F_C = feed consumption (g)

$$\mathrm{FCR} = \frac{\mathrm{F_C}}{\Delta \mathrm{W}}$$

Equation 19 - Feed conversion ratio, where F_C = feed consumption (g) and W = mass (g)

Hypoxia Resilience (Survival) Analysis

The LOE response in fish during severe hypoxia is closely followed by mortality, and while no mortality was observed in my experiment, time to LOE data were compared using standard survival analysis procedures (Roze et al., 2013; Speers-Roesch et al., 2013; Zambonino-Infante et al., 2013). The survival analysis is referred to as the 'hypoxia resilience analysis' (HRA) herein to avoid confusion. The HRA procedure consisted of fitting a Cox proportional hazard's model to assess differences between population, hypoxia category and HCT, with fish mass included in the model as a covariate. The proportional hazard's assumption was assessed *via* a visual examination of Schoenfield residuals (Figures 46, 47, 48) and the accompanying chi-square test-statistic for each population, hypoxia category and HCT.

An additional assessment of the HRA was conducted using Kaplan-Meier analysis. A log-rank test was used to assess differences between survival curves for the three hypoxia categories, with rho set to 0.

Additional data analysis

To satisfy the assumptions of linearity for Pearson's product-moment correlation, it was necessary to perform a $\log_{10}$ transformation of the response (x) variable for the residual LT_{50} from the second HCT (Figure 21). A linear regression was fit to time vs. $(mass)^2$ in the first HCT, and $\log_e(time)$ vs. mass in the second HCT (Figure 22). The adjusted R^2 for the first and second HCT from this analysis was 0.166 and 0.270, respectively. These

same transformations were used to compute Pearson's product-moment correlation for the relationship between time to LOE and mass for the first and second HCTs.

Supplementary Results

Food Consumption & Growth Rates

Tropical fish were marginally but significantly heavier (36.04 ± 7.70 g) than sub-tropical fish (32.03 ± 6.47 g) at the commencement of the trials ($F_{1, 354}$ = 30.93, $P < 0.0001$).

The main results from the growth trial are presented in Figures 19 and 42. The average rate of food consumption throughout the experiment was 3.14 ± 0.57% body mass · d^{-1}. There were no differences in feeding rate between populations ($F_{1, 2}$ = 14.69, P = 0.062) or hypoxia categories ($F_{2, 2}$ = 1.0, P = 0.50). The average FCR was 1.19 ± 0.18. Average FCE throughout the experiment was 85.92 ± 10.80 %. There was no effect of hypoxia category on either FCR or FCE, however FCE was higher for the sub-tropical population, and accordingly FCR was lower (Figure 42).

Given the positive correlation between fish mass and LOE, hypoxia tolerant fish were significantly heavier at the beginning and end of the growth trial (final mass = 123.6 ± 4.7 g) than either hypoxia intermediate fish (113.1 ± 1.5 g) or hypoxia sensitie fish (109.2 ± 7.1 g), ($F_{2, 342}$ = 15.910, $P < 0.0001$, initial mass; $F_{2, 338}$ = 14.338, $P < 0.0001$, final mass). There were no differences in final fish mass between the two populations ($F_{1, 338}$ = 1.404, P = 0.237). There were significant differences in AGR for population ($F_{1, 12}$ = 7.325, P =

0.019), and hypoxia category ($F_{2, 12}$ = 5.406, P = 0.021). There was no difference in SGR between the different hypoxia categories ($F_{2, 12}$ = 0.495, P = 0.621), however sub-tropical fish (2.94 ± 0.60 % · d^{-1}) had a significantly higher SGR than tropical fish (2.56 ± 0.68 % · d^{-1}), ($F_{1, 12}$ = 31.638, P = 0.0001). Condition factor was not different between hypoxia categories initially ($F_{2, 342}$ = 0.062, P = 0.940), but was significantly higher for tropical fish at the completion of the growth trial ($F_{2, 338}$ = 7.667, P = 0.001). Condition factor was significantly greater in tropical (1.34 ± 0.08, final condition factor) compared with sub-tropical (1.26 ± 0.07, final condition factor) fish at the start and the completion of the growth trial ($F_{1, 342}$ = 104.88, P < 0.0001, initial condition factor; $F_{1, 338}$ = 120.61, P < 0.0001, final condition factor).

Hypoxia resilience analysis

Hypoxia categories were significantly different in both the first (χ^2 = 139, df = 2, P < 0.0001) and second (χ^2 = 61.9, df = 2, P < 0.0001) HCTs, when assessed through Kaplan-Meier analysis.

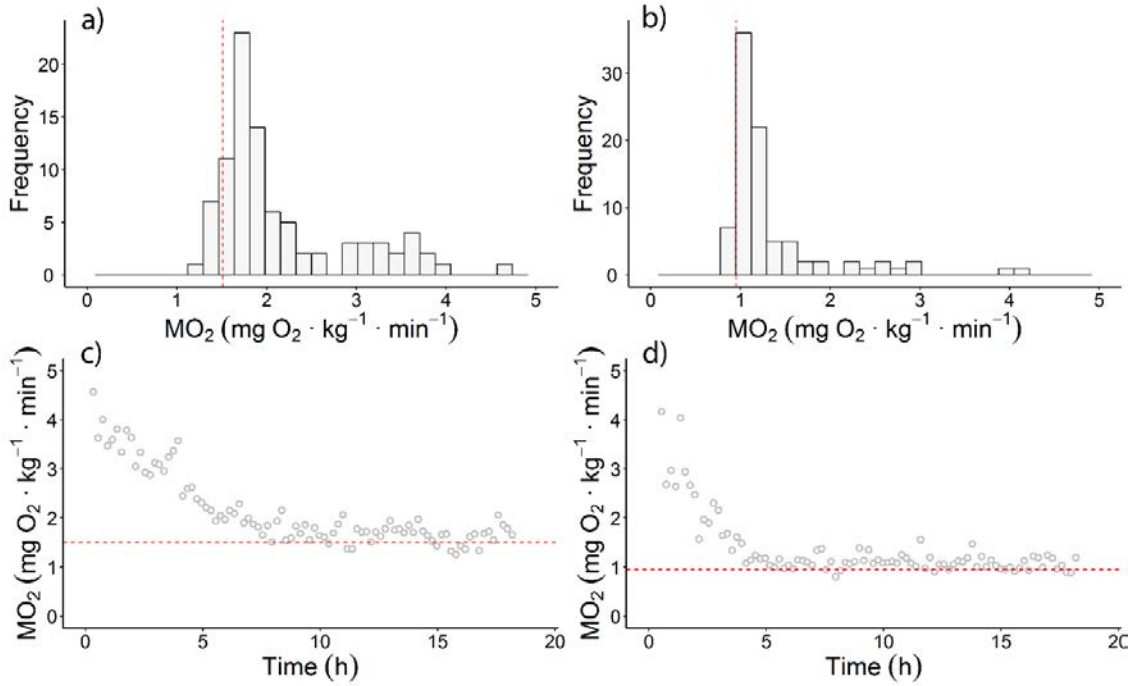

Figure 33 - Frequency histograms (upper panels) for O_2 consumption rates ($\dot{M}O_2$) from two individual fish over the ~18 h acclimation period. The dashed red vertical line indicates the SMR calculated using the quantile method, with q set to 0.1 (Chabot et al., 2016b). Oxygen consumption rates ($\dot{M}O_2$) plotted against time (lower panels) during the respirometer acclimation period for individual fish. The SMR is indicated by the dashed red horizontal line. Upper and lower panels indicate data from the same fish.

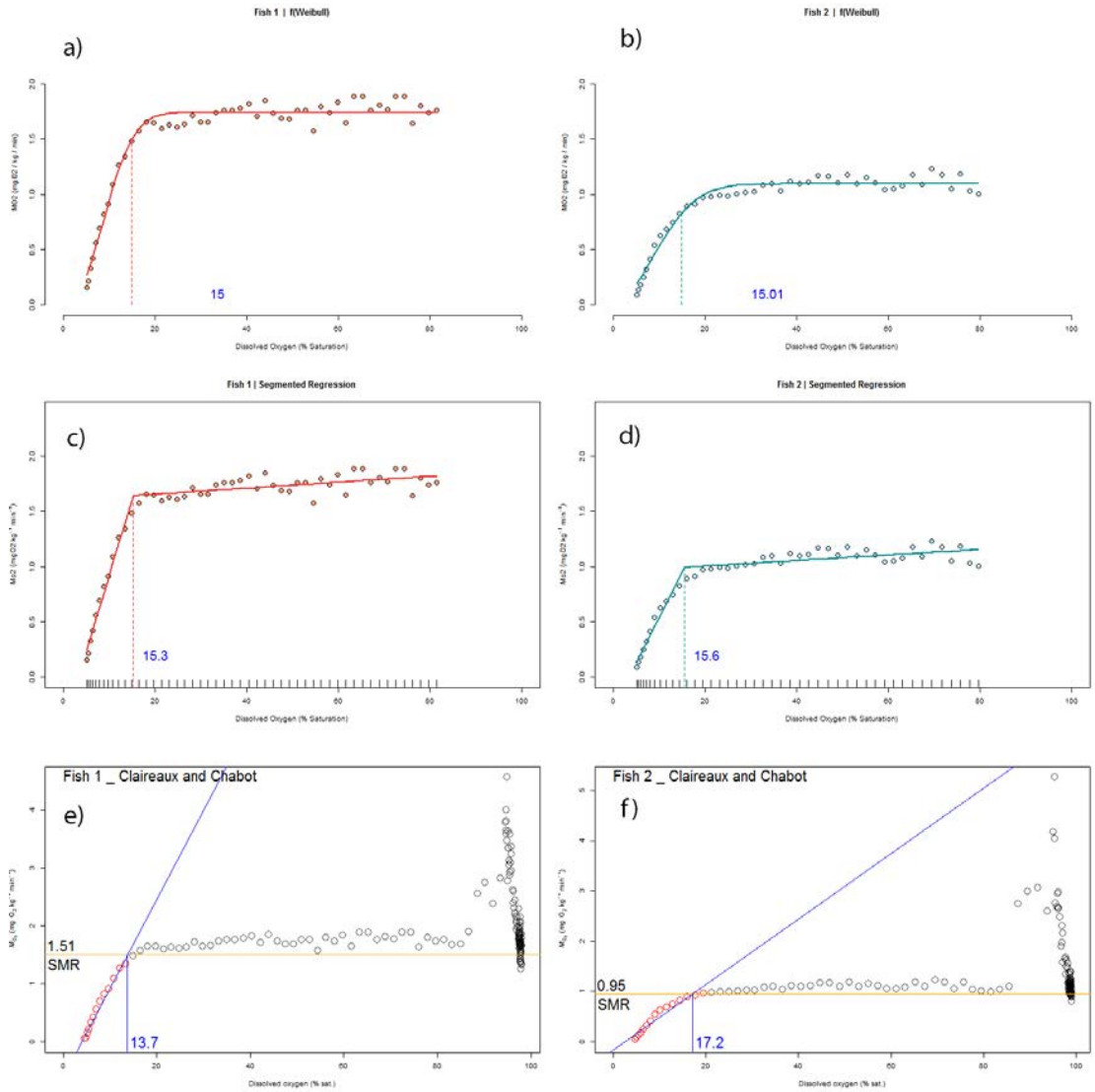

Figure 34 - Relationship between O_2 consumption rate and dissolved O_2 for two individual barramundi (panels a, c and e represent data from one individual, and panels b, d and f represent data from another individual). The O_{2CRIT} was calculated using three different methods: non-linear regression (Marshall et al., 2013; panels a and b), broken-stick regression (Muggeo, 2008; panels c and d) or least-squares regression accompanying SMR (Claireaux and Chabot, 2016; panels e and f).

Table 5 – Time to loss of equilibrium (mean ± standard deviation) from the first and second hypoxia challenge tests (HCT), separated according to population and hypoxia category. Means were calculated for each combination of hypoxia category and population from the median time to loss of equilibrium for each day of data collection (N = 3 for all measurements). Super-script letters indicate significant differences for each population * category for HCT 1 (ANOVA; $F_{5, 12}$ = 196.3, P < 0.0001), identified from Tukey's Honest Significant Difference (HSD) test. There were significant differences between each combination of population * category for HCT 2 (ANOVA; $F_{5, 12}$ = 3.12, P = 0.0494), however Tukey's HSD test failed to elucidate any specific differences.

	HCT 1		HCT 2	
	Sub-Tropical	Tropical	Sub-Tropical	Tropical
Sensitive	70 ± 22 [a]	81 ± 3 [a]	100 ± 25	108 ± 19
Intermediate	149 ± 10 [b]	147 ± 8 [b]	122 ± 8	113 ± 27
Tolerant	342 ± 13 [d]	289 ± 18 [c]	186 ± 25	184 ± 80

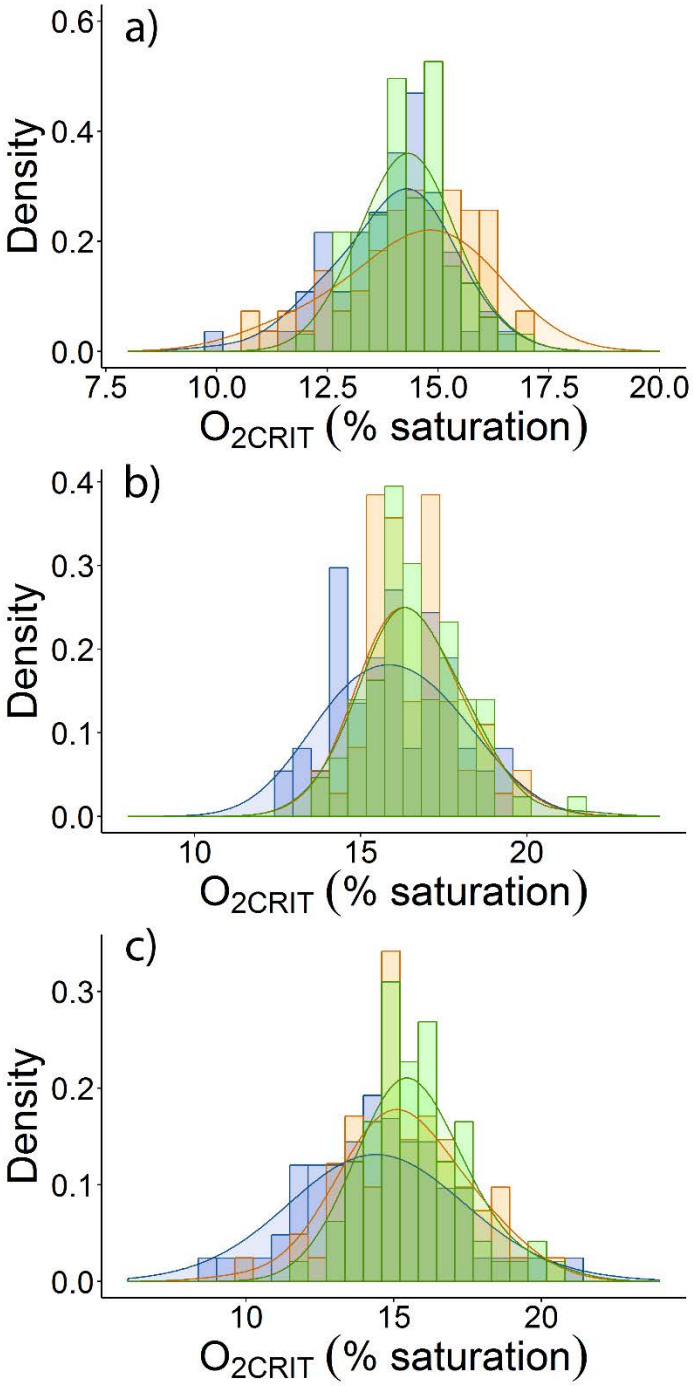

Figure 35 - Density histograms for O_{2CRIT}, calculated using non-linear regression (NLR; panel a), broken-stick regression (BSR; panel b) and least-squares regression accompanying SMR (LSR_{SMR}; panel c). Blue, orange and green bars and density functions represent hypoxia sensitive, intermediate, and tolerant groups, respectively.

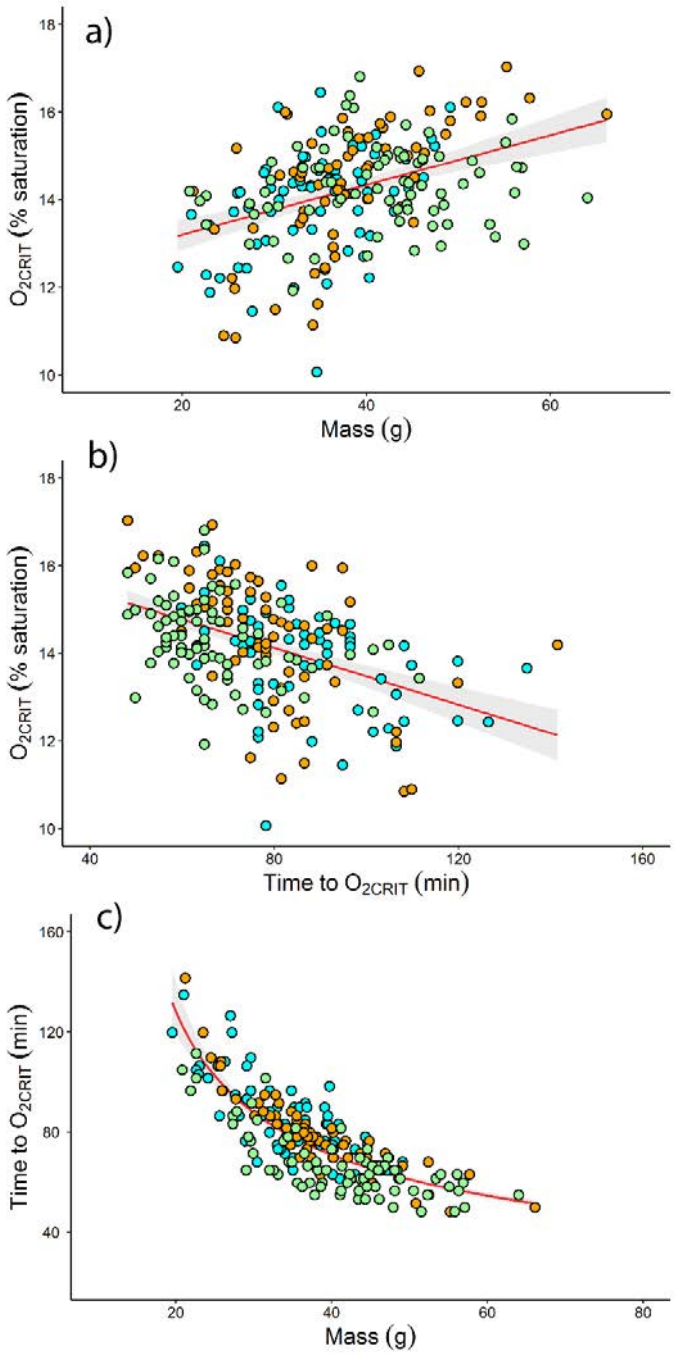

Figure 36 - Relationship between O_{2CRIT} (calculated using non-linear regression) and mass (panel a). Relationship between O_{2CRIT} and the time from sealing the respirometers to the O_{2CRIT} for each individual fish (panel b). Relationship between fish mass (g) and the time from sealing respirometers to the O_{2CRIT} (panel c). Blue, orange and green circles represent hypoxia sensitive, intermediate, and tolerant groups, respectively. Pearson's correlation test results were: r = 0.408, df = 209, $P < 0.0001$ (O_{2CRIT} ~ mass), r = -0.453, df = 209, $P < 0.0001$ (O_{2CRIT} ~ time to O_{2CRIT}), r = -0.822, df = 209, $P < 0.0001$ (time to O_{2CRIT} ~ mass; where mass was $\log_e$ transformed prior to correlation analysis).

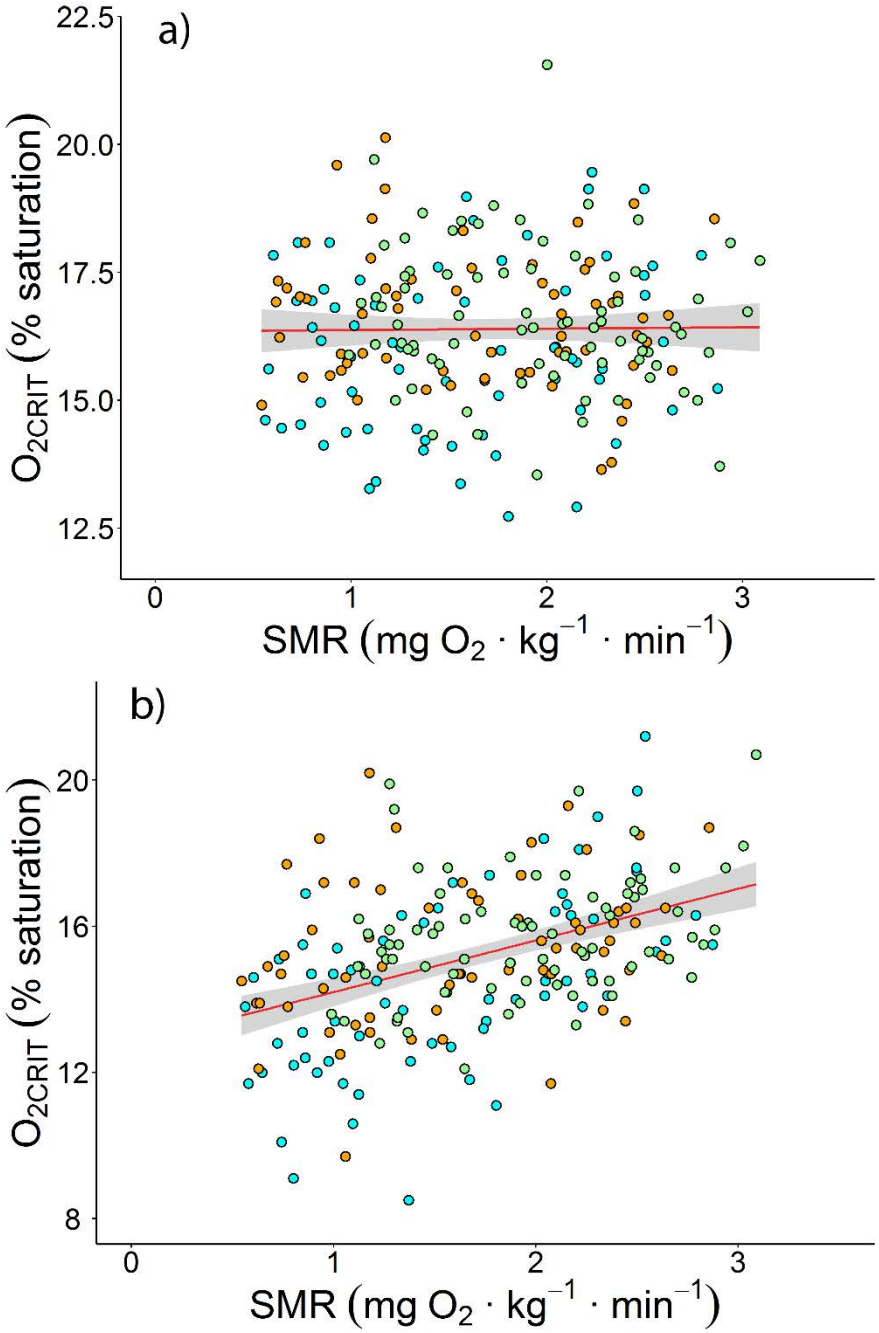

Figure 37 - Alternative analysis for the relationship between O_{2CRIT} and SMR. The O_{2CRIT} was calculated using broken-stick regression (BSR; panel a) and least-squares regression accompanying SMR (LSR_{SMR}; panel b). Blue, orange and green circles represent hypoxia sensitive, intermediate, and tolerant groups, respectively. Pearson's correlation test results were: r = 0.011, df = 209, P = 0.868 (BSR), r = 0.428, df = 209, P < 0.0001 (LSR_{SMR}).

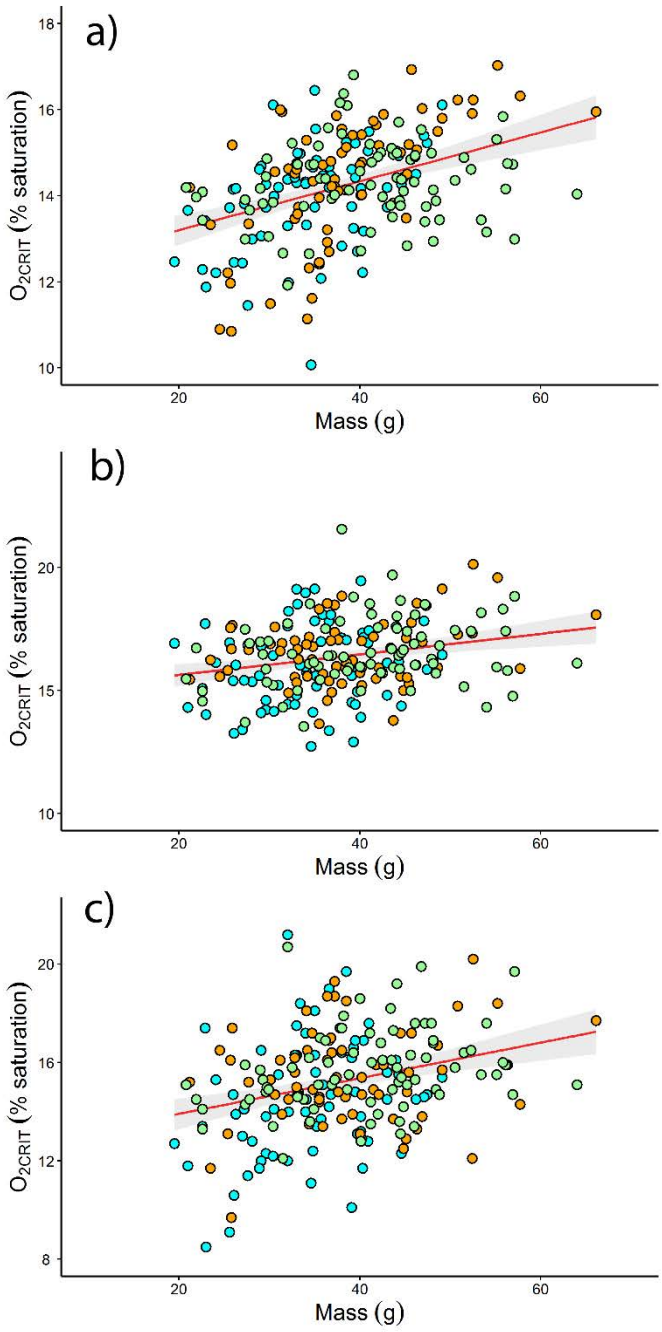

Figure 38 - Alternative analyses for the relationship between O_{2CRIT} and fish mass. O_{2CRIT} was calculated using non-linear regression (NLR; panel a), broken-stick regression (BSR; panel b) or least-squares regression accompanying SMR (LSR_{SMR}; panel c). Blue, orange and green circles represent hypoxia sensitive, intermediate, and tolerant groups, respectively. Pearson's correlation test results were: r = 0.408, df = 209, $P < 0.0001$ (NLR), r = 0.248, df = 209, $P = 0.0003$ (BSR), r = 0.303, df = 209, $P < 0.0001$ (LSR_{SMR}).

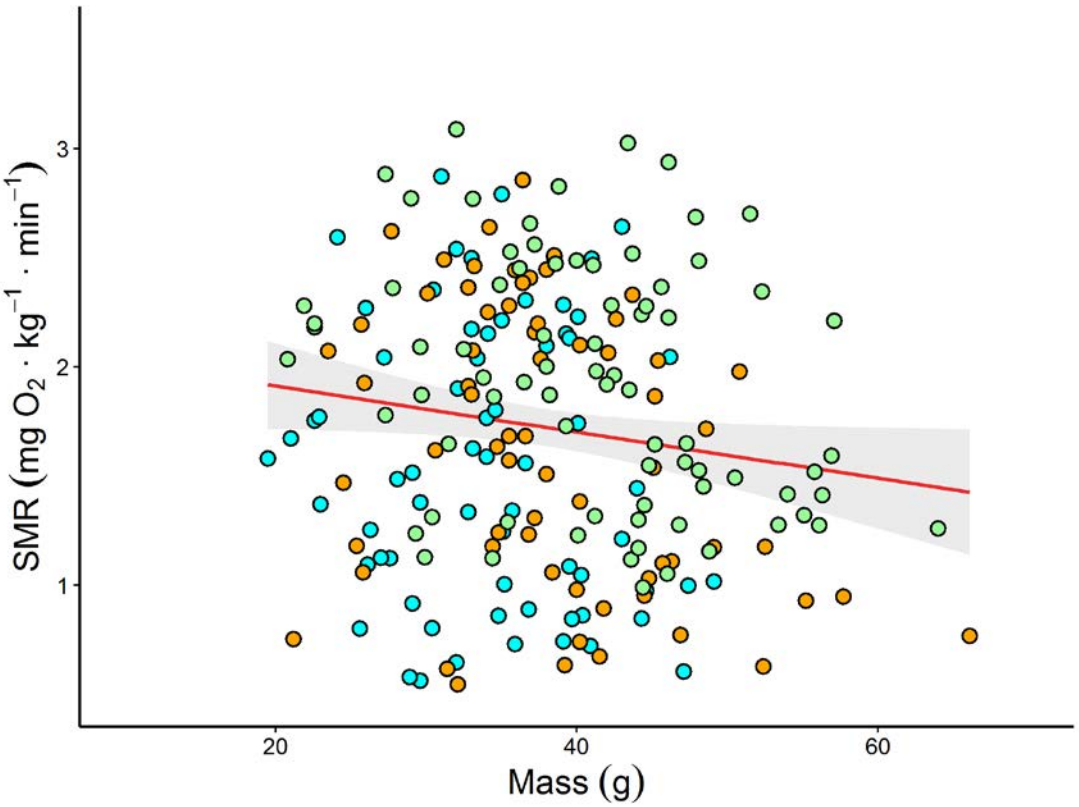

Figure 39 - Relationship between mass-specific SMR and fish mass (g). Blue, orange and green circles represent hypoxia sensitive, intermediate, and tolerant groups, respectively. Pearson's correlation test results: r = -0.145, df = 209, *P* = 0.035.

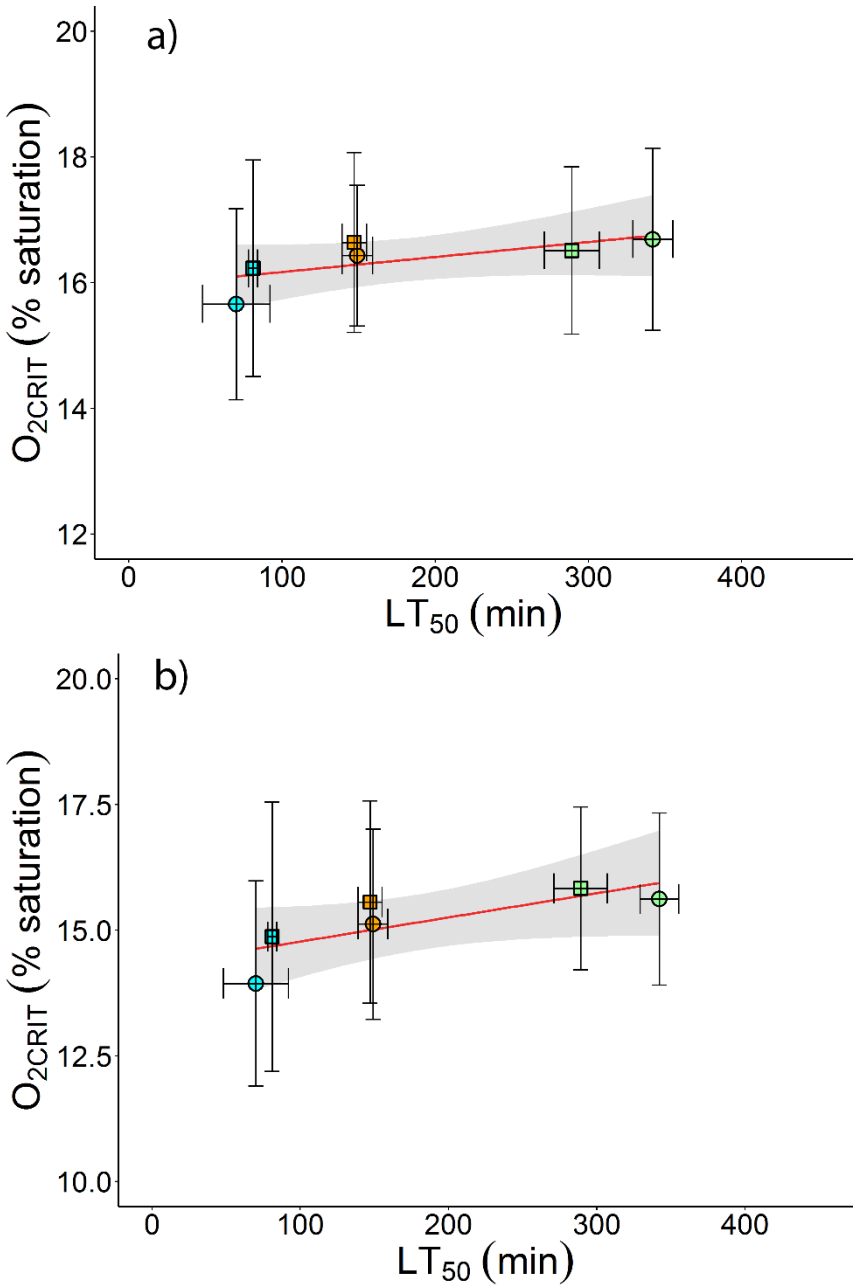

Figure 40 - Alternative analysis for the relationship between O_{2CRIT} and LT_{50} (cf. Figure 18a). O_{2CRIT} was calculated using segmented/broken-stick regression (BSR; panel a) or least-squares regression accompanying SMR (LSR_{SMR}; panel b). Error bars (horizontal and vertical) represent standard deviation. Blue, orange and green symbols represent hypoxia sensitive, intermediate, and tolerant groups, respectively. Squares and circles represent sub-tropical and tropical populations, respectively. Pearson's correlation test results: r = 0.701, df = 4, *P* = 0.121 (BSR), r = 0.774, df = 4, *P* = 0.071 (LSR_{SMR}).

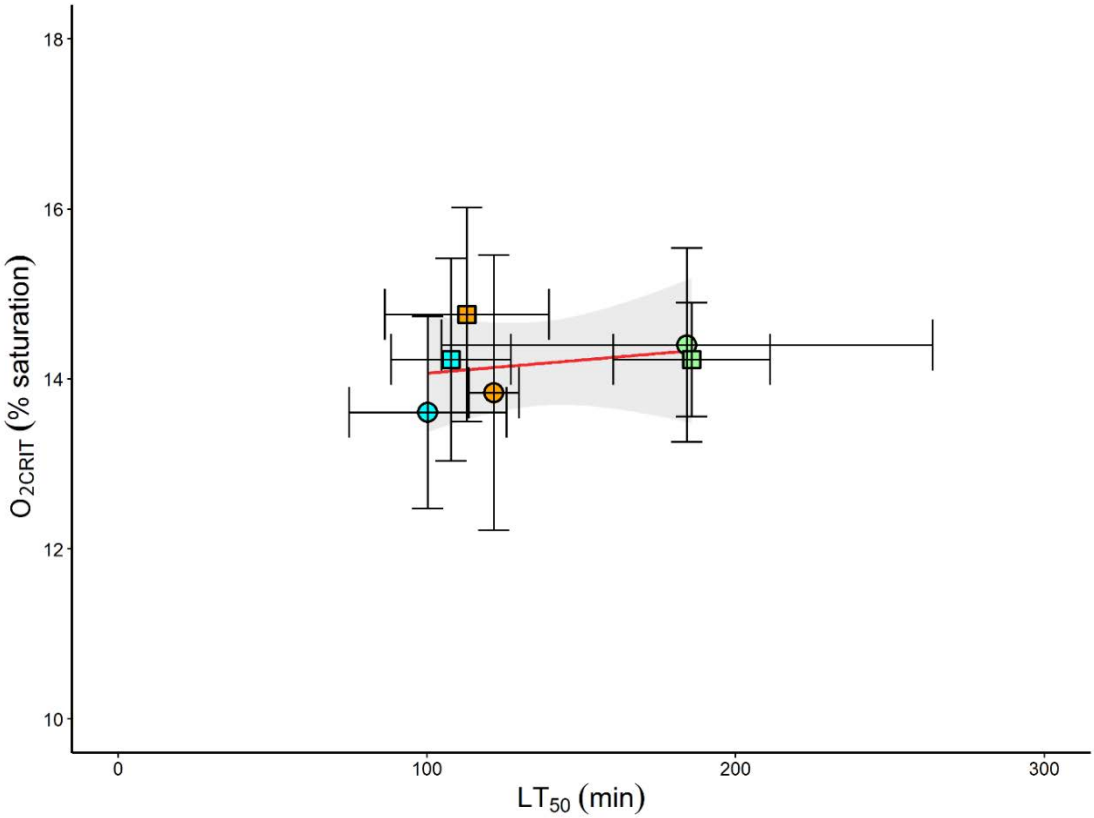

Figure 41 - Alternative analysis for the relationship between O_{2CRIT} (Non-linear regression) and average LT_{50} (based on time to LOE) for juvenile barramundi. The LT_{50} for each population and treatment were obtained from the second HCT (cf. Figure 18a). Error bars represent standard deviation. Squares and circles represent sub-tropical and tropical populations, respectively. Blue, orange and green symbols represent hypoxia-sensitive, -intermediate and -tolerant fish, respectively. The red line ± 95% confidence limits (shaded grey area) is a fitted linear regression. Results from Pearson's correlation test were: r = 0.296, df = 4, *P* = 0.569.

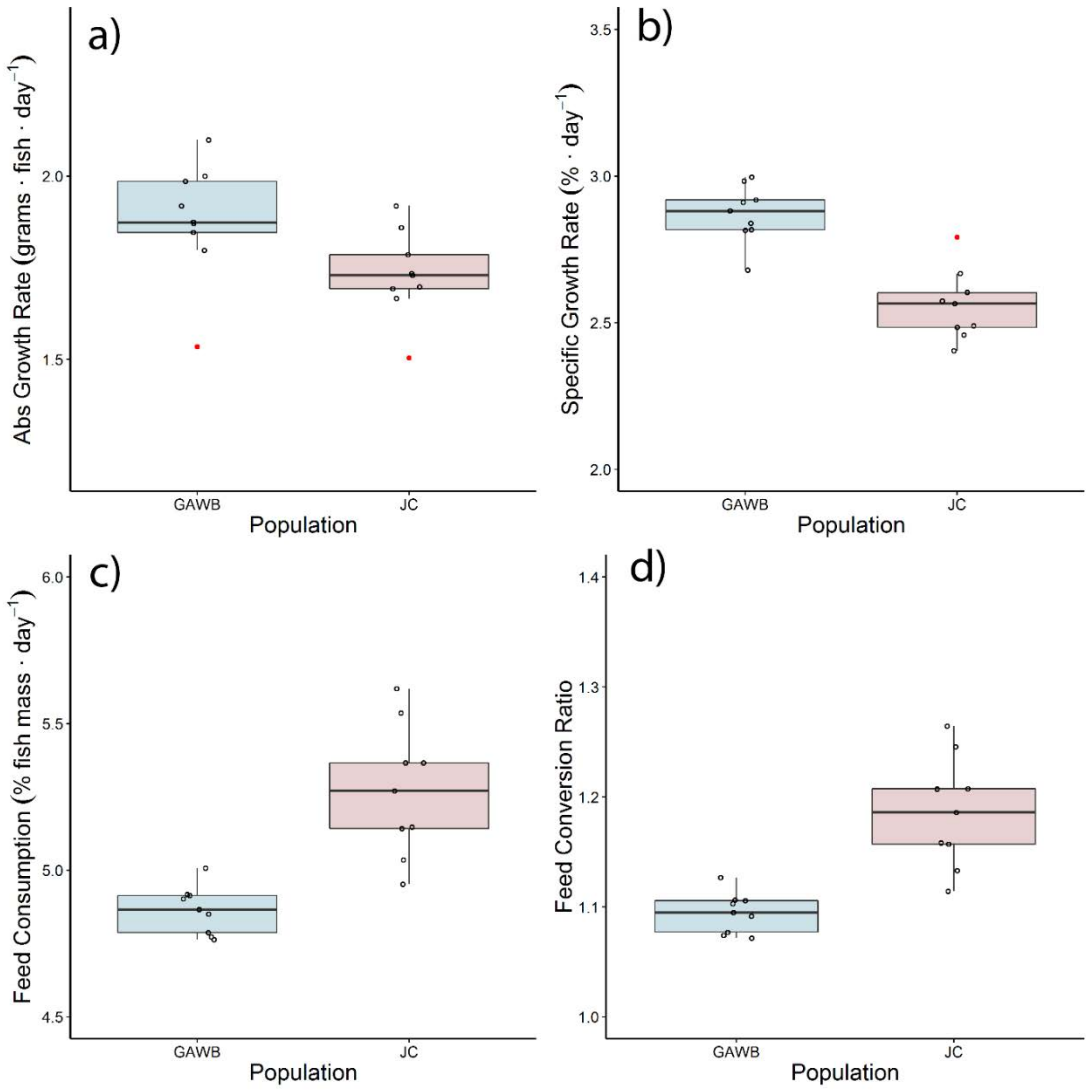

Figure 42 - Growth and food consumption rates for barramundi during the 45 d growth trial. Panels represent: a, absolute growth rate (grams · fish^{-1} · d^{-1}); b, specific growth rate (% · d^{-1}); c, feed consumption (% average fish mass · d^{-1}); d, feed conversion ratio (unit less). Boxes represent the first and third quartiles, and the black line inside the box indicates the median. Upper and lower whiskers represent the maximum and minimum, and red dots indicate outliers. Blue, and red boxes represent sub-tropical (GAWB; Gladstone) and tropical (JC; Innisfail) populations, respectively.

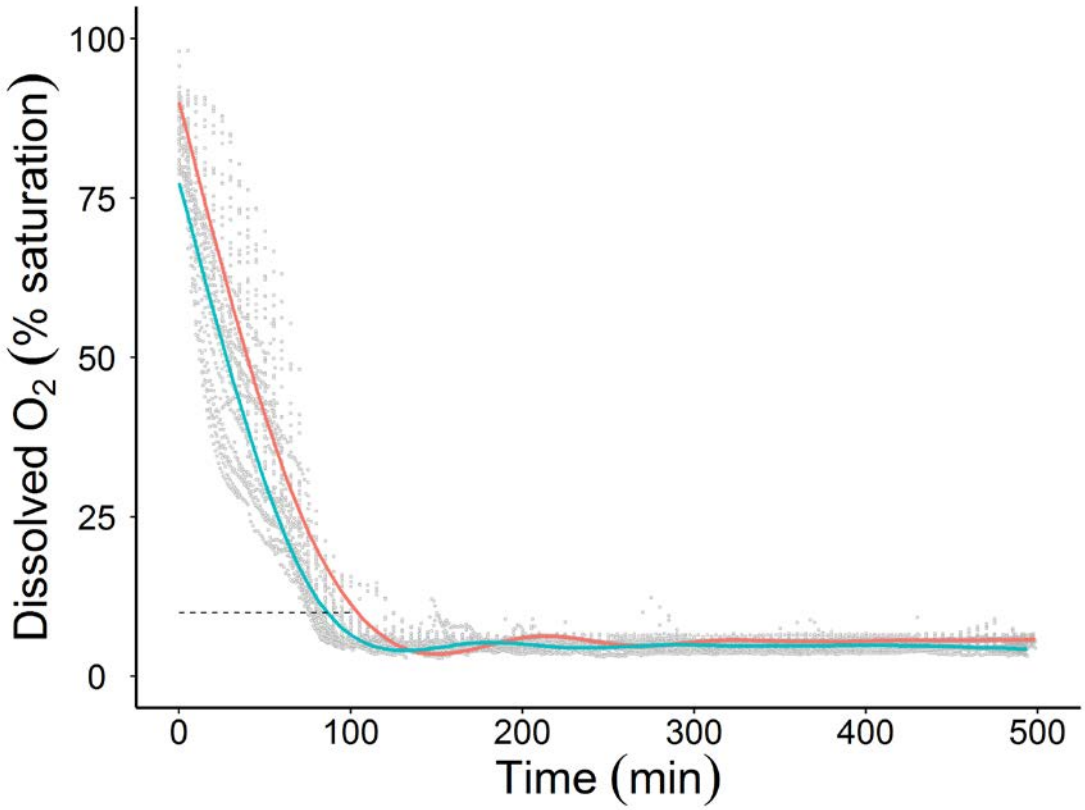

Figure 43 - Dissolved O_2 in the experimental tanks during the first (red line) and second (blue line) HCT. The lines fit to the data are non-parametric (loess) curves, with the smoothing parameter fixed at 0.3. The horizontal dashed line indicates where DO = 10% saturation. Time to LOE was calculated for each tank when DO fell below this level.

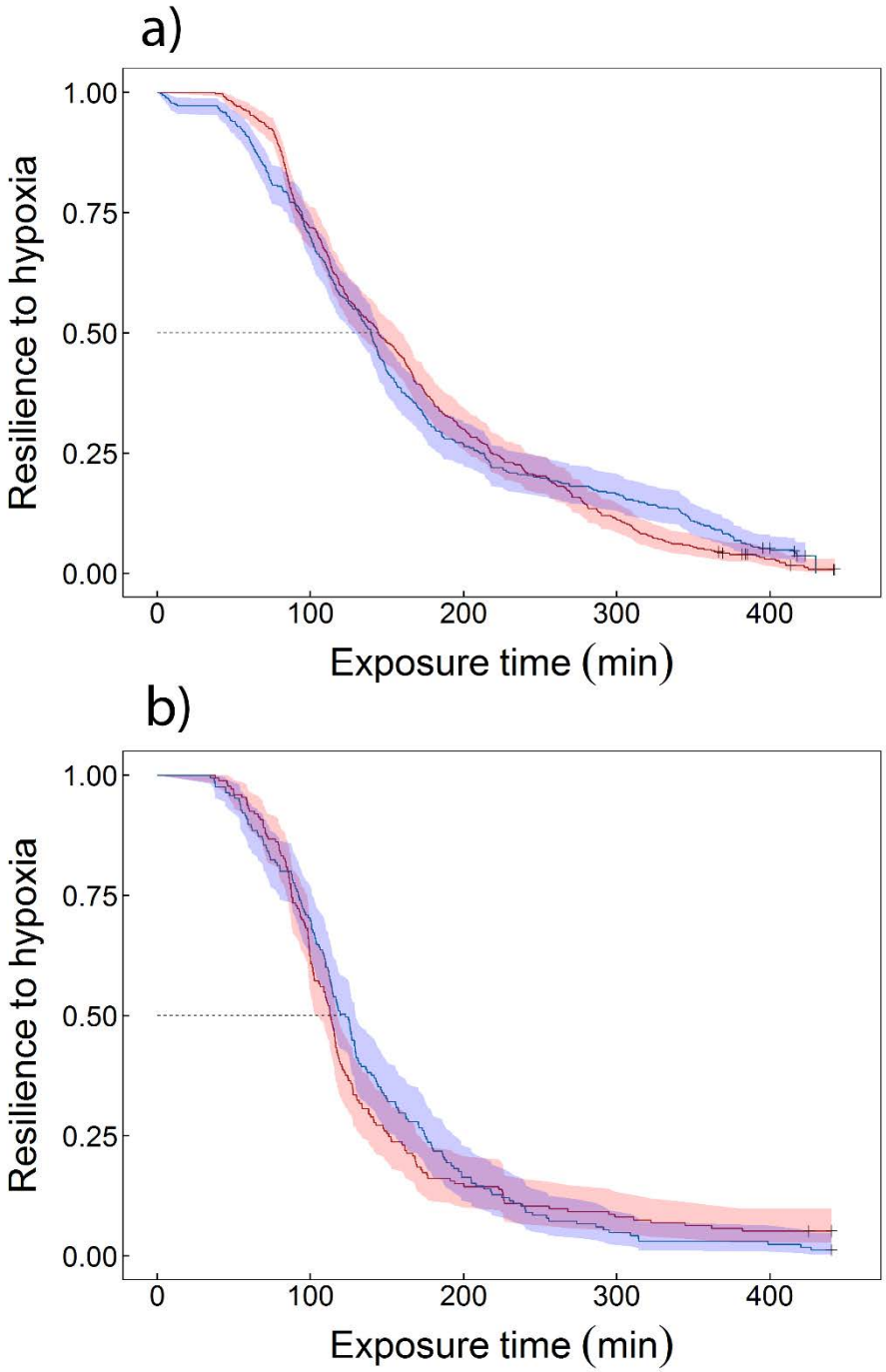

Figure 44 - Survival functions (solid line ± shaded 95% confidence limits) for the first (panel a) and second (panel b) HCT, where blue and red lines represent the sub-tropical and tropical populations, respectively.

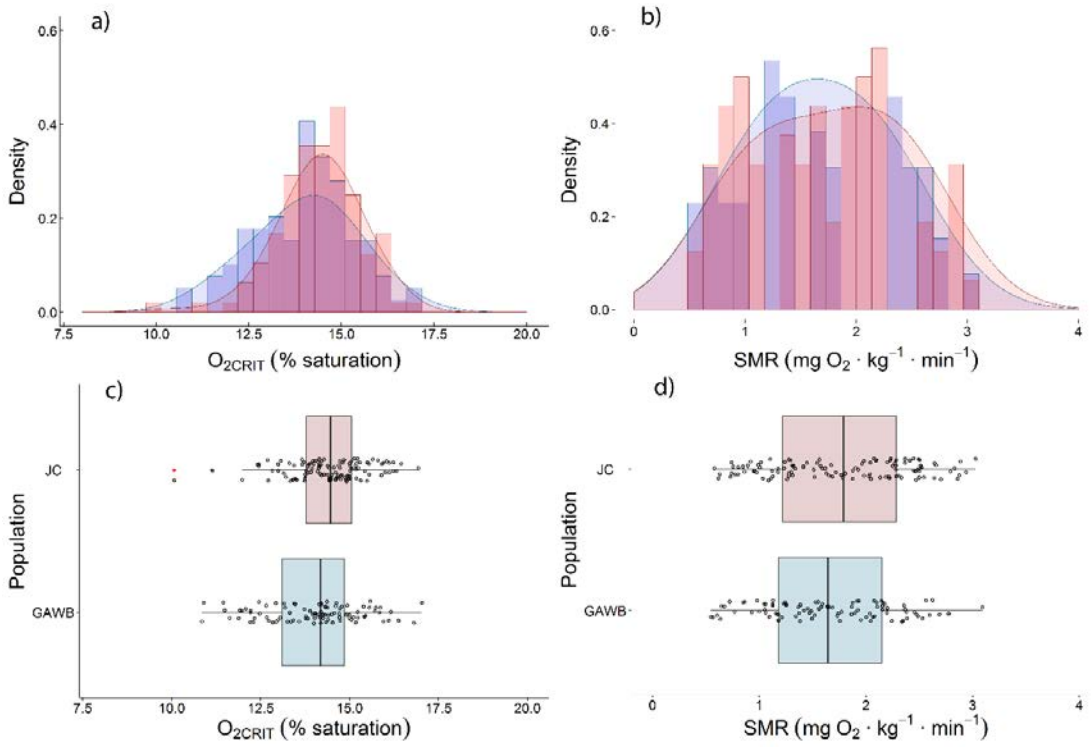

Figure 45 - Density histograms (panels a and b) and horizontal boxplots (panels c and d) for the critical O_2 level (panels a and c) and standard metabolic rate (panels b and d). Blue and red bars and boxes represent sub-tropical (GAWB) and tropical (JC) populations, respectively

Table 6 - Table of results for the assessment of the proportional hazard's assumption from Cox PH models. The models are indicated by the blue text as follows: HCT (full model); HCT0 (1st HCT); HCT1 (2nd HCT)

co-variate	rho	χ^2	*P*
	HCT	HCT	HCT
weight	0.108589517	21.52800087	3.49E-06
populationJC	0.061652204	4.642325352	0.031192709
categoryINT	0.166441573	32.48727562	1.20E-08
categoryTOL	0.138129945	21.25470693	4.02E-06
HCT	-0.092190836	14.72538341	0.000124361
GLOBAL	NA	98.03092476	0
	HCT0	HCT0	HCT0
weight	0.154748361	22.21582626	2.44E-06
populationJC	0.036887217	1.149534477	0.283646621
categoryINT	0.119483663	12.0887823	0.000507261
categoryTOL	0.092527325	7.1233327	0.007608714
GLOBAL	NA	99.69830518	0
	HCT1	HCT1	HCT1
weight	0.234556212	27.06831575	1.96E-07
populationJC	0.052209578	0.951763375	0.329270851
categoryINT	0.172244589	10.17600239	0.001422803
categoryTOL	0.169072031	9.085249776	0.002576793
GLOBAL	NA	40.7542749	3.02E-08

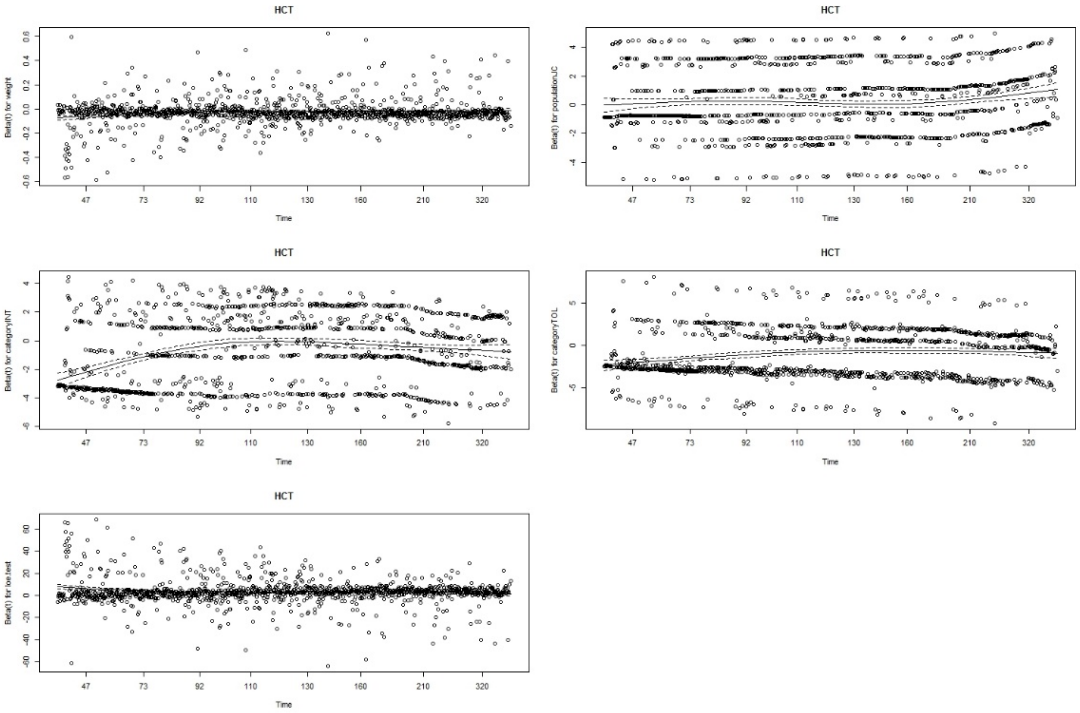

Figure 46 - Schoenfield residuals plots for the HCT Cox Proportional Hazard's model. Panels represent: top-left (mass); top-right (population_JC); mid-right (hypoxia category_TOL); bottom-left (HCT); mid-left (hypoxia category_INT)

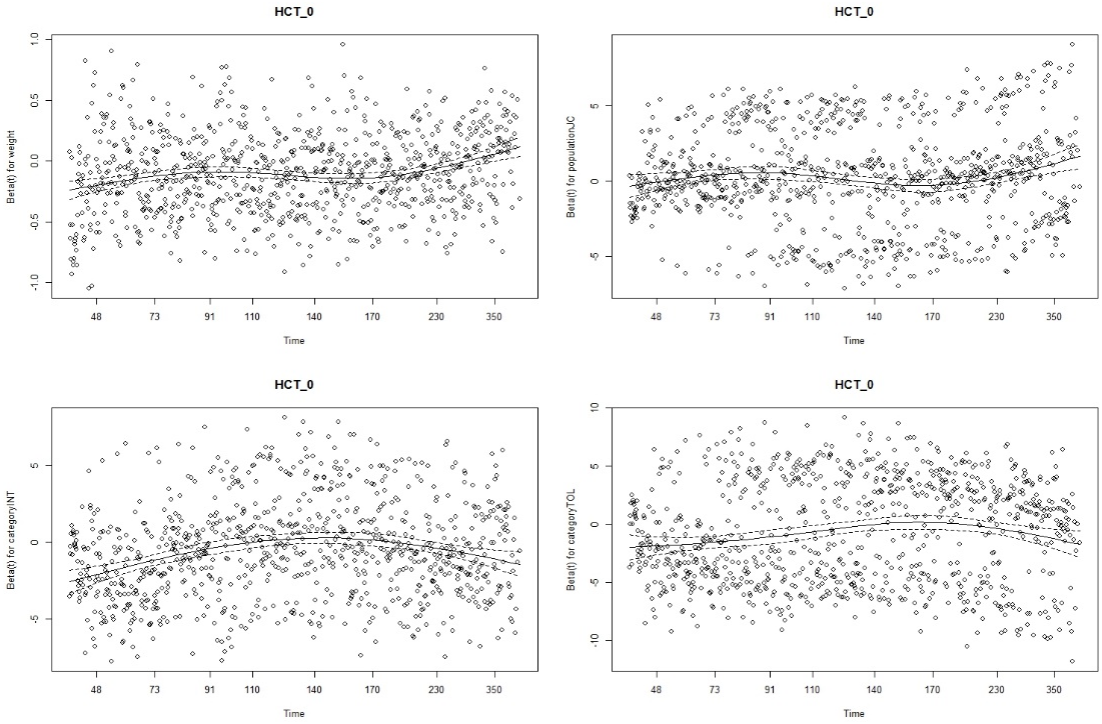

Figure 47 - Schoenfield residuals plots from the first HCT. Panels represent: top-left (mass); top-right (population_JC); bottom-right (category_TOL); bottom-left (category_INT)

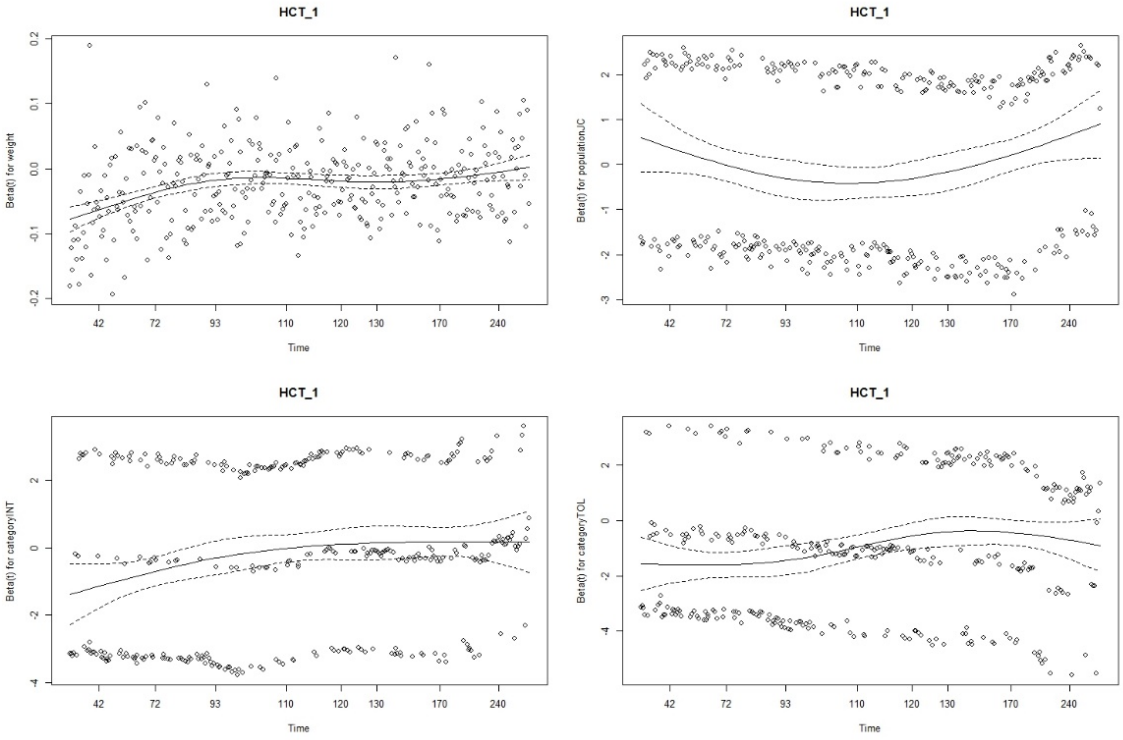

Figure 48 - Schoenfield residuals plots for the second HCT. Panels represent: top-left (mass); top-right (population_JC); bottom-right (category_TOL); bottom-left (category_INT)

Table 7 – O_{2CRIT} comparisons (NLR) from linear mixed effects model

```
Linear mixed-effects model fit by REML
 Data: Data
       AIC      BIC    logLik
  649.1343 675.7184 -316.5672

Random effects:
 Formula: ~1 | day
        (Intercept)  Residual
StdDev:   0.7241758 0.9867929

Fixed effects: pcrit.weibull1 ~ population * treatment
                              Value Std.Error  DF  t-value p-value
(Intercept)                13.666412 0.4062721 183 33.63857  0.0000
populationJC                0.566685 0.5437427  22  1.04219  0.3086
treatmentINT                0.262341 0.5782869  22  0.45365  0.6545
treatmentTOL                0.713896 0.5431096  22  1.31446  0.2022
populationJC:treatmentINT   0.242761 0.7713144  22  0.31474  0.7559
populationJC:treatmentTOL -0.708642 0.7453041  22 -0.95081  0.3520
 Correlation:
                           (Intr) ppltJC trtINT trtTOL pJC:IN
populationJC               -0.747
treatmentINT               -0.703  0.525
treatmentTOL               -0.748  0.559  0.526
populationJC:treatmentINT   0.527 -0.705 -0.750 -0.394
populationJC:treatmentTOL   0.545 -0.730 -0.383 -0.729  0.514

Standardized Within-Group Residuals:
        Min          Q1         Med          Q3         Max
-3.90625977 -0.52159730 -0.05017574  0.68640324  2.12801459

Number of Observations: 211
Number of Groups: 28
> anova(mod_lme)
                    numDF denDF  F-value p-value
(Intercept)             1   183 8648.022  <.0001
population              1    22    1.523  0.2302
treatment               2    22    0.659  0.5274
population:treatment    2    22    0.891  0.4245
```

Table 8 - Confidence intervals for model parameters from Table 7 (O_{2CRIT} comparisons)

	2.5%	97.5%
.sig01	0.4252681	0.8855060
.sigma	0.8938697	1.0977599
(Intercept)	12.9349090	14.3946715
populationJC	-0.4082384	1.5448455
treatmentINT	-0.7795884	1.2994851
treatmentTOL	-0.2592698	1.6913994
populationJC:treatmentINT	-1.1399704	1.6314207
populationJC:treatmentTOL	-2.0490708	0.6272964

Table 9 - SMR comparisons from linear mixed effects model

```
Linear mixed-effects model fit by REML
 Data: Data
      AIC      BIC    logLik
  364.013 390.5971 -174.0065

Random effects:
 Formula: ~1 | day
        (Intercept)  Residual
StdDev:   0.4178228 0.4853814

Fixed effects: smr ~ population * treatment
                              Value Std.Error  DF   t-value p-value
(Intercept)                1.4129243 0.2277355 183  6.204233  0.0000
populationJC               0.2689990 0.3049606  22  0.882078  0.3873
treatmentINT               0.1242701 0.3237386  22  0.383859  0.7048
treatmentTOL               0.4974539 0.3046868  22  1.632673  0.1168
populationJC:treatmentINT -0.1323977 0.4323365  22 -0.306238  0.7623
populationJC:treatmentTOL -0.2463700 0.4182609  22 -0.589034  0.5618
 Correlation:
                           (Intr) ppltJC trtINT trtTOL pJC:IN
populationJC               -0.747
treatmentINT               -0.703  0.525
treatmentTOL               -0.747  0.558  0.526
populationJC:treatmentINT  0.527 -0.705 -0.749 -0.394
populationJC:treatmentTOL  0.544 -0.729 -0.383 -0.728  0.514

Standardized Within-Group Residuals:
        Min          Q1         Med          Q3         Max
-2.30599347 -0.58840247 -0.09121674  0.63024452  2.28369234

Number of Observations: 211
Number of Groups: 28
> anova(mod_lme)
                    numDF denDF  F-value p-value
(Intercept)             1   183 396.0381  <.0001
population              1    22   0.4740  0.4984
treatment               2    22   1.8447  0.1817
population:treatment    2    22   0.1735  0.8419
```

Table 10 - Confidence intervals for model parameters from Table 9 (SMR comparisons)

	2.5%	97.5%
.sig01	0.26154942	0.5061208
.sigma	0.43957066	0.5395872
(Intercept)	1.00311297	1.8231193
populationJC	-0.28038904	0.8174711
treatmentINT	-0.45780269	0.7085036
treatmentTOL	-0.05096417	1.0457929
populationJC:treatmentINT	-0.91154838	0.6452193
populationJC:treatmentTOL	-0.99856683	0.5068391

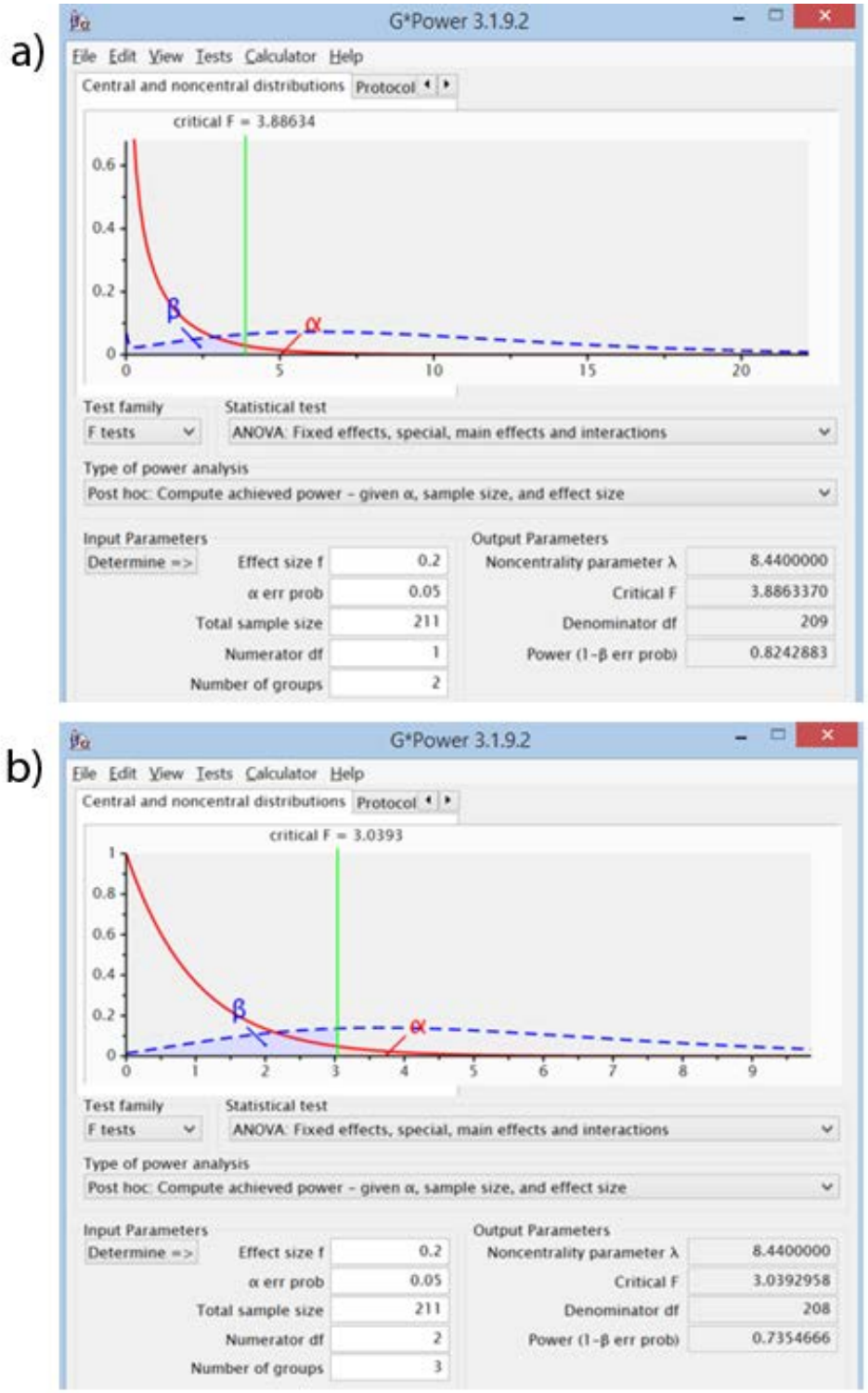

Figure 49 - Computed power with conditions: *N* = 211, α = 0.05, f = 0.2. Panels a and b display power analysis for numerator d.f. = 1 and 2, respectively (where the number of groups = 2 and 3, respectively, corresponding to the number of groups for population (a) and treatment (b) comparisons), where computed power is equal to 0.82 and 0.74, respectively. Calculations were performed using the software package G*Power 3.1.9.2.

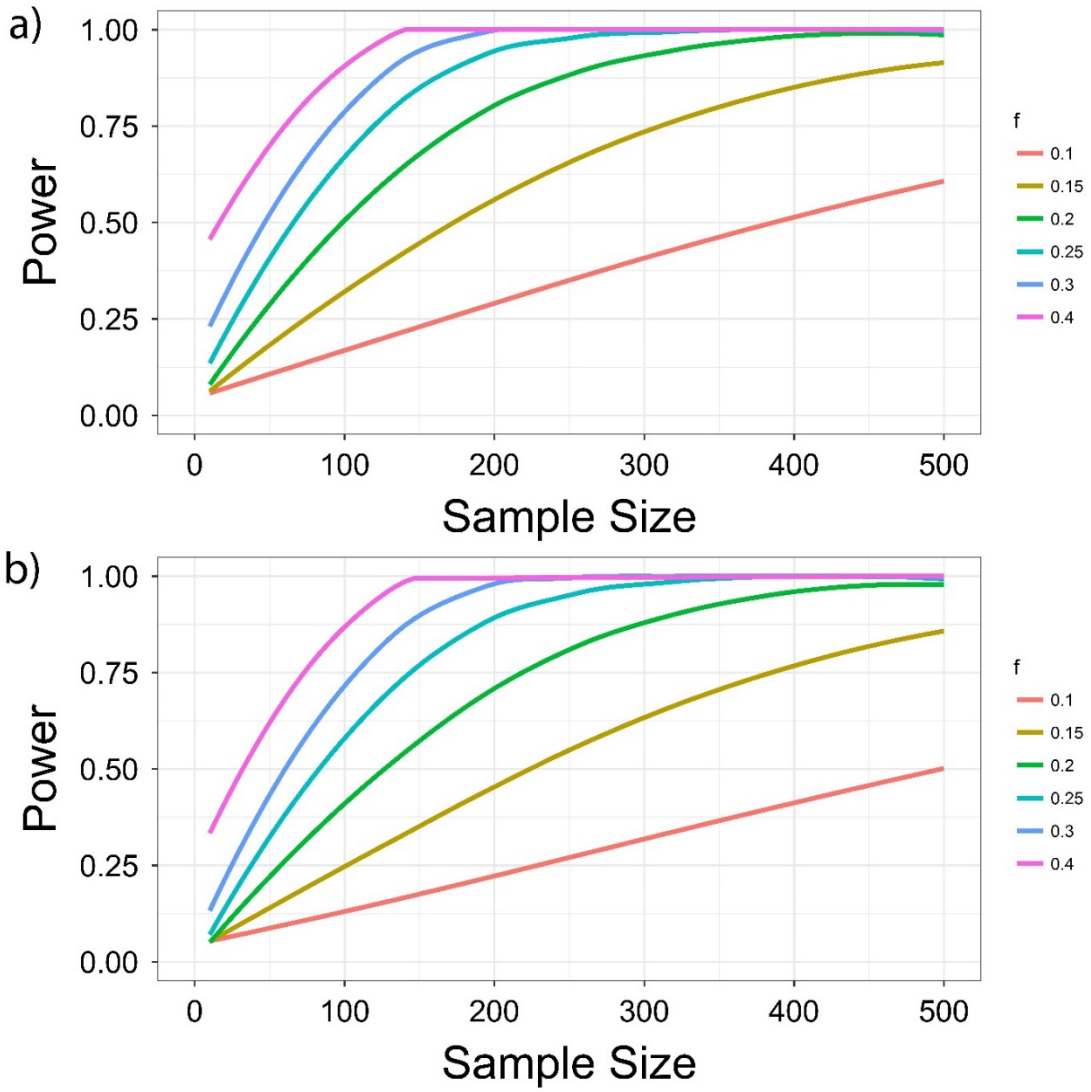

Figure 50 - Statistical power plotted against total sample size for a range of effect sizes (0.1 to 0.4), where the numerator d.f. is equal to 1 (panel a) and 2 (panel b), respectively. Calculations were performed using the software package G*Power 3.1.9.2.

APPENDIX C – SUPPORTING INFORMATION FOR CHAPTER 5

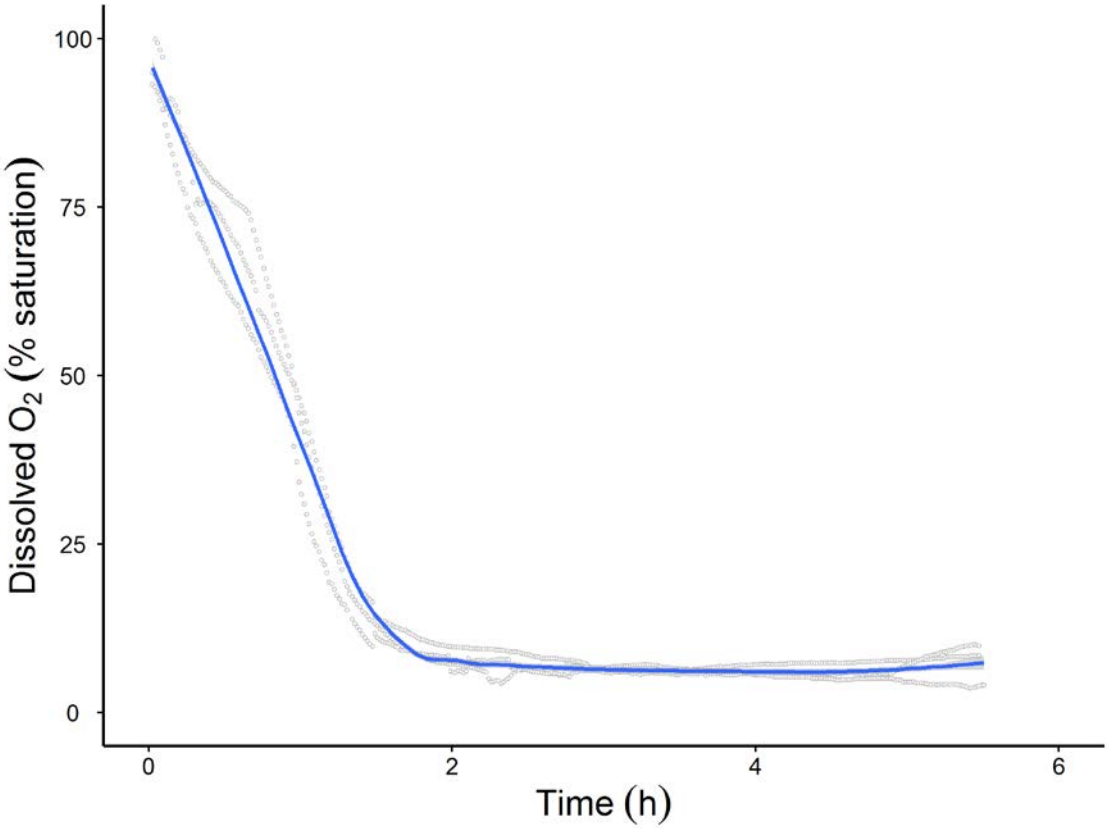

Figure 51 – Dissolved O_2 plotted against time during the hypoxia challenge tests (HCT). The blue line is a fitted local polynomial regression.

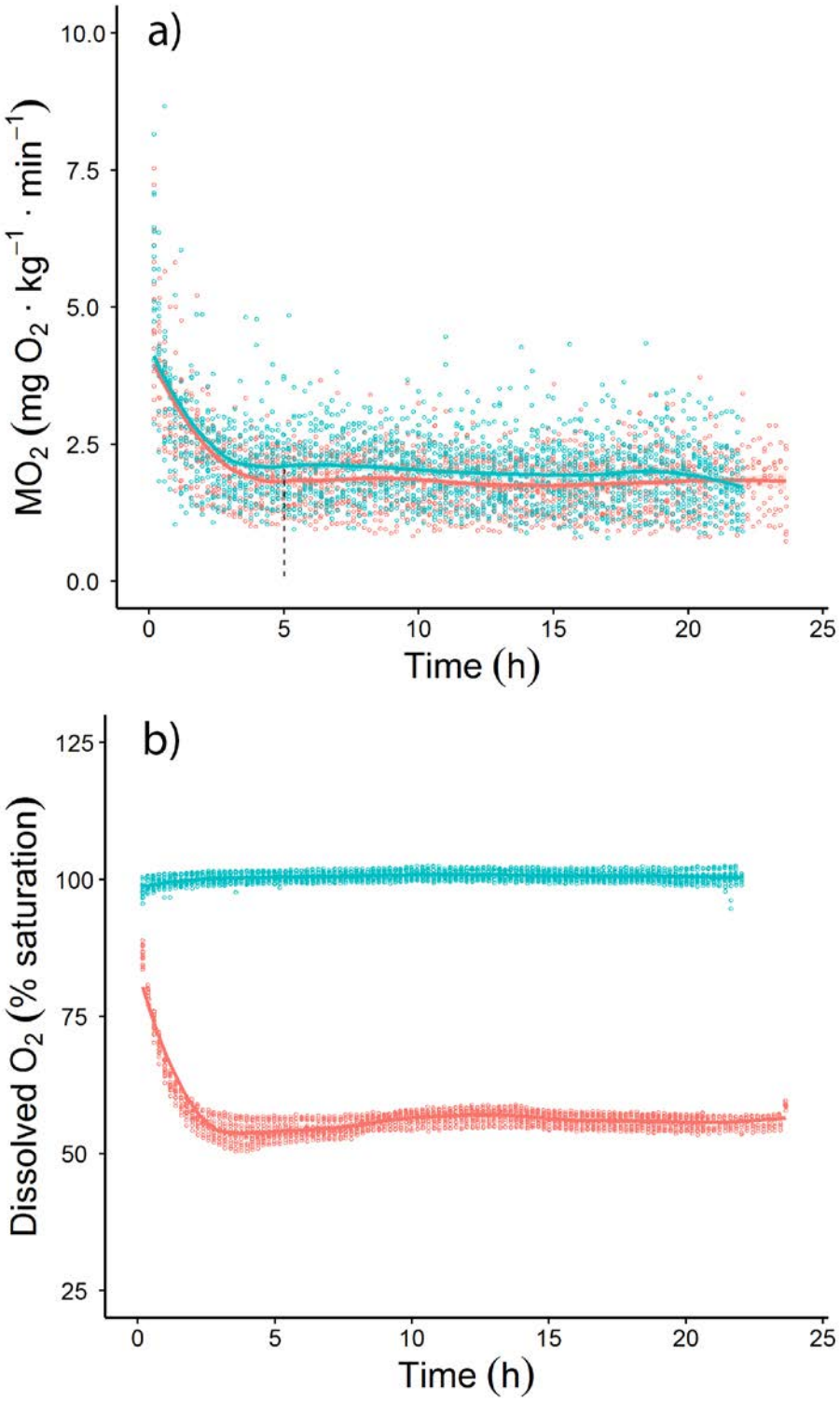

Figure 52 – Oxygen consumption rates (panel a) and dissolved O_2 (panel b) plotted against time during the determination of handling stress trial. Blue and red lines and dots represented normoxic and hypoxic treatments, respectively. The vertical dashed line in panel a (5 h after the fish's introduction to the respirometer) represents the time where recovery from handling stress was deemed complete.

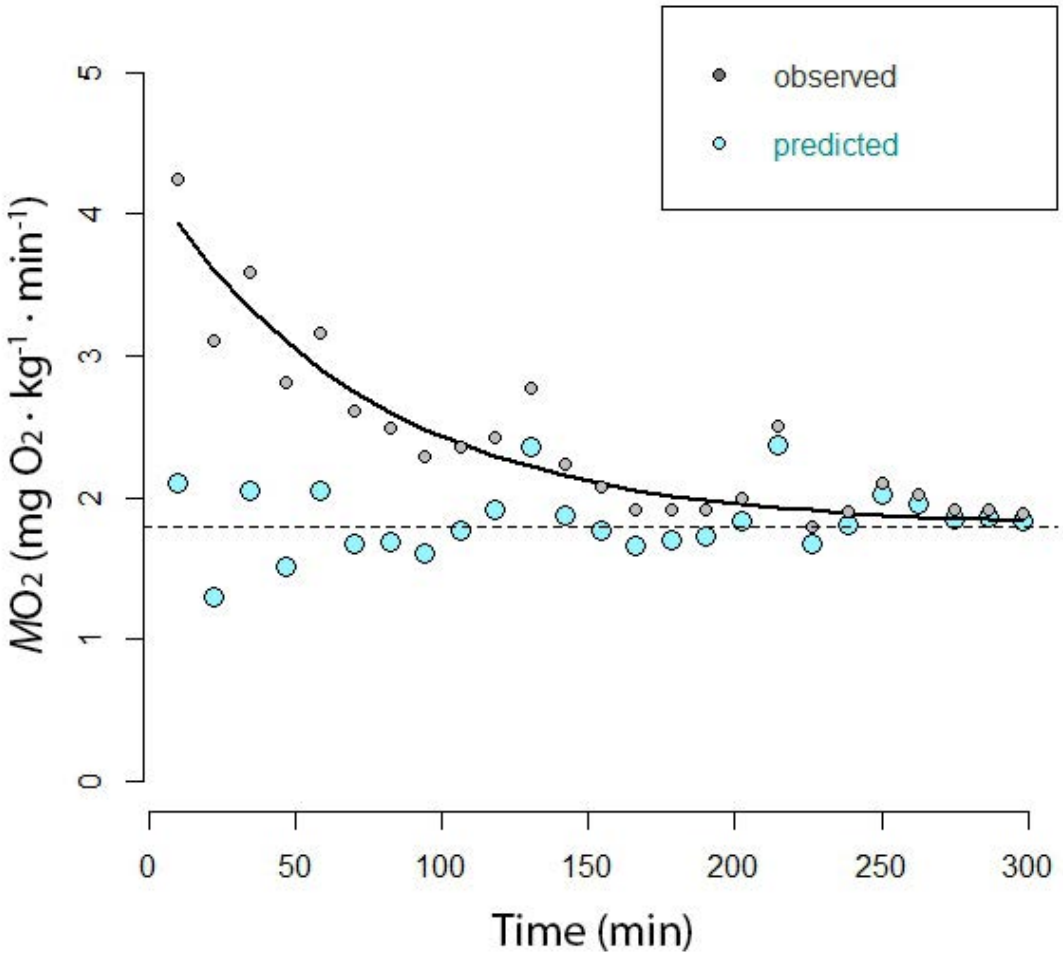

Figure 53 – Oxygen consumption rate ($\dot{M}O_2$) plotted against time from introduction to the respirometer (grey circles) for an unfed fish. The solid black line is an exponential decay function fitted to the observed data. The solid blue circles are the predicted $\dot{M}O_2$ (observed $\dot{M}O_2$ – the corresponding predicted value along the exponential decay function). The horizontal, dashed line represents the SMR.

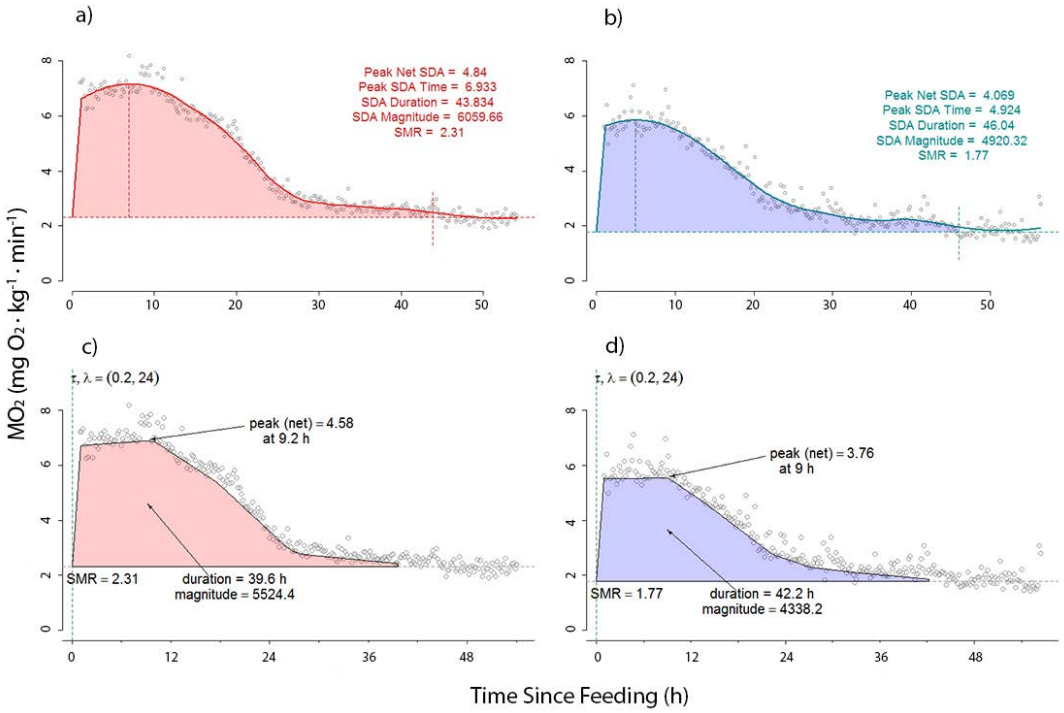

Figure 54 – Oxygen consumption rate ($\dot{M}O_2$; grey circles) plotted against time for two fed fish: panels a and c are data from the same fish, and likewise panels b and d are data from the same fish. A local polynomial regression (loess curve) was fit to the data in panels a and b, and additive quantile regression smoothing (rqss) was fit to the data in panels c and d (Chabot et al, 2016).

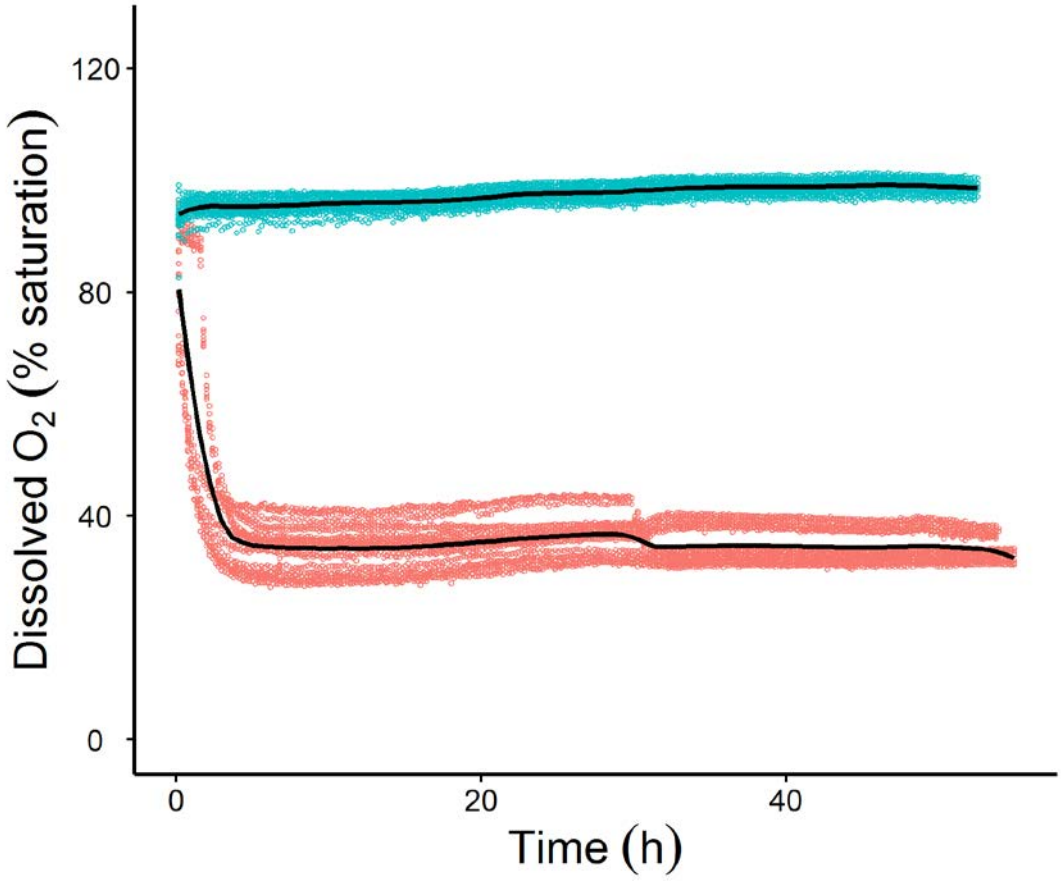

Figure 55 - Dissolved O_2 (% saturation) plotted against time from introduction to the respirometer for *Chapter 5*. Blue and red circles represent DO for the normoxic and hypoxic treatments, respectively. The solid black lines are local polynomial regressions.

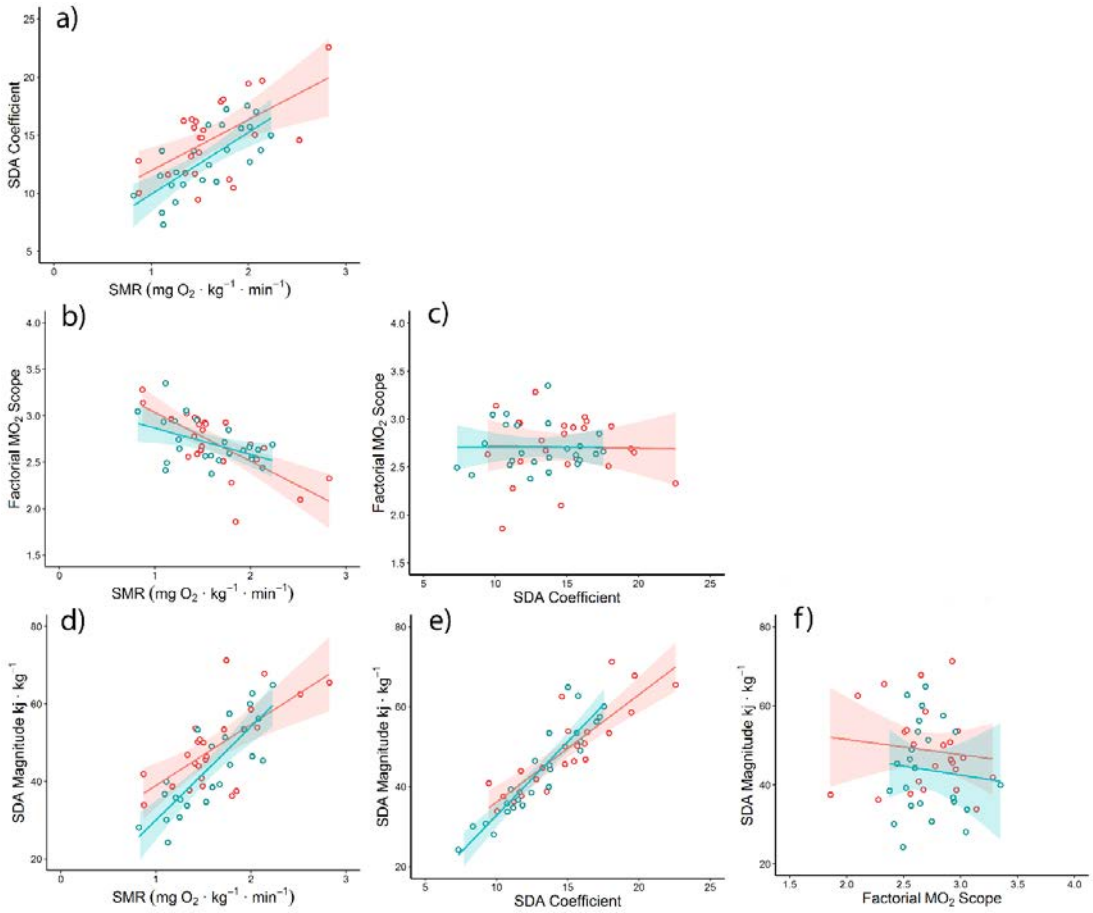

Figure 56 – Relationships between metabolic measurements in SDA trials, plotted according to dissolved O_2 treatment: red circles and line = normoxia, blue circles and line = hypoxia. The solid lines and shaded confidence regions are fitted linear regressions. The overall results from Pearson's correlation tests were: r = 0.646, df = 46, $P < 0.0001$ (panel a); r = -0.614, df = 46, $P < 0.0001$ (panel b); r = -0.015, df = 46, $P = 0.921$ (panel c); r = 0.740, df = 46, $P < 0.0001$ (panel d); r = 0.885, df = 46, $P < 0.0001$ (panel e); r = -0.103, df = 46, $P = 0.486$ (panel f).

APPENDIX D – SUPPORTING INFORMATION FOR CHAPTER 3

The O_{2CRIT} and haematology (haematocrit, Hct; haemoglobin, Hb) measurements from chapter 3 have been re-analysed using the nlme package in R v. 3.2.2 and the results are presented below.

The fixed effects in the model are treatment (normoxia vs. hypoxia) and population (Gladstone (sub-tropical) vs. Broome (tropical)), where tank number and consecutive number of days within the experiment are introduced into the model as random effects. The O_{2CRIT} data analysis is presented first, followed by the haematology (haematocrit and haemoglobin).

The first model assesses the main effects of treatment and population on the response variable (O_{2CRIT}, Hct or Hb), with consecutive number of days introduced as a random effect, and tank number as an additional random effect nested within the number of consecutive days (see Tables 11, 13 and 15).

The results from the first model indicate that there is no significant effect of population on O_{2CRIT} (Table 11) or Hct (Table 13), however population is significant for Hb (Table 15) with the tropical population consistently displaying a lower Hb than the sub-tropical population throughout the experiment. Treatment (DO) is significant for all of O_{2CRIT}, Hct and Hb (Tables 11, 13 and 15). There are no significant interactions between the two main factors (treatment and population) for any of O_{2CRIT}, Hct or Hb.

The second model assesses the control measurements at days 0, 8 and 16 and the hypoxic measurements at days 8 and 16 for the response variables (O_{2CRIT}, Hct and Hb) independently through a variable named '**treatment.id**', which contains the following levels: 1 = normoxia (0 days), 2 = hypoxia (8 days), 3 = normoxia (8 days), 4 = hypoxia (16 days), 5 = normoxia (16 days) (see Tables 12, 14 and 16). The population term was removed from the second model, however the results of adding population into the model for each of O_{2CRIT}, Hct and Hb are presented in Figures 59, 62 and 65, respectively. Tank number is maintained in the model as a random effect. Consecutive days within the experiment is no longer in the model as a random effect, as each combination of normoxia and consecutive days within the experiment is now assessed independently as a fixed effect and is therefore already accounted for in the model design.

Critical O_2 Level (O_{2CRIT})

Table 11 - Output from mixed effects model examining treatment and population effects on O_{2CRIT}

```
> mod.o2crit<-lme(o2.crit~treatment*population, random=~1|consecutive.days/tank.number,
+                         data=mo2.data)
>
> summary(mod.o2crit)
Linear mixed-effects model fit by REML
 Data: mo2.data
      AIC      BIC    logLik
  424.819 441.5801 -205.4095

Random effects:
 Formula: ~1 | consecutive.days
        (Intercept)
StdDev:    1.116116

 Formula: ~1 | tank.number %in% consecutive.days
        (Intercept) Residual
StdDev:    1.212329 2.612836

Fixed effects: o2.crit ~ treatment * population
                                      Value Std.Error DF   t-value p-value
(Intercept)                        18.409094  1.029275 63 17.885494  0.0000
treatmentnormoxia                   2.946468  1.213380 16  2.428314  0.0273
populationgladstone                -0.894477  1.049249 16 -0.852492  0.4065
treatmentnormoxia:populationgladstone -0.139523  1.539971 16 -0.090601  0.9289
 Correlation:
                                   (Intr) trtmnt ppltng
treatmentnormoxia                  -0.571
populationgladstone                -0.517  0.438
treatmentnormoxia:populationgladstone  0.352 -0.639 -0.681

Standardized Within-Group Residuals:
        Min          Q1         Med          Q3         Max
-2.10856528 -0.60482879 -0.06546061  0.71057579  2.41779548

Number of Observations: 85
Number of Groups:
                consecutive.days tank.number %in% consecutive.days
                               3                                22
> anova(mod.o2crit)
                    numDF denDF  F-value p-value
(Intercept)             1    63 665.4509  <.0001
treatment               1    16   9.5304  0.0071
population              1    16   1.5600  0.2296
treatment:population    1    16   0.0082  0.9289
```

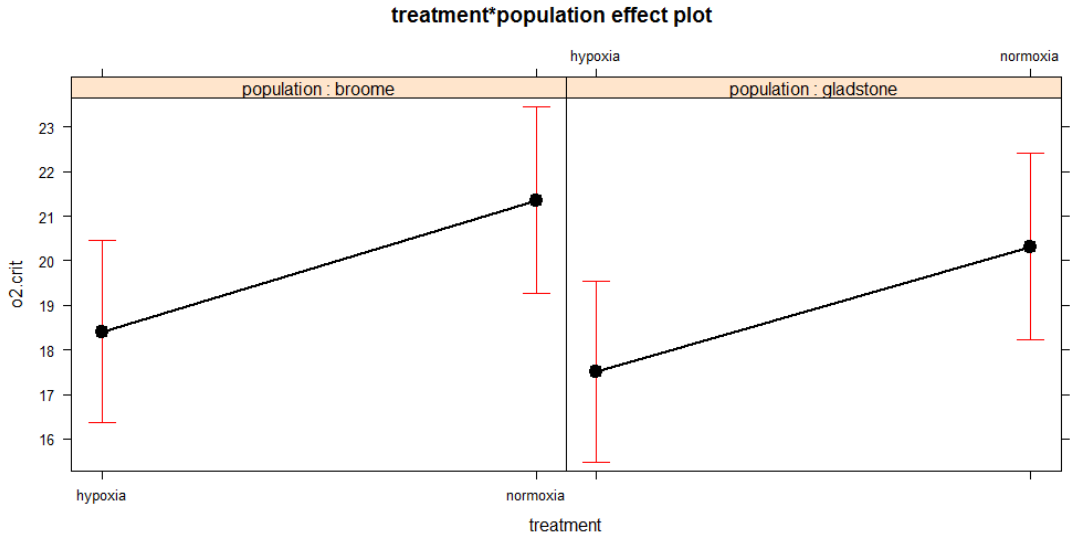

Figure 57 - Effect plot from mixed effects model assessing population and treatment effects on O_{2CRIT}. Black circles and red vertical lines represent the mean and standard error respectively.

Table 12 - Output from mixed effects model examining the effect of each combination of treatment and number of consecutive days within the experimental system on O_{2CRIT}

```
> mod.o2crit.2<-lme(o2.crit~treatment.id, random=~1|tank.number,
+                   data=mo2.data)
>
> summary(mod.o2crit.2)
Linear mixed-effects model fit by REML
 Data: mo2.data
       AIC      BIC    logLik
  417.3564 434.0306 -201.6782

Random effects:
 Formula: ~1 | tank.number
        (Intercept) Residual
StdDev:    1.128432 2.611233

Fixed effects: o2.crit ~ treatment.id
                  Value Std.Error DF   t-value p-value
(Intercept)   20.462500 0.7045083 63 29.045079  0.0000
treatment.id2 -0.858333 0.9963252 17 -0.861499  0.4010
treatment.id3  0.807500 1.4090167 17  0.573095  0.5741
treatment.id4 -3.945156 1.0177873 17 -3.876209  0.0012
treatment.id5  0.545000 1.4090167 17  0.386795  0.7037
 Correlation:
              (Intr) trtm.2 trtm.3 trtm.4
treatment.id2 -0.707
treatment.id3 -0.500  0.354
treatment.id4 -0.692  0.489  0.346
treatment.id5 -0.500  0.354  0.250  0.346

Standardized Within-Group Residuals:
        Min          Q1         Med          Q3         Max
-2.21432104 -0.64467346  0.02194127  0.68852238  2.57814936

Number of Observations: 85
Number of Groups: 22
> anova(mod.o2crit.2)
             numDF denDF   F-value p-value
(Intercept)      1    63 2701.9910  <.0001
treatment.id     4    17    5.4537  0.0052
```

```
> posthoc<-glht(mod.o2crit.2, linfct = mcp(treatment.id = "Tukey"))
> summary(posthoc)

         Simultaneous Tests for General Linear Hypotheses

Multiple Comparisons of Means: Tukey Contrasts


Fit: lme.formula(fixed = o2.crit ~ treatment.id, data = mo2.data,
    random = ~1 | tank.number)

Linear Hypotheses:
           Estimate Std. Error z value Pr(>|z|)
2 - 1 == 0  -0.8583     0.9963  -0.861  0.90763
3 - 1 == 0   0.8075     1.4090   0.573  0.97811
4 - 1 == 0  -3.9452     1.0178  -3.876  < 0.001 ***
5 - 1 == 0   0.5450     1.4090   0.387  0.99506
3 - 2 == 0   1.6658     1.4090   1.182  0.75459
4 - 2 == 0  -3.0868     1.0178  -3.033  0.01951 *
5 - 2 == 0   1.4033     1.4090   0.996  0.85250
4 - 3 == 0  -4.7527     1.4243  -3.337  0.00713 **
5 - 3 == 0  -0.2625     1.7257  -0.152  0.99987
5 - 4 == 0   4.4902     1.4243   3.153  0.01320 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
(Adjusted p values reported -- single-step method)
```

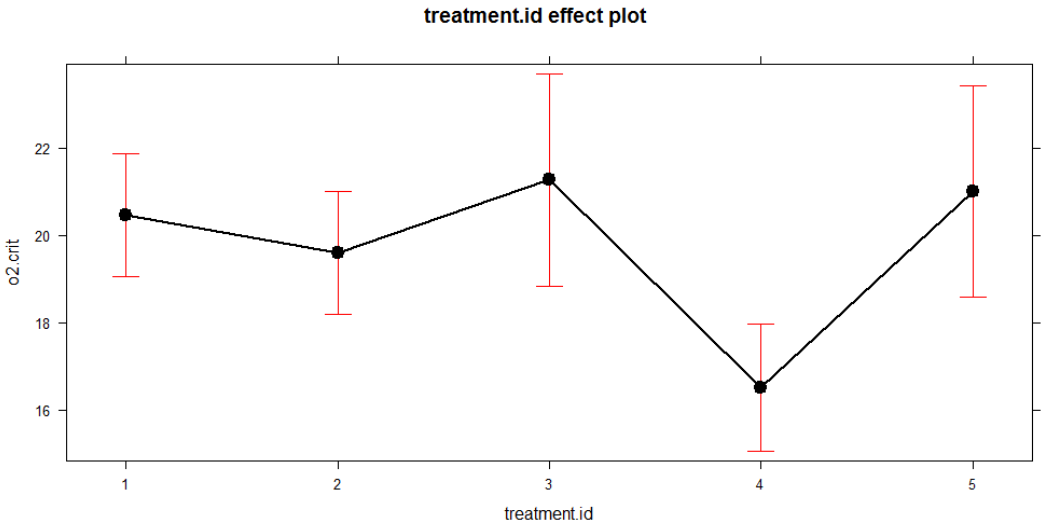

Figure 58 – Effects plot for the mixed effects model from Table 7. Black circles and red vertical lines represent the mean and standard error, respectively.

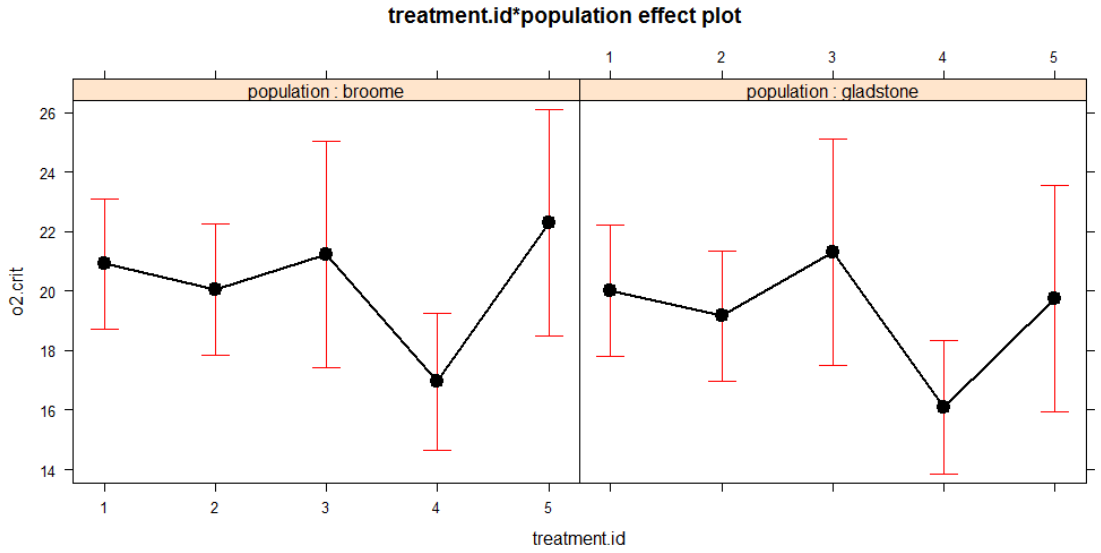

Figure 59 – Effects plot for the mixed effects model from Table 12 with population added to the model as an additional fixed effect.

Haematocrit (Hct)

Table 13 - Output from mixed effects model examining treatment and population effects on haematocrit

```
> mod.hct<-lme(haematocrit~treatment*population, random=~1|consecutive.days/tank.number,
+              na.action="na.omit", data=haem.data)
> summary(mod.hct)
Linear mixed-effects model fit by REML
 Data: haem.data
       AIC      BIC    logLik
  509.2988 527.2493 -247.6494

Random effects:
 Formula: ~1 | consecutive.days
        (Intercept)
StdDev:   0.7683773

 Formula: ~1 | tank.number %in% consecutive.days
        (Intercept) Residual
StdDev:   0.6021027  2.90563

Fixed effects: haematocrit ~ treatment * population
                                        Value Std.Error DF  t-value p-value
(Intercept)                          25.712438 0.7716992 72 33.31925  0.0000
treatmentnormoxia                    -1.046853 0.9904700 22 -1.05693  0.3020
populationgladstone                   1.781908 0.8405714 22  2.11988  0.0455
treatmentnormoxia:populationgladstone -1.735345 1.2568615 22 -1.38070  0.1812
 Correlation:
                                     (Intr) trtmnt ppltng
treatmentnormoxia                    -0.583
populationgladstone                  -0.536  0.418
treatmentnormoxia:populationgladstone  0.356 -0.634 -0.669

Standardized Within-Group Residuals:
        Min          Q1         Med          Q3         Max
-2.27101694 -0.71041266 -0.01024377  0.57979552  3.02967939

Number of Observations: 100
Number of Groups:
                consecutive.days tank.number %in% consecutive.days
                               3                                28
> anova(mod.hct)
                    numDF denDF   F-value p-value
(Intercept)             1    72 2238.4945  <.0001
treatment               1    22    6.1479  0.0213
population              1    22    2.5900  0.1218
treatment:population    1    22    1.9063  0.1812
```

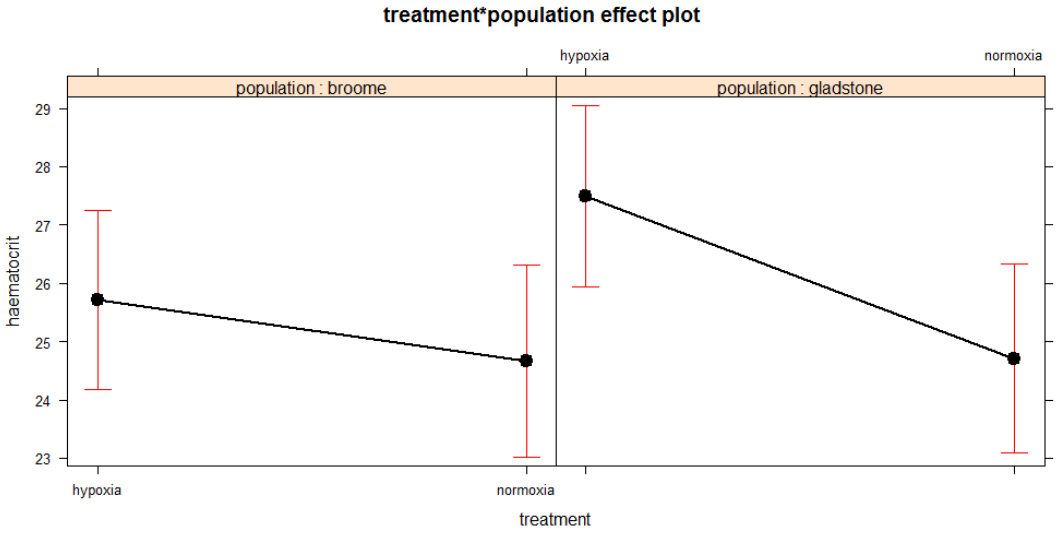

Figure 60 - Effect plot from mixed effects model assessing population and treatment effects on haematocrit. Black circles and red vertical lines represent the mean and standard error respectively.

Table 14 - Output from mixed effects model examining the effect of each combination of treatment and number of consecutive days within the experimental system on haematocrit

```
> mod.hct.2<-lme(haematocrit~treatment.id, random=~1|tank.number,
+                na.action="na.omit", data=haem.data)
> summary(mod.hct.2)
Linear mixed-effects model fit by REML
 Data: haem.data
       AIC      BIC    logLik
  506.0449 523.9221 -246.0225

Random effects:
 Formula: ~1 | tank.number
        (Intercept) Residual
StdDev:   0.8808174 2.892197

Fixed effects: haematocrit ~ treatment.id
                  Value Std.Error DF   t-value p-value
(Intercept)   24.957023 0.6218881 72 40.13105  0.0000
treatment.id2  0.497738 0.8854204 23  0.56215  0.5795
treatment.id3 -0.582023 1.3491718 23 -0.43139  0.6702
treatment.id4  2.597671 0.8916461 23  2.91334  0.0078
treatment.id5 -0.457023 1.3491718 23 -0.33874  0.7379
 Correlation:
              (Intr) trtm.2 trtm.3 trtm.4
treatment.id2 -0.702
treatment.id3 -0.461  0.324
treatment.id4 -0.697  0.490  0.321
treatment.id5 -0.461  0.324  0.212  0.321

Standardized Within-Group Residuals:
        Min          Q1         Med          Q3         Max
-2.24225506 -0.66399541 -0.04113488  0.52021212  3.29100127

Number of Observations: 100
Number of Groups: 28
> anova(mod.hct.2)
             numDF denDF  F-value p-value
(Intercept)      1    72 5922.264  <.0001
treatment.id     4    23    3.057  0.0371
```

```
> posthoc<-glht(mod.hct.2, linfct = mcp(treatment.id = "Tukey"))
> summary(posthoc)

         Simultaneous Tests for General Linear Hypotheses

Multiple Comparisons of Means: Tukey Contrasts


Fit: lme.formula(fixed = haematocrit ~ treatment.id, data = haem.data,
    random = ~1 | tank.number, na.action = "na.omit")

Linear Hypotheses:
           Estimate Std. Error z value Pr(>|z|)
2 - 1 == 0   0.4977     0.8854   0.562   0.9793
3 - 1 == 0  -0.5820     1.3492  -0.431   0.9924
4 - 1 == 0   2.5977     0.8916   2.913   0.0276 *
5 - 1 == 0  -0.4570     1.3492  -0.339   0.9970
3 - 2 == 0  -1.0798     1.3531  -0.798   0.9277
4 - 2 == 0   2.0999     0.8975   2.340   0.1254
5 - 2 == 0  -0.9548     1.3531  -0.706   0.9529
4 - 3 == 0   3.1797     1.3571   2.343   0.1244
5 - 3 == 0   0.1250     1.6932   0.074   1.0000
5 - 4 == 0  -3.0547     1.3571  -2.251   0.1529
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
(Adjusted p values reported -- single-step method)
```

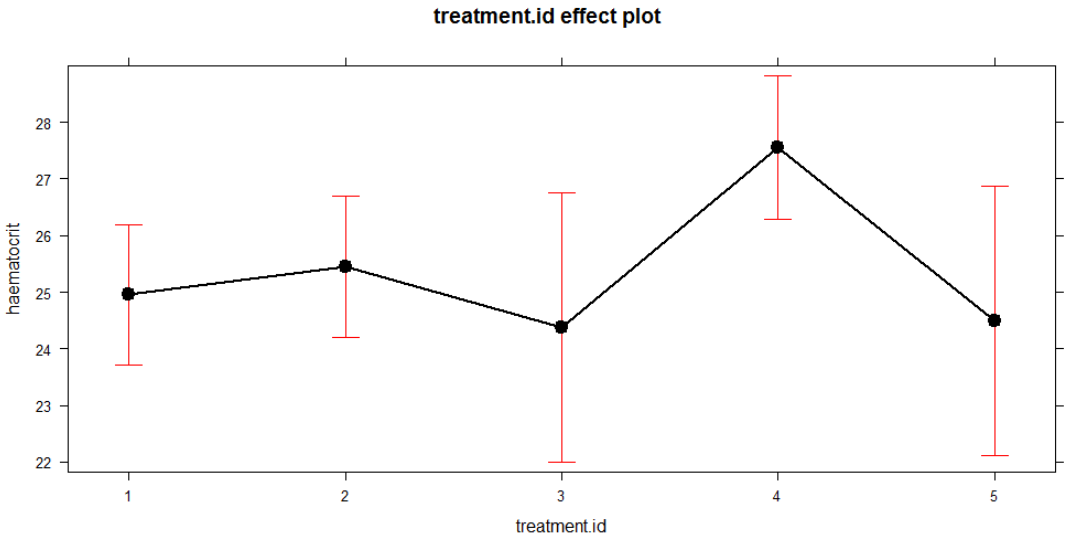

Figure 61 - Effects plot for the mixed effects model from Table 14. Black circles and red vertical lines represent the mean and standard error, respectively.

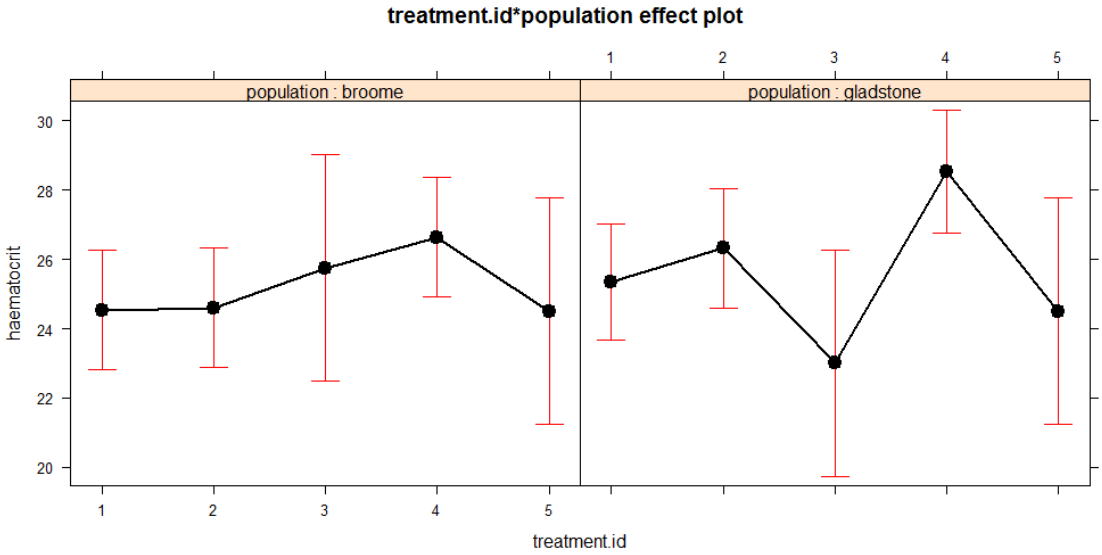

Figure 62 - Effects plot for the mixed effects model from Table 9 with population added to the model as an additional fixed effect.

Haemoglobin (Hb)

Table 15 - Output from mixed effects model examining treatment and population effects on haemoglobin

```
> mod.hb<-lme(hb.calibrated~treatment*population, random=~1|consecutive.days/tank.number,
+             na.action="na.omit", data=haem.data)
> summary(mod.hb)
Linear mixed-effects model fit by REML
 Data: haem.data
       AIC      BIC    logLik
  757.3636 775.5997 -371.6818

Random effects:
 Formula: ~1 | consecutive.days
        (Intercept)
StdDev:    2.097942

 Formula: ~1 | tank.number %in% consecutive.days
         (Intercept) Residual
StdDev: 0.0008005298 9.249339

Fixed effects: hb.calibrated ~ treatment * population
                                    Value Std.Error DF  t-value p-value
(Intercept)                      75.23753  2.195992 76 34.26130  0.0000
treatmentnormoxia                -2.59983  2.866758 22 -0.90689  0.3743
populationgladstone               6.29781  2.450772 22  2.56973  0.0175
treatmentnormoxia:populationgladstone -4.91826  3.645713 22 -1.34905  0.1910
 Correlation:
                                 (Intr) trtmnt ppltng
treatmentnormoxia                -0.594
populationgladstone              -0.548  0.420
treatmentnormoxia:populationgladstone  0.366 -0.635 -0.672

Standardized Within-Group Residuals:
        Min          Q1         Med          Q3         Max
-3.38065223 -0.52212366  0.01457686  0.49638166  3.51861278

Number of Observations: 104
Number of Groups:
                consecutive.days tank.number %in% consecutive.days
                               3                                28
> anova(mod.hb)
                    numDF denDF   F-value p-value
(Intercept)             1    76 2518.5283  <.0001
treatment               1    22    5.0896  0.0343
population              1    22    5.0448  0.0351
treatment:population    1    22    1.8199  0.1910
```

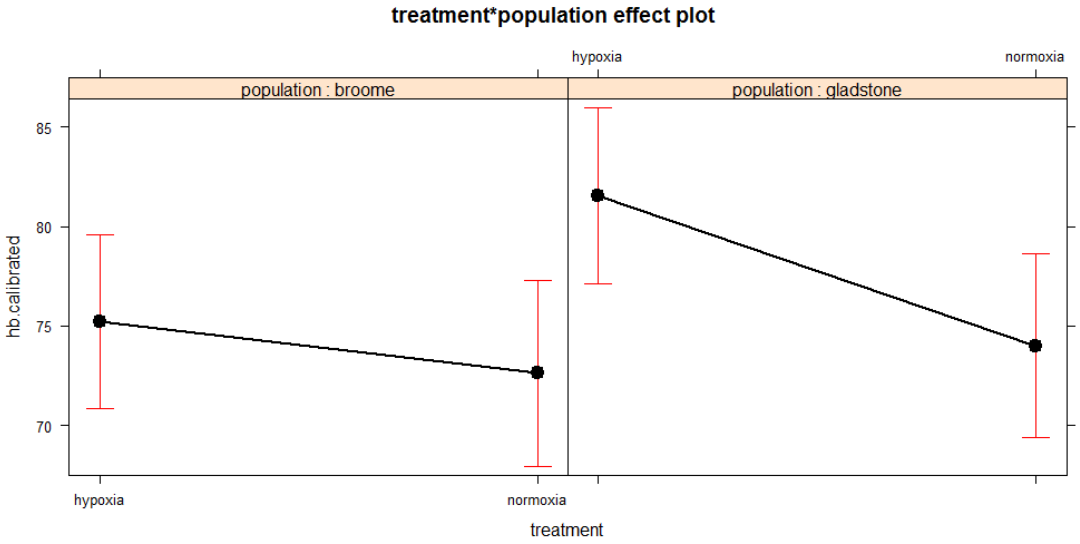

Figure 63 - Effect plot from mixed effects model assessing population and treatment effects on haemoglobin. Black circles and red vertical lines represent the mean and standard error respectively.

Table 16 - Output from mixed effects model examining the effect of each combination of treatment and number of consecutive days within the experimental system on haemoglobin

```
> mod.hb.2<-lme(hb.calibrated~treatment.id, random=~1|tank.number,
+               na.action="na.omit", data=haem.data)
> summary(mod.hb.2)
Linear mixed-effects model fit by REML
 Data: haem.data
       AIC      BIC    logLik
  755.5566 773.7225 -370.7783

Random effects:
 Formula: ~1 | tank.number
        (Intercept) Residual
StdDev:   0.5761127 9.511981

Fixed effects: hb.calibrated ~ treatment.id
                Value Std.Error DF   t-value p-value
(Intercept)   72.75496  1.720589 76 42.28492  0.0000
treatment.id2  3.25622  2.474382 23  1.31597  0.2011
treatment.id3 -0.68608  3.799487 23 -0.18057  0.8583
treatment.id4  8.29414  2.496818 23  3.32188  0.0030
treatment.id5  3.08329  3.799487 23  0.81150  0.4254
 Correlation:
              (Intr) trtm.2 trtm.3 trtm.4
treatment.id2 -0.695
treatment.id3 -0.453  0.315
treatment.id4 -0.689  0.479  0.312
treatment.id5 -0.453  0.315  0.205  0.312

Standardized Within-Group Residuals:
        Min          Q1         Med          Q3         Max
-3.49766354 -0.52075708  0.01277156  0.58506599  3.50644694

Number of Observations: 104
Number of Groups: 28
> anova(mod.hb.2)
             numDF denDF  F-value p-value
(Intercept)      1    76 6562.864  <.0001
treatment.id     4    23    3.171  0.0326
```

```
> posthoc<-glht(mod.hb.2, linfct = mcp(treatment.id = "Tukey"))
> summary(posthoc)

         Simultaneous Tests for General Linear Hypotheses

Multiple Comparisons of Means: Tukey Contrasts


Fit: lme.formula(fixed = hb.calibrated ~ treatment.id, data = haem.data,
    random = ~1 | tank.number, na.action = "na.omit")

Linear Hypotheses:
           Estimate Std. Error z value Pr(>|z|)
2 - 1 == 0   3.2562     2.4744   1.316  0.66952
3 - 1 == 0  -0.6861     3.7995  -0.181  0.99975
4 - 1 == 0   8.2941     2.4968   3.322  0.00739 **
5 - 1 == 0   3.0833     3.7995   0.812  0.92335
3 - 2 == 0  -3.9423     3.8259  -1.030  0.83401
4 - 2 == 0   5.0379     2.5369   1.986  0.26131
5 - 2 == 0  -0.1729     3.8259  -0.045  1.00000
4 - 3 == 0   8.9802     3.8405   2.338  0.12536
5 - 3 == 0   3.7694     4.7908   0.787  0.93103
5 - 4 == 0  -5.2108     3.8405  -1.357  0.64316
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
(Adjusted p values reported -- single-step method)
```

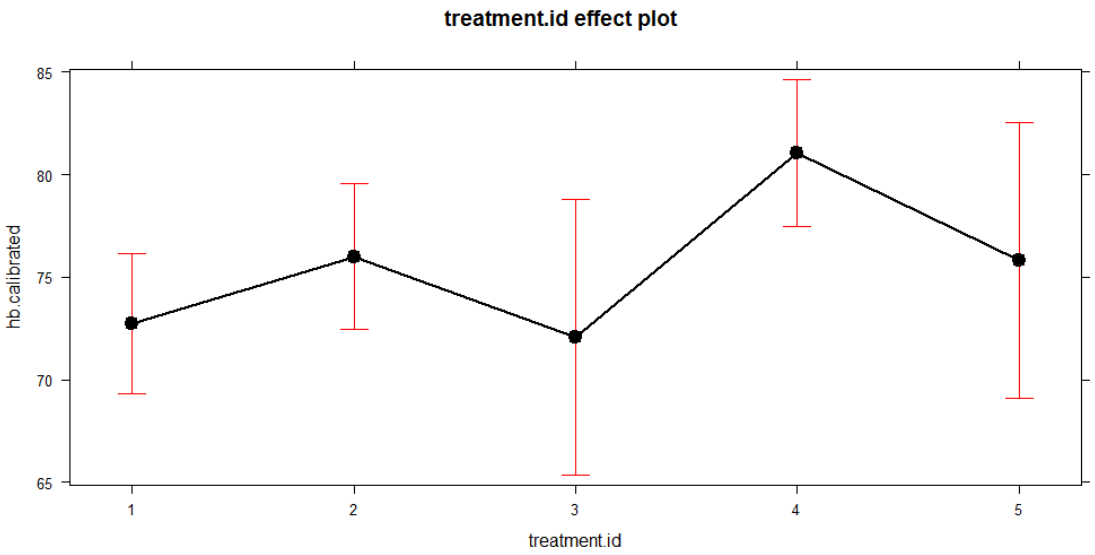

Figure 64 - Effects plot for the mixed effects model from Table 16. Black circles and red vertical lines represent the mean and standard error, respectively.

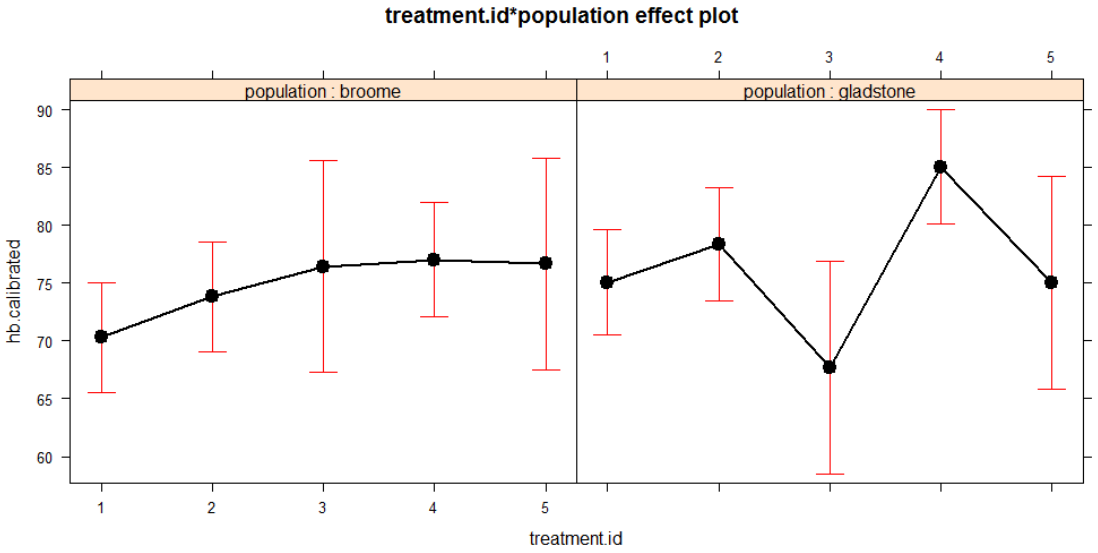

Figure 65 - Effects plot for the mixed effects model from Table 11 with population added to the model as an additional fixed effect.