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# **The Respiratory and Gut Physiology of Fish: Responses to Environmental Change**

Submitted by Nicholas John Rogers to the University of Exeter  
as a thesis for the degree of  
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## Thesis Abstract

Many of the habitats occupied by fish are highly dynamic, naturally demonstrating substantial abiotic fluctuations over diurnal, tidal or seasonal cycles. It is also the case that throughout their 545 million year evolutionary history, fish have existed in aquatic environments very different to those of the present day. However, the past several decades have seen unprecedented rates of environmental change, at local and global scales, arising from human activities. The two major themes of the present thesis are: 1) Respiratory responses of fish to changes in environmental oxygen and temperature in the context of exploring intra- and inter-specific trait variation and its ecological implications 2) The effects of environmental factors (oxygen, carbon dioxide, temperature and seawater chemistry) on the intestinal precipitation and excretion of calcium carbonate by marine teleosts.

In the first study (chapter two) a comprehensive database of fish critical  $PO_2$  ( $P_{crit}$ ) data compiled from the published literature is presented. The systematic review of this literature provided the opportunity to critically examine methodologies for determining  $P_{crit}$  as well as its usefulness as an indicator of hypoxia tolerance in fish. The second study (chapter three) examines whether inter- and intra-specific variation in thermal and hypoxia tolerance in two reef snapper species (*Lutjanus carponotatus* and *Lutjanus adetii*) reflects their distributions across the contrasting biophysical environments of the reef flat and reef slope surrounding Heron Island on the Great Barrier Reef. *L. carponotatus* was clearly the most thermally and hypoxia tolerant of the two species, demonstrating a  $\sim 3.5$  °C wider thermal tolerance zone (higher  $CT_{max}$ , lower  $CT_{min}$ ) and  $\sim 26\%$  lower  $P_{crit}$  than *L. adetii*. These results suggest that the contrasting distribution of these species between flat and slope reef zones is reflected in their physiological tolerances. However, there was no evidence of intra-species variation in tolerance between flat and slope caught *L. carponotatus* individuals, indicating that this species does not form physiologically distinct subpopulations between these reef zones. The third study (chapter four) experimentally quantified the effect of hypercarbia (3000

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$\mu\text{atm}$ ) and hypoxia (50% air saturation) on gut carbonate production by the European flounder (*Platichthys flesus*). Both hypercarbia and hypoxia resulted in a significant increase in carbonate excretion rate (1.5-fold and 2.4-fold, respectively) and acted synergistically when combined. In the final study (chapter five), gut carbonate production was measured in the European flounder undergoing conditions simulating the 'calcite seas' of the Cretaceous. The results of this study support the hypothesis that ocean conditions prevalent during the Cretaceous period resulted in piscine carbonate production rates substantially higher (~14-fold) than the present day. Ultimately, this thesis directly links the environmental physiology of fish at the individual level to wider scale implications (past, present and future), ranging from local ecological patterns all the way up to global carbon cycles.

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# Table of Contents

The Respiratory and Gut Physiology of Fish: Responses to Environmental Change .....	i
Thesis Abstract .....	ii
Acknowledgments .....	iv
Table of Contents .....	v
List of Figures.....	x
<b>Chapter 1</b> .....	1
General Introduction.....	3
The Diversity, Distribution and Value of Fish .....	3
Environmental Change and Ecophysiology.....	4
Respiratory Responses to Environmental Change .....	8
Gut Carbonate Production by Marine Teleosts .....	12
Global Significance of Piscine Carbonate Production .....	16
Environmental Change and Carbonate Production.....	19
Summary .....	21
<b>Chapter 2</b> .....	23
A physiological trait database of hypoxia tolerance ( $P_{crit}$ ) in fish. ....	25
Abstract .....	25
Introduction.....	26
Methods.....	29
Literature Search .....	29
Database Construction.....	30
Results.....	32
Database Coverage .....	32
$P_{crit}$ Methodology .....	33
Biotic and Abiotic Interactions .....	34
Discussion .....	37

---

$P_{crit}$ Methodology .....	37
$P_{crit}$ as a Hypoxia Tolerance Trait.....	42
Biotic and Abiotic Interactions .....	44
Future Applications .....	49
Acknowledgments .....	50
Appendix.....	51
<b>Chapter 3</b> .....	<b>57</b>
Thermal and hypoxia tolerance as traits of fishes using shallow-water coral- reef habitats. ....	59
Abstract .....	59
Introduction.....	60
Materials and Methods .....	65
Study Site.....	65
Ambient Temperature and Dissolved Oxygen Measurements.....	66
Experimental Animals and Thermal Acclimation .....	66
Respirometry.....	68
Determination of SMR, MMR and $AS_{abs}$ .....	70
Determination of $P_{crit}$ .....	71
Determination of $CT_{max}$ , $CT_{min}$ and Thermal Tolerance Zone .....	72
Statistical Analysis .....	74
Results.....	74
Ambient Temperature and Dissolved Oxygen.....	74
SMR, MMR and $AS_{abs}$ .....	76
$CT_{max}$ and $CT_{min}$ .....	79
$P_{crit}$ .....	81
Discussion .....	82
Ambient Temperature and Dissolved Oxygen.....	82
Thermal Acclimation and Tolerance.....	82
Hypoxia Tolerance .....	85
Limitations of Experimental Approach.....	87

---

Tolerance Traits and Inter-habitat Distribution .....	89
Acknowledgements .....	91
Appendix.....	92
ANOVA Summary .....	92
<b>Chapter 4</b> .....	<b>93</b>
Respiratory responses and gut carbonate production during hypoxia and hypercarbia in the European flounder ( <i>Platichthys flesus</i> ). .....	95
Abstract .....	95
Introduction.....	96
Materials and Methods .....	101
Animals and Experimental Series .....	101
Ambient PO <sub>2</sub> and PCO <sub>2</sub> Control.....	102
Respirometry ( <i>series I and II</i> ).....	102
P <sub>crit</sub> Determination ( <i>series I</i> ) .....	105
Gill Ventilation Measurements ( <i>series III</i> ).....	107
CaCO <sub>3</sub> Collection and Analysis ( <i>series IV</i> ) .....	109
Blood Sampling and Analysis ( <i>series IV</i> ) .....	110
Measurement of Drinking Rate ( <i>series V</i> ) .....	111
Statistical Analysis .....	112
Results.....	113
Metabolic Responses.....	113
Ventilatory Responses .....	116
Blood.....	118
Intestinal CaCO <sub>3</sub> Excretion .....	121
Drinking Rate .....	123
Discussion .....	124
Metabolic and Ventilatory Responses.....	124
Blood Measurements .....	129
Gut Carbonate Production and Drinking Rate.....	132
Global Significance .....	136

---

Limitations and Future Work .....	137
Appendix.....	139
ANOVA Summary .....	139
Licence Details.....	139
<b>Chapter 5</b> .....	141
Gut carbonate production in the European flounder ( <i>Platichthys flesus</i> ) under Cretaceous ocean conditions.....	142
Abstract .....	143
Introduction.....	144
Materials and Methods .....	149
Experimental Animals and Temperature Acclimation.....	149
Treatments.....	149
Carbonate Collection and Analysis .....	150
Closed Respirometry .....	152
Statistical Analysis .....	152
Results.....	153
Carbonate Excretion Rate.....	153
Precipitate Ion Analysis.....	154
Crystal Morphology .....	157
Discussion .....	158
Carbonate Excretion Rate.....	158
Precipitate Chemistry and Morphology .....	160
Implications and Future Work .....	162
Appendix.....	165
ANOVA Summary .....	165
License Details.....	165
<b>Chapter 6</b> .....	167
General Discussion and Conclusions.....	168
Thesis Context .....	169
Overview of Major Findings and Implications.....	171

---

General Limitations .....	176
Future Perspectives .....	178
<b>Chapter 7</b> .....	<b>181</b>
Bibliography .....	182

---

## List of Figures

Figure 1. (A) Schematic diagram of the key mechanisms behind intestinal carbonate precipitation and excretion by marine teleosts. Bicarbonate ions ( $\text{HCO}_3^-$ ) originating from both endogenous and extracellular sources, are secreted from epithelial cells into the lumen of the intestine. The accumulation of  $\text{HCO}_3^-$  results in alkalinisation of the gut fluid and subsequently the precipitation of imbibed calcium ( $\text{Ca}^{2+}$ ), as well as some magnesium ( $\text{Mg}^{2+}$ ), to form solid carbonate pellets which are then excreted. (Modified from: Wilson, 2014). (B) Photograph of an unfed European flounder (*Platichthys flesus*) in seawater at 15 °C. White coloured carbonate pellets, excreted over a period of 24 hours, can clearly be observed on the tank floor. .... 15

Figure 2. The equilibria that govern the carbonate system of the marine inorganic carbon cycle. (1) The transfer of  $\text{CO}_2$  between the atmosphere and the ocean's surface (2) Reaction of carbon dioxide and water to form bicarbonate and  $\text{H}^+$  (3) The disassociation of bicarbonate into carbonate and  $\text{H}^+$  (4) The process of calcification (Millero, 2007). .... 18

Figure 3. Diagram illustrating the effects of hypoxia on the standard metabolic rate (SMR), routine metabolic rate (RMR), maximum metabolic rate (MMR) and aerobic scope (AS) of an oxyregulator. .... 29

Figure 4. Example of the term-based search of the SCOPUS® abstract and citation database used to identify papers likely to contain a  $P_{\text{crit}}$  measurement. .... 30

Figure 5. Map illustrating the geographic range currently covered by the database. Red pins indicate the origin of each fish stock for which  $P_{\text{crit}}$  was reported as indicated by the respective study. .... 32

Figure 6. A breakdown of methods used throughout the published literature for measuring  $P_{\text{crit}}$  in fish. Routine metabolic rate (RMR), standard metabolic rate (SMR). 'N<sub>2</sub> equilibration' refers to stripping dissolved oxygen by bubbling the

---

water with nitrogen whereas 'O<sub>2</sub> consumption' refers to the depletion of ambient oxygen through the fish's own respiration. .... 33

Figure 7. The effect of temperature on inter-species P<sub>crit</sub> (black dashed line) and intra-species P<sub>crit</sub> (solid lines). .... 35

Figure 8 (previous five pages). Plots of the species and their respective mean P<sub>crit</sub> (± SE) values that were incorporated into the 'control' dataset. Numbers contained within each bar indicate the temperature (°C) at which P<sub>crit</sub> was determined. Data are ordered by P<sub>crit</sub> (highest to lowest) and grouped by temperature range and water type (marine / freshwater). .... 56

Figure 9. A simplified profile of a typical reef flat - slope zonation. The habitat ranges of the two snapper species *Lutjanus carponotatus* and *Lutjanus adetii* are indicated..... 64

Figure 10. Map illustrating the location of Heron Island and the reef flat and slope fish capture sites (red rectangles)..... 65

Figure 11. A timeline of the temperature acclimation and experimental procedures undergone by each fish from capture to release. .... 67

Figure 12. Diagram of the intermittent flow-through respirometry system used to measure oxygen consumption (MO<sub>2</sub>) rates in snapper. .... 69

Figure 13. A typical trace of metabolic rate (mgO<sub>2</sub> kg<sup>-1</sup> h<sup>-1</sup>) over time in a warm acclimated snapper during an experiment to determine aerobic scope. Three phases are indicated, A: acclimation to the respirometry chamber, B: determination of standard metabolic rate (SMR) and C: determination of maximum metabolic rate (MMR) following a chase to exhaustion..... 71

Figure 14. A typical trace of metabolic rate (mgO<sub>2</sub> kg<sup>-1</sup> h<sup>-1</sup>) in a warm acclimated snapper undergoing progressive hypoxia. The dotted line indicates the critical PO<sub>2</sub> (P<sub>crit</sub>) where two linear regressions (oxyregulating and oxyconforming) intersect. The cluster of MO<sub>2</sub> measurements enclosed in the dotted circle (A) were recorded during overnight acclimation to the

---

respirometry chamber. These measurements were excluded from the calculation of  $P_{crit}$ ..... 72

Figure 15. Diagram of apparatus used to determine  $CT_{max}$ . For determination of  $CT_{min}$  the heating element was replaced with an aquarium chiller but the set-up was otherwise identical. .... 73

Figure 16. Traces showing average (black), minimum (blue), and maximum (red) daily values of temperature and dissolved oxygen at the (a) inner reef flat, (b) outer reef flat, and (c) reef slope (~6m depth) at Heron Island, Great Barrier Reef, during 2013 and 2014. Dotted lines represent minimum and maximum values recorded during the entire period. Table provides the mean of the daily mean, maximum and minimum values as well as the mean and maximum daily range over the entire measurement period at each site. .... 75

Figure 17. Mean ( $\pm$  SEM) standard metabolic rate (SMR) and maximum metabolic rate (MMR) in cold and warm acclimated *Lutjanus adetii* (black), *Lutjanus carponotatus* caught on the slope (grey) and *Lutjanus carponotatus* caught on the flat (white). SMR and MMR were mass corrected across species and habitat (see methods). Columns labelled with different letters indicate a significant difference between means ( $P < 0.05$ , two-way ANOVA)..... 77

Figure 18. Mean ( $\pm$  SEM)  $CT_{max}$ ,  $CT_{min}$  and thermal tolerance zone ( $CT_{max} - CT_{min}$ ) in cold and warm acclimated *Lutjanus adetii* (black), *Lutjanus carponotatus* caught on the slope (grey) and *Lutjanus carponotatus* caught on the flat (white). Columns labelled with different letters indicate a significant difference between means ( $P < 0.05$ , two-way ANOVA)..... 80

Figure 19. Mean ( $\pm$  SEM)  $P_{crit}$  for warm acclimated *Lutjanus adetii* (black), *Lutjanus carponotatus* caught on the slope (grey) and *Lutjanus carponotatus* caught on the flat (white). Columns labelled with different letters indicate a significant difference between means ( $P < 0.05$ , one-way ANOVA). .... 81

---

Figure 20. Diagram summarising the predicted relationships between declining ambient PO <sub>2</sub> and the metabolic, respiratory and osmoregulatory responses in marine teleosts.....	100
Figure 21. Diagram of the fibre-optic intermittent flow through respirometry system used for measuring flounder oxygen consumption rate. Flounder were housed in a sealed chamber within an ambient tank of aerated seawater. Flush / re-circ pumps supplied water to the chamber and dissolved oxygen was measured via oxygen sensor spots connected to a fibre optic oxygen transmitter (OXY-4, Presens, Germany) and recorded on a PC running AutoResp software (Loligo® Systems, Denmark). .....	104
Figure 22. Typical traces of PO <sub>2</sub> and PCO <sub>2</sub> over time during hypoxia only (A) and hypoxic hypercarbia (B) P <sub>crit</sub> trials. ....	106
Figure 23. Illustration of exhalent and inhalant cannula placement for indirect measurement of gill ventilation volume (V <sub>v</sub> ) in European flounder (image modified from original by Hans Hillewaert, 2007). Suture sites of the exhalent cannula are indicated as black stars. Water samples for measurement of dissolved oxygen were withdrawn using a 2.5 ml syringe and ventilation volume calculated by application of the Fick principle.....	108
Figure 24. Diagram of the system used to facilitate daily collection of excreted CaCO <sub>3</sub> precipitates from individual flounder under hypoxic and hypercarbic conditions. Flounder drawn by H. L. Todd (1895). ....	110
Figure 25. Mean (± SEM) P <sub>crit</sub> of European flounder ( <i>Platichthys flesus</i> ) exposed to progressive hypoxia (white) and progressive hypoxic hypercarbia (black). Columns labelled with different letters indicate a significant difference between means (P < 0.05, two-tailed t-test assuming equal variance). ....	113
Figure 26. Plot of average routine metabolic rate (MO <sub>2</sub> ) in flounder (N = 8) across oxygen tensions under progressive hypoxia (A) and hypoxic hypercarbia (B). Dashed vertical lines indicate the mean P <sub>crit</sub> within each treatment.....	114

---

Figure 27. Means ( $\pm$  SEM) of routine metabolic rate ( $MO_2$ ) in flounder (N = 8) during chronic (7 day) exposure to normoxia and three levels of hypoxia. Data points labelled with different letters indicate a significant difference in  $MO_2$  between oxygen tensions (one-way ANOVA,  $P < 0.05$ )..... 115

Figure 28. Mean ( $\pm$  SEM) gill ventilation frequency ( $V_f$ ) in flounder at various oxygen tensions under normocarbica (400  $\mu$ atm) or hypercarbia (3000  $\mu$ atm). Asterisk indicates a significant difference at that oxygen tension ( $P < 0.05$ , two-way ANOVA) relative to normoxia under both  $PCO_2$  treatments..... 116

Figure 29. Mean ( $\pm$  SEM) gill ventilation volume ( $V_v$ ) and  $O_2$  extraction efficiency ( $EO_2$ ) in flounder at various oxygen tensions under normocarbica (400  $\mu$ atm) or hypercarbia (3000  $\mu$ atm). Asterisk indicates a significant difference at that oxygen tension ( $P < 0.05$ , two-way ANOVA) relative to normoxia under both  $PCO_2$  treatments. .... 117

Figure 30. Mean ( $\pm$  SEM) of various caudal puncture blood parameters in flounder (N = 8) held for 7 days under normoxia, hypoxia (50% air saturation), hypercarbia (3000  $\mu$ atm) and combined treatments (Table 8). Columns labelled with different letters indicates a significant difference between treatments (two-way ANOVA,  $P < 0.05$ )..... 119

Figure 31. Davenport diagram summarising blood acid-base status of flounder at 15 °C after 7 days under four different ambient  $PO_2$  and  $PCO_2$  treatments (Table 8, N = normoxia, H = hypoxia (50% air saturation), NH = normoxic hypercarbia (3000  $\mu$ atm), HH = combined hypoxic hypercarbia). Numbered isopleths indicate  $PCO_2$  (mmHg) based on pK equation and  $CO_2$  plasma solubility values from Boutlier et al., 1984)..... 120

Figure 32. Mean ( $\pm$  SEM) intestinal  $CaCO_3$  excretion rate in flounder (N = 8) under different ambient  $PO_2$  and  $PCO_2$  treatments (Table 8). Columns labelled with different letters indicates a significant difference between treatments (two-way ANOVA,  $P < 0.05$ )..... 121

---

Figure 33. Mean ( $\pm$  SEM) drinking rate in flounder (N = 5) under normoxia (>90% air saturation) and hypoxia ( $50 \pm 5\%$  air saturation). Columns labelled with the same letter indicate no significant difference between treatments (two-tailed t-test assuming equal variance,  $P < 0.05$ ). ..... 123

Figure 34. Diagram of the system used to facilitate daily collection of excreted calcium carbonate precipitates from individual flounder held in artificial seawater simulating modern day and Cretaceous seawater chemistries. Two identical set-ups permitted carbonate collection from a total of 8 flounder per treatment. .... 151

Figure 35. Mean ( $\pm$  SEM) carbonate precipitate excretion rate in European flounder (N = 8) under various seawater (SW) and temperature treatments (Table 11). Columns labelled with different letters indicate a significant difference between treatments (post-hoc Tukey test). Significant main and interactive effects of temperature and SW chemistry were detected (two-way ANOVA,  $P < 0.05$ , Table 13). ..... 153

Figure 36. Mean ( $\pm$  SEM) intestinal precipitation rates of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  ions of European flounder under various seawater (SW) and temperature treatments (Table 11). Columns labelled with different letters indicate a significant difference between treatments. Significant main effects of temperature and SW chemistry were detected (two-way ANOVA,  $P < 0.05$ , Table 13). ..... 155

Figure 37. Mean ( $\pm$  SEM) mol% of the minor cation  $\text{Mg}^{2+}$  and anion  $\text{SO}_4^{2-}$  of the predominantly  $\text{CaCO}_3$  precipitates excreted by European flounder under various seawater chemistry (SW) and temperature treatments (Table 11). Columns labelled with different letters indicate a significant difference between treatments (post-hoc Tukey test). Significant main effects of temperature and SW chemistry were detected (two-way ANOVA,  $P < 0.05$ , Table 13). ..... 156

Figure 38. Secondary electron (SE) images of the crystalline structure of the carbonate precipitates produced by European flounder under artificial

---

seawater chemistries (Table 11) simulating that of the modern day (A,B) and the Cretaceous period (C,D). ..... 157

Figure 39. Diagram summarising the overarching concepts presented in this thesis..... 171

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# Chapter 1



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## General Introduction

### The Diversity, Distribution and Value of Fish

Fish exhibit the greatest species diversity of any vertebrate group and occupy almost every aquatic habitat on earth (Jobling, 1995). At the time of writing, over 32,000 fish species have been identified and previously unknown species, particularly those of the deep sea, are being discovered at a rate of ~150 per year (Froese & Pauly, 2015). As a paraphyletic group, fishes are systematically divided into three major classes, the Agnatha (jawless fishes: the hagfishes and lampreys), Chondrichthyes (cartilaginous fishes: sharks and rays) and Osteichthyes (bony fishes). The osteichthyes are sub divided into the lobe-finned fishes (coelacanth and lungfish) and the ray finned fishes (almost exclusively teleosts), which represents the vast majority (>95%) of all extant fish species (Helfman *et al.*, 2009) and are the primary focus of the present thesis.

Fish diversity is roughly equally divided between marine species (~58%) and freshwater species (~41%), with around 1% of species considered to be diadromous (Cohen, 1970). Species of fish can be found in desert springs where water temperatures often exceed 40 °C (*Cyprinodon julimes*; Carson *et al.*, 2014) and beneath the Antarctic ice-sheet where seawater temperatures fall as low as -1.9 °C (*Notothenioidei*; Clarke & Johnstone, 1999). Most fish diversity is concentrated in the tropics, particularly the Indo Pacific region for marine species, and in tropical South America, Africa and Southeast Asia for freshwater species (Helfman *et al.*, 2009). Conservative model estimates suggest a present day, total marine fish biomass of 900 million tonnes (Jennings *et al.*, 2008; Wilson *et al.*, 2009). The majority of this biomass appears to occur around the continental shelves (50% in 17% of total ocean area) with especially high densities along coastal upwelling zones in the mid-latitudes (Jennings *et al.*, 2008). However, recent acoustic measurements suggest that the mesopelagic fishes (Myctophidae) that live in the twilight zone of the open ocean (200 – 1000 m depth), may be at least an order of magnitude

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more populous than previously recognised and hence likely dominate global fish biomass (Irigoien *et al.*, 2014).

The diversity and abundance of fish populations and the ecosystem services that they generate are of significant ecological, economic and societal value (Holmlund & Hammer, 1999). Fish account for the majority of higher trophic levels in aquatic ecosystems and as such exert vital top-down regulation of food web dynamics and nutrient cycling (Vanni *et al.*, 1997; Tait & Dipper, 1998). Thus, fish are a key component for maintaining ecosystem health and resilience (Mumby *et al.*, 2007; Eriksson *et al.*, 2009; Llope *et al.*, 2011). Fish also make major contributions to physical processes such as sediment cycles and carbon fluxes, both at local and global scales (Larkin & Slaney, 1997; Wilson *et al.*, 2009; Perry *et al.*, 2015). In 2012, total world fisheries production was 158 million tonnes (74% finfish) with a total export value of \$129.2 billion (FAO, 2014). Furthermore, it is estimated that 1 billion people, largely in developing countries, rely on fish as their primary animal protein source (FAO, 2014). Additional socio-economic values of fish include the aquarium trade (Wood, 2001), recreational fishing and tourism (Farr, 2013).

## Environmental Change and Ecophysiology

Different fish species and populations inhabit markedly different environments and, as a group, fish exhibit a wide array of adaptive traits (Jobling, 1995, Helfman *et al.*, 2009). Many of the aquatic habitats occupied by fish are themselves highly dynamic environments with substantial abiotic and biotic fluctuations over diurnal, tidal or seasonal cycles. It is also the case that, over their 545 million year evolutionary history (Janvier, 1999), fish have existed in aquatic environments very different to those of the modern age with long term environmental changes occurring throughout the geological past (Pomar & Hallock, 2008). In addition, aquatic environments are currently undergoing unprecedented rates of change at local and global scales as the result of human activities (Doney, 2010; Woodward *et al.*, 2010). Oxygen, carbon dioxide and temperature are major, highly interactive, abiotic variables in

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aquatic environments and they represent the main forms of environmental change discussed throughout the following thesis.

Water contains 20-40 times less oxygen (per unit volume) than air, and oxygen solubility varies inversely with water temperature and salinity (Graham, 1990). Low oxygen solubility combined with slow oxygen diffusion rates in water (~10,000 times slower than in air at 20 °C) means that fluctuations in oxygen levels are a common feature of many aquatic environments. Oxygen depletion occurs when oxygen demand by aerobic organisms or chemical processes exceeds the supply of oxygen from adjacent layers of water, the atmosphere or photosynthesis. Systems that are enclosed or semi-enclosed with limited water exchange or long water retention times, as well as regions where a strong degree of water column stratification occurs, are naturally prone to episodes of hypoxia (Friedrich *et al.*, 2014). The spatial scale at which hypoxia can occur in aquatic environments ranges from huge oceanic oxygen minimum zones (millions of km<sup>2</sup>; Diaz & Breitburg, 2009) to within microhabitats of just a few centimetres such as between respiring coral branches on tropical reefs (Nilsson *et al.*, 2007). The respiratory consumption of oxygen produces CO<sub>2</sub> as a major by-product, hence hypoxia in aquatic systems usually occurs in conjunction with hypercarbia (elevated environmental PCO<sub>2</sub>; Ultsch, 1996; Burnett, 1997; Gilmour, 2001). For example, water PCO<sub>2</sub> in marine tide pools has previously been shown to increase by more than 2.5-fold as PO<sub>2</sub> declines to almost zero over the course of a few hours of night emersion (Truchot & Duhamel-Jouve, 1980).

Eutrophication is a principle driver of hypoxia in aquatic systems and occurs when a nutrient input (nitrogen or phosphorous) leads to a bloom in primary productivity; the subsequent microbial decomposition of which consumes dissolved oxygen via aerobic respiration. Although many aquatic systems can become naturally eutrophic, increased nutrient loading originating from anthropogenic sources (e.g. fertilizer and sewage) is leading to eutrophication at increasing scales and frequency (Zhang *et al.*, 2010). As of 2008, eutrophication associated 'dead zones' (dissolved oxygen  $\leq 2 \text{ mlO}_2 \text{ l}^{-1}$ ) had been reported in over 400 coastal systems around the world, having spread

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exponentially since the 1960s (Diaz & Rosenberg, 2008). Similarly, eutrophication associated hypoxia has become widespread in freshwater systems from the latter half of the 21<sup>st</sup> century to the present, especially in those systems in close proximity to areas of high population density or intensive agriculture (Carpenter *et al.*, 1999; Smith, 2003).

Out of the plethora of anthropogenic drivers of environmental change, the emission of greenhouse gases (predominantly CO<sub>2</sub>) is arguably the most pervasive in terms of its impacts. Since the start of industrial revolution (1750), atmospheric CO<sub>2</sub> concentrations have increased by almost 43% and there is now overwhelming scientific consensus that this is the dominant cause of the observed 0.85 °C rise in average global temperatures over the same period (IPCC, 2014). Climate model projections indicate further temperature rises by the end of the 21<sup>st</sup> century of between 0.3 and 4.8 °C depending on future emission scenarios (IPCC, 2013). Such warming is predicted to cause significant shifts in the earth's climate and a multitude of knock-on effects including changes in oceanic circulation and precipitation patterns, sea level rise and increased occurrence of extreme weather events (IPCC, 2014). Due to high CO<sub>2</sub> solubility in seawater, the oceans are a major sink of CO<sub>2</sub>, and oceanic uptake accounts for nearly a third of anthropogenic carbon added to the atmosphere (Doney *et al.*, 2009). This uptake of CO<sub>2</sub> leads to reduced pH and significant alterations to the carbonate chemistry of seawater, a process known as 'ocean acidification' (Caldeira & Wickett, 2003). Since preindustrial times, ocean surface water pH has declined by a global average of 0.1 (a 26% acidification) and is expected to decrease by a further 0.3 – 0.4 by 2100, under a 'business as usual' emission scenario (Orr *et al.*, 2005).

Climate change is likely to promote hypoxia in aquatic environments for several reasons. Firstly, increasing water temperature results in reduced oxygen solubility as well as increased respiration rates (microbial and macrofaunal) and hence faster oxygen depletion (Diaz & Breitburg, 2009). Additionally, warming is likely to exaggerate and increase the persistence of stratification, particularly in closed systems such as lakes (Ficke *et al.*, 2007). In areas where precipitation rates are predicted to increase, greater land run-

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off and hence nutrient loading of rivers, lakes and coastal waters will intensify eutrophication and associated hypoxia (Rabalais *et al.*, 2009). Furthermore, changes in oceanic circulation are predicted to result in a 1 to 7% reduction in the global ocean O<sub>2</sub> inventory ('ocean deoxygenation') over the next century leading to a significant expansion of oxygen minimum zones (Keeling *et al.*, 2010).

Ice-core data suggests that atmospheric CO<sub>2</sub> concentrations are currently higher than at any other time in past 800,000 years (Luthi *et al.*, 2008). In the more distant geological past however (50 – 300 MYa), sedimentary and fossil records indicate that CO<sub>2</sub> levels and global temperatures have been substantially higher than at present (Pomar & Hallock, 2008). Several sudden warming events also appear to have occurred around 55, 120 and 183 million years ago, likely arising from sudden releases of methane hydrates from beneath the seabed (Zachos *et al.*, 2001; Kemp *et al.*, 2005). In addition, large fluctuations in atmospheric O<sub>2</sub> concentration have occurred over the past 300 million years. Aquatic oxygen levels are thought to have been 25 to 50% lower than present throughout the Jurassic, Triassic and early Cretaceous; which were also periods of spectacular teleost fish diversification (Randall *et al.*, 2014). A more detailed discussion of environmental conditions prevalent during the Cretaceous period is incorporated into chapter 5 of the present thesis.

The interactions between fish and their environment, specifically how the physiology of fishes is affected by and regulated in response to environmental factors (*fish ecophysiology*), provides the key to understanding the survival and maintenance of fish populations in changing aquatic environments (Rankin & Jensen, 1993). Not only does this provide fundamental biological insights, but such understanding, and the predictive capacity it generates, is increasingly critical given the extent of environmental change currently ongoing and the desire to conserve the ecosystem services that fish provide (Seebacher & Franklin, 2012; Cooke *et al.*, 2013). Reviewing all the interactions between the physiology of fish and their environment is far beyond the scope of this introduction. Instead, the remaining sections focus on the

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major themes of the present thesis: respiratory responses and gut carbonate production, and the linkages therein.

## Respiratory Responses to Environmental Change

### *Hypoxia*

The simplest response to hypoxia is avoidance (i.e. escape behaviours) but where this is not feasible fish demonstrate an array of respiratory adjustments in order to minimize any imbalance between oxygen demand and oxygen delivery. Most fish species can be considered 'oxyregulators' in that they initially maintain a stable rate of oxygen consumption and hence aerobic metabolism as environmental  $PO_2$  declines. Considering that ATP production via aerobic metabolism is 15- to 30-fold more efficient than anaerobic metabolism per unit of substrate consumed, the advantages of oxyregulation as a strategy for maintaining cellular energy balance are clear (Richards, 2009). The  $PO_2$  at which a fish can no longer maintain a stable oxygen consumption rate is referred to as the 'critical  $PO_2$ ' ( $P_{crit}$ ) and marks the transition between oxyregulation and oxyconforming. The significant inter and intra-specific variation in  $P_{crit}$  is the focus of chapter two in the present thesis.

Hyperventilation is arguably the primary physiological mechanism by which fish attempt to maintain oxygen consumption in the face of declining ambient  $PO_2$  (Perry *et al.*, 2009). The hypoxic ventilatory response (HVR) promotes branchial oxygen uptake by increasing the volume of water passing over the gills through combinations of both increases in ventilation frequency and amplitude. Like  $P_{crit}$ , the HVR is highly variable between species and is itself dynamic in nature - changing depending on the pattern, intensity and duration of the hypoxic exposure (Perry *et al.*, 2009; Porteus *et al.*, 2011). Fish typically demonstrate a hyperbolic relationship between ventilation and ambient  $PO_2$ , the shape of which generally reflects the position of the  $O_2$ -haemoglobin disassociation curve (Porteus *et al.*, 2011). Given the density and viscosity of water as the ventilated medium, there is a significant energetic cost associated with ventilation in fish. For example, in rainbow trout (*Oncorhynchus mykiss*) performing minimal exercise at normoxia, the relative oxygen cost of the

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branchial pump has been shown to account for 10 – 15% of total oxygen uptake (Farrell & Steffensen, 1987). Therefore, maintaining metabolic rate by hyperventilation becomes a 'battle of diminishing returns' as ambient PO<sub>2</sub> declines and eventually the costs associated with increasing ventilation will exceed the benefits obtained (Perry *et al.*, 2009). During severe hypoxia, many fish species, especially those that naturally inhabit stagnant freshwater habitats, perform a behaviour called aquatic surface respiration whereby they ventilate their gills at the oxygen enriched water-air interface (Kramer & McClure, 1982). A few fish species have even evolved to directly extract oxygen from the atmosphere via highly vascularized air breathing organs modified from the gastrointestinal tract, swim bladder or pharynx (Chapman & McKenzie, 2009).

To maintain oxygen extraction and tissue delivery, two major blood parameters, haematocrit and haemoglobin-O<sub>2</sub> binding, can be modified in fish undergoing hypoxia. An acute increase in haematocrit occurs as the result of circulating stress hormones (catecholamines) that activate receptors in the spleen to trigger the release of red blood cells into circulation. In response to chronic hypoxia exposure (>7 days), an increase in haematocrit independent of the spleen has been observed in rainbow trout as the result of hormonal stimulation of the kidney by erythropoietin (EPO; Lai *et al.*, 2006). Increased concentration of red blood cells enhances the oxygen carrying capacity of the blood, but due to the resulting increase in blood viscosity, comes at the cost of more energetically expensive cardiac pumping (Wells, 2009). Indeed chronic hypoxic exposure (40 days) produced no increase in haematocrit in either turbot (*Scophthalmus maximus*) or seabass (*Dicentrarchus labrax*) indicating that these species employ more energetically favourable strategies for increasing blood oxygen carrying capacity (e.g. increasing haemoglobin-O<sub>2</sub> affinity; Pichavant *et al.*, 2003).

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There appear to be two principle modulators of haemoglobin-O<sub>2</sub> affinity. Firstly, allosteric regulation of oxygen affinity can be achieved via changes in the molar ratio of Hb to organic phosphates. These phosphates (primarily ATP and GTP) are bound to specific sites within the Hb tetramers and serve to stabilize their structure in the low affinity conformation. A decrease in the concentration of these phosphates therefore results in increased haemoglobin-O<sub>2</sub> affinity and hence enhanced oxygen uptake at the gills (Val, 2000). Secondly, the circulation of catecholamines as an acute hypoxic response activates  $\beta$ -adrenergic Na<sup>+</sup>/H<sup>+</sup> exchange protein that is located on the membrane of red blood cells. By facilitating the outward movement of H<sup>+</sup> and the inwards movement of Na<sup>+</sup> ions this process results in the alkalinisation of intracellular pH and hence increased haemoglobin-O<sub>2</sub> affinity via the Bohr effect (Nikinmaa, 1983). Furthermore, the inward flow of Na<sup>+</sup> into the red blood cell results in an osmotic influx of water causing cell swelling. This swelling produces an equilibrium that favours non phosphate-bound Hb and therefore further promotes haemoglobin-O<sub>2</sub> affinity (Nikinmaa, 2001; Wells, 2009).

### *Hypercarbia*

The permeability of the gills to respiratory gases means that elevated water PCO<sub>2</sub> is paralleled by elevated blood PCO<sub>2</sub>. This initially gives rise to a respiratory acidosis which must be compensated in order to protect protein functioning. The low capacitance of water for O<sub>2</sub> means that fish must maintain high ventilation volumes, even at normoxia, to meet oxygen demand (Gilmour, 2001). This, combined with the high solubility of CO<sub>2</sub> in water, means that fish are hyperventilated with respect to CO<sub>2</sub>. Indeed fish typically exhibit arterial PCO<sub>2</sub> of 1 - 4 mmHg; 10-fold lower than that of humans (Ultsch, 1996; Perry & Gilmour, 2006). At these low levels there is limited capacity for further ventilatory reductions in blood PCO<sub>2</sub> in response to hypercarbia. Hence, fish predominantly control blood pH through adjustments of blood HCO<sub>3</sub><sup>-</sup> via differential Na<sup>+</sup>/H<sup>+</sup> and Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchange at the gills (Claiborne *et al.*, 2002; Evans *et al.*, 2005). Through the accumulation of blood HCO<sub>3</sub><sup>-</sup>, fish demonstrate a high capacity for acid-base regulation, generally restoring

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normal blood pH within 10 - 24 hours of moderate hypercarbia exposure (Melzner *et al.*, 2009).

### *Temperature*

The vast majority of teleost species demonstrate no ability to regulate body temperature independent of their environment and as such, fish can generally be considered as ectotherms. Rare exceptions to this include the partial endothermy exhibited by some members of the Scombroidei family (e.g. tunas and billfishes) as well as the full-body form of endothermy recently observed in the mesopelagic opah, (*Lampris guttatus*; Wegner *et al.*, 2015). As ectotherms, virtually all biochemical, physiological and life history activities of fish are affected by temperature (Portner & Peck, 2010). Temperature governs the rate of the reactions that constitute metabolism and hence increasing temperature results in increased oxygen demand. Based on an interspecific curve of 69 teleost fish species across a temperature range of 0 - 30 °C, Clarke & Johnson (1999) derived an average inter-specific  $Q_{10}$  for resting metabolism of 1.83 and a median intra-specific  $Q_{10}$  of 2.4. It has been hypothesized that the respiratory and circulatory capacity for meeting oxygen demand is the primary determinant of the thermal tolerance limits of aquatic ectotherms, a concept referred to as oxygen- and capacity-limited thermal tolerance (OCLTT; Portner & Knust, 2007; Portner & Farrell 2008). Whilst OCLTT arguably provides a useful conceptual framework, recent studies have presented a number of exceptions that call into question the generality of the OCLTT hypothesis (Overgaard *et al.*, 2012, Clark *et al.*, 2013; Wang *et al.*, 2014; Ern *et al.*, 2014). Other factors that may dictate thermal tolerance include nervous and mitochondrial function, as well as protein and membrane stability (Wang *et al.*, 2014).

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## Gut Carbonate Production by Marine Teleosts

With very few exceptions (Raymond, 1993), teleost fish in the marine environment osmoregulate in order to maintain an internal salt and water balance that is hypo-osmotic to their surrounding environment (320 mOsm kg<sup>-1</sup> compared with ~1000 mOsm kg<sup>-1</sup>). To prevent dehydration via osmotic water loss and the passive uptake of ions, marine teleosts must continuously drink the external seawater. Drinking rates reported in the literature range from 1 - 5 ml kg<sup>-1</sup> h<sup>-1</sup> (Marshall & Grosell, 2006). The intake and subsequent processing of seawater by marine teleosts for osmoregulatory purposes has been studied in great detail since the classic experiments of Krogh, Smith and Keys (Krogh, 1929; Smith, 1932; Keys 1931).

Marine teleosts have evolved numerous physiological mechanisms that integrate the functions of the gills, kidney, urinary bladder and gastrointestinal tract, in order to achieve a net retention of imbibed water and the excretion of excess ions (Marshall & Grosell, 2005). The intestine plays a prominent role within this osmoregulatory system as the site of water absorption and the site where unabsorbed divalent cations are eliminated. Water is absorbed across the entire length of the intestine at rates of between 2 and 6  $\mu\text{l cm}^{-2} \text{h}^{-1}$ , accounting for up to 85% of the imbibed seawater (Wilson *et al.*, 1996, 2002; Grosell, 2006). The movement of water across the intestinal epithelium is passive and secondary to the active net transport of solutes in the same direction, a process referred to as 'solute-linked water transport'. In the marine teleost intestine, this process is primarily driven by apical Na<sup>+</sup>-Cl<sup>-</sup> and Na<sup>+</sup>-K<sup>+</sup>-2Cl<sup>-</sup> cotransporters (House & Green, 1965; Lotan & Skadhauge, 1972; Skadhauge, 1982). The significant salt load in the blood that results from this process is excreted at the gills via specialised mitochondria-rich cells (Foskett & Scheffey, 1982; Marshall & Grosell, 2005).

There is a second mechanism involved in fluid absorption and osmoregulation in the intestines of marine teleosts that has only come to light more recently - the intestinal secretion of bicarbonate (HCO<sub>3</sub><sup>-</sup>). It was Walsh *et al.* in 1991, following studies in the Gulf toadfish (*Opsanus beta*), who first reported that

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imbibed seawater becomes alkaline (pH 8.4 - 9.0) and rich in  $\text{HCO}_3^-$  as it passes along the intestine (Walsh *et al.*, 1991). The high concentration of  $\text{HCO}_3^-$  in the intestine occurs as a result of apical  $\text{Cl}^- / \text{HCO}_3^-$  exchange which plays a significant role in the active uptake of  $\text{Cl}^-$  by the intestine (Wilson *et al.* 1996; Grosell & Jensen, 1999). Indeed, this anion exchange has been shown in the European flounder (*Platichthys flesus*) to contribute up to 70% of net  $\text{Cl}^-$  uptake and water absorption by the intestine (Grosell *et al.*, 2005). There are two major sources of this intestinal  $\text{HCO}_3^-$ . Firstly there is the endogenous source whereby  $\text{HCO}_3^-$  is produced via the hydration of  $\text{CO}_2$  within the epithelial cells of the intestine, a cellular reaction that is catalysed by carbonic anhydrase. The intestinal epithelial cells are rich in mitochondria and intestinal tissue demonstrates high mass-specific metabolism such that intracellular  $\text{CO}_2$  production alone is sufficient to sustain high  $\text{HCO}_3^-$  secretion rates (Grosell, 2011). The second source is extracellular whereby  $\text{HCO}_3^-$  is transported from the blood, a process that is facilitated by basolateral  $\text{Na}^+/\text{HCO}_3^-$  cotransporters (Taylor *et al.*, 2010).

The seawater consumed by marine teleosts is rich in the divalent cations  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (~10 and ~53 mM respectively). The alkalinisation of imbibed seawater and the secretion of  $\text{HCO}_3^-$  results in the precipitation of  $\text{Ca}^{2+}$  (as well as some  $\text{Mg}^{2+}$ ) as insoluble carbonates which are then excreted (Figure 1). This process is highly important in preventing the build-up of these divalent cations which could potentially reach toxic levels and lead to an osmotic gradient that would be detrimental to fluid absorption (Wilson *et al.*, 2002). In addition, the precipitation process limits the intestinal absorption of calcium that would otherwise be excreted in urine via the kidneys. The extremely low urine flow rates in marine teleosts puts them at high risk of developing kidney stones if urinary calcium becomes too concentrated (Wilson & Grosell, 2003).

The carbonate precipitates formed in the gut are excreted as mucus-coated pellets or are incorporated with faeces when the fish are feeding (Walsh *et al.*, 1991). Formation of these precipitates occurs at a surprisingly high rate. Collection and titration of excreted carbonates have revealed production rates ranging from 18 to 40  $\mu\text{molC kg}^{-1} \text{h}^{-1}$  in the temperate European flounder and

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subtropical Gulf toadfish (Wilson *et al.* 2009). It has previously been suggested that natural intra- and interspecific variation in carbonate production rate occurs primarily due to differences in body mass and temperature (Jennings & Wilson, 2009). Carbonate production by fish is assumed to be proportional to their metabolic rate. Because passive ion and water fluxes occur mainly at the gills, drinking rate and active ion transport (the two factors thought to determine carbonate production rate) are proportional to gill ventilation and perfusion which are in turn proportional to the rate of oxygen uptake, i.e. metabolic rate (Gonzalez & McDonald, 1994). Mass specific metabolism in aquatic animals is inversely related to body size, increasing ~1.6-fold per 10-fold decrease in body mass. As previously discussed, increasing temperature results in an exponential increase in metabolism, typically 2.4-fold for every 10 °C rise (Clark & Johnston, 1999). Experimental data of carbonate excretion rate in European flounder and Gulf toadfish across a range of temperature and body mass have shown a similar relationship to that typically observed for metabolic rate over the same range (Wilson *et al.*, 2009).

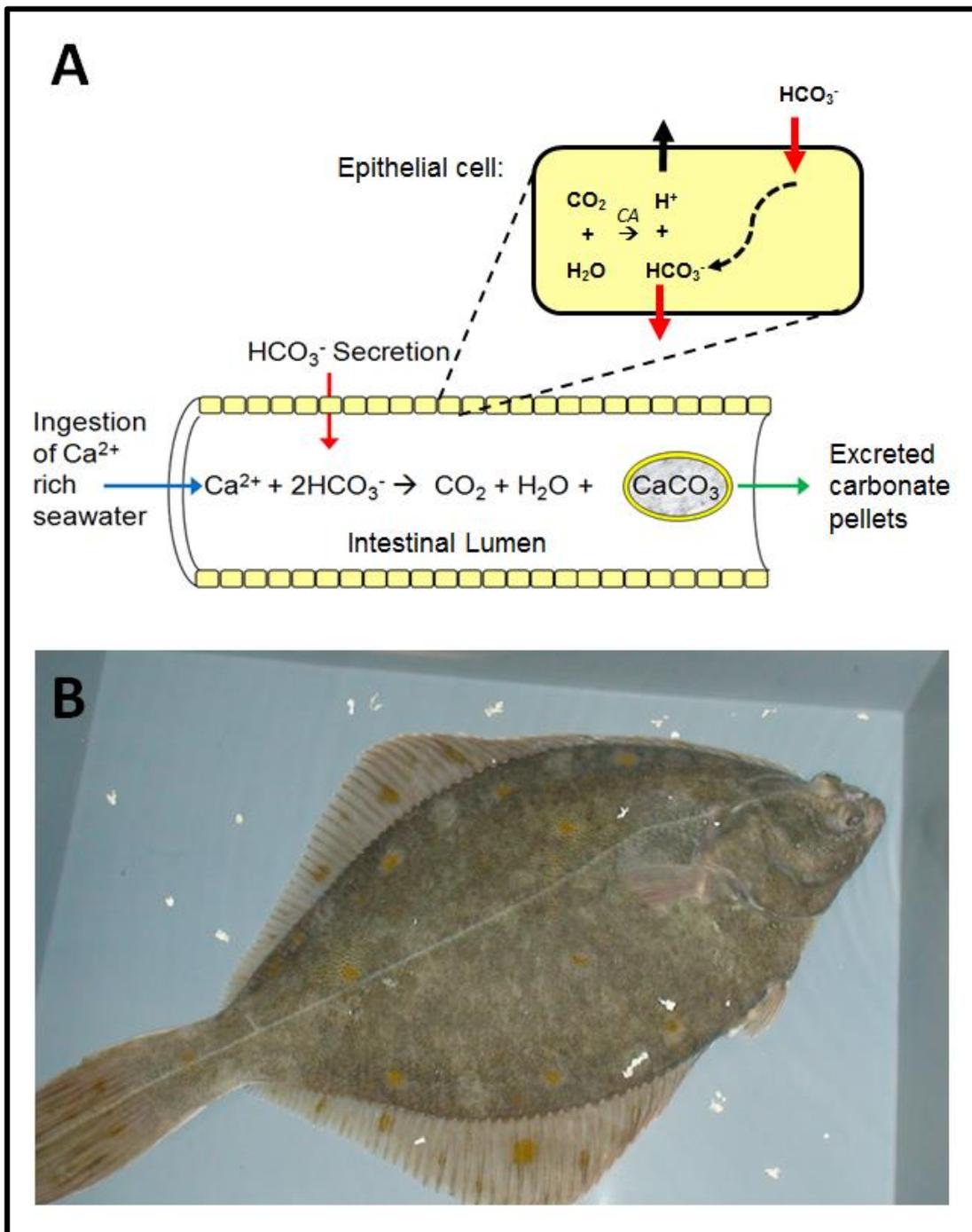


Figure 1. (A) Schematic diagram of the key mechanisms behind intestinal carbonate precipitation and excretion by marine teleosts. Bicarbonate ions ( $\text{HCO}_3^-$ ) originating from both endogenous and extracellular sources, are secreted from epithelial cells into the lumen of the intestine. The accumulation of  $\text{HCO}_3^-$  results in alkalinisation of the gut fluid and subsequently the precipitation of imbibed calcium ( $\text{Ca}^{2+}$ ), as well as some magnesium ( $\text{Mg}^{2+}$ ), to form solid carbonate pellets which are then excreted. (Modified from: Wilson, 2014). (B) Photograph of an unfed European flounder (*Platichthys flesus*) in seawater at 15 °C. White coloured carbonate pellets, excreted over a period of 24 hours, can clearly be observed on the tank floor.

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## Global Significance of Piscine Carbonate Production

The oceanic carbon cycle consists of both an organic and inorganic pump that combine to effectively regulate CO<sub>2</sub> fluxes between the oceans and the atmosphere. The organic pump occurs via autotrophs that use light energy to convert dissolved CO<sub>2</sub> into organic molecules. Some of this organic carbon eventually sinks into the deep ocean where it is sequestered in the sediments (Field *et al.*, 1998). Dissolved forms of carbon occur in the ocean mostly as carbonate and bicarbonate ions. An important component of the marine inorganic carbon cycle is the combination of dissolved carbonate with dissolved calcium to form solid calcium carbonate, a process known as calcification (Figure 2). This is a biogenic reaction that occurs primarily in the formation of the carbonate shells in marine plankton such as coccolithophorids. Upon death, these calcium carbonate skeletons sink through the water column and are either dissolved or deposited in ocean sediments (Feely *et al.*, 2004).

The discovery that marine teleosts rapidly produce large quantities of calcium carbonate, led on to the suggestion that fish may play a significant role in the global oceanic carbonate budget, an idea that had not previously been considered (Walsh *et al.*, 1991). Wilson *et al.* (2009) attempted to quantify the calcium carbonate contribution of marine fish on a global scale. Two independent models were used to determine global fish biomass and describe size composition and abundance across the oceans. Estimates of fish biomass were then combined with average local sea temperatures and individual fish CaCO<sub>3</sub> excretion rates to produce an estimate of global piscine carbonate production. This approach revealed conservative estimates that ranged from  $3.2 \times 10^{12}$  to  $8.9 \times 10^{12}$  mol year<sup>-1</sup> which represents between 2.7 to 15.4% of total global new CaCO<sub>3</sub> in the surface oceans (Wilson *et al.*, 2009). Applying less conservative but arguably realistic assumptions regarding the effects of variables such as temperature, feeding and body size on fish carbonate production, reveals model estimates up to three times greater (Wilson *et al.*, 2009; Wilson, 2014). These findings suggest that fish make a significant and previously unrecognised contribution to the marine inorganic carbon cycle.

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An important question arising from the discovery that fish produce a significant proportion of new surface ocean  $\text{CaCO}_3$  is what happens to it once it has been excreted. The fate of fish carbonates will be closely related to their chemistry. The chemical composition of fish produced  $\text{CaCO}_3$  precipitates differs substantially to that of the more traditionally recognised sources of ocean  $\text{CaCO}_3$ , namely the shells and skeletons of calcifying planktonic organisms. Walsh *et al.* found via X-ray diffraction, that the Mg content of toadfish carbonate pellets was 2.4-fold higher than the maximum as predicted by stoichiometry (Walsh *et al.*, 1991). Those authors suggest that this is the result of the high  $\text{Mg}^{2+}$  content of intestinal fluids that becomes trapped in the carbonate pellets during precipitation. More recent work shows that fish derived carbonates typically resemble the crystal make-up of high magnesium calcite but that individual crystal magnesium content ranges widely between 0.5 and 40 mol% (Wilson *et al.*, 2009; Perry *et al.*, 2011; Salter *et al.*, 2012).

High magnesium content means that fish carbonates are more soluble than the other major forms of oceanic  $\text{CaCO}_3$ : calcite and aragonite. Increasing pressure and declining temperature with depth causes the dissolution of  $\text{CaCO}_3$  and results in its under-saturation in the deep ocean. This leads to an increase in the concentration of dissolved  $\text{HCO}_3^-$  and  $\text{CO}_3^{2-}$  with depth. This is measured as an increase in the total alkalinity of seawater. The dissolution of a particular form of  $\text{CaCO}_3$  is predicted to occur once it reaches its chemical lysocline, a function of depth and temperature. The higher solubility of fish carbonates means that they are likely to dissolve at shallower depths than the more traditionally recognised forms of marine  $\text{CaCO}_3$  (Wilson *et al.*, 2009; Woosley *et al.*, 2012). Shallow dissolution of piscine carbonates may go some way to explain the controversial phenomenon whereby total alkalinity has been found to increase at depths much shallower than the predicted chemical lysocline of calcite and aragonite. The cause of this shallow  $\text{CaCO}_3$  dissolution is unclear and has been the subject of debate among oceanographers for a number of decades. It has previously been attributed to (1) dissolution in the guts of zooplankton that have been grazing on coccolithophorids, (2) dissolution in microenvironments, formed by bacterial oxidation of organic

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matter, that favour dissolution, (3) dissolution of more soluble forms of  $\text{CaCO}_3$  (Millero, 2007; Bissett *et al.*, 2011). Wilson *et al.* (2009) suggested that fish derived carbonates may account for up to 26% of increased total alkalinity in shallow waters, a prediction since supported by experimentally determined solubility data (Woosley *et al.*, 2012).

Carbonate sediments originating from shallow waters have been used widely as records of change in ocean chemistry and climate shifts in the geological past (Pomar *et al.*, 2008). However, the origin of these sediments has proved difficult to resolve and has been subject to long term debate (Morse *et al.*, 2007). Fish derived carbonate crystals have been identified as preserved in shallow tropical sediments of the Bahamian archipelago and estimates based on biomass and excretion rate data suggest that fish are a significant source (~14%) of carbonate sediment in these areas (Perry *et al.*, 2011). Such findings have major implications for models of ocean chemistry and climate in the geological past that are primarily based on data obtained from carbonate sediment cores. However, important questions remain about the long-term preservation potential of piscine carbonates. Specifically, how long they remain in the sediment record before either dissolving or undergoing chemical transformation (Wilson *et al.*, 2011).

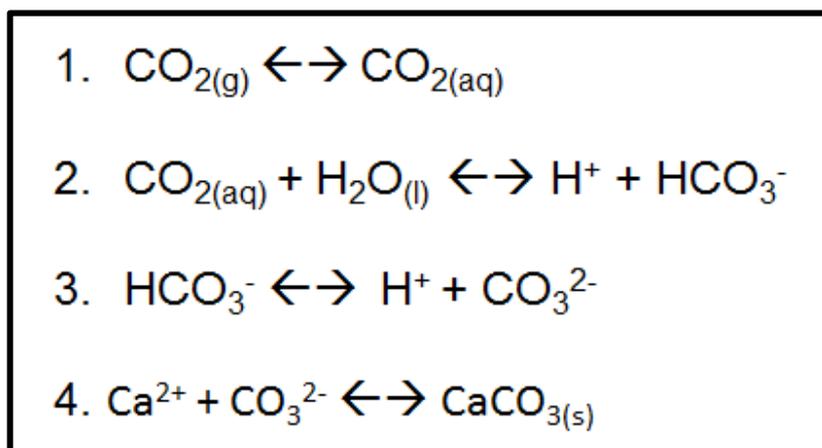


Figure 2. The equilibria that govern the carbonate system of the marine inorganic carbon cycle. (1) The transfer of  $\text{CO}_2$  between the atmosphere and the ocean's surface (2) Reaction of carbon dioxide and water to form bicarbonate and  $\text{H}^+$  (3) The disassociation of bicarbonate into carbonate and  $\text{H}^+$  (4) The process of calcification (Millero, 2007).

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## Environmental Change and Carbonate Production

The previously unrecognised contribution of fish to the marine inorganic carbon cycle is particularly topical given the growing concern regarding changes in the carbonate chemistry of the world's oceans (ocean acidification) as a result of their uptake of anthropogenic CO<sub>2</sub> emissions (Caldeira & Wickett, 2003). The carbonate chemistry of the oceans is highly sensitive to changes in pH brought about by the absorption of CO<sub>2</sub>. Dissolving CO<sub>2</sub> in seawater increases the H<sup>+</sup> concentration and these additional H<sup>+</sup> ions react with carbonate ions to form bicarbonate (Figure 2). The decline in the carbonate ion concentration results in a decrease in the saturation state of calcium carbonate minerals such as aragonite and calcite (Feely *et al.*, 2004; Orr *et al.*, 2005; Cao *et al.*, 2007). For calcifying organisms such as corals and coccolithophores, decreasing CaCO<sub>3</sub> saturation state make it increasingly challenging to form calcium carbonate shells and exoskeletons and leaves existing calcium carbonate structures vulnerable to dissolution (Doney *et al.*, 2009).

In contrast, Wilson *et al.* (2009) predict that production of carbonate by fish will accelerate in response to future increases in seawater PCO<sub>2</sub> and the accompanying increase in ocean surface temperatures. As previously discussed, calcium carbonate production in fish occurs via a very different mechanism to that of calcifying plankton and corals. Intestinal carbonate precipitation by fish is independent of dissolved carbonate and bicarbonate in the seawater. Instead teleost fish utilise endogenous bicarbonate produced via the hydration of metabolic waste CO<sub>2</sub> (Figure 1). Hence, during environmental hypercarbia, increased blood PCO<sub>2</sub> and compensatory increases in blood HCO<sub>3</sub><sup>-</sup> are likely to fuel enhanced intestinal HCO<sub>3</sub><sup>-</sup> secretion and therefore increase carbonate excretion rates by fish.

Increases in ventilation volume in response to increased oxygen demand (e.g. elevated temperature) or reduced oxygen availability (e.g. hypoxia) are also likely to result in increased intestinal carbonate production. The structure and function of the teleost gill that make it such an effective site for gas exchange

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also make it a primary site of osmosis and ionic diffusion – a phenomenon referred to as the ‘osmo-respiratory compromise’ (Sardella & Brauner, 2007). Elevated rates of water loss and ion uptake as a result of hyperventilation requires marine teleosts to drink and process a greater volume of seawater in order to avoid dehydration (Genz *et al.*, 2008). Carbonate production by marine teleosts is directly linked to the rate at which they drink seawater so any compensatory increase in drinking rate is likely to produce a similar increase in carbonate excretion rate (Wilson *et al.*, 2009).

Hence piscine carbonate production appears to be intimately linked to respiratory responses of fish to environmental changes in  $\text{PCO}_2$ ,  $\text{PO}_2$  and temperature. However, these predictions are primarily based on our understanding of the underlying physiological mechanisms behind carbonate production and so far there has been limited experimental quantification of these predicted effects. Such data are crucial in order to update estimates of global piscine carbonate production to include variability with regard to  $\text{O}_2$  and  $\text{CO}_2$ , and to predict the contribution of fish to the marine inorganic carbon cycle of the past, present and future.

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## Summary

The ubiquity, diversity and abundance of fish generates highly valuable ecosystem services. Many of the aquatic environments inhabited by fish are characterized by fluctuations, both spatially and temporally, in abiotic conditions such as  $PO_2$ ,  $PCO_2$  and temperature. Furthermore, aquatic environments have undergone significant change throughout the evolutionary history of fish, and are now undergoing unprecedented rates of change as the result of human activities. Fish demonstrate an array of respiratory responses to changes in  $PO_2$ ,  $PCO_2$  and temperature, our understanding of which is key to predicting the impacts of environmental change on fish populations. In addition, the intestinal precipitation and excretion of carbonate by marine teleosts, a key osmoregulatory strategy, is likely to be closely linked to their respiratory responses to these environmental variables. Piscine carbonate production has recently been recognised as a major component of the marine inorganic carbon cycle but current global estimates are based on experimental data from fish undergoing a limited range of environmental conditions. Therefore, there is significant scope for further research to experimentally quantify the effects of various environmental factors, and their interactions, on gut carbonate production by marine teleosts.

The series of studies reported in this thesis begin (chapter two) with a collation of previously published data on the critical oxygen thresholds ( $P_{crit}$ ) of fish. Such data provide a quantitative insight into the variation of hypoxia tolerance across species and allows for the identification of a range of biotic / abiotic interactions. In addition, by critically reviewing the methodologies and principles behind investigations of respiratory physiology in fish, chapter two sets the scene for the following three empirical studies, within which such measurements are a re-occurring theme. The first of these empirical studies (chapter three) aims to experimentally determine the thermal and hypoxia tolerance of two closely related coral-reef snapper species - *Lutjanus carponotatus* and *Lutjanus adetii*. The contrasting biophysical environments occupied by these fish, the reef flat and reef slope, provide a useful model system in which to examine the extent to which variation in their spatial

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distribution is reflected by inter- and intraspecific variation in some common physiological tolerance trait measures (aerobic scope, critical temperatures and  $P_{crit}$ ). Such studies, linking the respiratory physiology of organisms to environmental conditions in their natural range, are of increasing interest for predicting possible shifts in the distribution of populations as the result of future climate change. In the fourth chapter, this thesis moves on to examine how the respiratory responses of the European flounder (*Platichthys flesus*) to hypoxia and hypercarbia influences their intestinal precipitation and excretion of carbonate. Measurements of  $P_{crit}$ , ventilation volume, drinking rate and various blood parameters are made in order to elucidate the mechanistic link between carbonate excretion rate and environmental  $PO_2$  and  $PCO_2$ . Finally, the study presented in chapter five builds upon the results of the proceeding chapter in order to explore how additional environmental factors such as temperature and seawater chemistry affect the rate of carbonate excretion by European flounder. Treatment conditions in chapter five are designed to reflect conditions thought to be prevalent in the warm, calcite seas of the Cretaceous period. Thus, this study provides the first experimental evidence for how piscine carbonate production may have varied in the geological past. In summary, the following thesis can be characterized as a series of fish ecophysiology studies linked by two major themes, respiratory physiology and gut carbonate production, both of which are set in the context of past, present and future environmental change.

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# Chapter 2



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# **A physiological trait database of hypoxia tolerance ( $P_{crit}$ ) in fish.**

## **Abstract**

Hypoxia is a common feature of many aquatic habitats and is becoming an increasingly frequent and widespread environmental perturbation, primarily as the result of anthropogenic nutrient enrichment and climate change. Fish are typically among the most hypoxia sensitive of aquatic taxa and as such, developing a good understanding of the hypoxia tolerance limits of fish and how this varies between individuals and species is essential in order to make accurate predictions of future ecological impacts and to better inform management decisions. As a measure of oxygen extraction capacity, critical  $PO_2$  ( $P_{crit}$ : the oxygen level at which the oxygen consumption rate of an organism transitions from being independent of, to dependant on ambient  $PO_2$ ) has been widely utilized by fish physiologists as a hypoxia tolerance trait. Here, a comprehensive database of fish  $P_{crit}$  values compiled from the published literature is presented. This database incorporates 331 measurements of  $P_{crit}$  from a total of 96 published studies and covers 151 fish species from 58 families. The systematic review of this literature provided the opportunity to critically examine methodologies for determining  $P_{crit}$  as well as its usefulness as an indicator of hypoxia tolerance in fish. Additionally, various abiotic and biotic interactions with hypoxia and their effect on  $P_{crit}$  are reviewed. It is anticipated that the present database will eventually be incorporated into a widely accessible central repository of physiological trait data that will facilitate future studies of fish ecology, conservation and management.

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## Introduction

In recent decades there has been growing concern regarding the increasingly widespread and frequent occurrence of hypoxia in aquatic environments (Diaz, 2001; Diaz & Breitburg, 2009). Although periods of hypoxia develop naturally in many aquatic systems, anthropogenic influences have been shown to be a major driver of hypoxic events in both freshwater and marine habitats (Friedrich *et al.*, 2014). In particular, eutrophication associated with increased anthropogenic nutrient loading of lakes, rivers and coastal waters, leads to blooms of algae and phytoplankton, the deaths of which subsequently fuel microbial respiration and the depletion of dissolved oxygen (Smith *et al.*, 2003). In the marine environment, over 400 coastal systems have been reported as eutrophication-associated dead zones (Diaz & Rosenberg, 2008) and hypoxic events have been linked to multiple fishery collapses across the globe (Vaquer-Sunyer & Duarte, 2008). Global warming is likely to exacerbate hypoxia in aquatic systems due to increased microbial respiration rates and reduced oxygen solubility with increasing water temperatures (McBryan *et al.*, 2013). In addition, modifications of oceanic circulation linked to future climate change is predicted to result in greater stratification and 'deoxygenation' of the oceans (Keeling & Garcia 2002; Keeling *et al.*, 2009). Fish tend to be among the most hypoxia sensitive of aquatic taxa (Vaquer-Sunyer & Duarte, 2008) and as such fish populations are at particular risk as hypoxia becomes an increasingly common aquatic perturbation. Understanding the physiological responses of individual organisms to environmental stressors such as hypoxia provides the mechanistic link between environmental change and population level effects and is key to predicting future ecological impacts (Chown, 2012; Seebacher & Franklin, 2012; Cooke *et al.*, 2013).

Fish demonstrate numerous physiological responses to hypoxia including changes in ventilation, haemoglobin-O<sub>2</sub> binding and cardiovascular function (Richards *et al.*, 2009). Primarily these responses work to sustain aerobic ATP production during periods of hypoxia by promoting oxygen extraction from the environment. The majority of fish species maintain stable oxygen consumption rates across a wide range of ambient PO<sub>2</sub> and as such can be described as

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oxyregulators (Perry *et al.*, 2009). However, when oxygen falls to a level at which oxygen consumption rate can no longer be maintained, fish respond by oxyconforming whereby oxygen consumption declines linearly with ambient  $PO_2$  (Portner & Grieshaber, 1993). The  $PO_2$  at which oxygen consumption transitions from being independent of, to dependent on ambient oxygen, is referred to as the critical  $PO_2$  ( $P_{crit}$ ). As a whole-animal measure of oxygen extraction capacity that varies widely across species and between populations,  $P_{crit}$  is widely utilized by physiologists to describe the degree of hypoxia tolerance in fish (Ultsch *et al.*, 1978; Chapman *et al.*, 2002; Nilsson *et al.*, 2007; Mandic *et al.*, 2009; Roesch *et al.*, 2012).

Oxygen is the fuel that drives aerobic ATP production and as such the rate of oxygen consumption is equivalent to the rate of aerobic metabolism, at least when in a steady state. Standard metabolic rate (SMR) is the oxygen consumption rate of an entirely inactive, post absorptive fish and reflects its minimum cost of living at a given temperature (Beamish & Mookherjee, 1964). Routine metabolic rate (RMR) provides a similar estimate of the cost of living but takes into account energy expended on maintaining posture and making the small movements that are typical of most fish even when in a quiescent state (McBryan *et al.*, 2013). In contrast, maximum metabolic rate (MMR) is the highest rate of oxygen consumption that can be attained under defined environmental conditions (Clark *et al.*, 2013). The difference between SMR and MMR is referred to as aerobic scope and accounts for the aerobic demands of higher functions such as locomotion, growth, behaviour and reproduction (Farrell & Richards, 2009). In the context of this aerobic hierarchy, several levels of critical  $PO_2$  can be identified (Figure 3). MMR is first to become limited as ambient oxygen falls, from which point ( $P_{Cmax}$ ) a decline in MMR equates to a narrowing of aerobic scope. Secondly the  $P_{crit}$  for RMR is reached whereby oxygen supply cannot sustain even minimal levels of aerobic exercise. Finally, the  $P_{crit}$  for SMR indicates that oxygen supply cannot meet basic oxygen costs and anaerobiosis is required just to sustain life (Portner & Lannig, 2009). Clearly all three levels of  $P_{crit}$  have major

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implications for the fitness of fish living in environments prone to hypoxia and as such can be considered as functional traits (McGill *et al.*, 2006).

The term 'trait' refers widely to any morphological, physiological, behavioural, ecological or life-history expression of an organisms adaptations to its environment (Goldstein & Meador, 2005). The examination of trait variation across communities and its ecological implications are increasingly becoming the basis for predicting and potentially mitigating the effects of environmental change on biodiversity (Chown, 2012). Such trait-based approaches are facilitated by the collection and dissemination of trait data. Large scale multi-trait databases have been compiled for various taxa including plants (Kattge *et al.*, 2011), mammals (Jones *et al.*, 2009), marine polychaetes (Faulwetter *et al.*, 2014) and North American freshwater fish (Frimpong & Angermeier, 2009). As a quantifiable measure of hypoxia tolerance that is measured on individuals and is applicable at population level;  $P_{crit}$  is likely to be a useful trait for incorporation into trait-based approaches to the conservation physiology of fish (Frimpong & Angermeier, 2009).

The field of fish physiology has generated a large body of literature on  $P_{crit}$  across a wide range of species and under highly variable abiotic and biotic conditions (Perry *et al.*, 2009). Owing to individual peculiarities and the discrete nature of each study the usefulness of these data is not immediately tangible. The aims of the present work were to 1) assemble from published literature a database of the  $P_{crit}$  values reported for fish in a suitable format for future incorporation into multi-trait based analyses 2) perform preliminary analysis of the collated data in order to identify how biotic and abiotic factors (particularly temperature) interact with hypoxia and affect  $P_{crit}$  3) critically review methodologies for measuring  $P_{crit}$  and its usefulness for quantifying hypoxia tolerance in fish.

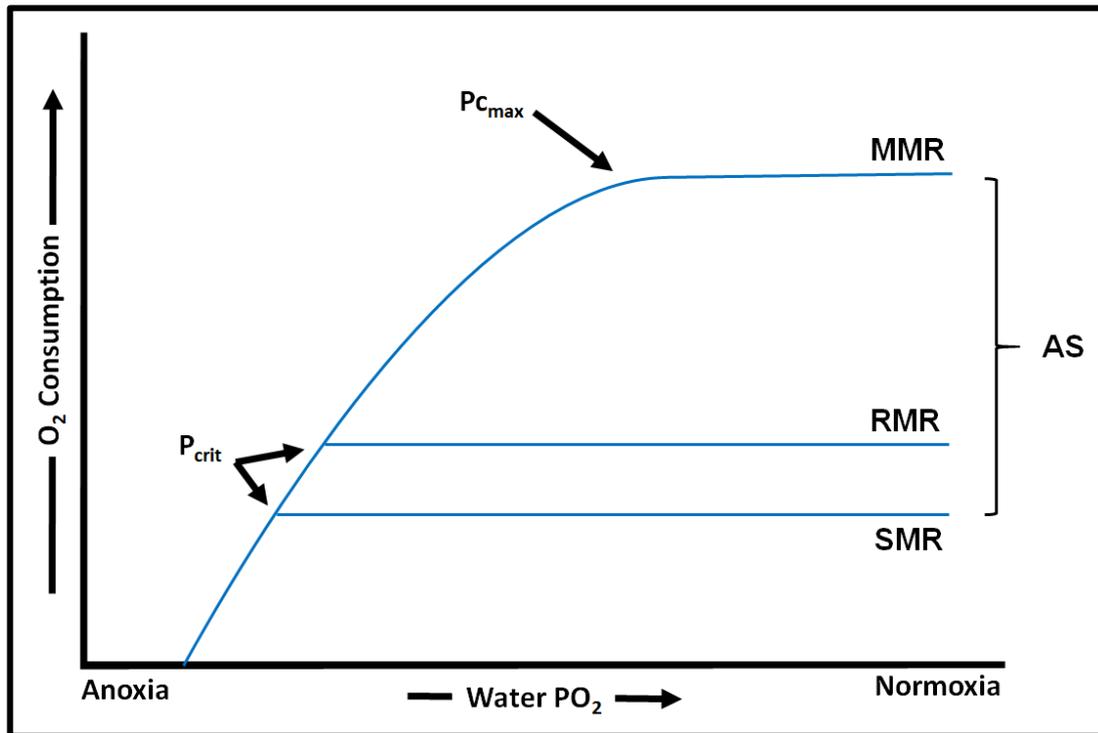


Figure 3. Diagram illustrating the effects of hypoxia on the standard metabolic rate (SMR), routine metabolic rate (RMR), maximum metabolic rate (MMR) and aerobic scope (AS) of an oxyregulator.

## Methods

### Literature Search

Two major citation and abstract indexes, Scopus<sup>®</sup> and Web of Science<sup>®</sup>, were used to collect relevant peer-reviewed literature. Titles, abstracts and keywords were searched using the terms: “critical oxygen” OR “critical PO<sub>2</sub>” OR “oxygen threshold” OR “P<sub>crit</sub>” OR “oxyregulate” OR “oxyconform” OR “hypoxia tolerance”. These search terms yielded > 900 results which were reduced to ~ 400 by excluding irrelevant research areas (Figure 4). These articles were individually assessed for relevance based on their title and abstract. Ultimately, 144 papers were downloaded for closer inspection and of these 96 included a measurement of P<sub>crit</sub> in at least one fish species.

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( TITLE-ABS-KEY ( "critical oxygen" ) OR TITLE-ABS-KEY ( "critical PO2" ) OR TITLE-ABS-KEY ( "oxygen threshold" )
OR TITLE-ABS-KEY ( pcrit ) OR TITLE-ABS-KEY ( oxyregulation ) OR TITLE-ABS-KEY ( oxyconforming ) ) OR TITLE-
ABS-KEY ( "hypoxia tolerance" ) AND ( LIMIT-TO ( SUBJAREA , "AGRI" ) OR LIMIT-TO ( SUBJAREA , "ENVI" ) )
AND ( EXCLUDE ( SUBJAREA , "MEDI" ) OR EXCLUDE ( SUBJAREA , "EART" ) OR EXCLUDE ( SUBJAREA , "IMMU" )
OR EXCLUDE ( SUBJAREA , "ENGI" ) OR EXCLUDE ( SUBJAREA , "MATE" ) OR EXCLUDE ( SUBJAREA , "CENG" ) OR
EXCLUDE ( SUBJAREA , "MATH" ) OR EXCLUDE ( SUBJAREA , "COMP" ) OR EXCLUDE ( SUBJAREA , "DECI" ) OR
EXCLUDE ( SUBJAREA , "NEUR" ) OR EXCLUDE ( SUBJAREA , "CHEM" ) OR EXCLUDE ( SUBJAREA , "ENER" ) OR
EXCLUDE ( SUBJAREA , "PHYS" ) OR EXCLUDE ( SUBJAREA , "SOCI" ) )
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Figure 4. Example of the term-based search of the SCOPUS® abstract and citation database used to identify papers likely to contain a  $P_{crit}$  measurement.

## Database Construction

In order to maximise the future usability of the database and to ensure it fully reflects the variation in abiotic / biotic conditions under which  $P_{crit}$  has previously been measured in fish, it was necessary to extract multiple parameters from each study. For each  $P_{crit}$  entry, 66 columns summarise information on the species and origin, acclimation parameters, sample information, experimental method, results, statistical analyses, general comments and bibliographic information (Table 1). The database is constructed as a single Microsoft Excel file with individual columns for each parameter and rows for each  $P_{crit}$  determination in a particular species or treatment group. As such a single study may occupy several rows depending on the number of treatment groups and/or species for which  $P_{crit}$  is reported. Each entry contains full citation details including a DOI hyperlinked to the relevant published .pdf file. Values for  $P_{crit}$  are reported in a variety of different oxygen units across the literature (mmHg, Torr, % air saturation,  $\text{mgO}_2 \text{ L}^{-1}$ ,  $\mu\text{M}$ ) but are converted here to a partial pressure of oxygen (kPa) based on oxygen solubility values reported in Green & Carrit (1967) and assuming standard atmospheric pressure at sea level (760 mmHg) if not otherwise reported. Similarly, all values of oxygen consumption rate were converted to  $\text{mgO}_2 \text{ kg}^{-1} \text{ h}^{-1}$ . To enable unbiased inter-species comparisons, a secondary ‘control’ dataset was produced which included only those  $P_{crit}$  measurements made in fish: 1) in an unfed or post-absorptive state 2) undergoing no additional (to hypoxia) abiotic stressor 3) temperature acclimated for >2 days.

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<b>Species and Origin</b>	<b>Stock Acclimation</b>	<b>Sample Characteristics</b>	<b>Experimental Method</b>	<b>Results</b>	<b>Statistical Analysis</b>	<b>Comments and Reference</b>
Family	Holding Time	Sample Size	Respirometry Type	Oxy - conf. / reg.	Statistical Method	Comments
Genus	Acclimation Temperature	Mean Mass	BMR/RMR/SMR/MMR	MO <sub>2</sub>	Pcrit Calculation Method	Reference
Species	Acclimation Salinity	Mass SD	Determination Method	Critical PO <sub>2</sub>	SMR Determination	Year
Origin	PO <sub>2</sub> Units	Mass SEM	Swimming Speed	Critical PO <sub>2</sub> Range		Corresponding Author
Lat. and Long.	Acclimation PO <sub>2</sub>	Mass Range Upper	Hypoxia Method	Critical PO <sub>2</sub> SD		DOI
	Acclimation pH	Mass Range Lower	Rate of Hypoxia Onset	Critical PO <sub>2</sub> SEM		Full Citation
	Acclimation time	Mean Length	PO <sub>2</sub> Setpoint Time	Critical PO <sub>2</sub> Unit		
	Diet	Length SD	Minimum PO <sub>2</sub>	Air Breathing Threshold		
	Energy content	Length SEM	PO <sub>2</sub> Unit	Common PO <sub>2</sub> Unit		
	Ration unit	Length Range Upper	Salinity			
	Ration size	Length Range Lower	Temperature			
	Photoperiod (L:D)	Life Stage	pH			
	Feeding regime	Sex	PCO <sub>2</sub>			
		Last Feed	Photoperiod (L:D)	Access to Air		

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Table 1. List of the parameters incorporated into the database alongside each reported P<sub>crit</sub> value.

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## Results

### Database Coverage

Of the 96 studies reviewed, 331 measurements of  $P_{crit}$  across 151 species (58 families) were incorporated into the database. Geographic coverage includes at least one entry from every continent, although North America, Europe and Australasia are by far the most heavily represented and when combined account for 87 % of  $P_{crit}$  entries (Figure 5). Freshwater and marine (including euryhaline) species account for 40 and 60 % of  $P_{crit}$  entries, respectively. Water temperatures at which  $P_{crit}$  values were determined ranged between  $-1.5$  and  $36$  °C with a mean of  $21.7$  °C  $\pm$  7.6 (S.D.). Values for  $P_{crit}$  over the entire dataset ranged between 1.02 (*Pseudocrenilabrus multicolor victoriae*) and 16.2 kPa (*Solea solea* larvae) with a mean  $P_{crit}$  in the 'control' dataset of 5.15 kPa  $\pm$  2.21 (S.D.). Plots of species and their reported  $P_{crit}$  values from the 'control' dataset are provided in the appendix of this chapter (Figure 8).

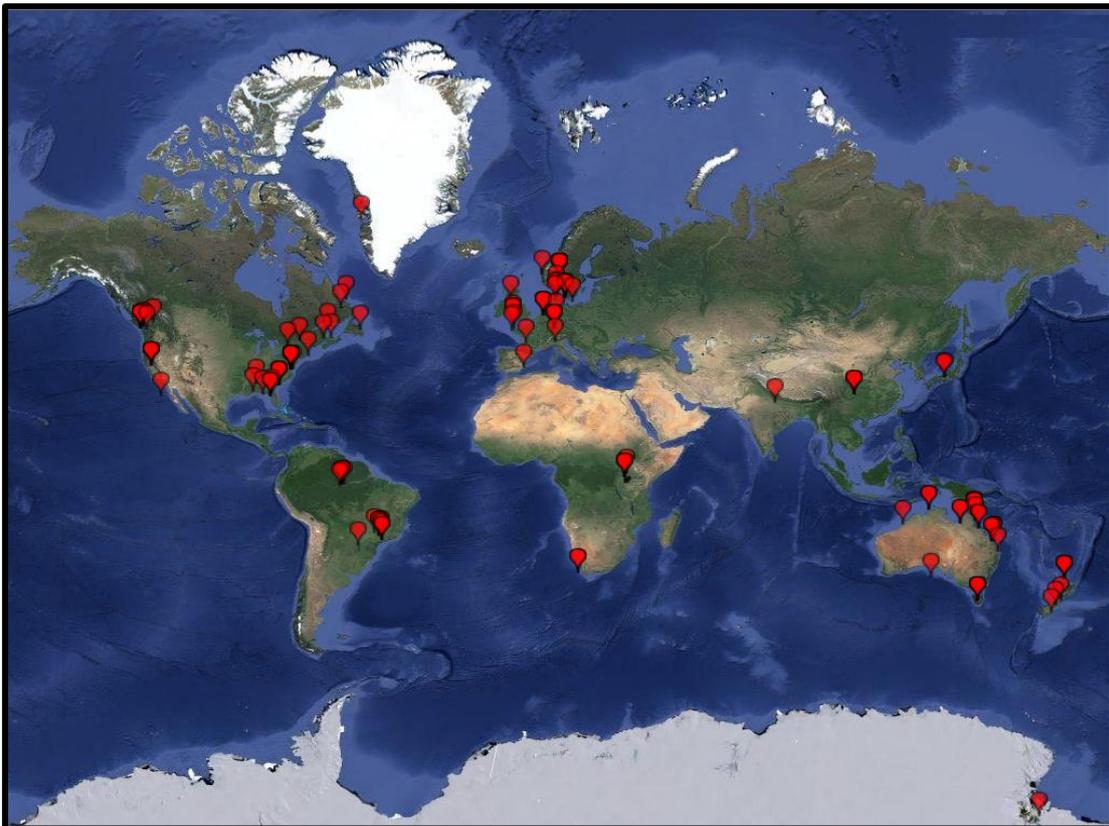


Figure 5. Map illustrating the geographic range currently covered by the database. Red pins indicate the origin of each fish stock for which  $P_{crit}$  was reported as indicated by the respective study.

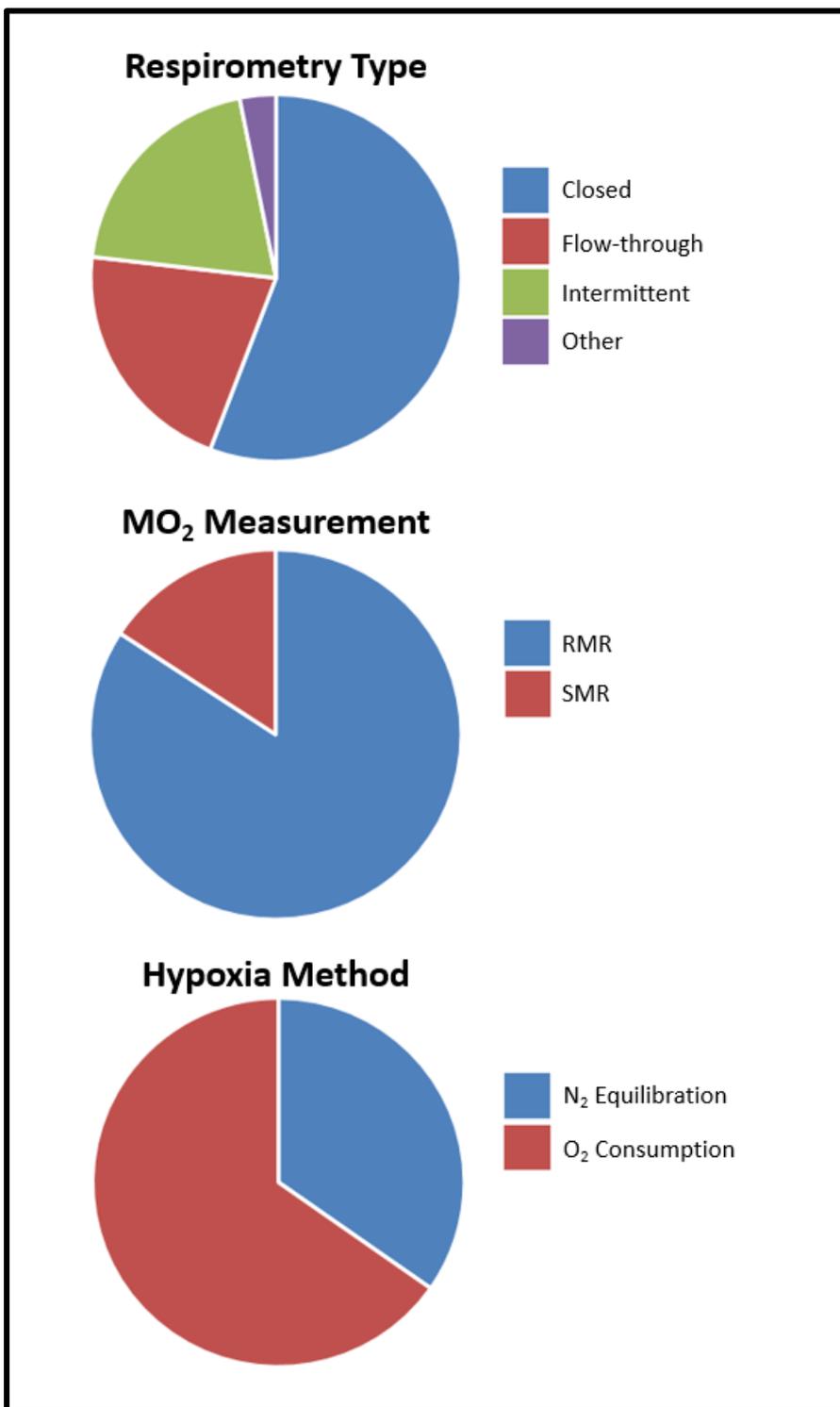


Figure 6. A breakdown of methods used throughout the published literature for measuring P<sub>crit</sub> in fish. Routine metabolic rate (RMR), standard metabolic rate (SMR). 'N<sub>2</sub> equilibration' refers to stripping dissolved oxygen by bubbling the water with nitrogen whereas 'O<sub>2</sub> consumption' refers to the depletion of ambient oxygen through the fish's own respiration.

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## Biotic and Abiotic Interactions

The effect on  $P_{crit}$  of numerous abiotic and biotic factors have been investigated in the literature (Table 2). In particular the interaction between temperature and hypoxia has been the focus of multiple studies. Of the 30 species included in the database for which  $P_{crit}$  measurements have been made over a range of temperatures (including measurements made in separate studies of the same species) all but four showed a strong positive relationship between  $P_{crit}$  and temperature. However, examination of the 'control' dataset revealed no significant relationship between temperature and inter-species  $P_{crit}$  (Figure 7). Other biotic and abiotic factors predominantly reported to increase intra-species  $P_{crit}$  include feeding, trace metal contamination, acidification, mycobacteriosis infection and anaemia, whilst hypoxic preconditioning and succession between life stages appear to reduce  $P_{crit}$  (Table 2).

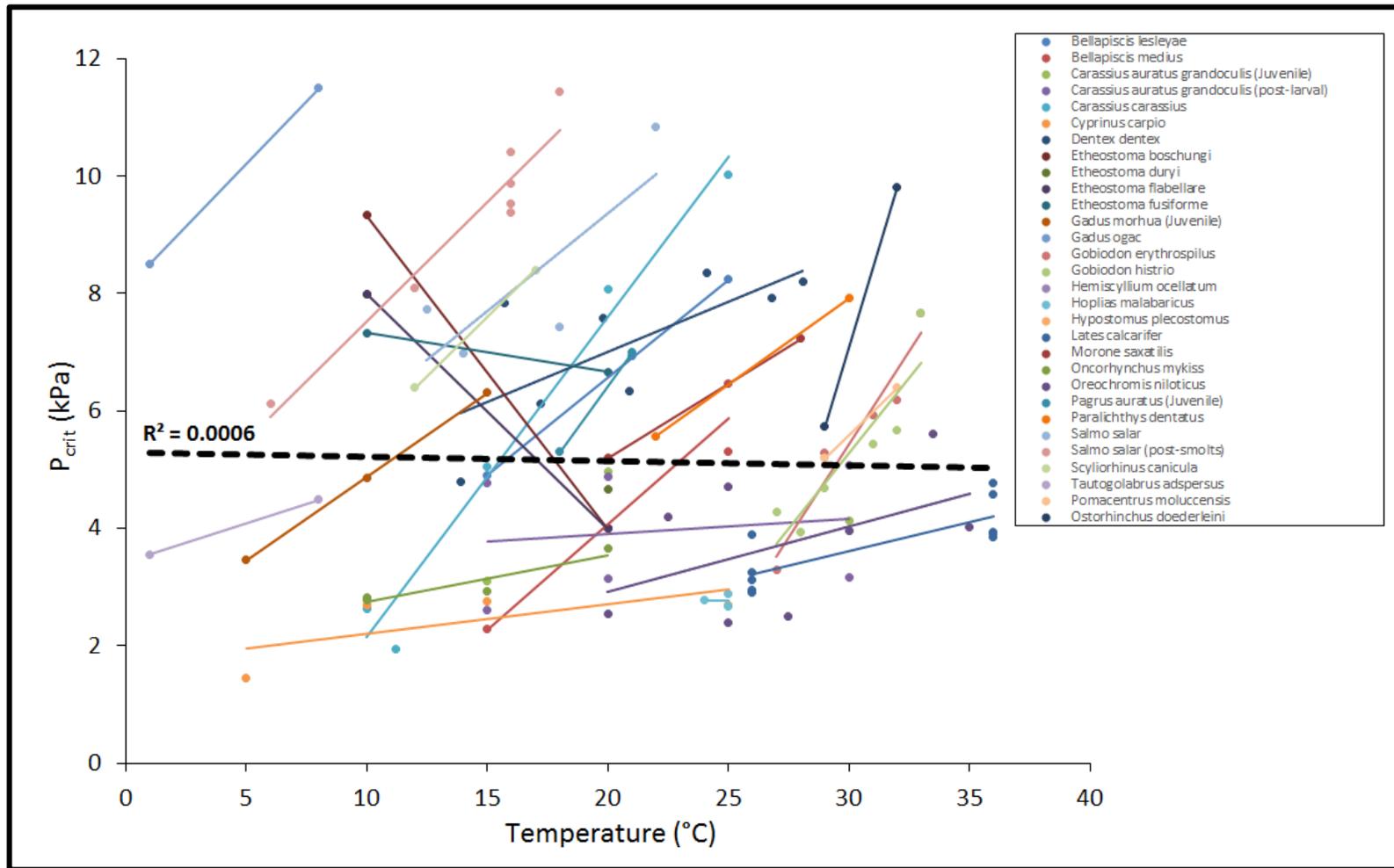


Figure 7. The effect of temperature on inter-species P<sub>crit</sub> (black dashed line) and intra-species P<sub>crit</sub> (solid lines).

Variable	Species	Effect on P <sub>crit</sub>	Reference
<b>Increasing temperature</b>			
	<i>Gadus morhua</i>	Increase	Schurmann & Steffensen, 1997
	<i>Lates calcarifer</i>	Increase	Collins <i>et al.</i> , 2013
	<i>Scyliorhinus canicula</i>	Increase	Butler & Taylor, 1975
	<i>Salmo salar</i>	Increase	Barnes <i>et al.</i> , 2011
	<i>Salmo salar</i>	Increase	Remen <i>et al.</i> , 2013
	<i>Dentex dentex</i>	Increase	Valverde <i>et al.</i> , 2006
	<i>Tautogolabrus adspersus</i>	Increase	Corkum & Gamperl, 2009
	<i>Gadus ogac</i>	Increase	Corkum & Gamperl, 2009
	<i>Bellapiscis medius</i>	Increase	Hilton <i>et al.</i> , 2008
	<i>Bellapiscis lesleyae</i>	Increase	Hilton <i>et al.</i> , 2008
	<i>Morone saxatilis</i>	Increase	Lapointe <i>et al.</i> , 2014
	<i>Carassius carassius</i>	Increase	Sollid <i>et al.</i> , 2005
	<i>Gobiodon histrio</i>	Increase	Sorensen <i>et al.</i> , 2014
	<i>Gobiodon erythrospilus</i>	Increase	Sorensen <i>et al.</i> , 2014
	<i>Oreochromis niloticus</i>	Increase	Fernandes & Rantin, 1989
	<i>Cyprinus carpio</i>	Increase	Ott <i>et al.</i> , 1980
	<i>Oncorhynchus mykiss</i>	Increase	Ott <i>et al.</i> , 1980
	<i>Pomacentrus moluccensis</i>	Increase	Nilsson <i>et al.</i> , 2010
	<i>Ostorhinchus doederleini</i>	Increase	Nilsson <i>et al.</i> , 2010
	<i>Carassius auratus grandoculis</i>	No effect	Yamanaka <i>et al.</i> , 2013
	<i>Etheostoma boschungii</i>	Decrease	Ultsch <i>et al.</i> , 1978
	<i>Etheostoma fusiforme</i>	Decrease	Ultsch <i>et al.</i> , 1978
	<i>Etheostoma flabellare</i>	Decrease	Ultsch <i>et al.</i> , 1978
	<i>Etheostoma rufilineatum</i>	Decrease	Ultsch <i>et al.</i> , 1979
<b>Increasing salinity</b>			
	<i>Cottus asper</i>	Decrease	Henriksson <i>et al.</i> , 2008
	<i>Leptocottus armatus</i>	No effect	Henriksson <i>et al.</i> , 2008
	<i>Cyprinus carpio</i>	Increase	Boeck <i>et al.</i> , 2000
	<i>Cyprinodon ariegatus</i>	Increase	Haney & Nordlie, 1997
<b>Increased PCO<sub>2</sub></b>			
	<i>Fundulus heteroclitus</i>	No effect	Cochran & Burnett, 1996
	<i>Leiostomus xanthurus</i>	No effect	Cochran & Burnett, 1996
	<i>Anguilla anguilla</i>	Increase	Cruz-Neto & Steffensen, 1997
	<i>Platichthys flesus</i>	Increase	Rogers (ch. 4), 2015
<b>Hypoxic pre-conditioning</b>			
	<i>Pagrus auratus</i>	No effect	Cook <i>et al.</i> , 2013
	<i>Salmo salar</i>	No effect	Remen <i>et al.</i> , 2013
	<i>Hemiscyllium ocellatum</i>	Decrease	Routley <i>et al.</i> , 2002
	<i>Spinibarbus sinensis</i>	Decrease	Dan <i>et al.</i> , 2014
	<i>Carassius auratus</i>	Decrease	Fu <i>et al.</i> , 2011
	<i>Poecilia latipinna</i>	Decrease	Timmerman & Chapman, 2004
<b>Reared in hypoxic environment</b>			
	<i>Pseudocrenilabrus multicolor</i>	Decrease	Reardon & Chapman, 2010
<b>Exercise pre-conditioning</b>			
	<i>Carassius auratus</i>	Decrease	Fu <i>et al.</i> , 2011
<b>Fed vs. Unfed</b>			
	<i>Astronotus ocellatus</i>	Increase	Boeck <i>et al.</i> , 2013
	<i>Oreochromis niloticus</i>	Increase	Mamun <i>et al.</i> , 2013
	<i>Perca fluviatilis</i>	Increase	Thuy <i>et al.</i> , 2010
<b>Fatty acid enriched diet</b>			
	<i>Solea solea</i> (larvae)	Decrease	McKenzie <i>et al.</i> , 2008
	<i>Solea solea</i> (juveniles)	Decrease	McKenzie <i>et al.</i> , 2008
<b>Increasing body mass</b>			
	<i>Hypostomus plecostomus</i>	Decrease	Perner & Fernandes, 1996
	<i>Astronotus ocellatus</i>	Decrease	Sloman <i>et al.</i> , 2006
	<i>Pomacentridae</i>	No effect	Nilsson & Ostlund-Nilsson, 2008
<b>Pre- to post-settlement (larvae)</b>			
	<i>Chromis atripectoralis</i>	Decrease	Nilsson <i>et al.</i> , 2007
	<i>Pomacentrus amboinensis</i>	Decrease	Nilsson <i>et al.</i> , 2007
<b>Larvae to juveniles</b>			
	<i>Carassius auratus grandoculis</i>	Decrease	Yamanaka <i>et al.</i> , 2013
<b>Juveniles to adults</b>			
	<i>Reinhardtius hippoglossoides</i>	Decrease	Dupont-Prinet <i>et al.</i> , 2013
<b>Increasing brood size (mouthbrooders)</b>			
	<i>Zoramia fragilis</i>	Increase	Ostlund-Nilsson & Nilsson, 2004
	<i>Zoramia leptacantha</i>	Increase	Ostlund-Nilsson & Nilsson, 2004
<b>Mycobacteriosis infection</b>			
	<i>Morone saxatilis</i>	Increase	Lapointe <i>et al.</i> , 2014
<b>Acidified water</b>			
	<i>Salmo gairdneri</i>	Increase	Ultsch <i>et al.</i> , 1980
	<i>Cyprinus carpio</i>	Increase	Ultsch <i>et al.</i> , 1980
<b>Metal exposure</b>			
	<i>Brycon amazonicus</i>	Increase	Monteiro <i>et al.</i> , 2013 (Hg <sup>2+</sup> )
	<i>Carassius carassius</i>	Increase	Schjolden <i>et al.</i> , 2007 (Cu <sup>2+</sup> )
	<i>Perca fluviatilis</i>	Increase	Bilberg <i>et al.</i> , 2007 (AgNO <sub>3</sub> )
	<i>Perca fluviatilis</i>	Increase	Bilberg <i>et al.</i> , 2007 (nano-Ag)
<b>Organophosphate exposure</b>			
	<i>Oreochromis niloticus</i>	Increase	Thomaz <i>et al.</i> , 2009
<b>Anaemia</b>			
	<i>Pagrus auratus</i>	Increase	Cook <i>et al.</i> , 2011

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Table 2 (previous page). Summary of biotic and abiotic factors and their interactions with the intra-species  $P_{crit}$  as reported by studies included in the database.

## Discussion

### $P_{crit}$ Methodology

The relationship between ambient  $PO_2$  and oxygen consumption in fish has been investigated since the beginning of the twentieth century and even at this early stage there was considerable discussion between physiologists regarding the validity of different methodologies (Keys, 1930). Since then technological developments, particularly methods for measuring dissolved oxygen content such as galvanic oxygen electrodes and more recently fibre-optic sensors, have made performing high resolution measurements of oxygen consumption in fish increasingly straight forward (Clark *et al.*, 2013). Nevertheless, the literature examined for the purpose of building this database is characterized by considerable variation in terms of methods employed to determine  $P_{crit}$ . This variability arises from the individual experimental constraints of each study as well as different definitions of  $P_{crit}$  as a physiological measurement between studies. These methodological differences and their implications are important to consider when it comes to interpreting collated  $P_{crit}$  data.

Closed respirometry, whereby the fish is placed within a sealed chamber from which water is intermittently sampled for measurement of dissolved oxygen content, provides the simplest method of measuring oxygen consumption rate (Steffensen, 1989):

$$MO_2 = (Vr \times \Delta O_2) \div (\Delta t \times bw)$$

Where:  $MO_2$  represents oxygen consumption rate,  $Vr$  is respirometer volume,  $t$  is time, and  $bw$  is fish mass (body weight).

Importantly, water needs to be recirculated within the chamber to ensure adequate mixing thus preventing the stratification of dissolved oxygen within the chamber (Keys, 1930). Variations of the closed method have been used in the majority of studies incorporated into the database (56%, Figure 6). For

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closed determinations of  $P_{crit}$ , hypoxia is generated by allowing the fish to deplete available oxygen through its own respiration therefore negating the need to artificially strip dissolved oxygen from the water through equilibration with nitrogen. For this reason, closed respirometry is particularly useful for conducting measurements of  $P_{crit}$  in the field or at remote locations where facilities such as a supply of  $N_2$  may not be readily available (Rosenberger & Chapman, 2000; Nilsson *et al.* 2007).

There are several important considerations regarding the use of closed respirometry for determining  $P_{crit}$ . For instance, the rate of hypoxia onset during closed respirometry is determined by the ratio of fish size (or oxygen consumption rate) to respirometer volume. A lack of control over the development of hypoxia can be problematic in comparative studies that use the same respirometer to measure  $P_{crit}$  in fish of contrasting size and/or metabolic rate. As an illustrative example, the depletion of oxygen levels from 20 to 1 kPa by Australian barramundi (*Lates calcarifer*) took between 1.5 and 4 hours depending on the temperature treatment in question (26 or 36 °C, Collins *et al.*, 2013). It is notable that there is very little if any standardisation in terms of the rate of hypoxia onset between  $P_{crit}$  studies irrespective of which respirometry method is employed. This is in contrast to measurements of other physiological threshold traits such as the determination of critical temperature, which tends to be made at consistent warming or cooling rates between studies (0.2 – 0.3 °C min<sup>-1</sup>; Beitinger *et al.*, 2000; Mora & Maya, 2006; Murchie *et al.*, 2011). It is unclear whether how quickly hypoxia develops will significantly affect  $P_{crit}$  but it is plausible that a longer time scale would allow for greater respiratory adjustment and hence reveal lower  $P_{crit}$  values than more acute hypoxic exposures.

A further issue associated with closed respirometry is the build-up of the waste products of metabolism, in particular  $CO_2$  (Keys, 1930; Steffensen 1989). Our own measurements of seawater pH change within a 6.25 l closed respirometer holding a ~ 300 g European flounder (*Platichthys flesus*) at 15 °C, revealed an increase in water  $PCO_2$  from ~0.3 mmHg (395  $\mu$ atm) to ~1 mmHg (1370  $\mu$ atm) as  $PO_2$  declined from normoxia to 5 kPa (pH change: 8.05 - 7.54). It has been

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argued that this level of CO<sub>2</sub> accumulation within a closed respirometer is unlikely to significantly impact on CO<sub>2</sub> excretion by fish given that they normally exhibit a blood PCO<sub>2</sub> of around 2 - 4 mmHg (Ishimatsu *et al.*, 2005; Nilsson *et al.*, 2007) Previously, more severe levels of hypercarbia (2.25 – 20 mmHg) have been shown to increase P<sub>crit</sub> in European eels (*Anguilla Anguilla*; Cruz-Neto & Steffensen, 1997) and European flounder (chapter 4) but no effect on P<sub>crit</sub> was observed in spot fish (*Leiostomus xanthurus*) or mummichog (*Fundulus heteroclitus*; Cochran & Burnett, 1996). Given the potential influence of hypercarbia it would be prudent to report any change in water PCO<sub>2</sub> alongside values for P<sub>crit</sub> that have been determined through closed respirometry, but this has rarely been the case throughout the existing literature.

Flow-through respirometry is a technique whereby oxygen content of the inflowing (O<sub>2,in</sub>) and outflowing (O<sub>2,out</sub>) water is continuously measured at a fixed water flow rate through the respirometer (V<sub>w</sub>). By application of the Fick principle oxygen consumption (MO<sub>2</sub>) is determined by:

$$MO_2 = V_w \cdot (O_{2,in} - O_{2,out}) \div bw$$

Although flow-through respirometry avoids the accumulation of metabolites in the chamber it suffers from problems primarily related to the ‘wash-out’ effect whereby a significant lag can develop between changes in the fish’s actual MO<sub>2</sub> and changes in observed O<sub>2,out</sub>. The degree of wash-out is difficult to resolve and depends on the dilution factor: a function of water mixing, volume and flow rate (Steffensen, 1989).

Intermittent flow-through respirometry is generally considered the ideal method of MO<sub>2</sub> determination in fish as it involves none of the problems associated with closed or flow-through techniques (Steffensen 1989; Clark *et al.*, 2013). The term ‘intermittent’ in this context refers to the transitioning between a closed phase for determination of MO<sub>2</sub>, and a flush phase for restoring O<sub>2</sub> to a set level and removing metabolites from the respirometer. As the equipment and software for automating flush/recirculation cycles and data acquisition in multiple chambers simultaneously have become more sophisticated and

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widely available, intermittent flow-through respirometry has been increasingly utilized. However,  $P_{crit}$  measurements via this preferred technique only account for around 20 % of values incorporated into the present database (Figure 6).

Flow-through techniques allow for the supply of hypoxic water to the respirometry chamber. This hypoxic water can be produced by bubbling with  $N_2$  via a solenoid valve linked to an  $O_2$  probe (Schurmann & Steffensen, 1997) or by bubbling with set gas mixtures of variable  $O_2$  and  $N_2$  content (chapter 4). Both methods allow for finer control of the hypoxic exposure compared to allowing the fish to deplete ambient oxygen levels dependent on its own  $MO_2$ . Progressive hypoxia can be generated in a step wise fashion such that multiple  $MO_2$  measurements can be made at a specific  $PO_2$  thereby increasing the likelihood of determining an  $MO_2$  that is representative of true SMR or RMR (Rantin *et al.*, 1993).

To determine  $P_{crit}$ ,  $MO_2$  is plotted against ambient  $PO_2$  in order to identify the inflection point at which  $MO_2$  transitions from being independent of ambient oxygen to dependant on ambient oxygen. Within this procedure, a great deal of subtle variation exists between studies. Most obvious is the differential use of SMR and RMR, with the majority (84 %) of studies reporting a  $P_{crit}$  for RMR (Figure 6). Arguably, the  $P_{crit}$  exhibited for RMR is more ecologically relevant given that this level of  $MO_2$  is likely to be exhibited most of the time in the field (Ultsch *et al.*, 1978; Portner, 2010). Indeed for some highly active species such as salmonids,  $P_{crit}$  determined during active swimming may be most useful in considering the ecological implications of hypoxia (Fry, 1957). Activity level may affect  $P_{crit}$  in unexpected ways such as in the Adriatic sturgeon (*Acipenser naccarii*) which exhibits a well-developed ability to oxyregulate ( $P_{crit} = 4.9 \pm 0.5$  kPa) when permitted to swim at a low sustained speed, but oxyconforms across the entire range of declining ambient oxygen when its activity is restricted in a static respirometer (McKenzie *et al.*, 2007). Some species exhibit a  $P_{crit}$  for RMR at a relatively high  $PO_2$  that is well above the  $P_{50}$  of their haemoglobin. In these instances,  $P_{crit}$  may indicate a behavioural change and not simply a physical limitation of oxygen supply (McBryan *et al.*, 2013).

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Of the studies that determine the  $P_{crit}$  for SMR, the methods used for quantifying SMR vary considerably. Some studies use the single lowest  $MO_2$  value recorded at normoxia whilst others take the average of a set number of the lowest  $MO_2$  values (Iverson *et al.*, 2010). More sophisticated and robust methods involve extrapolating the average  $MO_2$  measured at specified swimming speeds back to zero activity (Cook *et al.*, 2014) or the use of percentiles and frequency distributions to assess all normoxic  $MO_2$  data (Dupont-Prinet *et al.*, 2013). As the critical level for basal metabolism,  $P_{crit}$  determinations based on SMR should theoretically reflect a true physiological limitation of oxygen extraction capacity (McBryan *et al.*, 2013). Given that the  $P_{crit}$  for RMR is likely to be encountered at higher  $PO_2$  than that for SMR (Figure 3), intra- or inter-species comparisons between studies reporting different levels of  $MO_2$  may not be entirely valid. Whether SMR or RMR measurements are utilized to reflect normoxic  $MO_2$  it is essential that sufficient time is allowed for the fish to acclimate to the respirometry chamber. Otherwise, apparent reductions in  $MO_2$  as hypoxia develops may be an artefact of increasing habituation rather than true oxyconforming (Nilsson *et al.*, 2004).

The method employed to establish the point of intersection between continuous oxyregulation and oxyconforming  $MO_2$  data is also inconsistent between  $P_{crit}$  studies. The slope of these lines will determine the  $P_{crit}$  and *vice versa*. Therefore, determining which data points should be considered part of which line is critical to establishing an accurate estimate of  $P_{crit}$  (Yeager *et al.*, 1989). This can be achieved graphically by fitting a least-squares linear regression through datapoints that show a progressive decline in  $MO_2$  such that it intersects with a regression line fitted through normoxic  $MO_2$  data (Monteiro *et al.*, 2013). A number of mathematical methods for performing so called piece-wise or segmented linear regression analyses are available which provide greater robustness to estimates of  $P_{crit}$  and are used in the majority of studies incorporated into the present database (Nickerson *et al.*, 1989; Yeager *et al.*, 1989). These approaches assume that the response of  $MO_2$  to declining  $PO_2$  is biphasic and consists of two entirely linear elements with an abrupt transition between the two. Such assumptions are not necessarily met by real-

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world data and indeed concentration-dependent reaction kinetics make truly linear relationships between  $\text{MO}_2$  and  $\text{PO}_2$  unlikely (Marshall *et al.*, 2013). Recent developments in non-linear regression techniques are now being promoted as a more accurate approach to determining biological thresholds such as  $P_{\text{crit}}$  (Stinchcombe & Kirkpatrick, 2012; Marshal *et al.*, 2013).

Perhaps unsurprisingly, most studies of  $P_{\text{crit}}$  in fish have been concentrated around the major fish physiology research groups in Europe, North America, Canada and Australia (Figure 5). Arguably this introduces an element of bias into the database given the limited representation of habitats and species at a global scale. In addition the species studied tend to be those conducive to respirometry trials. In particular, large, active or highly sensitive species such as those of the Scombridae family (tuna, mackerels, bonitos) are generally underrepresented in the literature (Blank *et al.*, 2007).

### $P_{\text{crit}}$ as a Hypoxia Tolerance Trait

A low  $P_{\text{crit}}$  is generally associated with well adapted hypoxia tolerance because it indicates a high capacity for oxygen extraction and tissue delivery at low  $\text{PO}_2$  (Mandic *et al.*, 2009). Maintaining aerobic metabolism during hypoxia is advantageous because it is up to 30-fold more efficient than anaerobic ATP production (per unit substrate consumed) and avoids accumulation of the deleterious by-products (e.g.  $\text{H}^+$ ) of anaerobic metabolism (Richards, 2009). Hypoxia-induced physiological modifications that increase oxygen extraction capacity, such as increased gill surface area (Nilsson, 2007) and haemoglobin  $\text{O}_2$  binding (Brix *et al.*, 1999) are observed in fish that frequently encounter hypoxia, suggesting that maintaining aerobic metabolism is a primary hypoxic survival strategy (Mandic *et al.*, 2009). However, when ambient  $\text{PO}_2$  declines below  $P_{\text{crit}}$ , survival depends on the availability of substrate for  $\text{O}_2$ -independent ATP production (primarily glycolysis), and the ability to reduce metabolic demand (Richards, 2009).

How long a fish can maintain a balance between ATP demand and supply below its  $P_{\text{crit}}$  and thus delay the onset of cellular dysfunction, necrosis and subsequent death, is a key component of hypoxia tolerance (Nilsson &

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Ostlund-Nilsson, 2008; Speers-Roesch *et al.*, 2013). Speers-Roesch *et al.*, (2013) showed that  $P_{crit}$  does not entirely predict hypoxia tolerance at lower oxygen levels. Those authors exposed three species of sculpin (*Blepsias cirrhosis*, *Leptocottus armatus* and *Oligocottus maculosus*) known to exhibit different values of  $P_{crit}$ , to relative hypoxia (30 % of their respective  $P_{crit}$ ) and determined that time to loss of equilibration (LOE) was only consistent in two out of the three species. Similar relative hypoxia exposures in the epaulette shark (*Hemiscyllium ocellatum*) and shovelnose ray (*Aptychotrema rostrata*) revealed lower lactate accumulation in epaulette sharks indicating enhanced metabolic depression in this species (Speers-Roesch *et al.*, 2012). Furthermore, Nilsson & Ostlund-Nilsson (2008) showed that  $P_{crit}$  did not correlate with body mass in juvenile and adult damselfish (Pomacentridae) ranging between 10 mg and 40 g but that smaller fish were much less tolerant to hypoxia below  $P_{crit}$  due to their limited capacity for meeting ATP demand through anaerobic metabolism. These results illustrate the benefit of considering  $P_{crit}$  alongside other methods of determining hypoxia tolerance such as measurements of tissue specific lactate accumulation and determinations of LOE<sub>50</sub>, in order to assess overall hypoxia tolerance (Speers-Roesch *et al.*, 2013).

As a hypoxia tolerance trait,  $P_{crit}$  alone does not reflect specialist hypoxia survival strategies such as adaptations for emersion and air breathing. The inanga (*Galaxias maculatus*) which inhabits lowland streams prone to severe hypoxia, is a rare example of a fish species that appears to be an entirely obligate oxyconformer and thus demonstrates no discernible  $P_{crit}$  (Urbina *et al.*, 2012). However, a lack of scales and a large surface area to volume ratio infers a high capacity for cutaneous O<sub>2</sub> uptake whilst emersed and hence provides a short-term means to escape aquatic hypoxia (Urbina *et al.*, 2011). Air breathing as an extreme adaptation to hypoxia is seen in a number of fish species such as the bowfin (*Amia calva*) which possesses a swim bladder modified for use as an air-breathing organ (Randall *et al.*, 1981). Bowfin exhibit a relatively high  $P_{crit}$  of  $9.3 \pm 1$  kPa at 22 °C when denied access to air but are able to maintain their blood PO<sub>2</sub> during severe hypoxia (1.9 kPa) when given

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access to air and able to perform air-breathing (Porteus *et al.* 2014). Emersion, air-breathing and aquatic surface respiration thresholds were incorporated into the database, but only where they have been reported alongside  $P_{crit}$  measurements. Such examples demonstrate the limitation of  $P_{crit}$  as a universal and comparative measure of hypoxia tolerance between species and emphasises the benefit of multi-trait based approaches.

## Biotic and Abiotic Interactions

Environmental stressors such as hypoxia rarely occur in isolation and the interaction between stressors is of key concern in the context of predicting the ecological impacts of future environmental change (Crain *et al.*, 2008). As a typical threshold effect, the response of fish to hypoxia is likely to result in ‘ecological surprises’, whereby seemingly resilient populations suddenly collapse once a critical threshold is crossed (McBryan *et al.*, 2013). Additive or synergistic interactions with hypoxia could hasten the arrival of such thresholds meaning that small environmental shifts could result in large effects on the performance of a population. Theoretically, any abiotic or biotic factor that affects either oxygen supply (cardiorespiratory capacity) or oxygen demand (metabolic rate) of an individual and the balance therein, will have implications for its hypoxia tolerance. As an indicator of hypoxia tolerance, the effect of a wide range of abiotic and biotic interactions on  $P_{crit}$  in fish have been studied in the literature (Table 2) and the most prevalent of these are discussed here.

Temperature is by far the most widely studied abiotic interaction with hypoxia and is particularly relevant given predicted near-future global warming (Ficke *et al.*, 2007; Portner, 2010). As ectotherms, oxygen demand in fish increases roughly exponentially with temperature (inter-species mean  $Q_{10}$  of 1.83; Clarke & Johnstone, 1999) and the intrinsic link between temperature and hypoxia has become the basis of an overarching concept termed ‘oxygen and capacity limitation of thermal tolerance’ (OCLTT; Portner, 2001, 2010). Essentially this concept suggests that the thermal tolerance of ectotherms is dictated by their capacity for meeting aerobic demand. Whilst increased temperature elevates

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basal oxygen demand (SMR), hypoxia reduces oxygen supply, hence temperature and hypoxia are likely to act synergistically in fish. Within species, increasing temperature generally results in an increased  $P_{crit}$  (Table 2). However, the slope of the relationship between temperature and  $P_{crit}$  is highly variable between species (Figure 7). For example, Atlantic salmon (*Salmo salar*) exhibits a steep linear increase of  $P_{crit}$  in comparison to the shallower slope seen in the common carp (*Cyprinus carpio*) across a similar temperature range (Remen *et al.*, 2013; Ott *et al.*, 1980). A surprising exception to the generally positive intra-species correlation between temperature and  $P_{crit}$  was observed in four out of six species of darter (*Etheostoma*) for which  $P_{crit}$  was lower at 20 than 10 °C (Ultsch *et al.*, 1978). Variation between species' sensitivity to temperature in terms of hypoxia tolerance likely arises due to differences in their potential for thermal acclimation either through reducing the metabolic impact of increased temperature or by enhancing oxygen extraction capacity (Ott *et al.*, 1980; Portner, 2010). Species exhibit highly contrasting acclimation potential. At opposite ends of this spectrum, Crucian carp (*Carassius carassius*) have been observed to dramatically increase respiratory surface area through gill remodelling in response to temperature and hypoxia (Sollid *et al.*, 2005) whilst certain tropical reef fish species (*Ostorhinchus doederleini* and *Pomacentrus moluccensis*) demonstrate no thermal acclimation ability even over a relatively modest temperature range (29 - 32 °C; Nilsson *et al.*, 2010).

Unlike intra-species  $P_{crit}$ , there is no apparent relationship between temperature and inter-species  $P_{crit}$  (Figure 7) suggesting that evolutionary adaptation has nullified the thermal sensitivity of hypoxia tolerance across species. Previously it has been shown that the difference in RMR between a typical cold-water and warm-water fish is less than expected given the thermal sensitivity of RMR within individual species (intra-species median  $Q_{10} = 2.4$ ; Clarke & Johnstone, 1999). In addition, gill surface area appears to scale linearly with metabolic rate implying that natural selection equips fish with the oxygen extraction capacity required to match demand at higher temperatures (Nilsson & Ostlund-Nilsson, 2008). Selective pressures for small gills such as

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the osmosrespiratory compromise, gill parasites and risks associated with gill injury are likely to limit respiratory surface area so that oxygen extraction capacity does not exceed that required by a particular species for survival in its natural range (Nilsson, 2007). Thus, it is not possible to make generalisations regarding hypoxia tolerance across temperatures at the inter-species level.

Since the biological processes that consume O<sub>2</sub> also produce CO<sub>2</sub>, hypoxia and hypercarbia commonly co-occur in aquatic environments (Ultsch, 1996, Cruz-Neto & Steffensen, 1997, Gilmour, 2001). Despite this, the interactive effect of environmental hypercarbia on hypoxia tolerance has been relatively understudied. As previously discussed, there are conflicting reports within the available literature as to the effect of hypercarbia on the P<sub>crit</sub> of fish (Cochran & Burnett, 1996; Cruz-Neto & Steffensen, 1996). The most likely mechanism by which hypercarbia could negatively impact on hypoxia tolerance is through inducement of respiratory acidosis leading to the Bohr / Root effect on haemoglobin and reduced oxygen extraction capacity (Jensen *et al.*, 1993; Cruz-Neto & Steffensen, 1996). In this respect hypercarbia is partially akin to the far more extreme acidosis that can occur in poorly buffered freshwater environments subjected to acid precipitation or drainage. Acidification of the surrounding water (pH range 7.4 – 4.0) has been shown to lead to increased P<sub>crit</sub> in both rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*; Ultsch *et al.*, 1980). The time required to compensate for acid-base disturbance is highly variable between species and as such the effect of hypercarbia and acidification on hypoxia tolerance is likely to be largely dependent on the species in question as well as the severity and duration of the hypercarbic or acid exposure (Jensen *et al.*, 1993). The effect of CO<sub>2</sub> on hypoxia tolerance is further explored in chapter four of this thesis, through a direct experimental approach using European flounder.

Exposure to trace metal contamination appears to reduce hypoxia tolerance in fish. Specifically, exposure to elevated concentrations of copper (300 µg l<sup>-1</sup>), mercury (150 µg l<sup>-1</sup>) and silver (63 µg l<sup>-1</sup>) have been demonstrated to increase P<sub>crit</sub> in various species (Table 2). Primarily, the precipitation of such metals on

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lamellar surfaces stimulates the hypersecretion of gill mucus which acts as a barrier to diffusion of outside toxicants into the blood (McDonald & Wood, 1993; Wilson *et al.*, 1994). In addition, some trace metals appear to cause hyperplasia and hypertrophy of gill epithelia cells that results in the fusing and thickening of gill lamellae (Schjolden *et al.*, 2007; Bilberg *et al.*, 2010). As a consequence of such responses, respiratory function is compromised due to reduced diffusion area and increased diffusion distance (McDonald & Wood, 1993). The organophosphate trichlorfon has been shown to increase  $P_{crit}$  by inducing similar gill morphology changes as well as through the promotion of vasoconstriction that results in reduced lamellar blood flow (Thomaz *et al.*, 2009). These interactions between xenobiotic contaminants and hypoxia in fish are of clear concern, particularly given that both stressors predominantly threaten freshwater and coastal marine systems and are therefore likely to coincide (McDonald & Wood, 1993; Diaz & Rosenberg, 2008).

Determinations of  $P_{crit}$  in fish have almost universally been made in unfed, post-absorptive individuals which, whilst providing a useful basis for intra- and inter-species comparisons of absolute hypoxia tolerance, does not fully account for the digestive state typical of fish in their natural setting. A rise in oxygen consumption following ingestion of food, termed specific dynamic action (SDA), is required in order to meet the energetic costs associated with mechanical and biochemical digestion and assimilation (Jobling, 1993). Shortly after a meal, oxygen consumption in fish typically rises rapidly, reaching a peak two to three times higher than pre-fed levels within a few hours. The shape and duration of the SDA is highly dependent on the species in question as well as meal size and composition (Secor, 2009). Measurements of  $P_{crit}$  in fish undergoing SDA have revealed significant increases in  $P_{crit}$  compared to unfed controls showing that increased aerobic demand during digestion has negative consequences for hypoxia tolerance (Table 2). In Perch (*Perca fluviatilis*) force fed a 5% body mass ration,  $P_{crit}$  at twenty hours post-feeding was increased by 1.44-fold compared to sham fed individuals (Thuy *et al.*, 2010). Similarly, oscars (*Astronotus ocellatus*) fasted for 14 days showed a 1.6 fold lower  $P_{crit}$  than individuals fed a daily 1% body

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mass ration up to 24 hours prior to  $P_{crit}$  determination (Boeck *et al.*, 2013). In such experiments, the requirement for a stable  $MO_2$  on which to base a determination of  $P_{crit}$  means that measurements at peak SDA are not feasible and thus are likely to underestimate the effect of digestion on hypoxia tolerance (Thuy *et al.*, 2010).

Several studies have investigated the effect of hypoxia pre-conditioning on  $P_{crit}$  (Table 2). Broadly, short-term physiological acclimation to hypoxia appears to be achieved through either enhanced  $O_2$  extraction capacity or metabolic depression. In goldfish (*Carassius auratus*) 48 hours of severe (0.63 kPa) hypoxia induced dramatic increases in both lamellae surface area and blood haemoglobin content, leading to a 49% reduction in  $P_{crit}$  compared to individuals held at normoxia (Fu *et al.*, 2011). Similarly, sailfin molly (*Poecilia latipinna*) demonstrated increased haemoglobin and red blood cell concentrations, and a reduced  $P_{crit}$  following a six week exposure to severe hypoxia (Timmerman & Chapman, 2004). Depression of RMR at normoxia and a subsequent reduction in  $P_{crit}$  following chronic hypoxic exposure, has been observed in the epaulette shark (*Hemiscyllium ocellatum*; Routley *et al.*, 2002) and qingbo (*Spinibarbus sinensis*; Dan *et al.*, 2014). However, some less hypoxia tolerant species appear to demonstrate no physiological acclimation potential through hypoxic pre-conditioning. Daily exposure to 6 hours of moderate hypoxia (10.5 kPa) for 33 days had no effect on  $P_{crit}$  in post-smolt Atlantic salmon (*Salmo salar*; Remen *et al.*, 2013). Additionally, chronic (6 week) moderate hypoxia produced no change in the  $P_{crit}$  of juvenile snapper (Cook *et al.*, 2013; *Pagrus auratus*).

As hypoxia is likely to become an increasingly predominant aquatic perturbation in the future (Vaquer-Sunyer & Duarte, 2008; Keeling *et al.*, 2010), the degree of physiological plasticity for hypoxia tolerance will be a key determinant of species performance. The potential for long-term and intergenerational hypoxia acclimation with respect to  $P_{crit}$  has been largely unstudied. Reardon & Chapman (2010) demonstrated a strong element of developmental plasticity in the  $P_{crit}$  of the Egyptian mouthbrooder (*Pseudocrenilabrus multicolour*) when reared under hypoxic conditions In

48

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addition, intra-species population effects on  $P_{crit}$  across habitats of differing  $O_2$  regimes have been observed in several species, indicating that a high degree of adaptive capacity for  $P_{crit}$  exists within these populations (Timmerman & Chapman, 2004; Reardon & Chapman 2010; Fu *et al.*, 2011).

## Future Applications

The comprehensive  $P_{crit}$  database presented here provides the opportunity for a variety of further analyses with potential to offer fundamental physiological, as well as wider ecological insights. Elucidating the relationship between metabolic rate and  $P_{crit}$ , provides a relatively simple prospect for further meta-analysis. However, given the strong association between temperature and metabolic rate in ectotherms, such an analysis across the wide range of temperatures reported in the present database requires careful consideration of temperature as a major confounding factor. More complex analyses could involve combining species  $P_{crit}$  with phylogenetic data as a means to investigate the evolutionary relationships of hypoxia tolerance across species (Mandic *et al.*, 2009). Similarly, combining species  $P_{crit}$  with information on the spatial distribution of populations would help to establish the ecological relevance of  $P_{crit}$  as a physiological trait. Such an analysis would be particularly relevant to predicting the impacts on fish populations likely to arise from the increasingly widespread occurrence of hypoxic zones in aquatic environments around the globe (Friedrich *et al.*, 2014).

The integration of the present database with similar databases of other widely measured physiological parameters in fish will likely offer useful insights into interactions between traits. Such physiological data are of great value for improving the predictive capacity of models as an aid to the management and conservation of aquatic systems (Jørgensen *et al.*, 2012; Cooke *et al.*, 2014). Traits for which databases are currently under construction include SDA, aerobic scope, growth rate and critical temperature. On completion, the combined dataset will be made widely accessible via an online data repository facility such as that provided by Dryad (<http://datadryad.org/>). Thus it is

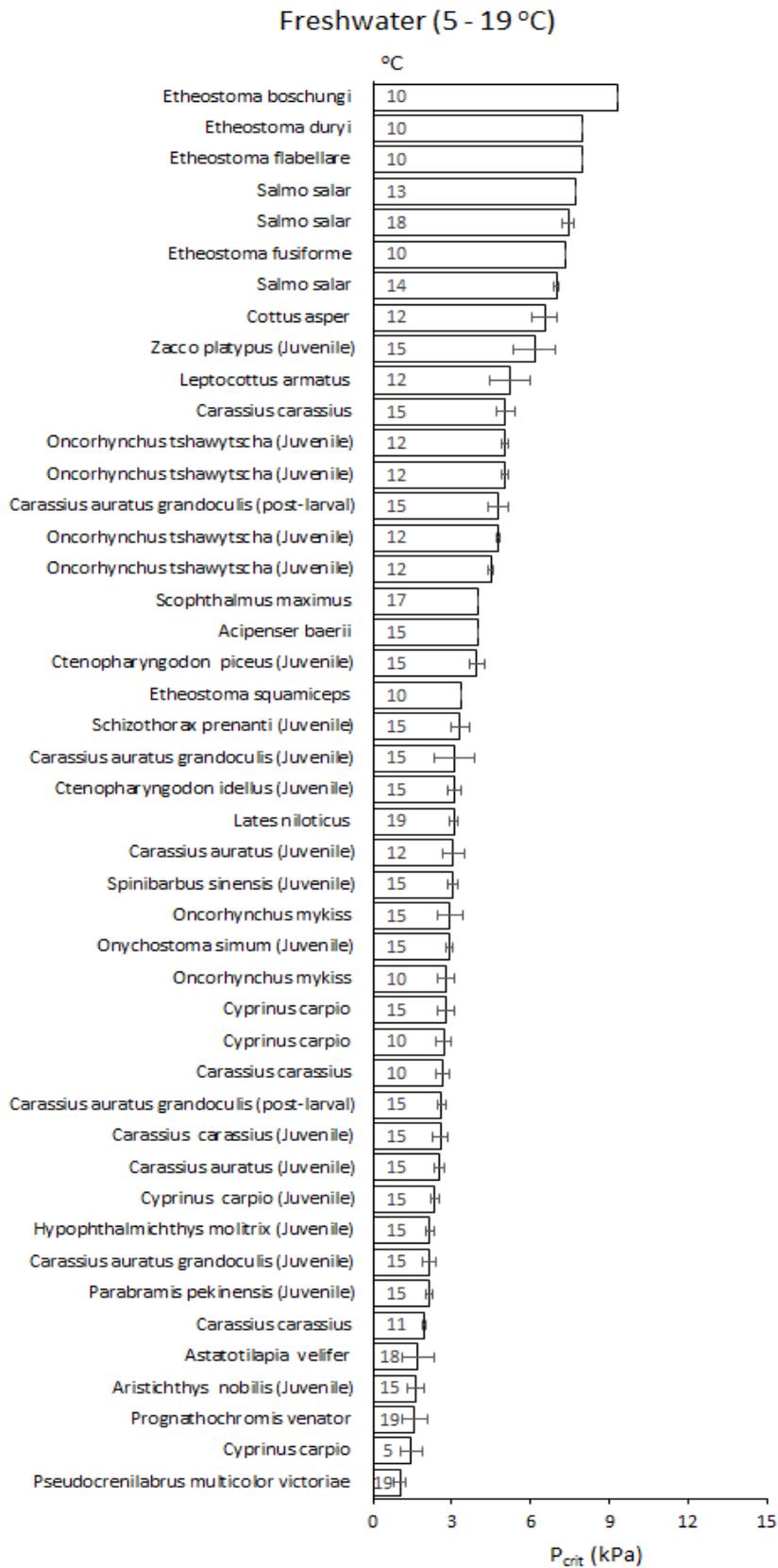
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envisaged that these data will prove to be a tangible link between the field of fish physiology and future studies of ecology, conservation and management.

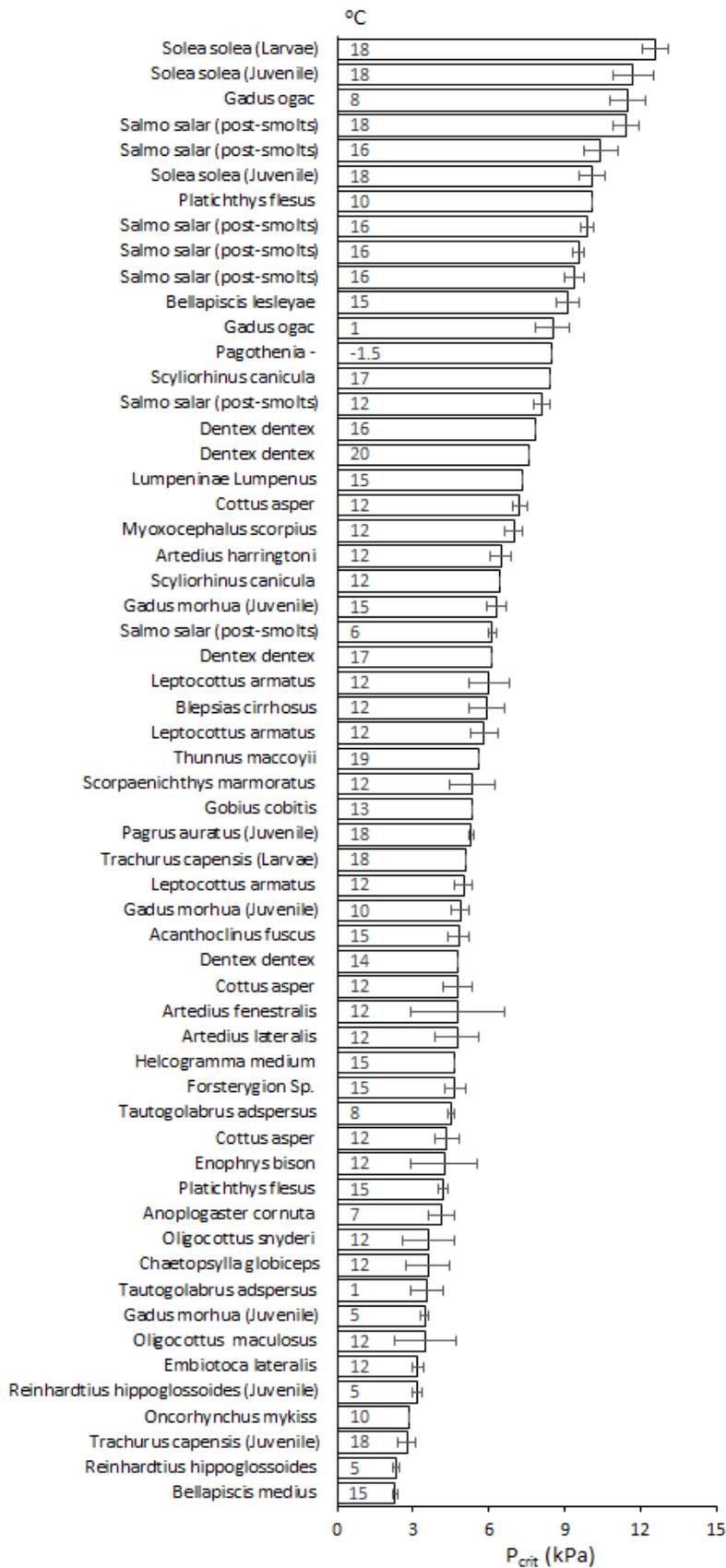
## **Acknowledgments**

This physiological database is a contribution of the European Union Cooperation in Science and Technology (COST) Action (FA1004) on the 'Conservation Physiology of Marine Fishes'. The same EU COST Action supported this work as a Short Term Scientific Mission (STSM). For more information, see: <http://fish-conservation.nu/>.

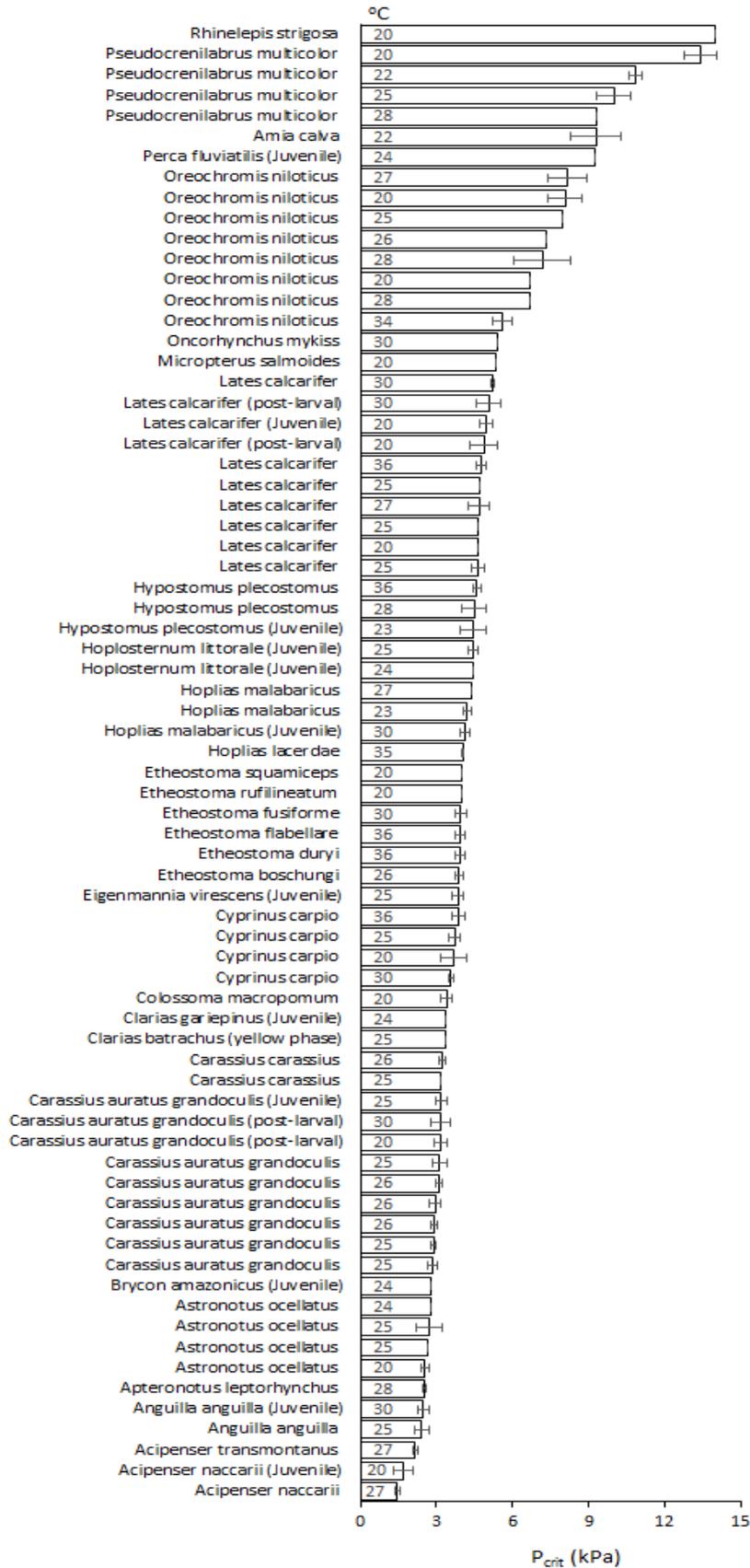
# Appendix



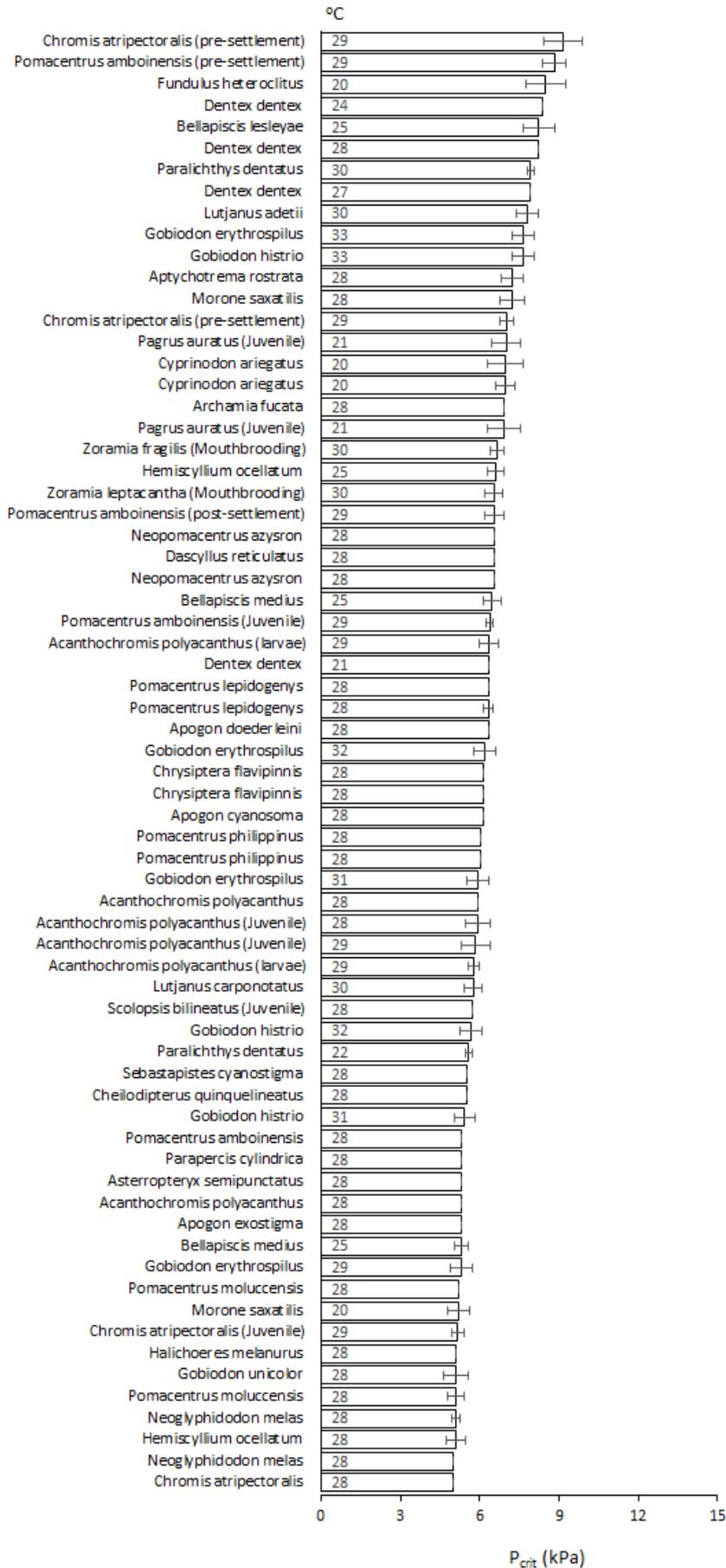
## Marine (-1.5 - 20 °C)



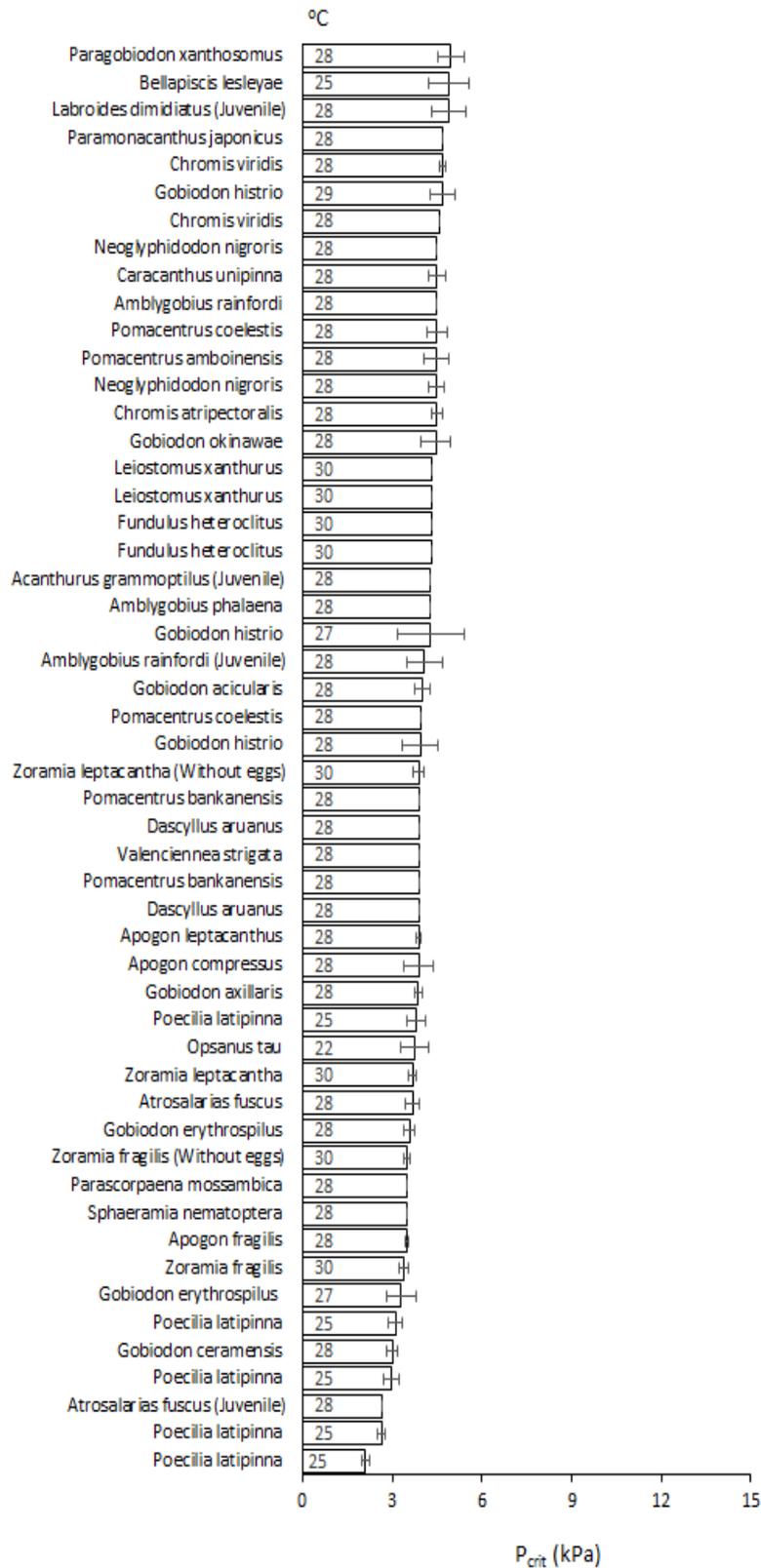
### Freshwater (20 - 36 °C)



## Marine (20 - 33 °C)



Marine (20 - 33 °C) cont.



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Figure 8 (previous five pages). Plots of the species and their respective mean  $P_{crit}$  ( $\pm$  SE) values that were incorporated into the 'control' dataset. Numbers contained within each bar indicate the temperature ( $^{\circ}$ C) at which  $P_{crit}$  was determined. Data are ordered by  $P_{crit}$  (highest to lowest) and grouped by temperature range and water type (marine / freshwater).

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# Chapter 3



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# Thermal and hypoxia tolerance as traits of fishes using shallow-water coral-reef habitats.

## Abstract

There is an increasing interest in understanding how species' traits affect their biogeography and biology. This study examines whether intra and inter-species differences in the physiological thermal and hypoxia tolerances of fish relate to their distributions across the contrasting biophysical environments of the coral reef flat and slope. Daily and seasonal trends in temperature and dissolved oxygen on the reef flat and slope surrounding Heron Island, Great Barrier Reef, were recorded over two years. These measurements revealed substantially greater fluctuations in oxygen and temperature on the flat compared to the slope, with daily temperature and hypoxia extremes occurring on the reef flat over tidal and diurnal cycles. The thermal and hypoxia tolerances of two reef snapper species were tested: Spanish flag snapper (*Lutjanus carponotatus*), a species found in both flat and slope habitats, and yellow-banded snapper (*Lutjanus adetii*), a species limited to the reef slope. Individuals of both species were acclimated to one of two temperatures, 20 and 30 °C. Aerobic scope (AS), critical oxygen tension ( $P_{crit}$ ), critical maximum ( $CT_{max}$ ) and minimum ( $CT_{min}$ ) temperature were measured as indicators of thermal and hypoxia tolerance. *L. carponotatus* was clearly the most thermally and hypoxia tolerant of the two species, demonstrating a ~3.5 °C wider thermal tolerance zone (higher  $CT_{max}$ , lower  $CT_{min}$ ) and ~26% lower  $P_{crit}$  than *L. adetii*. These results show that inter-species variation in the distribution of these fish between flat and slope reef zones is reflected in their physiological tolerances. We found no evidence of intra-species variation in tolerance between flat and slope *L. carponotatus* individuals, indicating that they do not form physiologically distinct subpopulations between these reef zones. This study provides evidence of inter species variation in physiological tolerance that underpins variation in distribution; the appreciation of which is necessary for predicting the impacts of future climate change scenarios on reef fish assemblages.

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## Introduction

Understanding the biotic and abiotic controls of species habitat preferences is a key goal of ecology, and it is well established that physiological capacity plays a crucial role in understanding the mechanisms underlying inter- and intra-species variations in range (Chown *et al.*, 2004). Consequently, the physiology of species represents an important trait that may be used to provide a generic understanding of how organisms are distributed across landscapes, and trait-based approaches that integrate physiology and ecology are increasingly commonly utilized (Chown, 2012). Among fishes, thermal and hypoxia tolerance traits have been shown to underpin habitat partitioning in sympatric fish species from a range of different environments (Ultsch *et al.*, 1978; Dent & Lutterschmidt, 2003; Hilton *et al.*, 2008; Eme & Bennett, 2009; Mandic *et al.*, 2009).

Trait-based approaches are increasingly being used for coral-reef fishes, including for understanding patterns of geographical range (Luiz *et al.*, 2013), feeding behaviour (Green & Cote, 2014) and tropicalisation of temperate regions (Feary *et al.*, 2014). Coral reefs are characterized by zonation and fish assemblages vary dramatically between reef zones as a result of significant differences in their biophysical environment (Done, 1983). However, the role of fish physiology in this zonation has been poorly studied, despite its likely importance given the highly contrasting, but interconnected, habitats found on coral reefs (Harborne, 2013). Here we examine the role of physiology, namely temperature and hypoxia tolerance, in relation to the distribution of fishes across two major hard-bottom reef zones on Indo-Pacific reefs: the flat and the slope (Figure 1). The reef slope extends seaward of the crest and descends towards the inter-reefal floor. Typically the slope exhibits dense coral growth subject to consistent gradients of decreasing water movement and light with increasing depth. Reef 'flat' is a broad term that encompasses a number of reef habitats found landward of a reef crest or ridge. Although not characteristic of all tropical reef systems, tidal flats are a common feature of Pacific reefs. On the Great Barrier Reef (GBR), flats are among the most extensive of habitats (Harborne, 2013) and are typically shallow with significant

60

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relative changes in depth across tidal cycles. Benthic structure varies considerably across reef flats, ranging from coral-dominated to rubble-dominated and is mainly determined by substrate and hydrodynamic regime which vary markedly with small changes in elevation or lateral position (Done, 1983). Both habitats support abundant and diverse, but distinct, fish assemblages (Williams, 1991).

Reef flats provide a functionally important habitat to a diverse assemblage of adult and juvenile fish. This assemblage is highly dynamic, changing across tidal cycles, with species richness and abundance decreasing as the tide drops (Ashworth *et al.*, 2006; Unsworth *et al.*, 2007; Harborne, 2013). In particular, it appears that large piscivorous fish leave the flat at low tide (Unsworth *et al.*, 2007; A. Harborne *pers. comm.*, 2014). Thus, reduced predation pressure is likely to be a key driver for smaller fish to adapt to life on reef flats. Indeed, juveniles are abundant on reef flats (Ashworth *et al.*, 2006; Pratchett *et al.*, 2008; Clark & Russ, 2012) and there is evidence to suggest that reef flats may act as nursery habitats for several fish species (McCormick & Makey, 1997; Craig *et al.*, 1997; Dorenbosh *et al.*, 2005 Wen *et al.*, 2013).

Shallow depths combined with significant tidal flow, result in a highly variable abiotic environment on reef flats. Over short temporal scales, the tide drives significant changes in depth, which in turn lead to fluctuations in temperature, dissolved oxygen and UV (Harborne, 2013). In this respect, reef flats are arguably akin to other intertidal marine habitats, such as rocky shores and estuaries, which are recognised as being highly dynamic environments (Tait & Dipper, 1998). Compared to the flat, the deeper waters of the reef slope are less influenced by the tide and are thus more abiotically stable (Pots & Swart, 1984). Species that exploit reef flats are therefore likely to require greater physiological tolerances than species which exclusively live on the reef slope.

The fluctuations in temperature on reef flats are likely to be particularly problematic for fish. As ectotherms, temperature is the primary abiotic factor influencing the physiological performance of fish (Brett & Groves 1979; Beitinger *et al.*, 2000). All species have an optimum temperature range and

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species differ in their ability to respond to thermal change whether that be short-term (e.g. over tidal cycles) or long-term (e.g. seasonal), and this ability is referred to as thermal tolerance (Madeira *et al.*, 2012). The capacity for fish to perform aerobically (aerobic scope), is believed to dictate thermal tolerance (Portner & Farrell., 2008; Portner & Lannig, 2009). Oxygen consumption is compromised at both ends of the thermal envelope due to the limited capacity of circulatory and ventilatory systems to meet oxygen demand (Portner, 2001). Failure to meet oxygen demand results in a reduction of aerobic scope which affects all higher functions such as locomotion, growth, behaviour and reproduction (Portner & Knust, 2007). Nilsson *et al.*, (2009) found that an increase in temperature from 29 to 33 °C almost entirely eliminated the aerobic scope of cardinalfishes whereas damselfish retained over half their aerobic scope. This illustrates the degree to which thermal tolerance can differ between species (and possibly within families) within the same reef assemblages.

Another major challenge for fish on reef flats is the periodic depletion of dissolved oxygen. Hypoxia is not commonly associated with coral reef systems, however oxygen has increasingly been recognised as a major abiotic selection pressure on reef fish (Nilsson & Ostlund-Nilsson., 2004; Nilsson *et al.*, 2007a). Oxygen saturation between respiring coral branches, where many fish species take refuge at night, has been shown to fall to an average of 20 % of air saturation and even as low as 2 % for short periods (Nilsson *et al.*, 2004.). Consequently, a wide range of reef fish has been identified as hypoxia tolerant (Nilsson *et al.*, 2007a). For example, in a study of 31 teleost species (7 families) from the GBR, all maintained their oxygen consumption rate down to between 20 and 30 % of air saturation and most appeared unaffected until oxygen fell below 10 % (Nilsson & Ostlund-Nilsson, 2004). This tolerance is comparable to tropical freshwater species that inhabit hypoxic waters and that are well known for their hypoxia tolerance (Verheyen *et al.*, 1994; Rosenberger & Chapman, 2000; Sloman *et al.*, 2006; Petry *et al.*, 2013).

Although not limited to reef flats, periods of hypoxia are likely to be exaggerated and more regularly encountered by fish living on the flat

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compared to the slope. Reasons for this include higher temperatures, the potential for entrapment in tidal pools and the need to shelter in corals at low tide (Harborne, 2013). Limited mixing with oceanic water at low tide combined with respiration by corals and other reef organisms, particularly at night when photosynthesis ceases, can lead to the development of widespread hypoxia on reef flats. During nocturnal low tides, ambient oxygen levels on reef flats have been found to drop as low as 15 % of air saturation (Kinsey & Kinsey, 1966; Routley *et al.*, 2002). The epaulette shark (*Hemiscyllium ocellatum*), a reef flat specialist, was one of the first tropical reef species in which hypoxia tolerance was identified (Wise *et al.*, 1998). In response to falling oxygen concentration, epaulette sharks were able to maintain a stable oxygen consumption rate down to around 27 % of air saturation (Routley *et al.*, 2002). This tolerance for hypoxia, the highest measured in any elasmobranch, shows adaptation to the frequent depletion of oxygen that can occur on reef flats (Nilsson & Renshaw, 2004).

In this study we examine the thermal and hypoxia tolerance of two closely related snapper species *Lutjanus carponotatus* and *Lutjanus adetii*. Both species are conspicuous, medium-sized carnivorous fish found in the Western Pacific and are abundant on the GBR (Allen, 1985). *L. adetii* appears to be restricted to the reef slope, congregating around outcrops during the day and feeding along the reef slope at night. In contrast, *L. carponotatus* inhabits both flat and slope habitats and is more abundant at shallow depths (< 30 m) than *L. adetii* (Allen, 1985; Newman *et al.*, 1996). Furthermore, there is some evidence to suggest that *L. carponotatus* forms two distinct sub-populations on the reef flat and slope. A 15 times higher parasite load (*Pomphorhynchus heronensis*) was found on reef flat *L. carponotatus* individuals compared to slope individuals (Cribb *et al.*, 2000), and because parasite transmission is concentrated locally, this implies that there is limited local movement of *L. carponotatus* between flat and slope habitats. The two *Lutjanus* species therefore provide an opportunity to examine not only inter-species, but in the case of *L. carponotatus* also the intra-species, variation in physiological tolerances across reef zones. We hypothesised that *L. carponotatus* would

have a greater thermal and hypoxia tolerance because of its ability to exploit reef flat habitats. Furthermore, by testing the thermal and hypoxia tolerance of *L. adetii* and *L. carponotatus*, we sought to examine whether these metrics could be considered as functional traits underlying the variation in habitat range of these two species, and potentially provide additional evidence of the benefit of considering physiology for exploring ecological patterns. Finally, concerns about rising sea surface temperatures have led to a growing body of literature on the effect of temperature on the physiology of reef fishes (Munday *et al.*, 2008, 2009; Gardiner *et al.*, 2010; Donelson *et al.*, 2010, 2011, 2012; Rummer *et al.*, 2014), and this study provides further evidence of the capacity of reef fishes to acclimate to different temperature regimes.

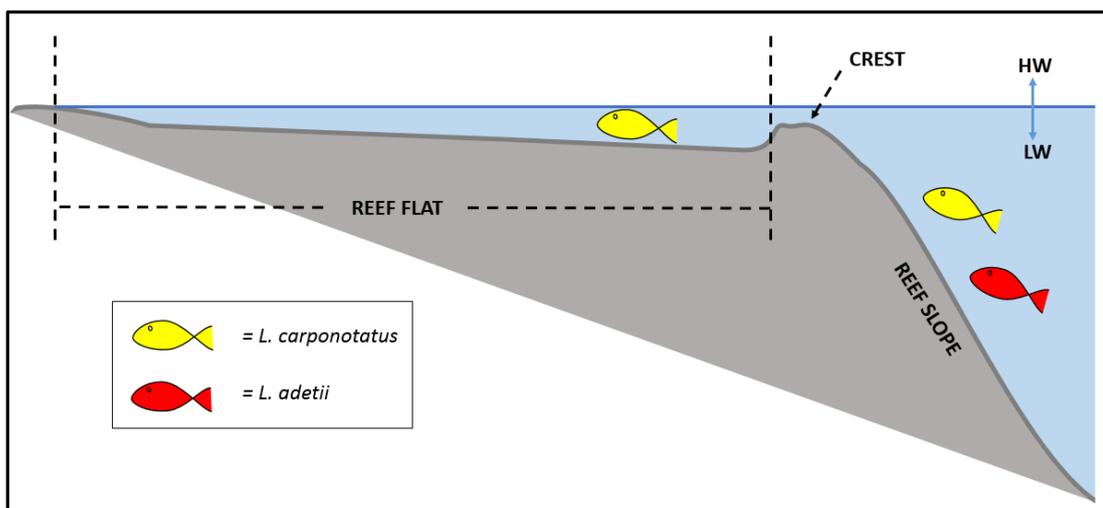


Figure 9. A simplified profile of a typical reef flat - slope zonation. The habitat ranges of the two snapper species *Lutjanus carponotatus* and *Lutjanus adetii* are indicated.

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## Materials and Methods

### Study Site

These experiments were conducted in November and December 2014 at Heron Island, which is a coral cay located in the southern GBR on the south-western side of Heron reef (Figure 10). The area supports a diverse fish assemblage and the waters around the island are split between Marine National Park, Conservation Park and Scientific Research management zones. The reef slope extends from the reef crest to depths of between 10 and 15 m. Surrounding the island is an extensive area of tidal reef flat up to 1 km in width and consisting of live and dead coral patches separated by sandy areas. At high tide the flats are ~2 m in depth, with some parts becoming aerially exposed at low tide (Grutter, 1998).



Figure 10. Map illustrating the location of Heron Island and the reef flat and slope fish capture sites (red rectangles).

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## Ambient Temperature and Dissolved Oxygen Measurements

Three depth loggers (Onset Hobo U20Titanium) were fixed in protective PVC housings at sites on the inner reef flat, outer reef flat, and reef slope (~6m depth) on the southern side of Heron Island to record temperature almost continuously during 2013 and 2014. Two oxygen loggers (Onset Hobo U26) and two conductivity / salinity data loggers (Onset Hobo U24) were fixed in two of the three reef zones at the same locations, and rotated among sites over the course of the two years to record daily and seasonal trends in dissolved oxygen concentrations (no continuous record at any one site). All loggers were retrieved every 3-6 months for cleaning and data downloads, and then replaced. The sensor on the oxygen logger was calibrated to 0% and 100% saturation following the manufacturer's instructions, and the sensor replaced every 6 months. The conductivity data were used to correct dissolved oxygen concentrations using the manufacturer's software.

## Experimental Animals and Thermal Acclimation

A total of 35 *L. adettii* and *L. carponotatus* were caught by hook-and-line on the reef slope around Harry's Bommie (S23 27 02, E151 55 03) and the reef flat on the south side of the island (S23 26 41, E151 54 54; Figure 10). Mean ambient water temperatures at all sites were ~25 °C. After capture, fish were held and recovered in a large (400cm x 400cm x 130 cm) holding tank of continuously flow-through aerated seawater pumped directly from the reef. Whilst in the holding tank, fish were fed daily *ad libitum* on chopped pilchard and squid. A transparent partition was used to keep flat and slope caught *L. carponotatus* separated. After a minimum of 3 days in the holding tank, fish were transferred to one of six 120 l acclimation tanks. One 160 l sump fed two acclimation tanks and water was circulated continuously between them in a closed loop. Sump tanks were heated (Thermo-control, EHEIM, Germany) or cooled (TK 1000, TECO, Italy) to the required acclimation temperature (temperature in each tank was logged every 5 minutes using a HOBO pendant temperature/light logger; Table 4) and a 100 % water change of the sump tank was conducted twice daily. Fish were acclimated to one of two temperatures,

~20 and ~30 °C, for four days prior to the first experiment. These acclimation temperatures were selected to reflect the upper and lower range of seasonal water temperatures on the reef. Fish were split into two groups (A and B) with each group sequentially following an identical protocol (Figure 11; 28 days in total).

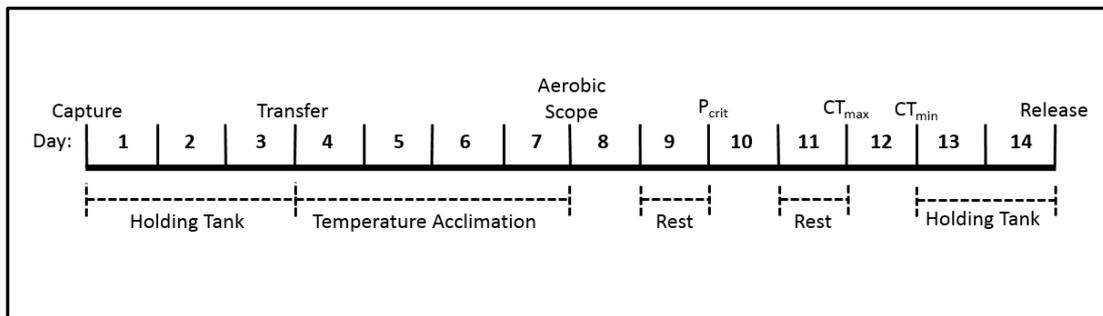


Figure 11. A timeline of the temperature acclimation and experimental procedures undergone by each fish from capture to release.

Species	Habitat	Acclimation		N	Mass (g)	
		Group				
<i>Lutjanus adetii</i>	Slope	High		7	366	± 88
		Low		6	265	± 16
<i>Lutjanus carponotatus</i>	Slope	High		6	532	± 109
		Low		6	481	± 197
<i>Lutjanus carponotatus</i>	Flat	High		5	292	± 146
		Low		5	274	± 149

Table 3. Number (N) and mean mass (± SD) of fish used in this series of experiments.

Acclimation Tank	Trial A (°C)		Trial B (°C)	
High 1	29.9	± 0.9	30.1	± 0.9
High 2	29.5	± 0.5	29.7	± 0.2
High 3	29.6	± 0.5	29.8	± 0.2
Low 1	20.7	± 0.6	20.6	± 0.5
Low 2	20.8	± 0.4	20.7	± 0.4
Low 3	20.6	± 0.5	20.6	± 0.6

Table 4. Mean temperature (°C ± SD) in the six acclimation tanks over the two trials.

## Respirometry

Intermittent flow-through respirometry as described by Steffensen (1989) was used to measure oxygen consumption rates (Figure 12). For the respirometry experiments, up to eight fish were transferred to individual 29 L chambers. The respirometry chambers were located within a 140 L ambient tank (two chambers per ambient tank). The water surface of the respirometry chamber was covered by two layers of bubble-wrap™ to minimise surface O<sub>2</sub> exchange (Spicer *et al*, 2007). Seawater of the correct temperature was pumped between sump tank, ambient tank and respirometry chamber in a closed loop. For measurement of metabolic rate (MO<sub>2</sub>), the flush pumps supplying the respirometry chambers were switched off and the fish allowed to consume oxygen within the chamber. By measuring the decline in PO<sub>2</sub> within the chamber the oxygen consumption rate (mgO<sub>2</sub> kg<sup>-1</sup> hr<sup>-1</sup>) of the fish was determined. This was calculated as follows:

$$MO_2 = \frac{\text{slope} \times \text{oxygen solubility} \times \text{respiratory volume}}{\text{fish mass}}$$

Where:

$$\text{slope} = \frac{PO_2 \text{ initial} - PO_2 \text{ final}}{\text{time}_{\text{initial}} - \text{time}_{\text{final}}}$$

And:

PO<sub>2</sub> (kPa), oxygen solubility (mgO<sub>2</sub> l<sup>-1</sup> kPa<sup>-1</sup>), volume (l), time (h), mass (kg).

Dissolved oxygen was measured using fibre-optic mini-sensors connected to a four-channel oxygen meter (Oxy-4 mini, Loligo® Systems, Denmark and FireStingO<sub>2</sub>, Pyro Science, Germany). Timing of the flush/measurement cycle was set such that the PO<sub>2</sub> within the chambers never fell by more than 10 % of the starting value and was fully restored to pre-measurement levels between measurement cycles. This generally entailed a 15 minute flush period followed by a 10 minute measurement period. The flush/measurement cycle was controlled and the metabolic rate data was recorded by Autoresp respirometry software (Loligo® Systems, Denmark). MO<sub>2</sub> values were rejected if the R<sup>2</sup> value of the slope was < 0.8. Respirometry chambers were thoroughly cleaned between trials and a negligible background rate of microbial O<sub>2</sub> consumption was measured within empty chambers (< 5 % of total consumption). Fish remained unfed for 24 hours prior to commencement of respirometry trials.

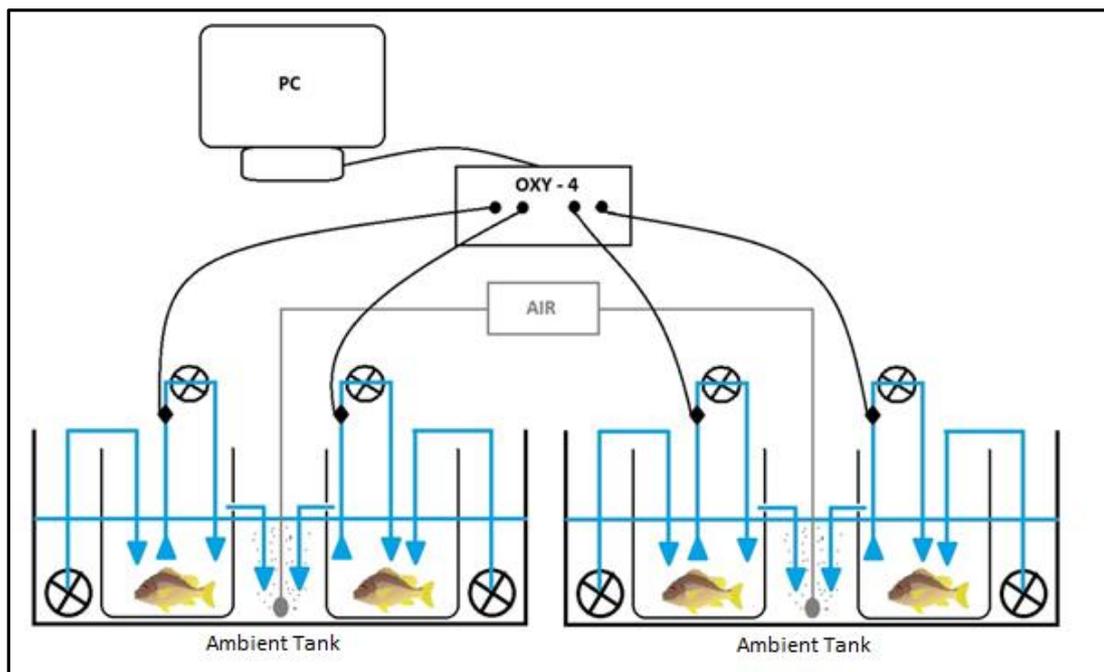


Figure 12. Diagram of the intermittent flow-through respirometry system used to measure oxygen consumption (MO<sub>2</sub>) rates in snapper.

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## Determination of SMR, MMR and AS<sub>abs</sub>

Standard metabolic rate (SMR), maximum metabolic rate (MMR) and absolute aerobic scope (AS<sub>abs</sub>) were used as measures of metabolic demand and aerobic performance. Fish were placed in the respirometry chambers in the early evening and MO<sub>2</sub> was continuously measured overnight. After a minimum of 12 hours, fish were removed and placed in a circular chase tank (1.2 m in diameter) of aerated seawater at the correct acclimation temperature. Here, the fish were chased by hand until they failed to respond to pinching of the dorsal fin. The fish were then air exposed for 20 seconds before being returned to the respirometry chamber where MO<sub>2</sub> was measured for further 5 hours (Roche *et al.*, 2013; MO<sub>2</sub> trace from a typical aerobic scope trial is illustrated in Figure 13). SMR was calculated as the mean of the lowest 10 % of MO<sub>2</sub> values recorded after outliers (where slope  $R^2 = < 0.8$ ) had been removed. MMR was taken as the single highest MO<sub>2</sub> value recorded following exhaustive exercise and a 20 second air exposure. Absolute aerobic scope (AS<sub>abs</sub>) was calculated by subtracting SMR from MMR (Clark *et al.*, 2013). SMR and MMR were mass normalised across species and habitat using the following equation:

$$\text{Log}_{10}(\text{MO}_2 \text{ adjusted}) = \text{Log}_{10}(\text{MO}_2 \text{ observed}) + b \left( \text{Log}_{10} \left( \frac{\text{Mass}_{\text{average}}}{\text{Mass}_{\text{observed}}} \right) \right)$$

Where:

$b$  = gradient of the relationship between  $\text{Log}_{10}(\text{observed mass})$  and  $\text{Log}_{10}(\text{MO}_2)$

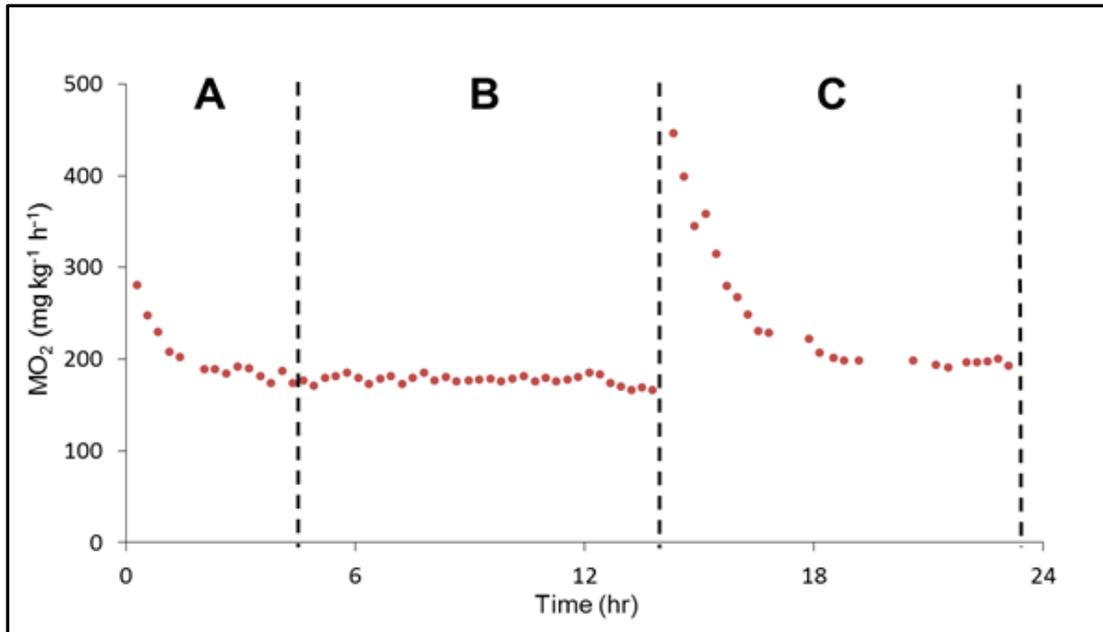


Figure 13. A typical trace of metabolic rate ( $\text{mgO}_2 \text{ kg}^{-1} \text{ h}^{-1}$ ) over time in a warm acclimated snapper during an experiment to determine aerobic scope. Three phases are indicated, A: acclimation to the respirometry chamber, B: determination of standard metabolic rate (SMR) and C: determination of maximum metabolic rate (MMR) following a chase to exhaustion.

### Determination of $P_{\text{crit}}$

$P_{\text{crit}}$  is the point at which a fish transitions from metabolism that is independent of ambient oxygen (oxyregulation) to metabolism that is dependent on ambient oxygen (oxyconformation), and it is widely used as measure of hypoxia tolerance (Portner & Grieshaber, 1993; Chapman *et al.*, 2002). To determine  $P_{\text{crit}}$ , fish were placed in the respirometry chambers in the evening and  $\text{MO}_2$  was continuously measured overnight. In the morning the flush pump was disconnected, effectively converting the system to a closed respirometer. In this way the fish's own oxygen consumption resulted in progressive hypoxia within the chamber.  $\text{MO}_2$  was calculated every 10 minutes as  $\text{PO}_2$  declined. Once fish exhibited prolonged oxyconformation or the  $\text{PO}_2$  dropped below 10 % air saturation, the chamber was flushed with aerated seawater and the fish allowed to recover at normoxia before it was returned to the acclimation tanks. To calculate  $P_{\text{crit}}$ ,  $\text{MO}_2$  was plotted against oxygen tension and a generalized linear model was fitted (Figure 14). This model was then updated using a

piecewise regression (Ryan & Porth, 2007) to determine the ‘breakpoint’ (i.e.  $P_{crit}$ ). This analysis was performed in R (v2.13.1) using the ‘segmented’ package (Muggeo, 2008).

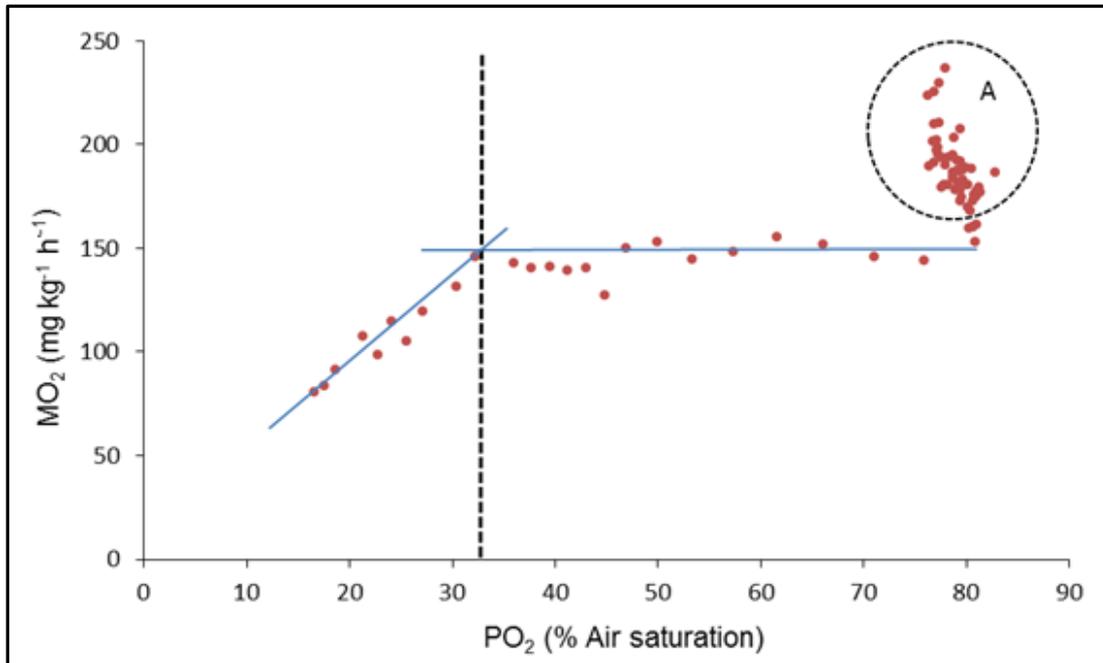


Figure 14. A typical trace of metabolic rate ( $\text{mgO}_2 \text{ kg}^{-1} \text{ h}^{-1}$ ) in a warm acclimated snapper undergoing progressive hypoxia. The dotted line indicates the critical  $\text{PO}_2$  ( $P_{crit}$ ) where two linear regressions (oxyregulating and oxyconforming) intersect. The cluster of  $\text{MO}_2$  measurements enclosed in the dotted circle (A) were recorded during overnight acclimation to the respirometry chamber. These measurements were excluded from the calculation of  $P_{crit}$ .

### Determination of $\text{CT}_{max}$ , $\text{CT}_{min}$ and Thermal Tolerance Zone

Critical maximum ( $\text{CT}_{max}$ ) and minimum temperature ( $\text{CT}_{min}$ ) were measured to determine the limits of each fish group’s thermal envelope. To determine  $\text{CT}_{max}$ , fish were transferred from their acclimation tanks to a 20 litre plastic bucket of aerated seawater at the correct acclimation temperature. The plastic bucket was immersed in a water bath (Julabo, GmbH, Germany) set at 50 or 60 °C for cold acclimated and warm acclimated fish respectively (Figure 15). This resulted in a heating rate within the plastic bucket of  $0.3 \pm 0.05 \text{ }^\circ\text{C min}^{-1}$  which is consistent with previous measurements of critical temperature (Beitinger *et al.*, 2000; Mora & Maya, 2006; Murchie *et al.*, 2011). Fish were

continuously monitored for loss of equilibrium. The  $CT_{max}$  was taken as the temperature (Temp-4, Loligo® Systems, Denmark) at which fish failed to regain equilibrium for a minimum of 20 seconds and showed no response to pinching of the dorsal fin (Beitinger *et al.*, 2000). Once the  $CT_{max}$  had been established, fish were returned immediately to their acclimation tanks where they recovered equilibrium and responses within one minute. The same setup and method was used to determine  $CT_{min}$  but in place of the heater an aquarium cooler (TK1000, TECO, Italy) set at 10 °C was used to recirculate water through the water bath. This resulted in a cooling rate of  $0.29 \pm 0.05$  °C min<sup>-1</sup>.  $CT_{min}$  trials were conducted on each fish at least 12 hours after its  $CT_{max}$  had been measured. Thermal tolerance zone was calculated by subtracting  $CT_{min}$  from  $CT_{max}$ .

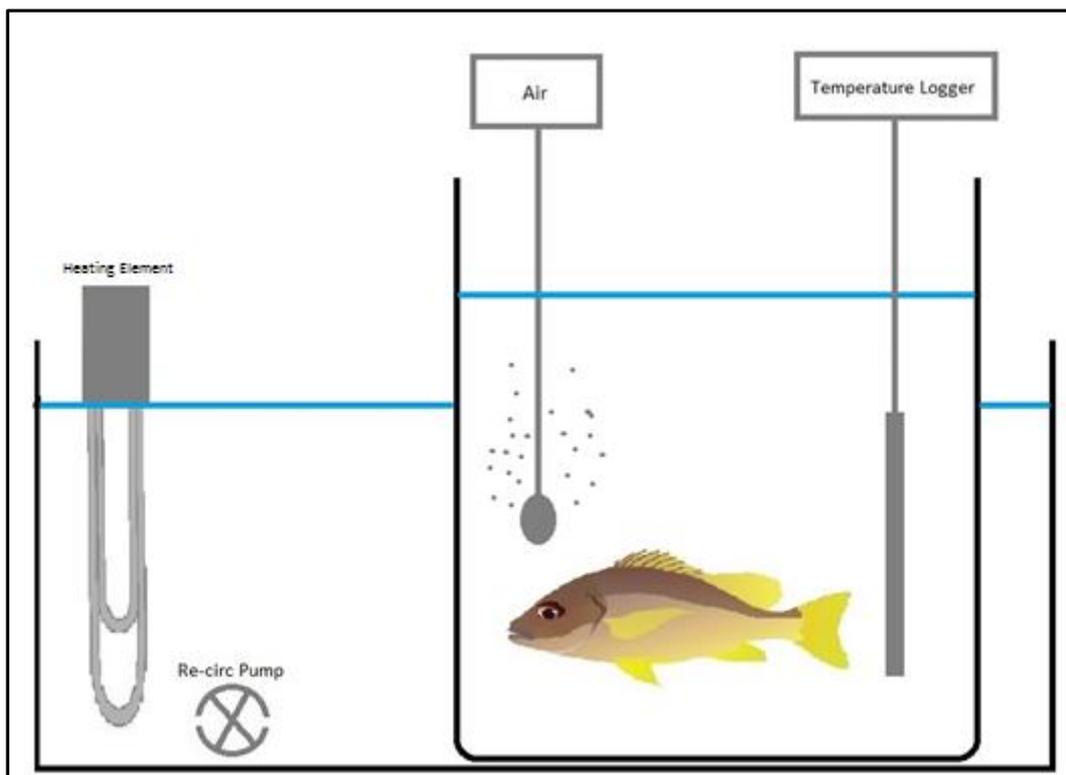


Figure 15. Diagram of apparatus used to determine  $CT_{max}$ . For determination of  $CT_{min}$  the heating element was replaced with an aquarium chiller but the setup was otherwise identical.

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## Statistical Analysis

Data are presented as means  $\pm$  SEM unless otherwise stated. Assumptions of normality and equal variance were tested via the Shapiro-Wilk test and Levene's test respectively ( $P > 0.05$ ). Significant differences between treatments were tested for by one-way or two-way ANOVA. Tukey's test was performed *post-hoc*. Results were accepted as significant at  $P < 0.05$ . Details of the statistical analyses are summarised in the appendix to this chapter (Table 6). Statistical analyses were carried out using SPSS version 17.0, and graphs were drawn using MS Excel 2010.

## Results

### Ambient Temperature and Dissolved Oxygen

The mean daily range in temperature recorded on the reef flat sites was approximately four times higher (4 and 3.7 °C on the inner and outer flat respectively) than on the slope (1 °C, Figure 16). Higher mean daily maximum and lower mean daily minimum were recorded on the two flat sites compared to the slope site. The greatest minima and maxima temperature extremes were recorded on the inner reef flat (14.6 and 33.8 °C respectively). The largest temperature range in a single day was recorded on the outer reef flat (9.2 °C) and was almost three times greater than the maximum daily range recorded on the reef slope (3.1 °C). The grand mean of daily temperature was consistent across flat and slope sites (~24 °C).

The average of daily mean dissolved oxygen (DO) concentration was higher on the reef slope (10 mg l<sup>-1</sup>) than on the inner and outer reef flat (8.7 and 7.5 mg l<sup>-1</sup> respectively, Figure 16). The mean daily range in DO was greater at both reef flat sites (18 mg l<sup>-1</sup>) compared to the slope site (11.5 mg l<sup>-1</sup>). Mean daily minimum DO was lowest on the outer flat (1.4 mg L<sup>-1</sup>) and was over four times lower than the mean daily minimum recorded on the slope (6.1 mg l<sup>-1</sup>).

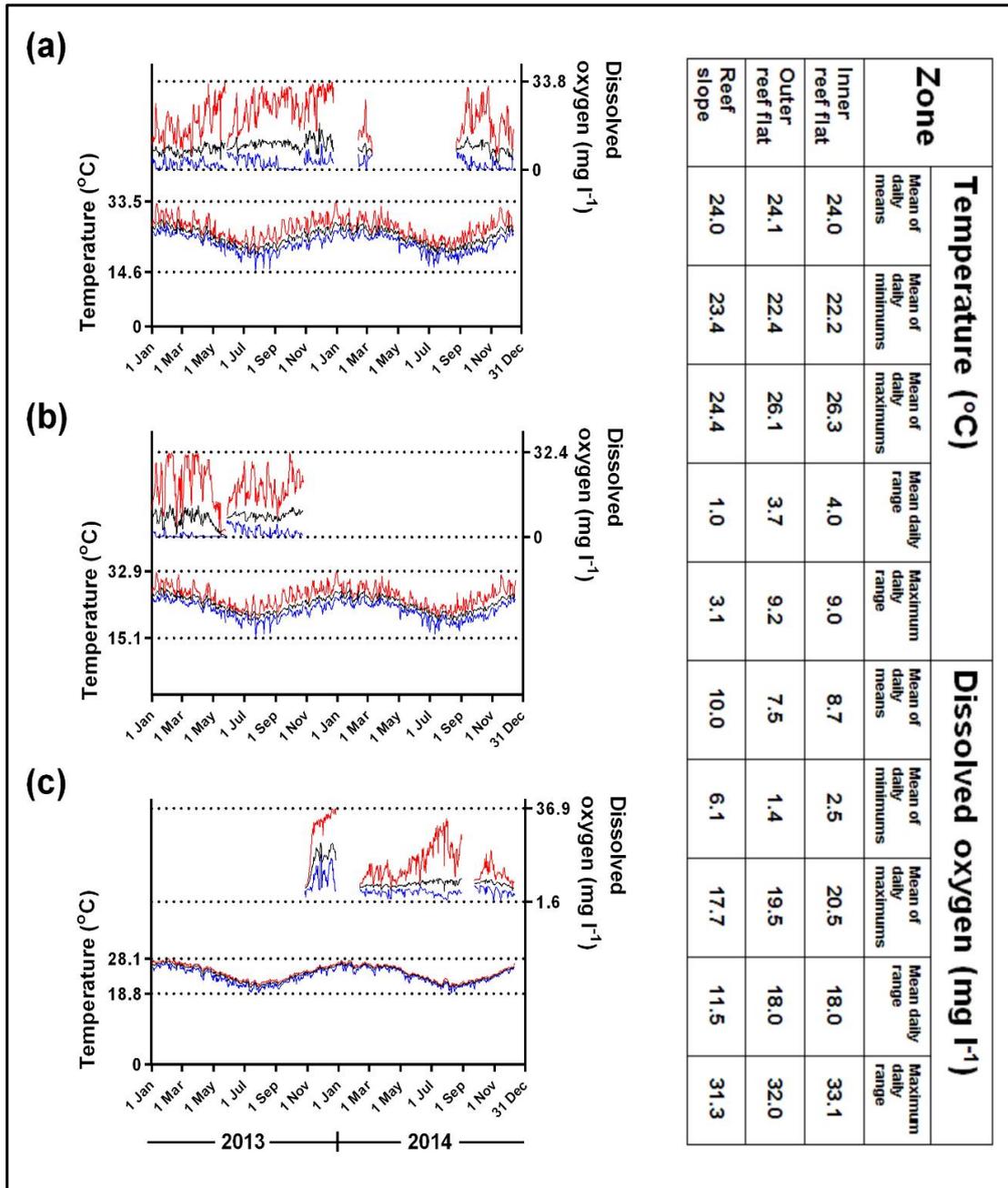


Figure 16. Traces showing average (black), minimum (blue), and maximum (red) daily values of temperature and dissolved oxygen at the (a) inner reef flat, (b) outer reef flat, and (c) reef slope (~6m depth) at Heron Island, Great Barrier Reef, during 2013 and 2014. Dotted lines represent minimum and maximum values recorded during the entire period. Table provides the mean of the daily mean, maximum and minimum values as well as the mean and maximum daily range over the entire measurement period at each site.

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### SMR, MMR and AS<sub>abs</sub>

There was no significant main effect on SMR or MMR across species / habitat but both were significantly elevated in warm acclimated compared to cold acclimated *Lutjanus* (Figure 17, Table 5). SMR averaged  $194 \pm 13$  and  $91 \pm 6$  mgO<sub>2</sub> kg<sup>-1</sup> h<sup>-1</sup> whilst MMR averaged  $581 \pm 24$  and  $381 \pm 28$  mgO<sub>2</sub> kg<sup>-1</sup> h<sup>-1</sup> in warm and cold acclimated *Lutjanus*, respectively. Across species and habitat the average Q10 with respect to SMR was 2.17. There was no significant main effect of species / habitat on AS<sub>abs</sub> but there was a significant main effect of acclimation temperature, with AS<sub>abs</sub> averaging  $290 \pm 26$  and  $386 \pm 15$  mgO<sub>2</sub> kg<sup>-1</sup> h<sup>-1</sup> in cold and warm acclimated *Lutjanus*, respectively. No significant interactions between species / habitat and acclimation temperature were detected for SMR, MMR or AS<sub>abs</sub> (Table 6).

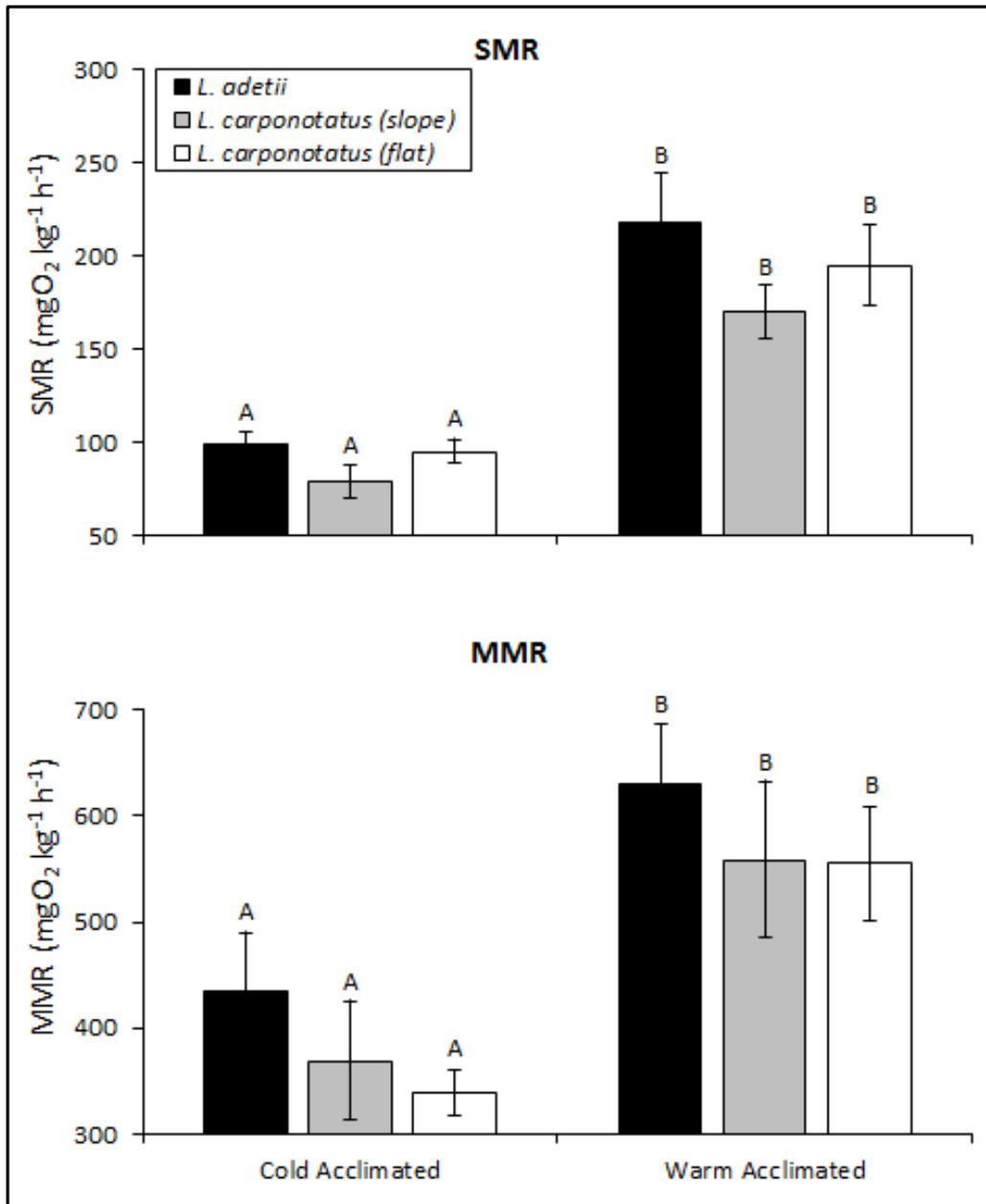


Figure 17. Mean ( $\pm$  SEM) standard metabolic rate (SMR) and maximum metabolic rate (MMR) in cold and warm acclimated *Lutjanus adetii* (black), *Lutjanus carponotatus* caught on the slope (grey) and *Lutjanus carponotatus* caught on the flat (white). SMR and MMR were mass corrected across species and habitat (see methods). Columns labelled with different letters indicate a significant difference between means ( $P < 0.05$ , two-way ANOVA).

Species	Habitat	Acclimation Group	N	Mass (g)	SMR (mgO <sub>2</sub> kg <sup>-1</sup> h <sup>-1</sup> )	MMR (mgO <sub>2</sub> kg <sup>-1</sup> h <sup>-1</sup> )	AS <sub>abs.</sub> (mgO <sub>2</sub> kg <sup>-1</sup> h <sup>-1</sup> )	SMR Q <sub>10</sub>
<i>Lutjanus adetii</i>	Slope	High	7	366 ± 89 <sup>ab</sup>	217.5 ± 26.5 <sup>a</sup>	628.8 ± 58.0 <sup>a</sup>	411.3 ± 61.2 <sup>a</sup>	2.20
		Low	6	265 ± 17 <sup>a</sup>	98.7 ± 7.4 <sup>b</sup>	434.7 ± 54.4 <sup>b</sup>	336.0 ± 53.9 <sup>b</sup>	
<i>Lutjanus carponotatus</i>	Slope	High	6	532 ± 109 <sup>b</sup>	170.1 ± 14.4 <sup>a</sup>	557.7 ± 73.0 <sup>a</sup>	387.6 ± 65.1 <sup>a</sup>	2.16
		Low	6	481 ± 197 <sup>ab</sup>	78.8 ± 8.6 <sup>b</sup>	369.1 ± 55.1 <sup>b</sup>	290.3 ± 62.7 <sup>b</sup>	
<i>Lutjanus carponotatus</i>	Flat	High	5	292 ± 147 <sup>ab</sup>	194.8 ± 21.5 <sup>a</sup>	555.3 ± 53.4 <sup>a</sup>	360.5 ± 44.8 <sup>a</sup>	2.05
		Low	5	274 ± 150 <sup>a</sup>	94.9 ± 5.8 <sup>b</sup>	340.0 ± 21.6 <sup>b</sup>	245.1 ± 16.7 <sup>b</sup>	

Table 5. Mean ( $\pm$  SEM) mass, standard metabolic rate (SMR), maximum metabolic rate (MMR) and absolute aerobic scope (AS<sub>abs.</sub>) in two species of snapper caught from two different habitats and acclimated to two different temperatures (high =  $\sim$ 30 °C, low =  $\sim$ 20 °C). SMR and MMR were mass corrected across species and habitat (see methods). For each variable, a different superscript denotes a significant difference ( $P < 0.05$ ) between means.

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## CT<sub>max</sub> and CT<sub>min</sub>

There was a significant main effect of both acclimation temperature and species / habitat on CT<sub>max</sub>, CT<sub>min</sub> and thermal tolerance zone (Figure 18, Table 6). Compared to cold acclimation, warm acclimation significantly increased the critical temperature of all three *Lutjanus* groups by an average of 3.2 and 2.2 °C for CT<sub>max</sub> and CT<sub>min</sub>, respectively. At both acclimation temperatures, *L. adetii* demonstrated a significantly lower CT<sub>max</sub> than *L. carponotatus* from either habitat. Cold acclimated *L. adetii* demonstrated a higher CT<sub>min</sub> than cold acclimated *L. carponotatus* from either habitat but there was no significant difference in warm acclimated CT<sub>min</sub> between species. At both acclimation temperatures, the thermal tolerance zone of *L. adetii* was significantly smaller than that of *L. carponotatus* by 3.4 and 3.5 °C for warm acclimated and cold acclimated fish, respectively. There was no significant difference in CT<sub>min</sub> or CT<sub>max</sub> at either acclimation temperature between slope and flat caught *L. carponotatus*. No significant interaction was detected between acclimation temperature and species / habitat (Table 6).

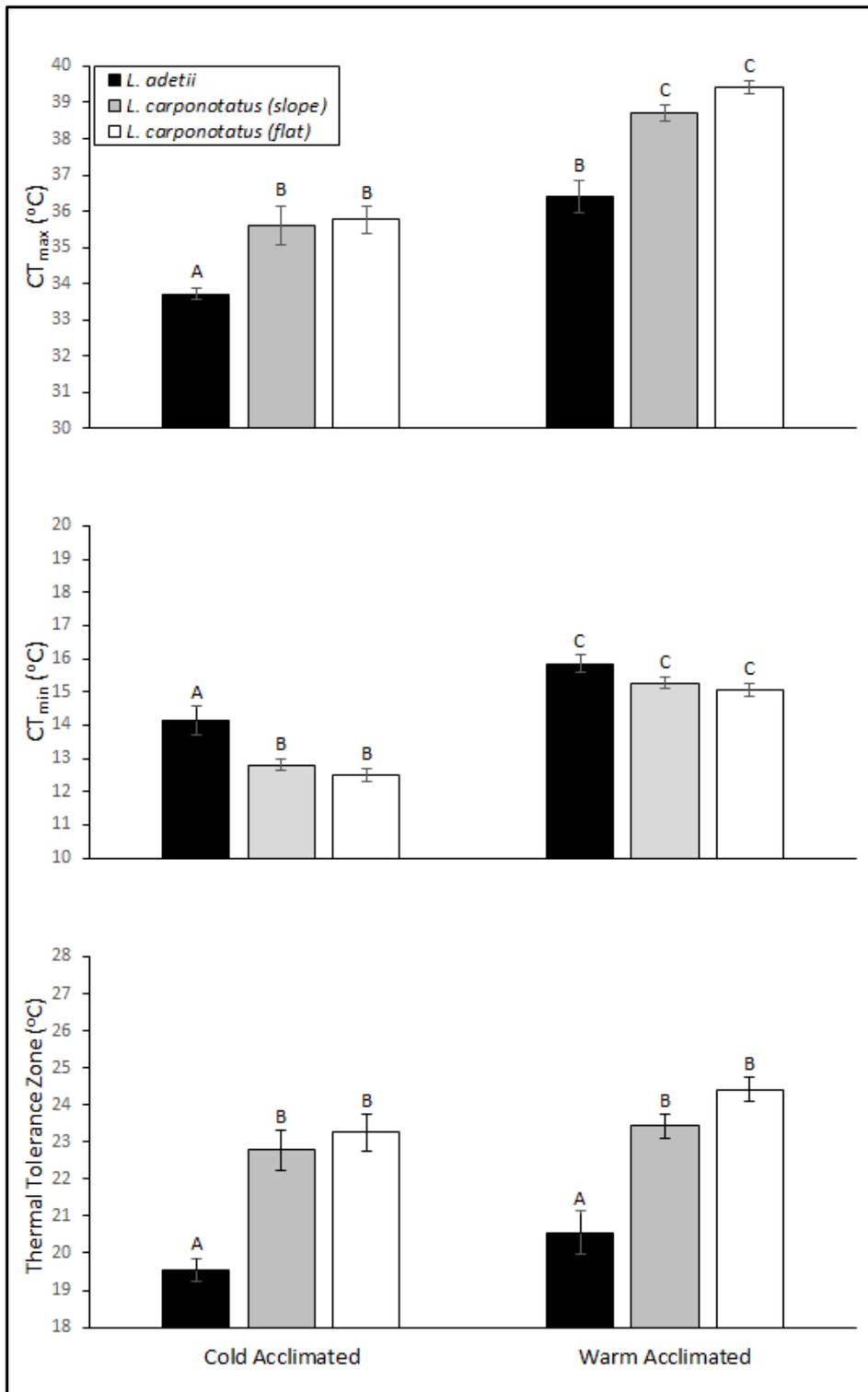


Figure 18. Mean ( $\pm$  SEM)  $CT_{max}$ ,  $CT_{min}$  and thermal tolerance zone ( $CT_{max} - CT_{min}$ ) in cold and warm acclimated *Lutjanus adetii* (black), *Lutjanus carponotatus* caught on the slope (grey) and *Lutjanus carponotatus* caught on the flat (white). Columns labelled with different letters indicate a significant difference between means ( $P < 0.05$ , two-way ANOVA).

$P_{crit}$

Oxygen consumption rates in the cold acclimated fish were insufficient to deplete ambient oxygen below 40% air saturation within the timeframe available for these trials and therefore  $P_{crit}$  could not be determined in cold acclimated individuals. Within warm acclimated individuals,  $P_{crit}$  differed significantly as a function of species / habitat,  $F(2,10) = 6.091$ ,  $P = 0.019$ . *L. adetii* demonstrated a significantly higher  $P_{crit}$  ( $38.4 \pm 2$  % Air Sat.;  $2.35 \text{ mgO}_2 \text{ l}^{-1}$ ) than *L. carponotatus* from either habitat (Figure 19). There was no significant difference in  $P_{crit}$  between *L. carponotatus* caught on the slope ( $28.3 \pm 1.6$  % Air Sat.  $1.73 \text{ mgO}_2 \text{ l}^{-1}$ ) and *L. carponotatus* caught on the flat ( $27.8 \pm 3.7$  % Air Sat.;  $1.7 \text{ mgO}_2 \text{ l}^{-1}$ ).

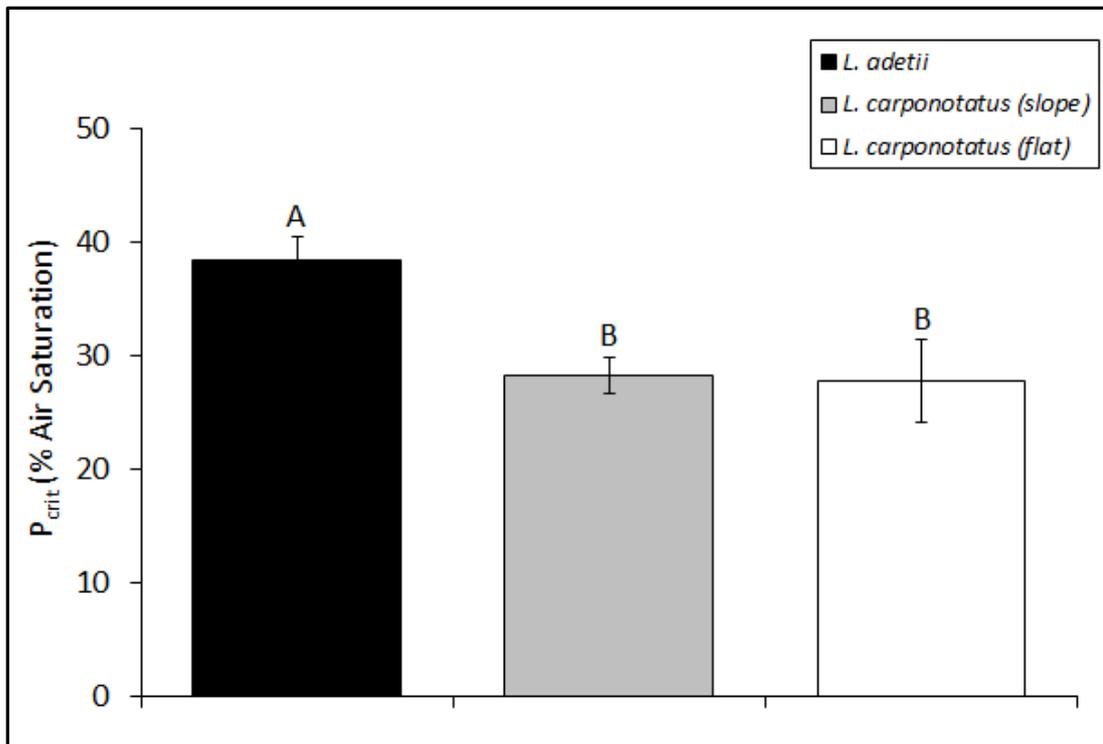


Figure 19. Mean ( $\pm$  SEM)  $P_{crit}$  for warm acclimated *Lutjanus adetii* (black), *Lutjanus carponotatus* caught on the slope (grey) and *Lutjanus carponotatus* caught on the flat (white). Columns labelled with different letters indicate a significant difference between means ( $P < 0.05$ , one-way ANOVA).

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## Discussion

### Ambient Temperature and Dissolved Oxygen

The reef flat surrounding Heron Island clearly exhibits much greater daily and seasonal fluctuations in both temperature and dissolved oxygen than the reef slope. The oxygen logger data shows that significant hypoxia is a daily occurrence on the reef flat with an average daily minimum DO of 1.4 and 2.5 mg L<sup>-1</sup> on the inner and outer flat respectively. Fluctuations in DO concentration do occur on the reef slope but episodes of hypoxia are much less frequent and severe relative to the flat. Likewise, the average daily temperature range recorded on the flat was greater than the maximum temperature range recorded in any single day on the reef slope over the two year measurement period. Previous studies have shown similarly dramatic changes in DO and temperature on reef flats over diel cycles (Kinsey & Kinsey, 1967; Potts & Swart, 1984; Ohde & Woesik 1999; Routley *et al.*, 2002; Nilsson *et al.*, 2007). However, to our knowledge these present data are the first high resolution records of daily and seasonal temperature and DO fluctuations across reef flat and slope zones over a long-term (2 year) timescale.

### Thermal Acclimation and Tolerance

The SMR measured in *L. adetii* and *L. carponotatus* indicates that the basic metabolic demands of these two species are very similar. As expected for aquatic ectotherms, there was a strong positive relationship between temperature and SMR in *L. adetii* and *L. carponotatus*. This relationship, as quantified by the Q<sub>10</sub>, was very similar between the three groups, indicating that there is little inter or intra-species difference in the metabolic thermal sensitivity of these fish. Based on an interspecific curve of 69 species across a temperature range of 0-30 °C, Clark and Johnson (1999) derived a between-species Q<sub>10</sub> of 1.83 in teleost fish. However, the median Q<sub>10</sub> value found within species was 2.4, indicating that evolutionary temperature adaptation has reduced between-species thermal sensitivity. Thus, the Q<sub>10</sub> of 2.05 - 2.16 and 2.2 for *L. carponotatus* and *L. adetii* respectively, indicates a slightly lower thermal sensitivity relative to the intra-species average for fish. Previously, it

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has been found that species experiencing large temperature fluctuations in their natural range, exhibit lower  $Q_{10}$  than comparable species occupying more thermally stable environments (Dent & Lutterschmidt, 2003; Seifert & Chapman, 2007). However, in *L. carponotatus* and *L. adetii*, which do appear to exploit habitats of differing thermal stability, there was no evidence for this correlation when considering  $Q_{10}$ .

The thermal envelope is indicated at either end by critical maximum and minimum temperatures, beyond which fish transition to passive, time-limited tolerance (Portner, 2001). Through thermal acclimation, fish can shift the limits of their thermal envelope (Beitinger *et al.*, 2000; Portner, 2001; Chung, 2001; Eme & Bennet, 2009; Huey *et al.*, 2012). The increased  $CT_{max}$  and  $CT_{min}$  measured under warm acclimation, and decreased  $CT_{max}$  and  $CT_{min}$  measured under cold acclimation, shows well-developed acclimation potential in all three *Lutjanus* groups. Despite shifting its limits, there was no significant net change in the width of the thermal envelope (thermal tolerance zone) between acclimation temperatures. The significantly smaller thermal tolerance zone (as a result of both lower  $CT_{max}$  and higher  $CT_{min}$ ) in *L. adetii* compared to *L. carponotatus*, indicates that *L. adetii* is the less thermally tolerant species of the two, at least in terms of coping with temperature extremes. The lack of significant difference in  $CT_{max}$  and  $CT_{min}$  between slope and flat *L. carponotatus*, suggests that these two groups are equally thermally tolerant.

The thermal tolerance limits of *L. carponotatus* ( $15.3 \pm 0.2 - 38.7 \pm 0.2$  °C in warm acclimated individuals) appear to be within the upper range for tropical marine species, whereas those of *L. adetti* ( $15.8 \pm 0.3 - 36.4 \pm 0.4$  in warm acclimated individuals) appear to fall within the lower range. Across 15 species of tropical reef fish from the eastern Pacific acclimated to  $26.5 \pm 0.5$  °C,  $CT_{max}$  ranged between  $34.7$  and  $40.8$  °C (Mora & Opsina, 2001). In the same species acclimated to the same temperature,  $CT_{min}$  ranged from  $10.8$  to  $16.3$  °C (Opsina & Mora, 2004). The one snapper species in this group (*Lutjanus guttatus*) had a  $CT_{min}$  of  $12 \pm 2$  and a  $CT_{max}$  of  $37.73 \pm 0.41$  °C; thus more closely resembles that of *L. carponotatus* than *L. adetii*. The bonefish (*Albula*

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*vulpes*), a species that exploits nearshore intertidal habitats throughout the Caribbean, has a  $CT_{max}$ , at a 30 °C acclimation temperature, of  $37.9 \pm 0.5$  °C (Murchie *et al.*, 2011), i.e., higher than *L. adetii* but lower than *L. carponotatus*. In a study by Eme & Bennett (2009), the thermal tolerance zones of tidepool resident and transient species (*Bathygobius fuscus* and *Ellochelon vaigiensis*) were compared to reef associated species (*Dascylus aruanus* and *Apogon novemfasciatus*). The tidepool species, which naturally experience diel-temperature extremes, showed substantially wider thermal tolerance zones than the reef associated species which experience more limited thermal fluctuations. This mirrors the significantly narrower thermal tolerance zone seen in the reef slope limited *L. adetii* compared to the reef flat exploiting *L. carponotatus*.

Within the thermal envelope of an ectotherm is its optimum temperature. According to the oxygen- and capacity-limited thermal tolerance hypothesis (OCLTT), the optimum temperature is the point at which aerobic scope is maximal (Portner & Farrel, 2008). In the present study, absolute aerobic scope was on average almost 100 mgO<sub>2</sub> kg<sup>-1</sup> h<sup>-1</sup> higher in warm acclimated fish than in cold acclimated fish. Interpreting this result as per the OCLTT hypothesis suggests that 30 °C is closer than 20 °C to the thermal optimum of the two *Lutjanus* species. The relationship between aerobic scope and temperature has been shown to be highly variable between aquatic ectotherms (Fry, 1957; Farrell, 2009). Broadly, this relationship occurs in two forms, 1) where maximal aerobic scope occurs at a specific point within the thermal envelope and declines with increasing and decreasing temperature (bell-shaped curve) and 2) aerobic scope increases gradually with temperature until close to the  $CT_{max}$ , beyond which aerobic scope rapidly declines (Clark *et al* 2013). As aerobic scope was only measured across two temperatures in the present study, it is not possible to determine from these results whether *Lutjanus* conforms to the former or latter of these scenarios.

Previously, it has been shown that aerobic scope can be modulated through acclimation such that it is independent of temperature. For example in juvenile barramundi (*Lates calcarifer*), in individuals acclimated to 38 °C for 5 weeks

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$AS_{abs}$  was equal to  $AS_{abs}$  in individuals acclimated to 29 °C for the same period. This was despite the cardiorespiratory capacity for much higher AS at the upper temperature as seen in acutely exposed individuals (Norin *et al.*, 2014). Metabolic plasticity such as this is advantageous for eurythermal fish like barramundi because it avoids expensive high performance at higher temperatures and poor performance at lower temperatures (Seebacher *et al.*, 2010; Portner, 2012; Norin *et al.*, 2014). The acclimation period allowed in the present study was short by comparison (4 days) but this is arguably reflective of short term fluctuations in temperature experienced by fish on the reef flat (Figure 16).

The average  $AS_{abs}$  of the two *Lutjanus* species (387 at 30 °C and 291 mgO<sub>2</sub> kg<sup>-1</sup> h<sup>-1</sup> at 20 °C) appears to be lower than previous measurements of  $AS_{abs}$  in coral reef fish, which range between 400 and 1000 mgO<sub>2</sub> kg<sup>-1</sup> h<sup>-1</sup> at 30 °C. However, these measurements of AS in tropical reef fish have so far been limited to a few species of small bodied, site-attached damselfish and cardinalfish as models for studying the impacts of climate change (Nilsson *et al.*, 2009; Gardiner *et al.*, 2010; Mundy *et al.*, 2013; Rummer *et al.*, 2013) and there is a need for further data on how AS varies more widely across reef fish assemblages. The lack of a significant difference in  $AS_{abs}$  between *L. adetii* and *L. carponotatus*, or between slope and flat habitats, suggests that the three groups do not differ significantly in aerobic performance and were equally capable of acclimation to both temperatures within the timeframe given. The wider thermal envelope of *L. carponotatus* indicates that aerobic scope measurements at temperatures lower than 20 and higher than 30 °C would reveal differences between the species.

### Hypoxia Tolerance

Oxyregulation, the maintenance of a stable metabolic rate in the face of reduced environmental PO<sub>2</sub>, is the most common response to hypoxia across teleost species (Ultsch *et al.*, 1978; Richards, 2009). The alternative response, oxyconforming across the entire range of PO<sub>2</sub>, is far less common but has been reported in a number of fish species (Tiffany *et al.*, 2010; Urbina *et al.*,

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2012; Tripathi *et al.*, 2013). Both strategies entail metabolic costs. In regulators, this cost is associated with metabolic demand to meet oxygen requirements such as increased ventilation (Perry *et al.*, 2009). In conformers, reduction in SMR equates to a loss of performance with declining PO<sub>2</sub> (Portner & Grieshaber, 1993). Both *L. carponotatus* and *L. adetii* maintained oxygen consumption across a wide range of PO<sub>2</sub> and can therefore be described as oxyregulators.

There was a clear difference between the two *Lutjanus* species in their ability to oxyregulate. *L. adetii* was unable to maintain its normoxic oxygen consumption rate below 38.4 % of air saturation (2.35 mgO<sub>2</sub> l<sup>-1</sup>), a more than 1.35 times higher oxygen level than the P<sub>crit</sub> seen in *L. carponotatus* (1.73 mgO<sub>2</sub> l<sup>-1</sup>). P<sub>crit</sub> has been widely used as measure of hypoxia tolerance (at least in oxyregulating fish) because it provides a quantifiable measure of a fish's ability to extract oxygen at low PO<sub>2</sub> (Richards, 2009). The striking difference in P<sub>crit</sub> between *L. adetii* and *L. carponotatus* clearly indicates that the latter is the more hypoxia tolerant of the two species. This tolerance suggests adaptation to life on the reef flat where, compared to the slope, there are frequent occurrences of hypoxia. That there was no difference in P<sub>crit</sub> between flat and slope caught *L. carponotatus*, suggests that hypoxia tolerance is not a limiting factor in slope individuals exploiting the flat.

The high P<sub>crit</sub> in *L. adetii* suggests an intolerance for hypoxia that is noteworthy in comparison not only to *L. carponotatus* but also the wider community of reef fishes. Out of 31 species across 7 families of GBR fish, all exhibited a P<sub>crit</sub> of between 20 and 30 % of air saturation at 28-30 °C (Nilsson & Ostlund-Nilsson, 2004). The two circumstances in which hypoxia is most frequently encountered by fish in coral reef systems and which are likely to be the drivers behind selection for hypoxia tolerance are; 1) on reef flats and in tidal pools during low tides (Routley *et al.*, 2002) and 2) between respiring coral branches at night (Nilsson *et al.*, 2007a). Neither of these niches appear to be occupied by *L. adetii* (Allen, 1985; Harborne *pers. obs.* 2014), and this is reflected in the relative lack of hypoxia tolerance in this species.

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Hypoxia tolerance is intrinsically linked to thermal tolerance. Oxygen solubility in water is negatively correlated with temperature and as previously discussed there is a strong positive relationship between temperature and metabolic rate in ectotherms such as fish (Clarke & Johnson, 1999). Thus, meeting oxygen demand becomes increasingly challenging as temperature rises. The cardiorespiratory capacity that facilitates the maintenance of metabolism during hypoxia is also crucial in facilitating the maintenance of aerobic scope at high temperatures (Portner, 2001; McBryan *et al.*, 2013). Therefore, it is not surprising to see the species with the greater thermal tolerance, *L. carponotatus*, also demonstrate the greater hypoxia tolerance.

### Limitations of Experimental Approach

The small sample sizes (5 – 7 fish per group) used throughout the present study were dictated by time constraints and logistical limitations imposed by working in the field. Whilst these sample sizes do fall at the lower end, they are still within the range typically reported in previously published comparative-physiology studies of reef fish (Routley *et al.*, 2002; Nilsson & Ostlund-Nilsson, 2004; Murchie *et al.*, 2011; Mundy *et al.*, 2013). Arguably of more concern is the restriction of the sampling sites to just two locations – one site per habitat type. If these sites were not entirely representative, biotically or abiotically, of the reef flat / slope as a whole, wider generalisations of physiological tolerance across each habitat may not be entirely valid. However, because both *Lutjanus* species do not demonstrate a strong degree of site attachment and appear to roam widely within their respective habitat, it may be reasonably assumed that individuals caught in one location were largely representative of the population present on the reef flat / slope. In addition, the location of the fish sample sites correspond closely to the locations at which ambient temperature and dissolved oxygen data were recorded (Figure 16). As such, these data represent the relevant abiotic conditions encountered by fish at the locations from which they were sampled.

Assumptions regarding the relative distributions of the two *Lutjanus* species between reef zones are currently based on previously reported as well as

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personal observations. Within the available literature, the distribution of *L. adetti* is consistently reported as limited to the deeper waters (>6 m) of the reef slope (Allen, 1985; Newman *et al.*, 1996). *L. carponotatus* individuals can be frequently observed in both flat and slope habitats around Heron Island and this is also well reflected in the available literature (Allen, 1985; Cribb *et al.*, 2011; Wen *et al.*, 2013; Harborne, 2013). However, qualifying these observations with survey data would greatly improve confidence in the major premises of the present study - in particular regarding the absence of *L. adetti* from the reef flat. In addition, surveying *L. carponotatus* abundance on the reef flat over tidal and diurnal cycles would confirm the extent to which individuals of this species actually encounter the temperature and oxygen extremes observed in this habitat (Figure 16).

The series of experiments presented here could have been enhanced by the addition of a third temperature group to act as an ambient 'control'. The two acclimation temperatures used were chosen such that their median reflected ambient water temperatures on the reef over the study period (~25 °C). Whilst a significant main effect of acclimation group was observed for both CT<sub>max</sub> and CT<sub>min</sub>, without an ambient control group for comparison it is difficult to interpret the degree of thermal acclimation that has occurred in either direction. It is also important to recognise that the maintenance of a constant temperature within each acclimation tank is unrepresentative of the thermal fluctuations encountered by these fish on the reef. This is especially the case for the reef flat, which demonstrates a mean daily temperature range of 4 °C (Figure 16). Previously, Chinese bream (*Parabramis pekinensis*) acclimated to diel-temperature cycling (20 ± 5 °C) have been shown to demonstrate wider thermal tolerance zones compared to individuals acclimated to a constant temperature of 20 °C (Jing Peng *et al.*, 2014). Furthermore, comparisons of gene expression in killifish (*Austrofundulus limnaeus*) undergoing fluctuating versus constant temperature regimes, has revealed major differences in their transcriptional responses. Genes found to be differentially expressed under the two thermal regimes included those involved in the production of heat shock proteins and chaperones, the regulation of cell proliferation and growth,

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and the maintenance of membrane integrity (Podrabsky & Somero, 2004). If the same is true in *L. adetii* and *L. carponotatus*, the results of the present study may not provide a true reflection of the physiological performance of these fish in the thermally dynamic environment of the reef flat.

### Tolerance Traits and Inter-habitat Distribution

For fish occupying the reef flat, the ability to tolerate significant fluctuations in temperature and dissolved oxygen over seasonal and diurnal cycles, will strongly influence their performance in this habitat. As such, hypoxia and thermal tolerance can be considered as functional traits for fishes on the reef flat (McGill *et al.*, 2006). Both traits certainly appear to be good predictors of variation in distribution between *L. adetii* and *L. carponotatus* across flat and slope zones. Based on the current data, it is not possible to speculate from an evolutionary perspective, whether high thermal and hypoxia tolerance were traits gained by *L. carponotatus* or lost by *L. adetti*. The phylogenetic relationships between 27 species from within the family *Lutjanidae*, including *L. adetii* and *L. carponotatus*, have previously been described (Miller & Cribb, 2007). Combining this phylogenetic analysis with hypoxia and thermal tolerance data from additional *Lutjanus* species may facilitate the determination of basal traits and provide insights into the evolution of physiological tolerance within this family. However, drawing robust conclusions from such analysis would likely require much higher sample numbers and a greater range of sampling sites than those presented in the current study.

Within the limits of the present study, it is not possible to establish cause and effect between the observed inter-specific differences in physiological tolerance and species distribution across the two reef zones. Numerous factors other than temperature and oxygen regime, for example prey availability, predator avoidance or larvae settlement dynamics, may dictate the distribution of a particular species. Furthermore, the differential hypoxia and thermal tolerance observed between *L. adetii* and *L. carponotatus* may arise in part through individual phenotypic plasticity rather than as a result of evolutionary adaptation alone. Phenotypic plasticity can be defined as the

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ability of a single genotype to produce multiple phenotypes in response to a change in the environment (Healy & Schulte., 2015). Previously, a strong element of developmental plasticity has been demonstrated for a variety of physiological tolerance traits in fish, including  $P_{crit}$  (Reardon & Chapman, 2010), critical temperature (Yongfeng He *et al.*, 2014) and aerobic scope (Donelson *et al.*, 2011; Grenchik *et al.*, 2013). An extension of the present study could involve comparisons of  $P_{crit}$ , critical temperature and aerobic scope in *L. adetii* and *L. carponotatus* individuals reared under temperature and oxygen regimes mimicking that of the reef flat or reef slope. Such an experiment would reveal the extent to which thermal and hypoxia tolerance are phenotypically plastic in these species rather than strictly conserved adaptive traits.

In terms of the physiological parameters measured, there is no evidence to suggest *L. carponotatus* individuals on the slope and flat form two *physiologically* distinct sub populations. The results of the present study suggest that individuals caught on the slope would be equally tolerant of the oxygen and temperature regime of the flat as individuals caught from the flat itself. As previously mentioned, contrasting parasite loads indicate limited movement of adult *L. carponotatus* individuals between slope and flat zones (Cribb *et al.*, 2000), i.e., *spatially* distinct subpopulations. However, *L. carponotatus* appears to recruit predominantly to the reef flat with recruits rarely found seaward of the reef crest (Wen *et al.*, 2013). Presumably, hypoxia and thermal tolerance is necessary for the survival of these recruits on the flat and these physiological traits are maintained in adults irrespective of which habitat they occupy later in their life history. Furthermore, it is likely that at least some fish do actually move between the two habitats, necessitating a hypoxia and thermal tolerance in all individuals.

Understanding the relationship between physiological traits and the inter-habitat distribution and acclimation potential of reef fish is increasingly relevant given the predicted environmental effects of global climate change (Rummer *et al.*, 2014). Tropical sea surface temperatures are projected to rise by 2 – 3 °C by 2100 (IPCC, 2013) and shallow water habitats such as the reef flat and

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upper reef slope are likely to be particularly susceptible to the effects of warming and sea level rise (Harborne, 2013). Given that *L. adetii* and *L. carponotatus* already encounter temperature and hypoxic extremes that are close to their tolerance limits, on the flat and slope respectively, both species could be sensitive to future increases in temperature and associated fluctuations in oxygen, leading to distributional shifts that may significantly affect key processes such as feeding, growth rates and mortality from predation. However, both species did demonstrate well adapted short term acclimation potential to the two trial temperatures in this study (20 and 30 °C) and recent work has shown that developmental plasticity (Donelson *et al.*, 2011; Grenchik *et al.*, 2013) and surprisingly rapid trans-generational acclimation (Donelson *et al.*, 2012) in some reef species may help to limit the impact of future temperature rises. Species such as *L. carponotatus* that are adapted to the eurythermal environment of the reef flat might be expected to demonstrate greater plasticity than the slope limited and less tolerant *L. adetii*. Further study of chronic temperature acclimation to relevant climate change scenarios and its effect on thermal and hypoxia tolerance are required to predict the future success and distribution of these species.

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## Appendix

### ANOVA Summary

Variable	Species / Habitat Main Effect			Acclimation Temp. Main Effect			Interaction		
	df	F	P	df	F	P	df	F	P
SMR	2,29	2.08	0.143	1,29	54.01	<0.001*	2,29	0.36	0.699
MMR	2,29	1.27	0.297	1,29	18.16	<0.001*	2,29	0.03	0.972
AS <sub>abs</sub>	2,29	0.77	0.472	1,29	4.32	0.047*	2,29	0.06	0.939
CT <sub>max</sub>	2,23	33.68	<0.001*	1,23	131.78	<0.001*	2,23	1.16	0.331
CT <sub>min</sub>	2,21	12.59	<0.001*	1,21	115.83	<0.001*	2,21	1.68	0.211
Tolerance Zone	2,21	37.01	<0.001*	1,21	5.93	0.024*	2,21	0.15	0.862
P <sub>crit</sub>	2,10	6.09	0.019*	n/a	n/a	n/a	n/a	n/a	n/a

Table 6. Summary of ANOVA results for all physiological parameters measured in the present study. \* indicate significant effects where  $P < 0.05$ .

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# Chapter 4



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# Respiratory responses and gut carbonate production during hypoxia and hypercarbia in the European flounder (*Platichthys flesus*).

## Abstract

The intestinal precipitation and excretion of  $\text{CaCO}_3$  by marine teleosts not only forms a key part of their osmoregulatory strategy but also makes a significant global contribution to the marine inorganic carbon cycle. Understanding how environmental factors such as hypoxia and hypercarbia affect fish carbonate production is therefore important to accurately model this process in the oceans of the past, present and future. In the European flounder (*Platichthys flesus*) hypoxia (50% air saturation) increased  $\text{CaCO}_3$  excretion rate by 2.4-fold. Further data suggest this effect was similar in scale to the hypoxic ventilatory response although compensatory increases in drinking rate were not detected. Blood pH regulation during hypercarbia (3000  $\mu\text{atm}$ ) further promoted carbonate precipitation by enhanced intestinal supply of  $\text{HCO}_3^-$ , resulting in a 1.5-fold increase in  $\text{CaCO}_3$  excretion. When combined, hypoxic and hypercarbic treatments acted synergistically, increasing  $\text{CaCO}_3$  excretion rate by 4.3-fold. These results directly link fish respiratory, osmoregulatory and acid-base physiology with the marine inorganic carbon cycle and have important implications for global models that estimate marine fish carbonate production.

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## Introduction

Over the past two decades, the vital osmoregulatory role of intestinal  $\text{CaCO}_3$  precipitation and excretion in marine teleosts has been increasingly recognised and a detailed understanding has been developed of the processes that facilitate it (Walsh *et al.*, 1991; Grosell *et al.*, 2001, 2005, 2009; Wilson *et al.*, 2002; Grosell & Genz, 2006; Cooper *et al.*, 2010; Whittamore *et al.*, 2010; Taylor *et al.*, 2010; Al-Jandal *et al.*, 2011; Ferlazzo *et al.*, 2012). In summary, bicarbonate ions ( $\text{HCO}_3^-$ ) originating from both endogenous and extracellular sources, are secreted from epithelial cells into the lumen of the intestine via apical exchange with  $\text{Cl}^-$ . The high concentration of  $\text{HCO}_3^-$  causes the alkalinisation of intestinal fluid within the lumen and results in the precipitation of imbibed  $\text{Ca}^{2+}$  to form insoluble  $\text{CaCO}_3$  precipitates which are then excreted as mucus coated pellets. This process of  $\text{HCO}_3^-$  secretion and  $\text{CaCO}_3$  precipitation is a major driver of intestinal water absorption and is also important for maintaining  $\text{Ca}^{2+}$  homeostasis (Wilson *et al.*, 2002; Wilson & Grosell, 2003; Whittamore *et al.*, 2010).

The surprisingly high rate of calcium carbonate production by marine teleosts coupled with their substantial global biomass has significant implications for our understanding of ocean chemistry and carbon cycling as well as carbonate sediment budgets and records (Wilson *et al.*, 2009; Perry *et al.*, 2011). Attempts to model piscine carbonate production at a global scale, which have suggested an up to 45% contribution by marine fish to global new oceanic calcium carbonate, have so far been based on a small number of experimental studies that have involved a limited range of species and environmental conditions (Wilson *et al.*, 2009).

Hypoxia and hypercarbia are important environmental factors to study in terms of their effect on piscine carbonate production. The depletion of dissolved  $\text{O}_2$  is common in marine systems and is often accompanied by an elevation in  $\text{CO}_2$  (as the waste product of respiration), particularly in those systems that are coastal, estuarine or high in biomass (Ultsch, 1996; Diaz & Rosenberg, 1995; Gilmour, 2001). Hypoxia is becoming an increasingly widespread

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perturbation in the world's oceans, primarily as the result of anthropogenic nutrient enrichment and climate change (Diaz & Rosenberg, 2008; Diaz & Breitburg, 2009). On top of this, seawater  $\text{PCO}_2$  is projected to rapidly increase in the future due the continued oceanic uptake of anthropogenic  $\text{CO}_2$  emissions (Caldeira & Wickett, 2003; Orr *et al.*, 2005). It is also the case that atmospheric levels of  $\text{O}_2$  and  $\text{CO}_2$  and hence ocean  $\text{PO}_2$  and  $\text{PCO}_2$ , have fluctuated substantially over geological time scales (Berner *et al.*, 2002; Falkowski, *et al.*, 2005; Berner *et al.*, 2006; Pomar & Hallock, 2008). For example, the prevailing view is that the Cretaceous period, which saw a spectacular radiation of the teleost fishes, was characterized by lower  $\text{PO}_2$  and higher  $\text{PCO}_2$  in comparison to modern day levels (Pomar & Hallock, 2008; Bice *et al.*, 2006; Bice & Norris, 2002; Near *et al.*, 2012). Therefore, an understanding of how hypoxia and hypercarbia influence the rate of gut carbonate production will not only assist with estimating the contribution of teleosts to the marine inorganic carbon cycle of the present day but also that of the past and the future.

How teleosts respond to hypoxia has been very well studied and a wide range of responses that allow survival at reduced  $\text{PO}_2$  have been identified (Richards, 2009). In terms of their metabolic response to hypoxia, most fish maintain a stable oxygen consumption rate as ambient  $\text{PO}_2$  declines (oxyregulation) until a critical threshold ( $P_{\text{crit}}$ ) is crossed, at which point oxygen consumption declines with ambient  $\text{PO}_2$  (oxyconforming, Ultsch *et al.*, 1978; Richards, 2009). The  $P_{\text{crit}}$  is determined by cardiac and respiratory capacity for meeting oxygen demand during hypoxia. The hypoxic ventilatory response (HVR) is arguably the primary mechanism by which teleosts facilitate the extraction of  $\text{O}_2$  from low  $\text{PO}_2$  water (Perry *et al.*, 2009). HVR refers to an increase in both the frequency and amplitude of gill ventilation in response to lowered ambient  $\text{PO}_2$ . This results in a greater volume of water passing through the gills and hence  $\text{O}_2$  uptake and  $\text{CO}_2$  excretion can be maintained across the gill epithelium. As with hypoxia tolerance, there is great inter- and intra- species variation in the HVR which is itself dynamic in nature and varies

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over time depending on the pattern and intensity of the hypoxia exposure (Porteus *et al.*, 2011).

The structure and function of the teleost gill that make it an effective site of gas exchange also make it a site of significant osmosis and ionic diffusion (Evans *et al.*, 2005). As such, an increase in ventilation volume (i.e. during the HVR) not only increases the rate of gas transfer across the gill epithelium but will also result in an increased flux of ions and water (Randall *et al.*, 1972). In the marine environment, where fish are hypoosmotic to the surrounding seawater, increased gill exposure results in higher rates of osmotic water loss and ion uptake. In order to balance osmotic water loss, marine teleosts continuously ingest seawater and then absorb water across the intestinal epithelium, a process driven by active solute uptake in the same direction and referred to as 'solute linked water transport' (Marshall & Grosell, 2006; Whittamore *et al.*, 2010). Water loss incurred by increasing ventilation volume must therefore require a compensatory increase in drinking rate and intestinal water absorption. This relationship has previously been demonstrated through measurements of drinking and intestinal  $\text{HCO}_3^-$  secretion at different water loss rates induced by varying environmental salinity (Genz *et al.*, 2008).

There are several potential mechanisms by which hypercarbia is likely to influence gut carbonate production. Firstly, although the primary driver of gill ventilation in fish is  $\text{O}_2$  there is evidence that a significant  $\text{CO}_2$  / pH drive also exists, at least in certain species (Gilmour, 2001). Marine and euryhaline teleosts that have been shown to increase ventilation volume (and presumably also increase drinking rate) in response to acute environmental hypercarbia include the Pacific sanddab (*Citharichthys sordidus*; McKendy, 2000), white sturgeon (*Acipenser transmontanus*; Crocker & Cech, 1998) Atlantic salmon (*Salmo salar*; McKendy, 2000) and rainbow trout (*O. Mykiss*; Perry & Gilmour 1996). Secondly, luminal  $\text{HCO}_3^-$  secretion is fuelled by the hydration of metabolic waste  $\text{CO}_2$  in the epithelial cells of the intestine as well as by direct transport of  $\text{HCO}_3^-$  from the blood (Grosell *et al.*, 2009; Whittamore *et al.*, 2010; Taylor *et al.*, 2011). During hypercarbia, when both intra-cellular  $\text{CO}_2$  and blood  $\text{HCO}_3^-$  are elevated, luminal supply of  $\text{HCO}_3^-$  and hence the rate of

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CaCO<sub>3</sub> precipitation are likely to increase (Wilson *et al.*, 2009; Heuer *et al.*, 2012).

Taken altogether, it appears that gill ventilation, drinking rate, acid-base regulation and gut calcium carbonate production will be closely linked in terms of their response to hypoxia and hypercarbia. Maintaining a stable metabolism under hypoxia through hyperventilation, is likely to result in greater osmotic water loss at the gills and increased intestinal supply of calcium via ingestion of seawater as a secondary compensatory response. In turn, this is likely to increase the rate of calcium carbonate precipitation and excretion via the intestine (Figure 20). In addition, hypercarbia is likely to further promote carbonate precipitation by increasing the supply of HCO<sub>3</sub><sup>-</sup> ions via enhanced secretion by the intestinal epithelium.

This study aims to test these predictions by measuring the metabolic, respiratory and osmoregulatory responses to hypoxia, hypercarbia and hypoxic hypercarbia, in the European flounder (*Platichthys flesus*). Flounder have been used extensively in previous experimental work on gut carbonate production in fish and as such a large body of data already exists for comparative purposes (Wilson *et al.*, 2002, 2009; Cooper *et al.*, 2010; Whittamore *et al.*, 2010). The sedentary behaviour exhibited by flounder make this species amenable to spending extended periods in respirometry chambers and to the regular collection of excreted carbonates. This behaviour, combined with easily accessible anatomy, lends this species well to experiments involving the placement of cannula, catheters and other surgeries. Furthermore, as a benthic species found extensively in coastal and estuary environments, flounder are likely to frequently encounter hypoxia and hypercarbia within their natural range (Steffensen *et al.*, 1982). For these reasons European flounder provide an ideal model species in which to conduct the following series of experiments.

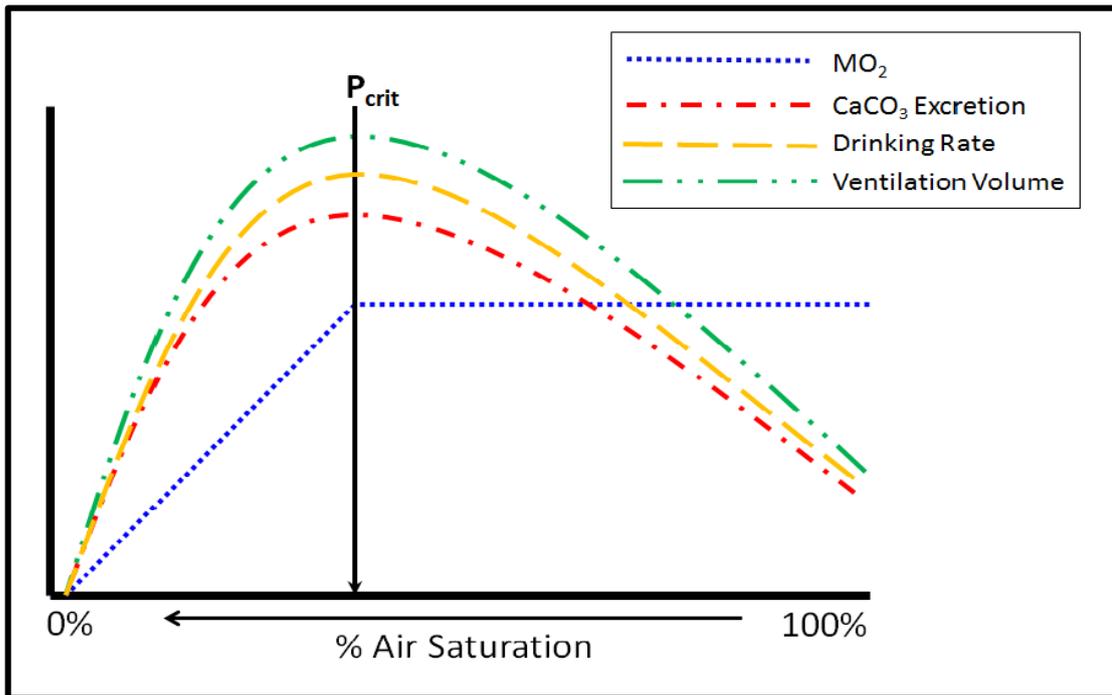


Figure 20. Diagram summarising the predicted relationships between declining ambient PO<sub>2</sub> and the metabolic, respiratory and osmoregulatory responses in marine teleosts.

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## Materials and Methods

### Animals and Experimental Series

European flounder (*Platichthys flesus*, 400 ± 200 g) were caught in the estuary of the River Taw, North Devon, UK and transported to the marine aquarium facilities at the University of Exeter. Here they were maintained in two 300 L holding tanks of flowing aerated artificial seawater (Tropic Marine, Tropical Marine Centre, Bristol, UK) as part of a recirculating seawater system maintained at a salinity of 33.8 ± 0.2 and 15 ± 0.3 °C, under a 14:10 h light-dark photoperiod. The flounder were maintained on a diet of live rag worm (*Nereidae*) fed weekly and cooked mussel fed three times per week. Food was withheld for 72 hours prior to the commencement of each experiment. For experiments requiring individual identification, each flounder was tagged with a PIT tag (AVID Identification Systems Inc., California, USA) injected subcutaneously at the anterior dorsal muscle. The experiments included in this study are split among five experimental series. The numbers of flounder, physiological parameters measured, and treatment conditions within each series are summarised in Table 7.

Experiment Series	N	Physiological Parameters Measured	Treatments	
			PO <sub>2</sub> (kPa)	PCO <sub>2</sub> (µatm)
Series I	8	P <sub>crit</sub>	21 - 2.1	400, 2250
Series II	8	Long-term MO <sub>2</sub> (7 days)	21, 15.75, 10.5, 5.25	400
Series III	6	V <sub>f</sub> , V <sub>v</sub> , EO <sub>2</sub>	21, 15.75, 10.5, 5.25	400, 3000
Series IV	8	Blood Parameters, CaCO <sub>3</sub> ppt. Excretion	21, 10.5	400, 3000
Series V	10	Drinking Rate	21, 10.5	400

Table 7. Summary of the physiological parameters measured and treatment conditions within each of the five experimental series incorporated into the present study (P<sub>crit</sub> = Critical PO<sub>2</sub>, MO<sub>2</sub> = routine oxygen consumption rate, V<sub>f</sub> = ventilation frequency, V<sub>v</sub> = ventilation volume, EO<sub>2</sub> = oxygen extraction efficiency)

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## Ambient PO<sub>2</sub> and PCO<sub>2</sub> Control

Seawater PO<sub>2</sub> and PCO<sub>2</sub> were controlled by continuous bubbling with a preset gas mixture of O<sub>2</sub>, N<sub>2</sub> and CO<sub>2</sub>. The correct proportional flow rate from cylinders of each gas was established using precision gas flow controllers (MC Series Mass Flow Controllers, Qubit Systems Inc., Ontario, Canada) connected to a PC running gas mixing software (C960 Gas Mixing Software, Qubit Systems Inc., Ontario, Canada). Total gas flow rate was set at 2.5 L min<sup>-1</sup> with N<sub>2</sub> set as the 'balance' gas. O<sub>2</sub> and CO<sub>2</sub> flow rates were adjusted to achieve the desired hypoxia and hypercarbia intensity. To confirm the correct PO<sub>2</sub> had been achieved, water samples were taken every 24 hours and dissolved oxygen measured independently using a Strathkelvin oxygen electrode and meter (Model 781, Strathkelvin Instruments, Scotland, UK). Seawater PCO<sub>2</sub> was confirmed by inputting daily measurements of seawater pH (pHC2401, Radiometer Analytical, France) and total alkalinity (double titration procedure, Titrando, Metrohm, Switzerland) into the seawater carbon calculator programme, CO<sub>2</sub> SYS. (Pierrot *et al.*, 2006). Seawater PCO<sub>2</sub> calculations were based on the NBS pH scale, equilibration constants from Dickson and Millero (1987) and KSO<sub>4</sub> dissociation constants from Dickson (1990).

## Respirometry (*series I and II*)

Intermittent flow-through respirometry as described by Steffensen (1989) was used to determine flounder metabolic rates (MO<sub>2</sub>) at various O<sub>2</sub> and CO<sub>2</sub> tensions (Figure 21). During the respirometry experiments, four flounder were held in individual 6.25 L water-tight chambers (Loligo® Systems, Denmark) each of which were placed in an individual 80 L ambient tank of seawater. Between measurements, each chamber was flushed by pumping (Eheim, Stuttgart, Germany) seawater from the ambient tanks through the chamber. For measurement of metabolic rate, the flush pumps were switched off and the flounder allowed to consume oxygen within the sealed chamber. By measuring the decline in PO<sub>2</sub> within the chamber the oxygen consumption rate (mgO<sub>2</sub> kg<sup>-1</sup> h<sup>-1</sup>) of the flounder was determined. This was calculated as follows:

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$$MO_2 = \frac{\text{slope} \times \text{oxygen solubility} \times \text{respiratory volume}}{\text{fish mass}}$$

Where:

$$\text{slope} = \frac{PO_2 \text{ initial} - PO_2 \text{ final}}{\text{time}_{\text{initial}} - \text{time}_{\text{final}}}$$

And:

PO<sub>2</sub> (kPa), oxygen solubility (mgO<sub>2</sub> l<sup>-1</sup> kPa<sup>-1</sup>), volume (l), time (h), mass (kg).

Oxygen was measured using fibre-optic mini-sensors connected to a four-channel oxygen meter (Oxy-4 mini, Presens, Germany). Timing of the flush/measurement cycle was set such that the PO<sub>2</sub> within the chambers never fell by more than 5% of the starting value and was fully restored to pre-measurement levels between measurements. This generally entailed a 10 minute flush period followed by a 5 minute measurement period. The flush/measurement cycle was controlled and the metabolic rate data was recorded by Autoresp respirometry software (Loligo® Systems, Denmark). Unfed (72 hours), post-absorptive flounder were acclimated to the respirometry chamber and flush/re-circ cycle for 12 hours before MO<sub>2</sub> values were recorded.

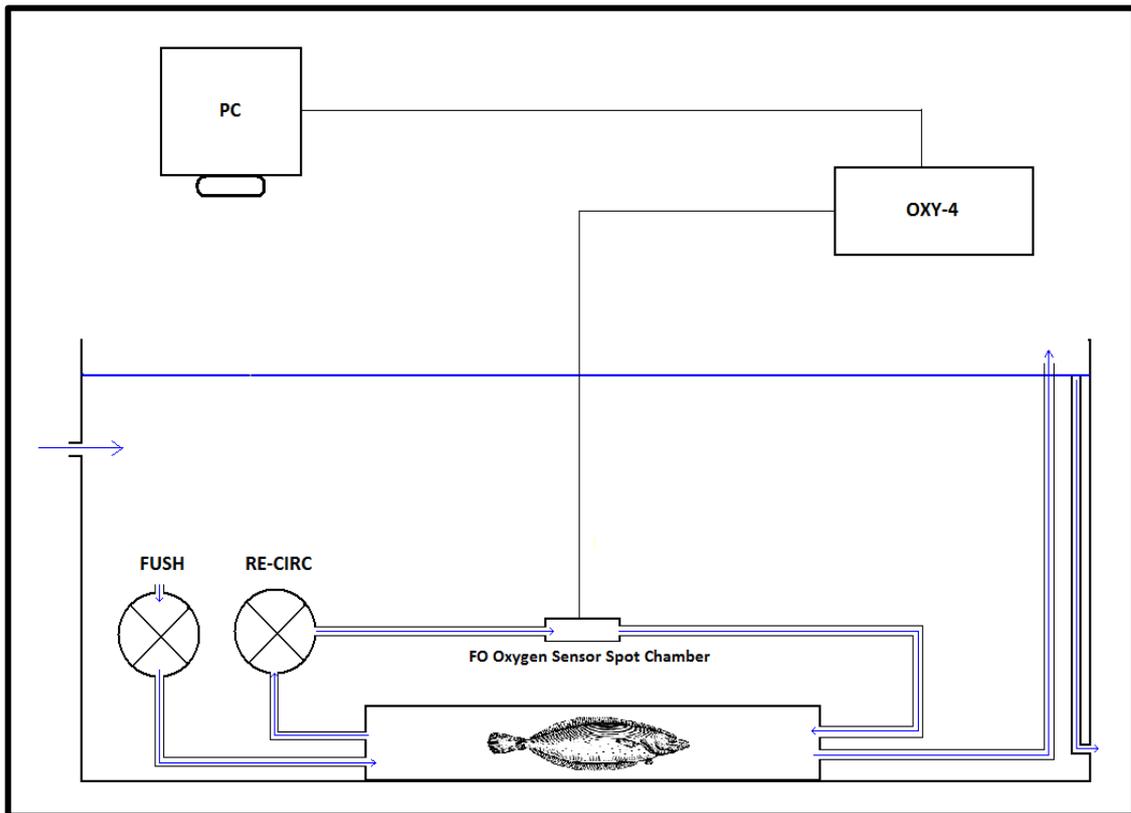


Figure 21. Diagram of the fibre-optic intermittent flow through respirometry system used for measuring flounder oxygen consumption rate. Flounder were housed in a sealed chamber within an ambient tank of aerated seawater. Flush / re-circ pumps supplied water to the chamber and dissolved oxygen was measured via oxygen sensor spots connected to a fibre optic oxygen transmitter (OXY-4, Presens, Germany) and recorded on a PC running AutoResp software (Loligo® Systems, Denmark).

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### $P_{crit}$ Determination (*series I*)

$P_{crit}$  was determined using the intermittent flow through respirometry system as previously described. In the first trial, flounder were exposed to progressive hypoxia at a constant  $PCO_2$  of  $\sim 400 \mu\text{atm}$ . In the second trial, flounder were exposed to progressive hypoxia combined with progressive hypercarbia. Progressive hypoxia and hypercarbia were achieved by bubbling the ambient tank with gas mixtures of progressively lower  $O_2$  and higher  $CO_2$  content, such that the  $PO_2$  declined from 100 to 10% air saturation and  $PCO_2$  increased from 400 to 2250  $\mu\text{atm}$  over the course of 9 hours (Figure 22). To calculate  $P_{crit}$ ,  $MO_2$  was plotted against oxygen tension and a generalized linear model was fitted. This model was then updated using a piecewise regression (Ryan & Porth, 2007) to determine the 'breakpoint' (i.e.  $P_{crit}$ ). This analysis was performed in R (v2.13.1) using the 'segmented' package (Muggeo, 2008).

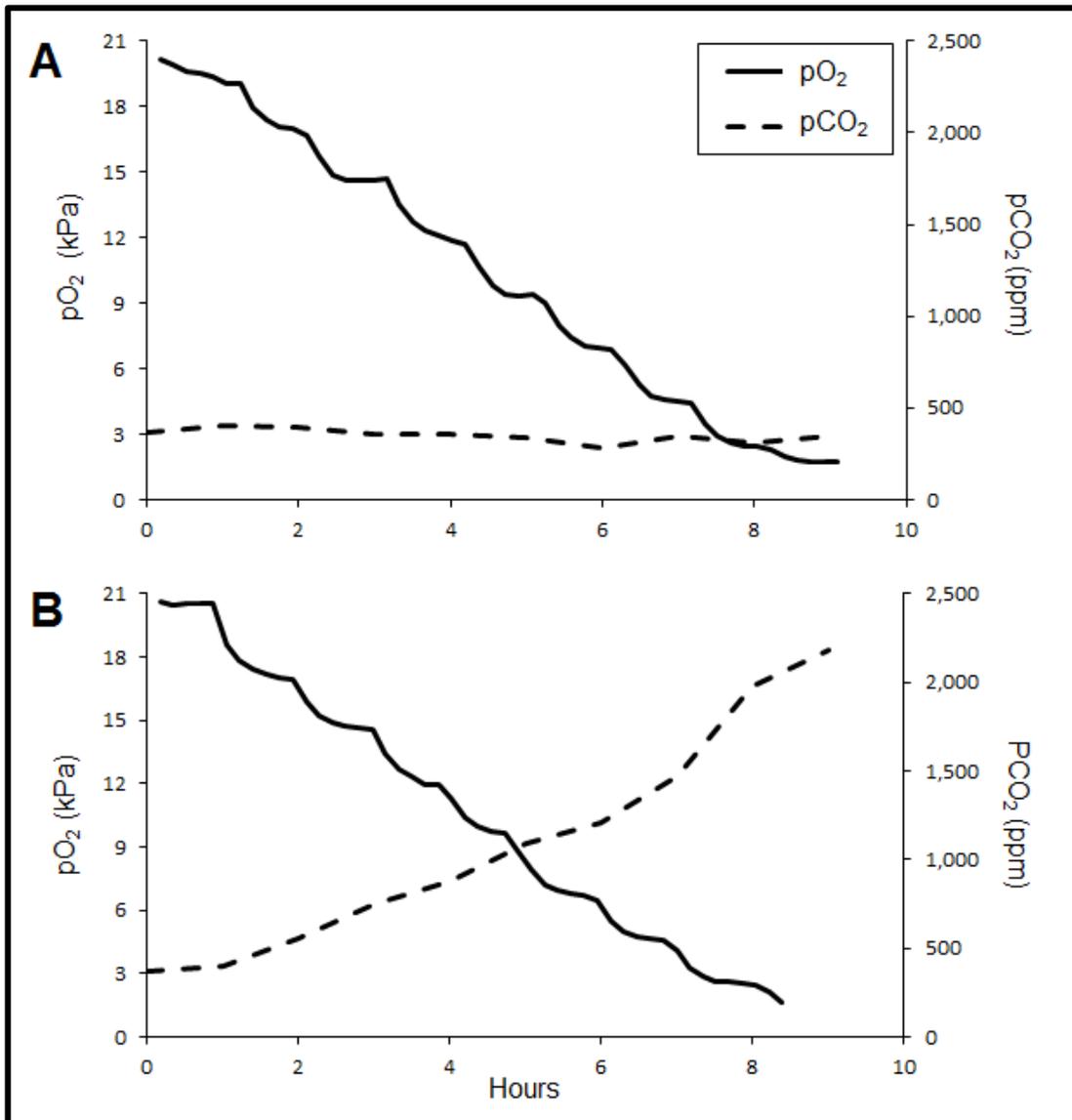


Figure 22. Typical traces of PO<sub>2</sub> and PCO<sub>2</sub> over time during hypoxia only (A) and hypoxic hypercarbia (B) P<sub>crit</sub> trials.

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### Gill Ventilation Measurements (*series III*)

Video recordings of gill ventilation frequency ( $V_f$ ) were made in 8 flounder during various dissolved oxygen and CO<sub>2</sub> treatments (Table 8). Each treatment lasted for 24 hours during which, three 10 minute recordings were made per flounder. Opaque tank walls obscured the observer from view throughout each recording. The number of opercula beats per minute were later determined from viewing the footage. The first 5 minutes of each recording was excluded from analysis to ensure full recovery from any disturbance caused on the commencement of filming.

Gill ventilation volume ( $V_v$ ) was measured indirectly by adaptation of the Fick principle (Fritts & Cournand 1958). Flounder were anaesthetised in seawater containing 160 mg l<sup>-1</sup> MS222 (pH-adjusted with NaOH) and transferred to a wet surgery table. Here the gills were continuously irrigated via the mouth with seawater containing 120 mg l<sup>-1</sup> MS222 (pH-adjusted with NaOH). A polyethylene cannula (ID 0.86 mm, OD 1.27 mm) was placed directly adjacent to the operculum to sample exhalent water. The gill end of the cannula was previously shaped (by heating) into a bend such that it entered under the ventral side of the operculum and followed the very outer edge of the subopercle towards the pectoral fin (Figure 23). This placement did not intrude into the buccal cavity or appear to restrict opercula movement in any way. Five holes (~2 mm diameter) were made along the portion of cannula that lay underneath the operculum so that the water sampled was a representative average of the exhaled water. The cannula was held in place by silk sutures at the subopercle, near the pelvic fin and towards the tail end of the lateral line. Flounder were then transferred to individual 20 l tanks and allowed to recover for 24 hours at normoxia. During this recovery period, flounder were closely monitored for signs of discomfort and to ensure that the position of the cannula was not interfering with normal ventilation behaviour.

Following recovery, cannulated fish were exposed to the exact same PO<sub>2</sub> and PCO<sub>2</sub> treatment regime as undergone during measurements of ventilation frequency. During each treatment, samples of inhalant water (sampled via a

cannula placed directly adjacent to the mouth) and exhalent water were taken simultaneously using 2.5 ml syringes. Dissolved oxygen was measured immediately in these samples using a Strathkelvin oxygen electrode and meter (Model 781, Strathkelvin Instruments, Scotland, UK). This procedure was repeated three times per fish per treatment with each repetition separated by a minimum of one hour. The total oxygen consumption rate ( $MO_2$ ) of each flounder was measured continuously throughout each treatment by intermittent flow through respirometry as previously described. Oxygen concentration of inspired water ( $PO_2_{insp}$ ) and expired water ( $PO_2_{exp}$ ) were combined with  $MO_2$  to calculate an estimate of ventilation volume ( $V_v$ ) as follows:

$$V_v = \frac{MO_2}{(O_2_{insp} - O_2_{exp})}$$

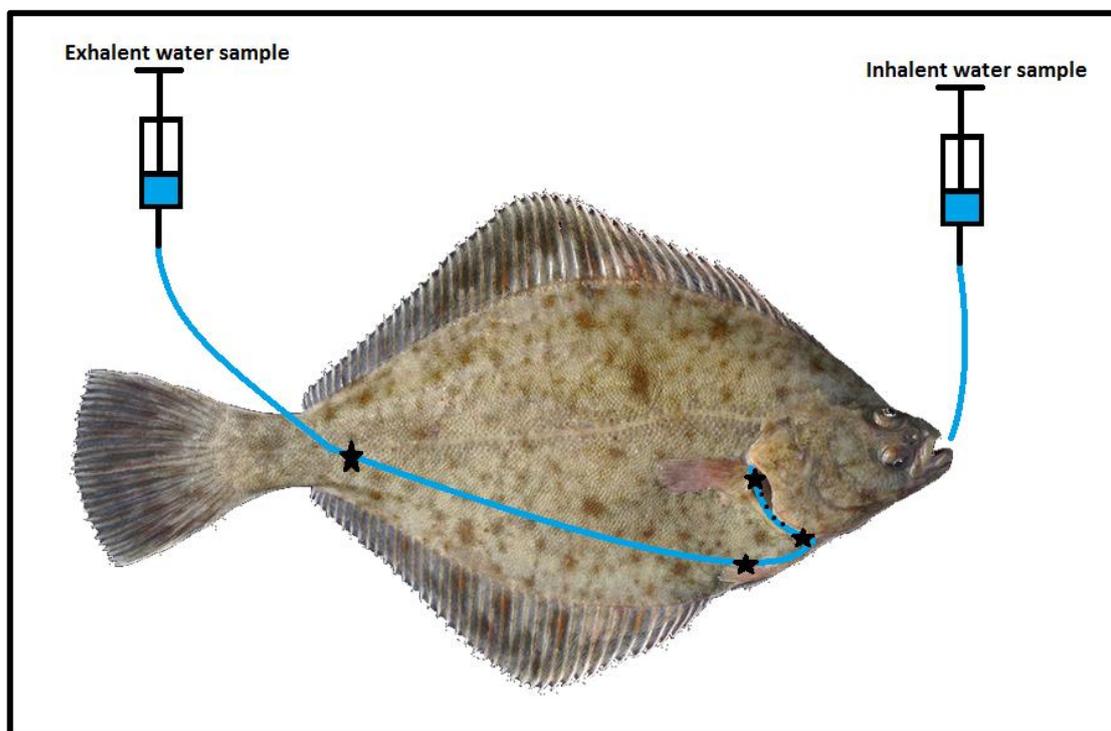


Figure 23. Illustration of exhalent and inhalent cannula placement for indirect measurement of gill ventilation volume ( $V_v$ ) in European flounder (image modified from original by Hans Hillewaert, 2007). Suture sites of the exhalent cannula are indicated as black stars. Water samples for measurement of dissolved oxygen were withdrawn using a 2.5 ml syringe and ventilation volume calculated by application of the Fick principle.

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## CaCO<sub>3</sub> Collection and Analysis (*series IV*)

To measure carbonate excretion rate, 8 flounder were held in individual 30 L tanks which were in turn placed in a 100 L ambient tank (2 flounder per ambient tank). PO<sub>2</sub> and PCO<sub>2</sub> were controlled within the ambient tank and individual flounder tanks by continuous bubbling with preset 'nitrox' gas mixtures as described previously (Figure 24). Ambient PO<sub>2</sub> and PCO<sub>2</sub> of the four treatments (normoxia, normoxic hypercarbia, hypoxia and hypoxic hypercarbia) are summarised in Table 8. Every 24 hours throughout each 7 day treatment, excreted calcium carbonate precipitates were collected by manually pipetting from the bottom of each flounder tank. These precipitates were then immediately and rapidly centrifuged and rinsed three times in deionised water, re-centrifuging each time. The deionised water was then removed and 5% sodium hypochlorite solution was added at an approximate 3:1 ratio based on the pellet size. The pellet was left in this solution for 24 hours at room temperature in order to remove the carbonate's mucus coating and other organic material whilst leaving all inorganic carbonate intact. The sodium hypochlorite solution was then removed and the pellet rinsed thoroughly with deionised water, again re-centrifuging each time. The cleaned carbonates were then dried for approximately 48 hours in an oven set to 40°C. This protocol is modified from Keatings *et al.*, (2006).

The dry weight of each carbonate sample was measured so that flounder carbonate excretion rate ( $\mu\text{mol kg}^{-1} \text{h}^{-1}$ ) could be estimated (assuming samples were 100% CaCO<sub>3</sub>). To obtain a more accurate measure of carbonate excretion rate each sample was sonicated (Vibra-Cell, Sonics and Materials) in 20 ml of ultrapure water (Maxima Ultrapure water, ELGA). The bicarbonate equivalents (HCO<sub>3</sub><sup>-</sup> + 2CO<sub>3</sub><sup>2-</sup>) content of a sub sample of these precipitates was then determined by double titration with HCl and NaOH as described in Wilson *et al.*, (2002) using an autotitration system (TIM845, Radiometer Analytical) with autosampler (SAL80, Radiometer Analytical).

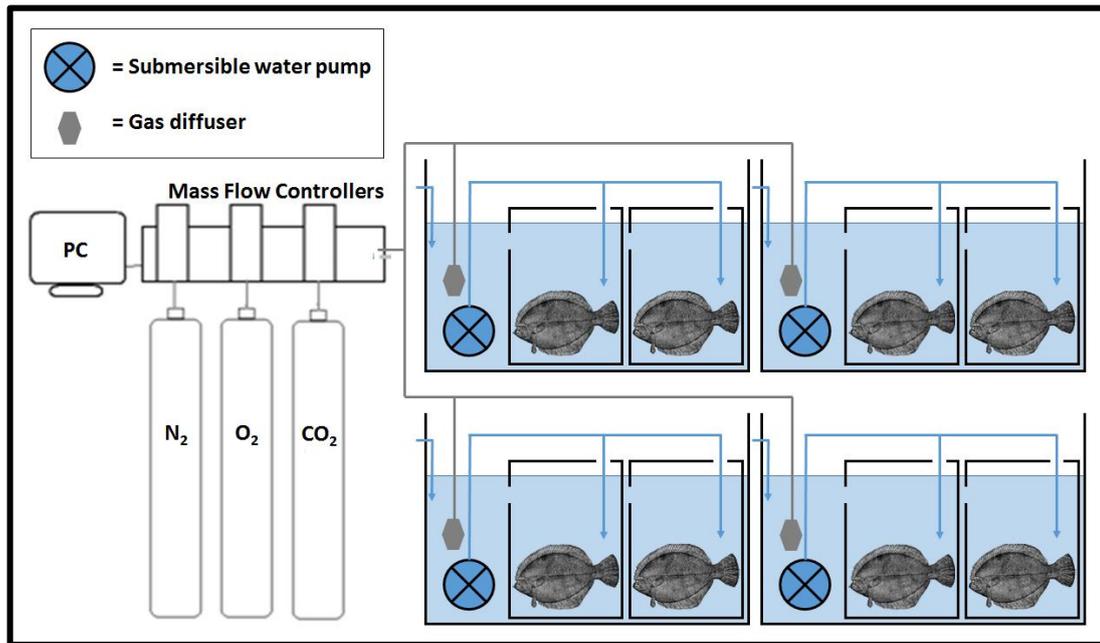


Figure 24. Diagram of the system used to facilitate daily collection of excreted  $\text{CaCO}_3$  precipitates from individual flounder under hypoxic and hypercarbic conditions. Flounder drawn by H. L. Todd (1895).

### Blood Sampling and Analysis (*series IV*)

At the end of each 7 day carbonate collection period, flounder were anaesthetised *in situ* with  $160 \text{ mg l}^{-1}$  MS222 (pH-adjusted with NaOH) to match ambient pH. Flounder were then transferred to a custom made wet table designed such that the head and gills remained submerged while the posterior and tail were emersed. The gills were continuously irrigated via the mouth with seawater containing  $120 \text{ mg l}^{-1}$  MS222 (pH-adjusted with NaOH) equilibrated with the identical  $\text{O}_2$  and  $\text{CO}_2$  tension as in the proceeding treatment. Gill irrigation flow was set at a rate consistent with previous measurements of ventilation volume and controlled using a pump (Eheim, Stuttgart, Germany) connected to a  $0.1 - 1.4 \text{ l min}^{-1}$  flow meter (Rotameter 1100). A 1 ml blood sample was then collected by caudal puncture into a heparinised syringe with a 21-gauge needle.

The blood sample was immediately transferred to a  $15^\circ \text{C}$  water bath where a 0.2 ml aliquot was taken for pH (Accumet Micro pH Electrode, Cole Parmer) and  $\text{PO}_2$  (Model 781, Strath Kelvin Instruments) measurement. Three micro-haematocrit tubes (Jaytech, UK) were filled and spun at 10,000 RPM for 5

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minutes, following which the volume percentage of red blood cells was measured on a haematocrit reader (Hawksley Mirco-Haematocrit Reader). The remaining blood sample was spun at 13,000 RPM for 5 minutes and 100  $\mu$ l of the separated plasma analysed for total CO<sub>2</sub> (Corning 965 Carbon Dioxide Analyser) and 30  $\mu$ l analysed for osmolality (Vapro Vapour Pressure Osmometer, Wescor).

### Measurement of Drinking Rate (*series V*)

Prior to measurements of drinking rate flounder were acclimated to one of two treatments ( $n = 10$ ), normoxia ( $> 90\%$  air saturation) and hypoxia ( $50 \pm 5\%$  air saturation), for 7 days in groups of 5 per acclimation tank. Flounder remained unfed throughout this period and salinity, dissolved oxygen and pH were monitored daily. Following acclimation, flounder were transferred to individual 20 l covered chambers of seawater equilibrated to the correct oxygen tension. After 12 hours, 10 l was removed from each chamber using a fixed volume syphon inserted through a small hole in the chamber's lid. 1 ml of <sup>51</sup>Cr labelled EDTA (Perkin Elmer, USA) was then injected under the water surface in each chamber using a syringe and cannula tubing (final concentration = 0.005 mCi). The time of <sup>51</sup>Cr-EDTA addition was recorded and triplicate 20 ml water samples were taken from all chambers. Following a 4 - 5 hours incubation time, further triplicate 20 ml water samples were taken before 8 l of water were siphoned from each tank. Flounder were then immediately terminated with a lethal dose of benzocaine (300 mg l<sup>-1</sup>). The remaining water was pumped from the chamber before the flounder carcass was removed and rinsed with freshwater. Each flounder was then weighed and a 1.5 ml blood sample was taken by caudal puncture. The gastrointestinal tract (GIT) was exposed by careful incision along the walls of the gut cavity. Two pairs of ligatures were applied to the GIT, one pair just before the anal sphincter and the second pair between the oesophagus and stomach opening. The GIT was then removed by cutting between each pair of ligatures. Water, blood and GIT samples were added to separate scintillation vials and counted on a gamma counter (WIZARD<sup>2</sup> Automatic Gamma Counter, Perkin Elmer, USA). Estimates of drinking rate (DR) were calculated as follows:

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$$DR [ml\ kg^{-1}h^{-1}] = \frac{Total\ GIT\ count}{(Mass\ [kg] \times Avr.\ water\ count\ [ml^{-1}] \times Incu.\ Time\ [h])}$$

## Statistical Analysis

Data are presented as means  $\pm$  SEM unless otherwise stated. Assumptions of normality and equal variance were tested via the Shapiro-Wilk test and Levene's test, respectively ( $P > 0.05$ ). Significant differences between treatments were tested for by t-test, one-way or two-way ANOVA where appropriate. The Tukey test was performed *post-hoc* and results were accepted as significant at  $P < 0.05$ . Two-way ANOVA results are summarised in the appendix to this chapter (Table 10). Statistical analyses were carried out using SPSS version 17.0, and graphs were drawn using MS Excel 2010.

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## Results

### Metabolic Responses

$P_{crit}$  was significantly increased under hypercarbia ( $M= 5.0$ ,  $SE= 0.32$ ) compared to normocarbica ( $M= 4.22$ ,  $SE= 0.18$ );  $F= 0.323$ ,  $t(14)= -2.14$ ,  $P= 0.025$  (Figure 25, Figure 26). There was a significant effect of oxygen tension on routine metabolic rate,  $F(3,12) = 6.819$ ,  $P = 0.048$ . Flounder demonstrated an elevated routine metabolic rate relative to normoxia under exposure to chronic (7 day) hypoxia ( $15.1 \pm 0.2$ ,  $10.3 \pm 0.3$  and  $6.3 \pm 0.4$  kPa, Figure 27).

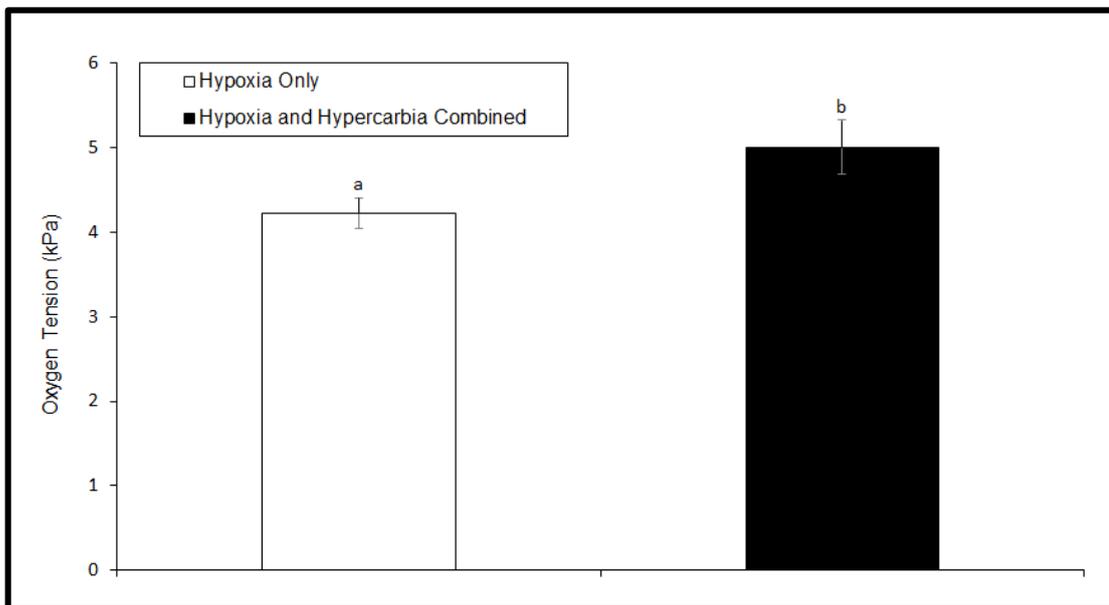


Figure 25. Mean ( $\pm$  SEM)  $P_{crit}$  of European flounder (*Platichthys flesus*) exposed to progressive hypoxia (white) and progressive hypoxic hypercarbia (black). Columns labelled with different letters indicate a significant difference between means ( $P < 0.05$ , two-tailed t-test assuming equal variance).

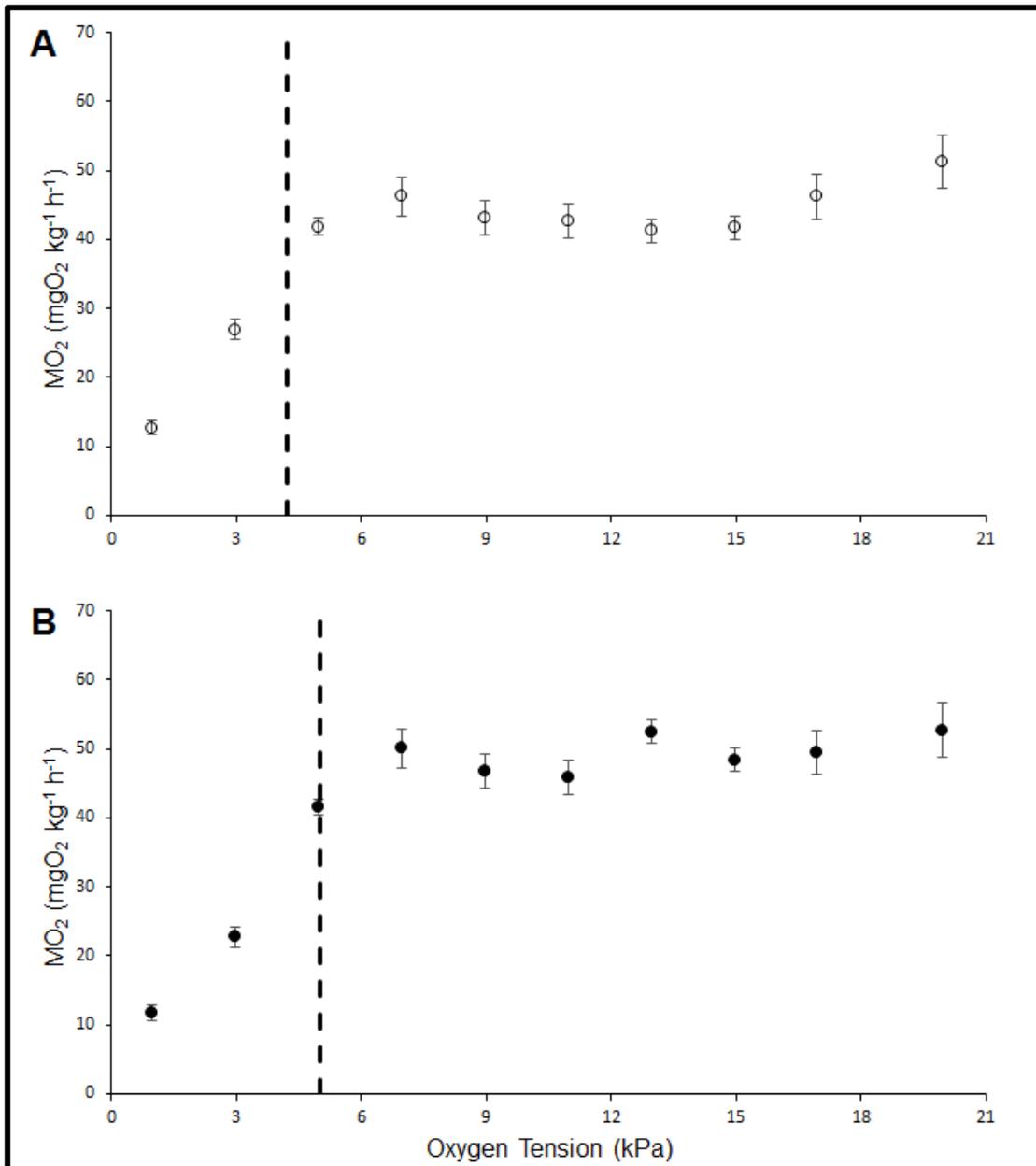


Figure 26. Plot of average routine metabolic rate ( $MO_2$ ) in flounder ( $N = 8$ ) across oxygen tensions under progressive hypoxia (A) and hypoxic hypercarbia (B). Dashed vertical lines indicate the mean  $P_{crit}$  within each treatment.

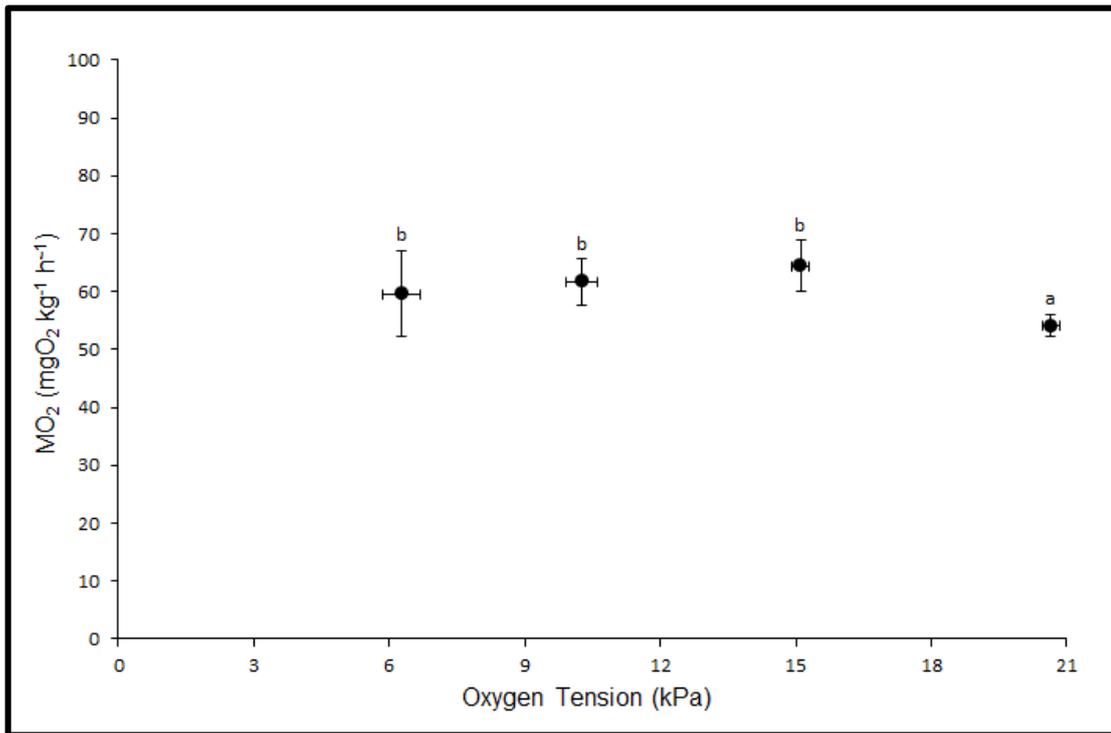


Figure 27. Means ( $\pm$  SEM) of routine metabolic rate ( $MO_2$ ) in flounder ( $N = 8$ ) during chronic (7 day) exposure to normoxia and three levels of hypoxia. Data points labelled with different letters indicate a significant difference in  $MO_2$  between oxygen tensions (one-way ANOVA,  $P < 0.05$ ).

## Ventilatory Responses

There was no significant change in gill ventilation frequency ( $V_f$ ) with declining  $PO_2$  until  $\sim 5$  kPa where it increased 1.4-fold relative to normoxia (Figure 28). Ambient  $PCO_2$  had no effect on  $V_f$ . Gill ventilation volume ( $V_v$ ) was significantly negatively correlated with ambient oxygen, increasing 2.4 and 5.5-fold relative to normoxia at 9.7 and 5 kPa respectively (Figure 29). There was no statistically significant effect of  $PCO_2$  on ventilation volume although there was a trend towards higher ventilation volumes at oxygen tensions below 10 kPa under combined hypoxic hypercarbia ( $P = 0.09$ ). There were significant independent effects of ambient  $PCO_2$  and  $PO_2$  on oxygen extraction efficiency ( $EO_2$ ) which declined with ambient oxygen and at elevated  $PCO_2$  (Figure 29).  $EO_2$  declined by 1.9-fold and 2.3-fold relative to normoxia at 5 kPa under normocarbica and hypercarbia respectively.

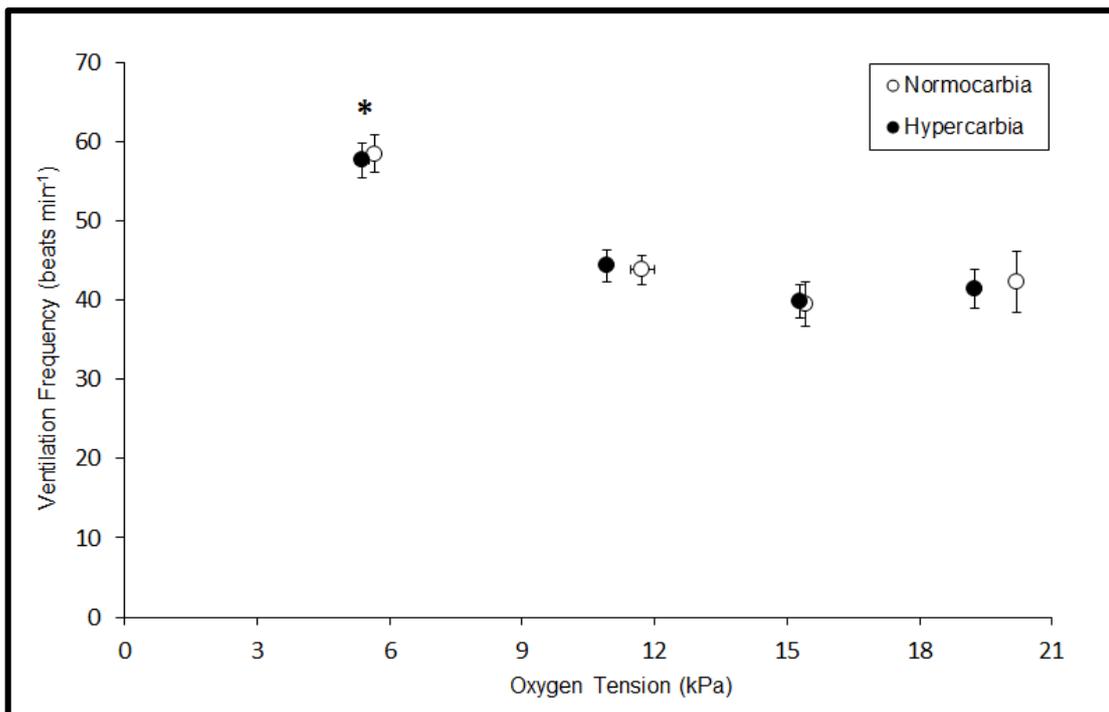


Figure 28. Mean ( $\pm$  SEM) gill ventilation frequency ( $V_f$ ) in flounder at various oxygen tensions under normocarbica (400  $\mu$ atm) or hypercarbia (3000  $\mu$ atm). Asterisk indicates a significant difference at that oxygen tension ( $P < 0.05$ , two-way ANOVA) relative to normoxia under both  $PCO_2$  treatments.

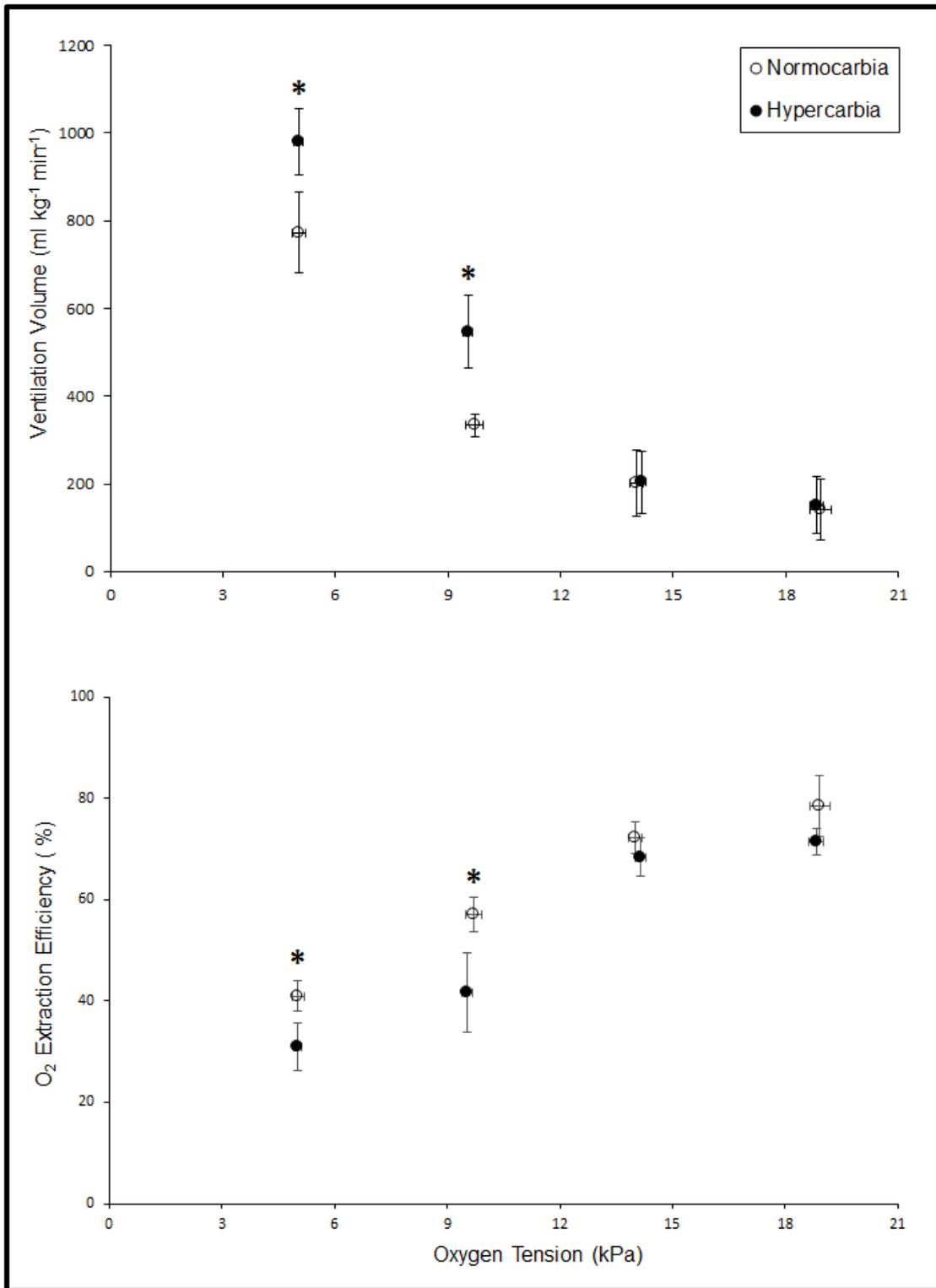


Figure 29. Mean ( $\pm$  SEM) gill ventilation volume ( $V_v$ ) and O<sub>2</sub> extraction efficiency ( $EO_2$ ) in flounder at various oxygen tensions under normocarbica (400  $\mu$ atm) or hypercarbica (3000  $\mu$ atm). Asterisk indicates a significant difference at that oxygen tension ( $P < 0.05$ , two-way ANOVA) relative to normoxia under both PCO<sub>2</sub> treatments.

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## Blood

Significant main effects of ambient  $PO_2$  were detected for blood  $PO_2$ ,  $HCO_3^-$ ,  $PCO_2$  and osmolality. Significant main effects of ambient  $PCO_2$  were detected for blood pH,  $HCO_3^-$  and  $PCO_2$ . Significant interactive effects of ambient  $PO_2$  and  $PCO_2$  included for blood pH,  $PCO_2$  and osmolality (Table 10). *Post-hoc* analysis revealed that hypoxia (~ 50% air saturation) had no significant effect on any of the blood parameters measured apart from increasing blood osmolality by an average of 26 mOsm  $kg^{-1}$  relative to normoxia (Figure 30). There is a trend towards a metabolic acidosis under hypoxia but the reduction in blood pH relative to normoxia was not statistically significant (Figure 31). Relative to normoxic normocarbica, hypercarbia (~ 3000  $\mu$ atm) resulted in a clear respiratory acidosis with reduced blood pH caused by an almost 3-fold increase in blood  $PCO_2$ , which was partially compensated by a similar fold-increase in blood  $HCO_3^-$ . Hypercarbia had no significant effect on either plasma osmolality or haematocrit. Combined hypoxic hypercarbia had no statistically significant effect on blood pH or  $PO_2$  relative to both normocarbica treatments. Blood  $HCO_3^-$  and  $PCO_2$  were significantly elevated under hypoxic hypercarbia relative to normocarbica, indicating a respiratory acidosis, but were significantly lower than under normoxic hypercarbia. There was no significant difference in blood osmolality or haematocrit under hypoxic hypercarbia relative to normoxic treatments.

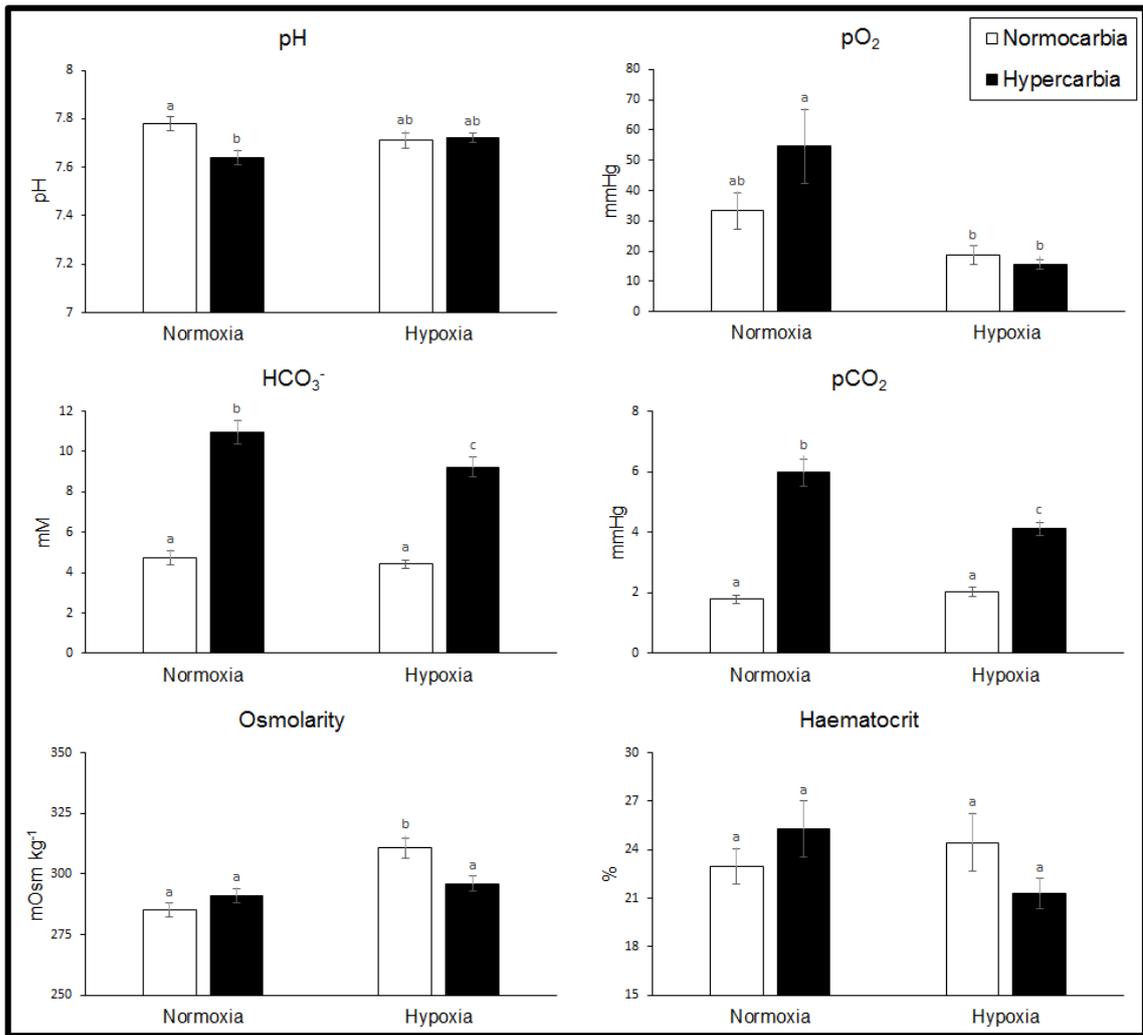


Figure 30. Mean ( $\pm$  SEM) of various caudal puncture blood parameters in flounder (N = 8) held for 7 days under normoxia, hypoxia (50% air saturation), hypercarbia (3000  $\mu$ atm) and combined treatments (Table 8). Columns labelled with different letters indicates a significant difference between treatments (two-way ANOVA, P < 0.05).

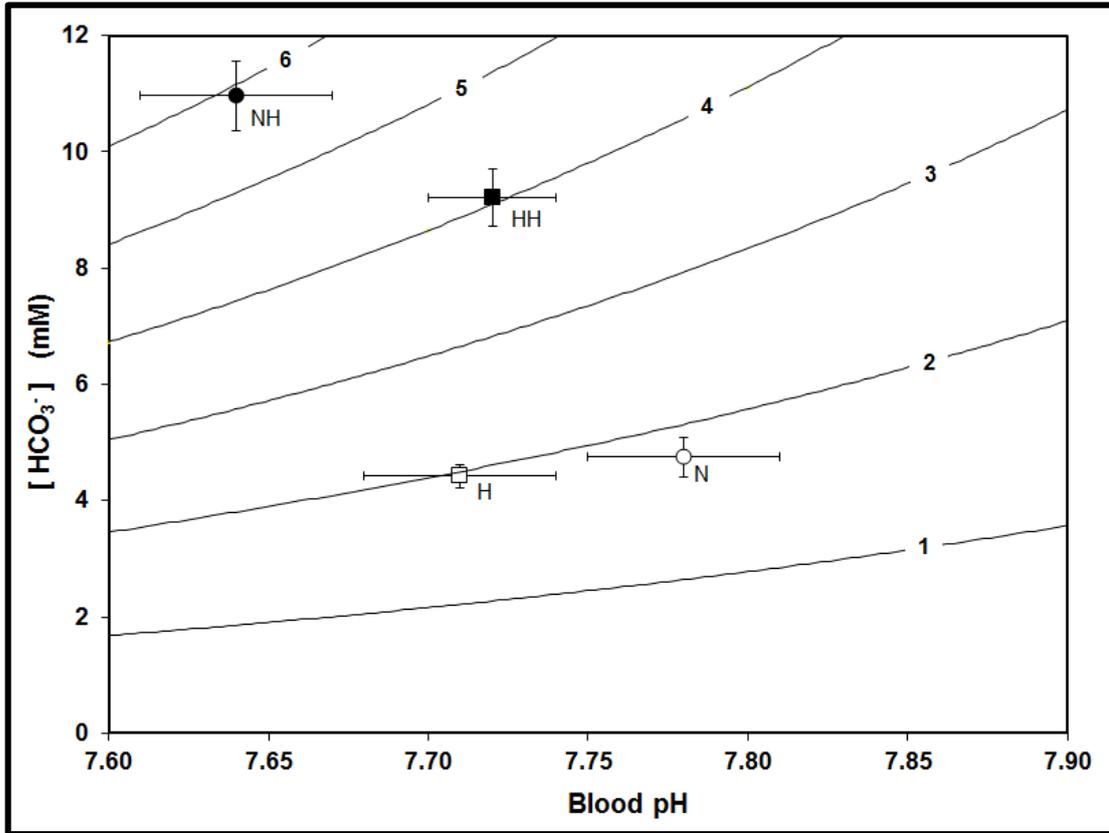


Figure 31. Davenport diagram summarising blood acid-base status of flounder at 15 °C after 7 days under four different ambient  $\text{PO}_2$  and  $\text{PCO}_2$  treatments (Table 8, N = normoxia, H = hypoxia (50% air saturation), NH = normoxic hypercarbia (3000  $\mu\text{atm}$ ), HH = combined hypoxic hypercarbia). Numbered isopleths indicate  $\text{PCO}_2$  (mmHg) based on pK equation and  $\text{CO}_2$  plasma solubility values from Boutlier *et al.*, 1984)

## Intestinal CaCO<sub>3</sub> Excretion

All treatments significantly increased CaCO<sub>3</sub> excretion rate relative to normoxic normocarbica. Hypercarbia (~ 3000  $\mu$ atm) and hypoxia (~ 50% air saturation) increased CaCO<sub>3</sub> excretion by 1.5 and 2.3-fold respectively and combined hypoxic hypercarbia resulted in a 4.3-fold increase relative to the control (Figure 32).

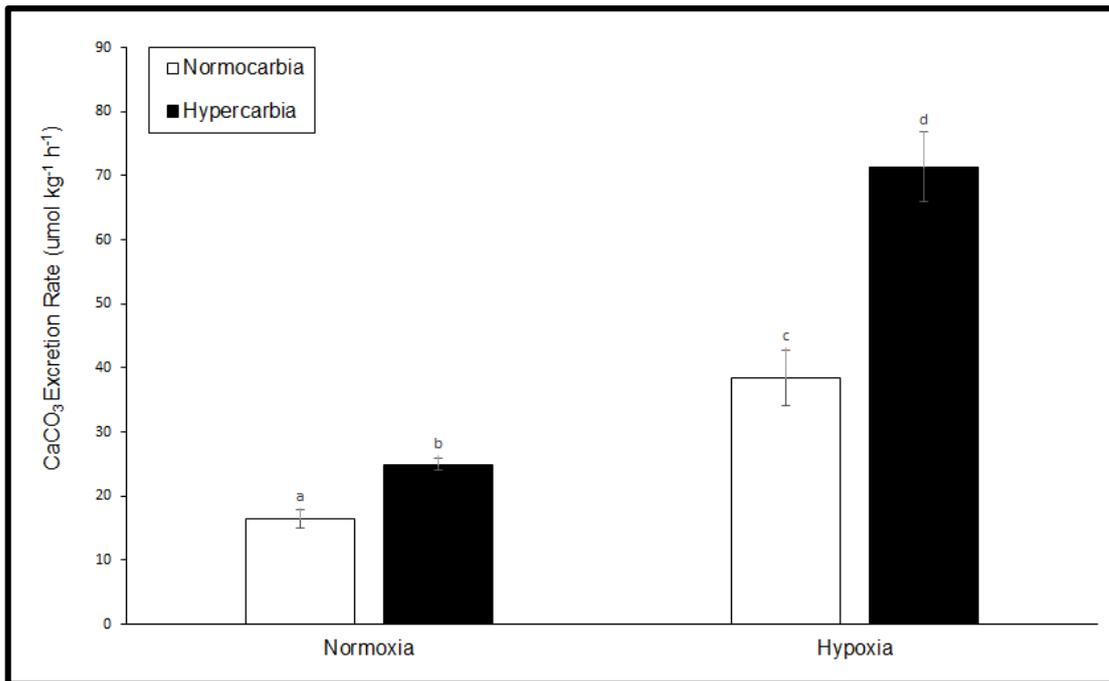


Figure 32. Mean ( $\pm$  SEM) intestinal CaCO<sub>3</sub> excretion rate in flounder (N = 8) under different ambient PO<sub>2</sub> and PCO<sub>2</sub> treatments (Table 8). Columns labelled with different letters indicates a significant difference between treatments (two-way ANOVA, P < 0.05).

	Normoxia		Hypoxia	
	Normocarbica	Hypercarbica	Normocarbica	Hypercarbica
Ambient PO <sub>2</sub> (kPa)	18.9 ± 0.3 <sup>a</sup>	18.8 ± 0.2 <sup>a</sup>	9.7 ± 0.2 <sup>b</sup>	9.5 ± 0.1 <sup>b</sup>
Ambient PCO <sub>2</sub> (ppm)	360 ± 30 <sup>a</sup>	3057 ± 76 <sup>b</sup>	360 ± 30 <sup>a</sup>	2996 ± 80 <sup>b</sup>
V <sub>f</sub> (opercula beats min <sup>-1</sup> )	42.4 ± 3.9 <sup>a</sup>	41.4 ± 2.4 <sup>a</sup>	43.8 ± 1.9 <sup>a</sup>	44.33 ± 2.04 <sup>b</sup>
V <sub>v</sub> (ml kg <sup>-1</sup> min <sup>-1</sup> )	140.3 ± 68.9 <sup>a</sup>	150.8 ± 65.1 <sup>a</sup>	333.0 ± 25.6 <sup>b</sup>	546.8 ± 83.6 <sup>b</sup>
EO <sub>2</sub> (%)	78.5 ± 4.8 <sup>a</sup>	71.4 ± 4 <sup>a</sup>	57.1 ± 2.5 <sup>b</sup>	41.6 ± 3.5 <sup>b</sup>
Blood PCO <sub>2</sub> (mmHg)	1.8 ± 0.1 <sup>a</sup>	6.0 ± 0.4 <sup>b</sup>	2.03 ± 0.2 <sup>a</sup>	4.12 ± 0.2 <sup>c</sup>
Blood HCO <sub>3</sub> <sup>-</sup> (mM)	4.8 ± 0.3 <sup>a</sup>	10.96 ± 0.6 <sup>b</sup>	4.42 ± 0.2 <sup>a</sup>	9.22 ± 0.5 <sup>c</sup>
Blood Osmolality (mOsm kg <sup>-1</sup> )	285 ± 3 <sup>a</sup>	291 ± 3 <sup>a</sup>	310 ± 4 <sup>b</sup>	296 ± 3 <sup>a</sup>
CaCO <sub>3</sub> Excretion (μmol kg <sup>-1</sup> h <sup>-1</sup> )	16.4 ± 1.4 <sup>a</sup>	25 ± 0.9 <sup>b</sup>	38.4 ± 4.4 <sup>c</sup>	71.4 ± 5.5 <sup>d</sup>

Table 8. Summary of key data related to CaCO<sub>3</sub> excretion in flounder under four PO<sub>2</sub> and PCO<sub>2</sub> treatments. Data labelled with a different superscript indicates a significant difference of that variable between treatments (two-way ANOVA, P < 0.05).

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## Drinking Rate

No significant difference in drinking rate was detected between flounder under normoxia (> 90% air saturation,  $M = 1.56$ ,  $SE = 0.33$ ) and hypoxia ( $50 \pm 5\%$  air saturation,  $M = 1.62$ ,  $SE = 0.49$ );  $F = 0.440$ ,  $t(18) = -1.03$ ,  $P = 0.459$  (Figure 33). All blood sample gamma counts were well within background levels showing that intestinal uptake of  $^{51}\text{Cr-EDTA}$  had not occurred.

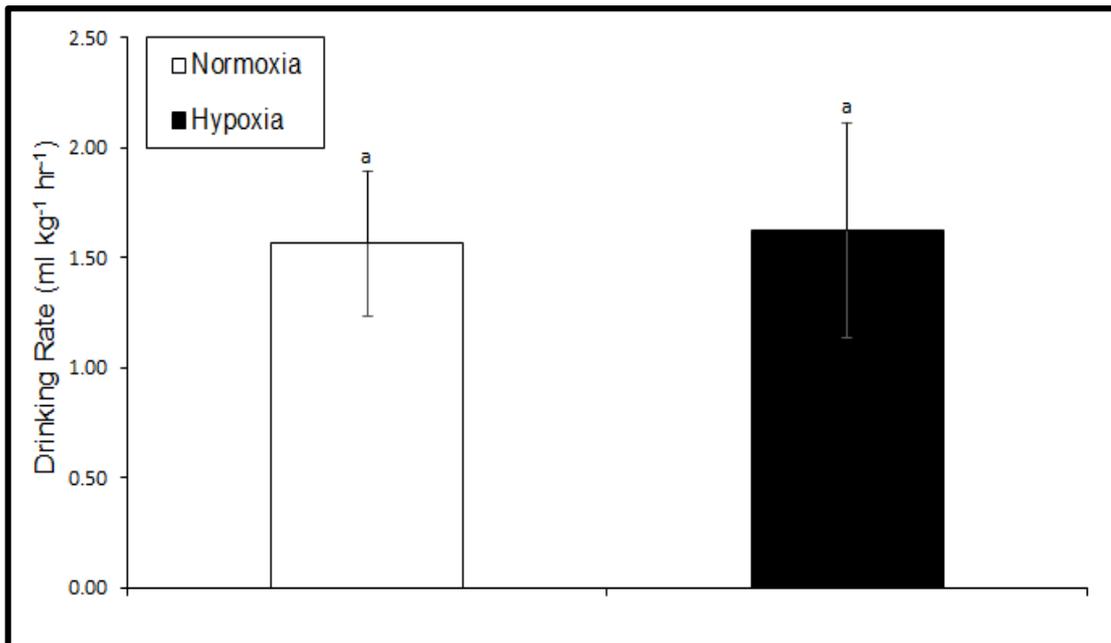


Figure 33. Mean ( $\pm$  SEM) drinking rate in flounder ( $N = 5$ ) under normoxia (>90% air saturation) and hypoxia ( $50 \pm 5\%$  air saturation). Columns labelled with the same letter indicate no significant difference between treatments (two-tailed t-test assuming equal variance,  $P < 0.05$ ).

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## Discussion

### Metabolic and Ventilatory Responses

European flounder displayed the classic two-part metabolic response to progressive hypoxia as seen in the majority of teleosts (Ultsch *et al.*, 1978; Richards, 2009). Figure 26 illustrates a clear ability to regulate  $\dot{M}O_2$  independent of ambient  $PO_2$ . This initial period of oxyregulation was followed by oxyconforming whereby  $\dot{M}O_2$  declined with ambient  $PO_2$ . A mean  $P_{crit}$  of 20% of air saturation demonstrated by flounder under progressive hypoxia in the present study, is within the range of values reported for other flatfish species at various acclimation temperatures: turbot (*S. maximus*, 13–20% saturation; Maxime *et al.*, 2000), sole (*S. solea*, 12–20 % saturation; Thillart *et al.*, 1994), starry flounder (*P. stellatus*, 30% saturation; Watters & Smith, 1973) and summer flounder (*P. dentatus*, 27%; Capossela *et al.*, 2012). However  $P_{crit}$  was significantly lower in the present study compared to values previously reported for European flounder (35 - 60% air saturation, Steffensen *et al.*, 1982). This difference may reflect the different experimental method utilized by Steffensen *et al.*, (1982) whereby oxygen consumption by the gills only was measured (thus neglecting cutaneous  $O_2$  uptake) as opposed to total  $O_2$  uptake as in the present study.

European flounder demonstrated a clear hypoxic ventilatory response (Figure 28, Figure 29). Hyperventilation is the most immediate and arguably most important physiological response to hypoxia in water-breathing fish (Perry *et al.*, 2009). Oxyregulation is achieved by maintaining arterial  $PO_2$  as ambient  $PO_2$  declines so that the transition to anaerobic metabolism can be delayed and increased gill ventilation achieves this by promoting branchial oxygen transfer. The 5.5-fold increase in ventilation volume demonstrated by European flounder at 5 kPa (Figure 29) is within the upper range of values reported for other flatfish species at similar ambient  $PO_2$  (Table 9) and is much higher than reported previously for European flounder (1.72-fold, Kerstens *et al.*, 1979; 1.91-fold, Steffensen *et al.*, 1982). The extent to which European flounder increased  $V_v$  in the present study rivals that of carp (5.75-fold, Lomholt

& Johansen, 1979), a species widely considered as highly hypoxia tolerant. However, inter and intra-species comparisons between studies are difficult given the multiplicity of factors that have been shown to influence the hypoxic ventilatory response in fish, including temperature, diet, stress, developmental plasticity and history of hypoxic exposure (Perry *et al.*, 2009).

Species	PO <sub>2</sub> (kPa)	ΔV <sub>v</sub> (fold-increase)	Reference
European flounder ( <i>Platichthys flesus</i> )	5	5.5	<i>Present study</i>
European flounder ( <i>Platichthys flesus</i> )	5.2	1.91	Steffensen <i>et al.</i> , 1982
Plaice ( <i>Pleuronectes platessa</i> )	5.2	1.65	Steffensen <i>et al.</i> , 1982
Starry flounder ( <i>Platichthys stellatus</i> )	6.7	3	Watters and Smith, 1973
Summer flounder ( <i>Paralichthys dentatus</i> )	5	6.4	Capossela <i>et al.</i> , 2012
English sole ( <i>Parophrys vetulus</i> )	6.7	5.21	Boesse, 1988
Rainbow trout ( <i>Oncorhynchus mykiss</i> )	6.3	2.42	Aota <i>et al.</i> , 1990
Carp ( <i>Cyprinus carpio</i> )	5.3	5.75	Lomholt & Johansen, 1979

Table 9. Summary of changes in gill ventilation volume (ΔV<sub>v</sub>) previously reported for various flatfish species at similar levels of hypoxia. Values for *Oncorhynchus mykiss* (hypoxia-intolerant) and *Cyprinus carpio* (hypoxia-tolerant) are included for comparison. ΔV<sub>v</sub> is expressed as a fold-increase relative to normoxia.

European flounder appear to rely predominantly on increasing ventilation amplitude (V<sub>amp</sub>) rather than frequency (V<sub>f</sub>) to achieve higher ventilation volumes during hypoxia. There was no significant increase in V<sub>f</sub> until ambient PO<sub>2</sub> had declined to 5 kPa and even then this only amounted to a 1.4-fold increase relative to normoxia (Figure 28). Although it was not directly measured, in the absence of other mechanisms such as ram ventilation it is reasonable to infer that increased V<sub>amp</sub> must have made up the 4.1-fold shortfall between V<sub>f</sub> and V<sub>v</sub>. It has been suggested that given the density and viscosity of water, increasing ventilatory stroke volume is a more energetically efficient strategy than increasing frequency (Perry & Wood, 1989; Gilmour, 2001). Indeed, of 35 teleost species in which both V<sub>f</sub> and V<sub>amp</sub> have been measured, 60% primarily or solely respond to hypoxia by increasing V<sub>amp</sub> (Perry *et al.*, 2009).

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Oxygen extraction efficiency ( $EO_2$ ) in European flounder was negatively correlated with  $V_v$  (Figure 29). During the hypoxic ventilatory response, a greater flow of water through the gills results in increased functional dead space and thus reduced  $EO_2$  (Hughes, 1966; Randal, 1982). The degree to which  $EO_2$  can be maintained during elevated gill ventilation is a key determinant of hypoxia tolerance (Steffensen *et al.*, 1982; Capossela *et al.*, 2012). An  $EO_2$  of 78% at normoxia in the present study compares very closely to that reported previously in European flounder (76 – 78%, Kerstens *et al.*, 1979; Steffensen *et al.*, 1982). However, the 2.4-fold decrease in  $EO_2$  between normoxia and 5 kPa is greater than previously reported in European flounder over a similar  $PO_2$  range (1.2 -1.5-fold Kerstens *et al.*, 1979; Steffensen *et al.*, 1982) but this likely corresponds to the more exaggerated HVR demonstrated by flounder in the present study.

Indirect measurement of  $V_v$  and  $EO_2$  via the Fick principle as performed in the present study is likely to result in an overestimate of these parameters because it does not factor in possible cutaneous  $O_2$  uptake. Previously it has been shown that cutaneous respiration accounts for 33% of total  $O_2$  uptake in European flounder at normoxia (Nonotte & Kirsch, 1981). However, measurements of cutaneous  $O_2$  uptake in plaice (*P. platessa*) showed that its proportional contribution to total  $O_2$  uptake was largely independent of ambient  $PO_2$  (Steffensen *et al.*, 1981). If the same is true in flounder, an overestimation of absolute values may not greatly affect the accuracy of the observed magnitude of change in  $V_v$  and  $EO_2$  (Capossela *et al.*, 2012).

The effect of simultaneous hypercarbia on the responses to hypoxia in teleosts has received surprisingly little attention given the evident correlation between  $PO_2$  and  $PCO_2$  in aquatic environments (Ultsch, 1996; Burnett, 1997; Gilmour, 2001). The biological demand for oxygen that leads to hypoxia produces  $CO_2$  as the main by-product of aerobic respiration. Where there is high aerobic biomass and when photosynthesis is limited, for example at night or in highly turbid water, hypoxic hypercarbia can quickly develop. Therefore, responses observed in fish exposed to hypoxia without simultaneous hypercarbia may not fully reflect how fish respond to hypoxia in the natural environment where

126

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reductions in ambient PO<sub>2</sub> are most likely to be accompanied with increased PCO<sub>2</sub>.

European flounder in the present study demonstrated reduced hypoxia tolerance (18.5% increase in P<sub>crit</sub>, Figure 25) under combined progressive hypoxic hypercarbia compared to progressive hypoxia only. Exposure to a progressive increase in PCO<sub>2</sub> from 400 μatm to ~ 2250 μatm over 8 – 9 hours is likely to have resulted in a significant blood acidosis that was not fully compensated (Claiborne *et al.*, 1997). Reduction in blood pH is associated with the Bohr and Root effects whereby the affinity and capacity of fish haemoglobin for oxygen is lowered thus impairing the oxygen extraction efficiency of the gills (Perry & Wood, 1989). As a result, hypoxemia of arterial blood and hence P<sub>crit</sub> would likely occur at a higher ambient PO<sub>2</sub> when ambient PCO<sub>2</sub> was increased simultaneously with progressive hypoxia.

Hypercarbia could influence gill ventilation indirectly via Bohr and Root effect induced hypoxemia, or via a direct CO<sub>2</sub> / pH stimulated ventilatory drive. CO<sub>2</sub> is highly soluble in water relative to O<sub>2</sub> due to the hydration reactions of CO<sub>2</sub> that produce HCO<sub>3</sub><sup>-</sup> and CO<sub>3</sub><sup>-</sup>. It is widely accepted that due to this difference in the capacity of water, ventilation in fish is directly responsive primarily to O<sub>2</sub> rather than CO<sub>2</sub> as is the opposite case in air breathing animals, (Perry & Wood, 1989). Indeed, because fish must hyperventilate relative to air-breathers to achieve the equivalent O<sub>2</sub> uptake, they have substantially lower blood PCO<sub>2</sub> (Ultsch, 1996). However, hyperventilatory responses to hypercarbia have been observed in teleosts in the absence of blood hypoxemia and during exposure to hyperoxia suggesting that CO<sub>2</sub> / pH can stimulate ventilation in fish independent of O<sub>2</sub> (Perry & Wood, 1989; Gilmour; 2001). Species reported to exhibit an O<sub>2</sub> independent CO<sub>2</sub> / pH ventilatory drive include rainbow trout (*O. mykiss*, Smith & Jones 1982; Thomas *et al.*, 1983), channel catfish (*I. punctatus*, Burlison & Smatresk, 2000), spotted gar (*L. oculatus*, Smatresk & Cameron, 1982) and white sturgeon (*A. transmontanus*, Crocker & Cech, 1998).

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In the present study,  $PCO_2$  was not a statistically significant factor in determining  $V_f$  or  $V_v$  across the whole range of ambient  $PO_2$  (Figure 28, Figure 29). However, it is difficult to rule out a positive effect of  $CO_2$  on  $V_v$  at ambient oxygen tensions of 10 kPa and below ( $P = 0.09$ ). That European flounder did not increase ventilation in response to normoxic hypercarbia suggests no direct  $CO_2$  ventilatory drive in these fish, at least at the  $PCO_2$  (~3000  $\mu$ atm) to which they were exposed. Significant hyperventilation at 10 kPa under hypercarbia relative to normocarbia is most likely an indirect response to  $CO_2$  via Bohr / Root effect induced hypoxemia. Indeed there was a significant negative effect of  $PCO_2$  on oxygen extraction efficiency across the entire range of ambient  $PO_2$  (Figure 29). Simultaneous measurements of arterial blood  $O_2$  content and ventilation during hypercarbia under normoxia, hypoxia and hyperoxia are required to fully elucidate the ventilatory response of European flounder to  $CO_2$ .

Under chronic (7 day) hypoxia above  $P_{crit}$  (15.1, 10.3, 6.3 kPa), European flounder maintained a slightly elevated  $MO_2$  relative to normoxia (13% increase, Figure 27). This increase in  $MO_2$  could in part reflect the energetic cost associated with the increased branchial muscle work during hyperventilation (Edwards, 1971; Steffensen & Lomholt, 1983; Farrell & Steffensen, 1987; Perry *et al.*, 2009). In addition, increased ion and water flux across the gills due to increased ventilation volumes will likely require upregulation of active ion pumps to prevent dehydration. Osmo/ion-regulation is energetically costly and is estimated to account for between 10 and 50% of the energy budget in fish (Boeuf & Payan, 2001). However the increase in  $MO_2$  under chronic hypoxia is at odds with observations in other benthic fishes in which hypoxic  $MO_2$  remained consistent with  $MO_2$  at normoxia (Steffensen *et al.*, 1982; Maxime *et al.*, 2000; Capossela *et al.*, 2010) and indeed with measurements in the present study of  $MO_2$  under acute progressive hypoxia (Figure 26). A possible explanation for this inconsistency is that under acute hypoxia, energy costs associated with hyperventilation are met by anaerobic metabolism (Maxime *et al.*, 2000) whereas under chronic hypoxic exposure the cost may be met from within the fish's aerobic scope.

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## Blood Measurements

European flounder did not maintain blood PO<sub>2</sub> (PO<sub>2</sub>) under chronic hypoxia relative to normoxia (Figure 30). Cech *et al.*, (1977) reported a PO<sub>2</sub> in winter flounder (*P. americanus*) of 31 ± 3 mmHg under normoxia and 16 ± 1 mmHg under hypoxia (40% air saturation) which is strikingly similar to that observed in the present study (33 ± 6 mmHg and 19 mmHg ± 3 under normoxia and 50% air saturation respectively). It should be noted that due to the close adjacency of caudal vasculature, blood collected by caudal puncture may represent either venous or arterial blood and hence samples may not be truly comparable. However, previous data obtained by cannulisation of the caudal vein and caudal artery of European flounder reveal only a small difference in blood PO<sub>2</sub> (38 - 46 mmHg arterial compared to 35 – 41 mmHg venous, Cooper & Wilson unpublished data).

Chronic exposure to elevated PCO<sub>2</sub> (~ 3000 µatm) appears to have no significant effect on blood PO<sub>2</sub> relative to normocarbica in European flounder. This corresponds to previous measurements listed in Gilmour *et al.*, 2001 which show that out of nine teleost species tested, all maintained or increased arterial PO<sub>2</sub> under acute hypercarbia. However, blood PO<sub>2</sub> is only a measure of the O<sub>2</sub> dissolved in the plasma and is not a function of haemoglobin O<sub>2</sub> content. Measurement of whole blood O<sub>2</sub> content is required to determine the status of haemoglobin and degree of any hypoxaemia associated with the Root / Bohr effect under elevated PCO<sub>2</sub>.

Typically, hyperventilation results in a decline in blood PCO<sub>2</sub> (due to increased CO<sub>2</sub> excretion) and hence a respiratory alkalosis under acute or progressive hypoxia (Thomas & Hughes, 1982; Claireaux & Dutil, 1992, Maxime *et al.*, 2000; Gilmour, 2001). However in the present study under chronic hypoxia, although there was a significant main effect of oxygen on blood PCO<sub>2</sub>, a significant decrease only occurred under hypoxic hypercarbia relative to normoxic hypercarbia. It is unclear why no reduction in PCO<sub>2</sub> was detected under normocarbic hypoxia given the substantial increase in ventilation volume observed at this oxygen tension (Figure 29). Previously, an adrenergic

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retention of CO<sub>2</sub>, through the release of catecholamines and the consequent activation of RBC Na<sup>+</sup>/H<sup>+</sup> exchange, has been observed in rainbow trout (*O. mykiss*) undergoing a respiratory alkalosis associated with hypoxia (Perry & Thomas, 1991).

As previously discussed, water breathing fish hyperventilate with respect to CO<sub>2</sub> in order to meet oxygen demand in a medium of relatively low O<sub>2</sub> capacity. Hence, blood PCO<sub>2</sub> in fish is low (~2 mmHg) relative to air breathers (~ 40 mmHg) and respiratory regulation of acid-base balance is of limited use (Ultsch, 1996; Perry & Gilmour, 2006). Instead, fish predominantly control blood pH through adjustments of blood HCO<sub>3</sub><sup>-</sup> via differential Na<sup>+</sup>/H<sup>+</sup> and Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchange at the gills (Claiborne *et al.*, 2002; Evans *et al.*, 2005). In the present study, blood PCO<sub>2</sub> in European flounder increased by 3.3-fold relative to normocarbica after chronic exposure to an ambient PCO<sub>2</sub> of 3000 µatm (Figure 30). This resulted in a partially compensated respiratory acidosis (Figure 31), indicated by a 0.14 reduction in blood pH and an almost 3-fold increase in HCO<sub>3</sub><sup>-</sup>. Under simultaneous hypoxic hypercarbia, European flounder demonstrated a fully compensated acidosis (2.3-fold increase in PvCO<sub>2</sub>, 1.9-fold increase in HCO<sub>3</sub><sup>-</sup> and no significant change in blood pH). The more fully compensated acidosis observed under hypoxia compared to normoxia at the same level of elevated ambient PCO<sub>2</sub>, indicates that hypoxic hyperventilation reduced the acid-base impact of hypercarbia by increasing CO<sub>2</sub> excretion rate.

Increased red blood cell levels enhances the oxygen carrying capacity of the blood and has been previously observed in fish as a response to both acute as well as chronic hypoxic and hypercarbic exposure (Perry *et al.*, 2009). Experiments in rainbow trout (*Oncorhynchus mykiss*) have shown acute changes in haematocrit that result from the release of red blood cells stored in the spleen in response to increased circulation of catecholamines; (Perry & Kinkhead, 1989) and chronic increases in haematocrit that are thought to occur through the stimulation of erythropoiesis in the kidney by erythropoietin (EPO) (Lai *et al.*, 2006). Haematocrit in European flounder was within the range (20 – 30%) reported previously in other flounder species (Wood *et al.*, 1979; 130

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Graham & Fletcher, 1983; Park *et al.*, 2012) and did not change significantly in response to either chronic hypoxia (50% air saturation) or chronic hypercarbia (3000  $\mu$ atm, Figure 30). This appears to correspond with measurements in turbot (*Scophthalmus maximus*) and seabass (*Dicentrarchus labrax*) which showed no increase in blood O<sub>2</sub> carrying capacity under chronic (40 day) hypoxia (Pichavant *et al.*, 2003). Whilst enhanced blood O<sub>2</sub> carrying capacity is likely to be beneficial during hypoxia, elevation in haematocrit also results in increased blood viscosity. For example, measurements in the winter flounder (*Pseudopleuronectes americanus*) showed that a haematocrit of 20% accounted for 50% of blood viscosity and increases in haematocrit resulted in a near exponential increase in viscosity (Graham & Fletcher, 1983). The higher the blood's viscosity the greater the energetic cost associated with cardiac pumping (Perry *et al.*, 2009) and therefore responses that do not impair cardiac function (such as regulation of Hb-oxygen affinity) may be favoured over increases in haematocrit (Wells, 2009).

The multifunctional nature of fish gills as the site of gas exchange, ion regulation, acid-base balance and nitrogenous waste excretion, inevitably leads to trade-offs where the interests of these functions diverge (Evans *et al.*, 2005). One such trade-off is referred to as the 'osmorepiratory compromise' and arises due to the competing interests of ion regulation and gas exchange whereby large surface area, small diffusion distances and high water and blood flow rates are beneficial to oxygen uptake but detrimental to regulating the flux of ions and water (Sardella & Brauner, 2007). The nature of the osmorepiratory compromise in fish can be observed by modifying the degree of either the ion regulatory or respiratory challenge, for example through manipulations of salinity, oxygen demand (i.e. exercise, temperature), or oxygen availability (i.e. hypoxia, hyperoxia). Henrikson *et al.*, (2008) showed that the euryhaline prickly sculpin (*Cottus asper*) had a 30% higher P<sub>crit</sub> in freshwater than when acclimated to seawater (with no observed change in plasma osmolality). This reduced hypoxia tolerance indicates a respiratory compromise associated with gill thickening necessary for freshwater tolerance

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in this species. In rainbow trout (*Oncorhynchus mykiss*), exercise induced increases in  $\text{MO}_2$  have been shown to yield increased ion losses (Gonzalez & McDonald 1991) and acclimation to soft water has been shown to impair branchial gas transfer due to the accumulation of chloride cells in the gills and resultant increase in diffusion distance (Greco *et al.*, 1995). Acute hypoxia typically increases the rate of ion and water flux at the gills (Thomas *et al.*, 1986; Robertson *et al.*, 2015). Additionally, in seabass (*Dicentrarchus labrax*) acclimated to mild hypoxia (70 – 80% air saturation) or mild hyperoxia (120 – 140% air saturation),  $\text{Na}^+$  and  $\text{Cl}^-$  efflux when exposed to an acute hypo-osmotic stress was almost  $100 \text{ nmol g}^{-1} \text{ min}^{-1}$  higher in the hypoxic group (Saroglia *et al.*, 2010). In the present study, hypoxia resulted in a slight increase relative to normoxia in the plasma osmolality of European flounder (Figure 30). Increased plasma osmolality is symptomatic of an elevated water loss and ion uptake that has not been fully compensated and is likely associated with the high ventilation volume observed in European flounder at 50% air saturation.

### Gut Carbonate Production and Drinking Rate

Under normoxia, at 15 °C and a salinity of 35, European flounder in the present study exhibited a carbonate excretion rate of  $16 \pm 1 \mu\text{mol kg}^{-1} \text{ h}^{-1}$  (Figure 32). This compares closely to the carbonate excretion rate previously reported in this species under similar conditions (Wilson *et al.*, 2009). At a hypoxic  $\text{PO}_2$  of 50% air saturation, European flounder increased carbonate excretion by 2.4 fold ( $38 \pm 4 \mu\text{mol kg}^{-1} \text{ h}^{-1}$ ). Strikingly, this increase exactly matches the 2.4 fold-increase in ventilation volume observed in these fish at the same level of hypoxia (Figure 29). These data support the prediction made at the outset of this study (Figure 20) and suggest that increases in carbonate production under hypoxia is directly proportional to increases in gill ventilation.

Given that the calcium in intestinal  $\text{CaCO}_3$  precipitates is derived from the imbibed seawater, it is assumed that intestinal carbonate production is proportional to drinking rate (Jennings & Wilson, 2009). As previously discussed, passive ion and water fluxes (which occur primarily at the gills) are

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directly proportional to gill ventilation (Gonzalez & McDonald, 1992) and teleost fish respond to increased water loss by increasing drinking rate (Marshall & Grosell 2005). In addition, European flounder undergoing hypoxia presumably also increased  $\text{HCO}_3^-$  secretion across the intestinal epithelium. Enhanced intestinal  $\text{HCO}_3^-$  secretion in response to osmoregulatory demand has been demonstrated previously in the Gulf toadfish (*Opsanus beta*) exposed to increasingly high salinities (Genz *et al.*, 2008).

The apical transport of  $\text{HCO}_3^-$  in the intestinal epithelium occurs in tandem with basolateral  $\text{H}^+$  transport (Grosell & Genz, 2006, Whittamore *et al.*, 2010). Hence, increases in these transport processes (such as under hypoxia) will lead to an increased acid load in the extracellular fluid. As previously described, no significant acidosis was detected in European flounder under normocarbic hypoxia (Figure 31) suggesting that net acid excretion (most likely branchial, Genz *et al.*, 2008) also increased with declining  $\text{PO}_2$ . Hypoxic hyperventilation and hence increased  $\text{CO}_2$  excretion could also play an indirect role in mitigating this acid-base imbalance. Previously it has been shown that blood flow to the gastrointestinal tract in fish is reduced while other systems are prioritised during periods of increased oxygen demand or reduced oxygen availability (Randal & Daxboeck 1982; Axelsson *et al.*, 2002). It could be argued that reduced blood flow to the GIT during hypoxia might limit intestinal  $\text{HCO}_3^-$  secretion. However, it seems unlikely that this vital fluid uptake mechanism would be sacrificed at a time of increased osmoregulatory demand.

The normoxic drinking rate determined in the present study ( $1.56 \pm 0.33 \text{ ml kg}^{-1} \text{ hr}^{-1}$ ) closely resemble previous determinations in E. flounder under similar conditions ( $1.3 - 1.5 \text{ ml kg}^{-1} \text{ hr}^{-1}$ , Carrick & Balment, 1982; Carroll *et al.*, 1994). It is puzzling that no significant increase in drinking rate was detected in European flounder at hypoxia relative to normoxia (Figure 33). Given the 2.4 fold-increase in carbonate excretion and ventilatory volume in European flounder at the same  $\text{PO}_2$ , a similar increase in drinking rate was expected (Figure 20). The proportion of ingested calcium precipitated by marine fish as carbonates in the gut has been reported to be within the range of 30 – 65%

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(Wilson & Grosell, 2003; Marshal & Grosell, 2005). At the lower end of this range there is scope for the increase in carbonate excretion rate observed under hypoxia in European flounder to be met through an increase in the proportion of imbibed  $\text{Ca}^{2+}$  that is precipitated. Indeed the salinity experiments of Genz *et al.*, (2008) showed that the fraction of  $\text{Ca}^{2+}$  excreted as precipitate increased from 26.8% at 35 ppt to 61.2% at 50 ppt, however this was also accompanied by an increase in drinking rate ( $2.56 - 3.75 \text{ ml kg}^{-1} \text{ hr}^{-1}$ ). As discussed by those authors, as ions that drive solute-linked water transport are absorbed, impermeable ions accumulate making it increasingly challenging to absorb water from the more concentrated fluid in the lumen. Thus there comes a point at which diffusive water loss must be compensated for by increased drinking rate (Genz *et al.*, 2008). Collection and ion analysis of the rectal fluid excreted by European flounder are required to compare the fractional precipitation of  $\text{Ca}^{2+}$  under normoxic and hypoxic conditions.

The  $^{51}\text{Cr}$ -EDTA method, both with and without a rectal catheter fitted, has been widely used to measure drinking rates in fish (Usher *et al.*, 1988; Hazon *et al.*, 1989; Perrot *et al.*, 1992; Carroll *et al.*, 1994; Fuentes & Eddy, 1997). In the absence of a rectal catheter such as in the present study, this method relies on the incubation time being set such that a detectable level of activity accumulates in the gut, but is not so long that  $^{51}\text{Cr}$ -EDTA is excreted during the incubation period. The 4 – 5 hour incubation time in the present study was chosen based on gut transit times previously reported for European flounder (Carroll *et al.*, 1994). Furthermore, low counts detected in the distal section (less than 3% of total) suggests minimal label loss. As a follow up study, a 2 hour incubation time and twice the  $^{51}\text{Cr}$ -EDTA dose were trialled to measure hypoxic drinking rate in European flounder but this did not produce significantly different results than those obtained in the original trial.

Drinking rate data appeared to be fairly stochastic in nature perhaps indicating the intermittency of drinking behaviour, an observation that has been noted previously (Carrick & Balment, 1983). Collection of excreted  $^{51}\text{Cr}$ -EDTA *via* a rectal catheter allows long term measurement of drinking rate and therefore may provide a more robust method for detecting changes in drinking rate

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between PO<sub>2</sub> treatments. Oesophageal cannulation provides an alternative direct method of measuring drinking rates but requires invasive surgery and results in artificial dehydration unless the imbibed fluid is returned to the gastrointestinal tract after sampling (Carrick & Balment, 1983). Previously, indirect estimates of drinking rate have been made successfully by measuring the volume of excreted rectal fluid and the concentration of Mg<sup>2+</sup> and SO<sub>4</sub><sup>2-</sup> in this fluid and the ambient water (Genz *et al.*, 2008). This method relies on the intestinal epithelium being mostly impermeable to MgSO<sub>4</sub>, an assumption for which there is some supporting evidence (Hickman, 1968; Grosell & Taylor, 2007). The degree and indeed presence of a drinking response to hypoxia in marine fish still remains to be resolved and the best method for doing so requires further consideration.

Presumably enhanced intestinal supply of HCO<sub>3</sub><sup>-</sup> explains the 1.5 fold-increase relative to the control in carbonate excretion rate observed in European flounder under hypercarbia (~3000 µatm, Figure 32). Two major sources of intestinal HCO<sub>3</sub><sup>-</sup> have been identified. Firstly there is the endogenous source whereby HCO<sub>3</sub><sup>-</sup> is produced via the hydration of CO<sub>2</sub> within the epithelial cells of the intestine, a cellular reaction that is catalysed by carbonic anhydrase. The second source is extracellular whereby HCO<sub>3</sub><sup>-</sup> is transported from the blood, a process facilitated by basolateral Na<sup>+</sup>/HCO<sub>3</sub><sup>-</sup> cotransporters (Grosell, 2006, Taylor *et al.*, 2011). Both sources were most likely increased under hypercarbia relative to normocarbia as indicated by the 3.3 and 3 fold-increase in PvCO<sub>2</sub> and plasma HCO<sub>3</sub><sup>-</sup> respectively. Previous studies on the isolated intestinal epithelium of European flounder showed that elevated PCO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> on the basal side resulted in increased apical secretion of HCO<sub>3</sub><sup>-</sup> (Grosell *et al.*, 2005). Furthermore, increased intestinal HCO<sub>3</sub><sup>-</sup> excretion has been observed under elevated PCO<sub>2</sub> (1900 µatm) in the Gulf toadfish (*Opsanus beta*), although surprisingly this was not accompanied by an increase in precipitate excretion rate (Heuer *et al.*, 2012). As those authors discuss, increased base excretion is counterproductive to regulating CO<sub>2</sub> induced respiratory acidosis and likely requires enhanced branchial acid extrusion or HCO<sub>3</sub><sup>-</sup> uptake, both of which are likely to entail some metabolic

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cost. Although not measured, it is unlikely that drinking rate changed under elevated  $\text{PCO}_2$  given that there was no change in ventilation volume and associated osmoregulatory demand under normoxic hypercarbia (Figure 29). The 4.3 fold-increase in carbonate excretion under combined hypoxia and hypercarbia (Figure 32) indicates an almost entirely additive effect of decreased ambient  $\text{PO}_2$  and increased  $\text{PCO}_2$  on intestinal carbonate production in European flounder.

### Global Significance

Whatever the exact mechanisms by which carbonate excretion increases under hypoxia and hypercarbia, the results of this study suggest that ambient  $\text{PO}_2$  and  $\text{PCO}_2$  are highly significant environmental factors to consider in terms of marine fish carbonate production. So far, temperature has been the only environmental parameter factored into modelling piscine carbonate production at a global scale (Jennings & Wilson, 2009; Wilson *et al.*, 2009). Already these models suggest a highly significant contribution by fish (up to 40%) to the marine inorganic carbon cycle (Wilson *et al.*, 2009). Given the widespread and frequent occurrences of hypoxia and hypercarbia in marine environments (Diaz & Rosenberg, 1995; Ultsch, 1996; Diaz & Breitburg 2009; Friedrich *et al.*, 2014) these models are likely to underestimate the extent of piscine carbonate production globally.

The results of this study support previous suggestions that rises in ocean  $\text{PCO}_2$  as a consequence of the absorption of anthropogenic  $\text{CO}_2$  emissions, will increase the rate of marine fish carbonate production in the future (Wilson *et al.*, 2009; Grosell, 2011). Oceanic uptake of  $\text{CO}_2$  is associated with reduced seawater pH and  $\text{CO}_3^{2-}$  concentration (Barker *et al.*, 2003). Predicted future increases in carbonate production by fish is in contrast to the prevailing prediction that calcification rates will decline in marine calcifiers such as plankton and corals that unlike fish are dependent on ambient seawater  $\text{CO}_3^{2-}$  for calcification (Feely *et al.*, 2004; Orr *et al.*, 2005; Fabry *et al.*, 2008; Kroeker *et al.*, 2010). Thus, the relative contribution of fish to marine carbonate production is likely to increase in the future. Hypoxia is also understood to be

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becoming an increasingly frequent and widespread perturbation in the world's oceans due to the combined effects of anthropogenic nutrient loading and climate change (Diaz & Breitburg, 2009; Diaz & Rosenberg, 2008). Reduced ocean PO<sub>2</sub> therefore might drive further increases in fish carbonate production in the future. However, global scale predictions must consider the ecological effects of PO<sub>2</sub> and PCO<sub>2</sub> perturbations as well as the impact of overfishing on fish biomass - which could ultimately lead to a reduced carbonate contribution by marine fish (Jennings & Wilson, 2009).

### Limitations and Future Work

Repeated measures within experimental series (I, II, III, and IV) may be seen as a significant limitation of the present study. In the absence of a true control group, it is not possible to establish the extent to which the repeated stress of sequential treatments may have influenced the physiological parameters being measured. Whilst a recovery period of at least seven days was incorporated between treatments (series I, II, IV) and fish condition was closely monitored throughout the experimental period, there was no formal assessment of the effectiveness of these measures in excluding carry-over effects between treatments. Ideally, individuals would have been randomly assigned to the various permutations of treatment sequence so that the effect of treatment order could be statistically determined. However, this approach was unviable within the constraints of the present study due to a limited facility for delivering more than one level of ambient PO<sub>2</sub> / PCO<sub>2</sub> simultaneously within different treatment tanks (Figure 24). Instead, a viable alternative approach could have been to repeat a normoxic normocarbic control between each hypoxic / hypercarbic treatment. If no significant differences were detected between the physiological parameters measured in these controls then the potential for carry-over effects between treatments may have been safely excluded.

It is important to avoid over generalising regarding the effects of hypoxia and hypercarbia on carbonate production in fish based on the results of this single species study. Significant inter and intra-specific variation exists in the ability of fish to oxyregulate, osmoregulate and maintain acid-base balance in the

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face of  $PO_2$  and  $PCO_2$  challenges (Gilmour, 2001; Perry & Gilmour, 2006; Perry *et al.*, 2009). Hence the effect of hypoxia and hypercarbia on carbonate production is likely to differ between species. The euryhaline European flounder as a benthic species and estuary dweller, is likely to exhibit well adapted responses to hypoxia and hypercarbia and in this respect is unlikely to be representative of the average marine teleost (Steffensen *et al.*, 1982). All the flounder used in the present study were obtained from a single collection site within the Taw estuary. This area demonstrates a very large tidal range (6-8 m at the estuary mouth), likely associated with significant daily  $PO_2$  and  $PCO_2$  fluctuations. Furthermore, the Taw is prone to estuarine eutrophication arising from high levels of anthropogenic nutrient input (Mair, 2009). Thus it is a significant possibility that individuals used in the present study represent a locally adapted / acclimated, rather than typical European flounder population.

Further study across a range of ambient  $PO_2$  and  $PCO_2$  is required to fully establish the relationship between these parameters and carbonate production in fish. In the case of  $PCO_2$ , measurement of carbonate excretion under relevant near-future  $CO_2$  scenarios are necessary to refine predictions of changes in the global carbonate contribution of fish. In order to account for inter- and intraspecific variation, global models of piscine carbonate production would benefit from similar studies across a representative range of marine teleost species and populations. There is also significant scope for investigating the interactive effects of other major abiotic variables such as temperature and salinity. Such experimental data is ultimately necessary to improve the resolution of piscine carbonate production models at both local and global scales.

## Appendix

### ANOVA Summary

Variable	O <sub>2</sub> tension			CO <sub>2</sub> tension			Interaction		
	df	F	P	df	F	P	df	F	P
<i>Ventilation</i>									
V <sub>f</sub>	3,56	21.55	< 0.001*	1,56	0.18	0.894	3,56	0.05	0.987
V <sub>v</sub>	3,32	23.19	< 0.001*	1,32	2.49	0.124	3,32	0.75	0.529
EO <sub>2</sub>	3,32	24.71	< 0.001*	1,32	6.20	0.018*	3,32	0.50	0.683
<i>Blood</i>									
pH	1,28	0.04	0.949	1,28	5.75	0.023*	1,28	6.38	0.017*
PO <sub>2</sub>	1,28	13.83	0.001*	1,28	1.60	0.216	1,28	2.92	0.098
HCO <sub>3</sub> <sup>-</sup>	1,28	5.41	0.027*	1,28	151.70	< 0.001*	1,28	2.47	0.127
PCO <sub>2</sub>	1,28	8.78	0.006*	1,28	133.06	< 0.001*	1,28	14.91	0.001*
Osmo.	1,28	19.83	0.001*	1,28	1.70	0.203	1,28	9.72	0.004*
Ht	1,28	0.72	0.402	1,28	0.08	0.786	1,28	3.41	0.075
<i>CaCO<sub>3</sub> ppt.</i>									
Excretion rate	1,27	84.14	0.001*	1,27	31.011	0.001*	1,27	10.74	0.003*

Table 10. Summary of two-way ANOVA results for physiological parameters measured in the present study. \* indicate significant effects where P < 0.05.

### Licence Details

All experimental procedures within this study were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986, PIL: 30/9871, PPL: 30/2735.



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# Chapter 5



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# **Gut carbonate production in the European flounder (*Platichthys flesus*) under Cretaceous ocean conditions.**

## **Abstract**

It has recently become clear that intestinal carbonate production by marine teleost fish has major implications for our understanding of fish physiology, ocean chemistry, carbon cycling as well as carbonate sediment budgets and records. The recognition of piscine carbonate production as a globally significant process leads to interesting questions as to how it may have varied in the geological past and the 'calcite seas' of the Cretaceous provide an intriguing case in point. Calcium carbonate excretion rates and the precipitate chemistries of the European flounder (*Platichthys flesus*) were measured under conditions simulating that of the Cretaceous oceans (high salinity, temperature and  $\text{PCO}_2$ ; and low Mg/Ca ratio and  $\text{PO}_2$ ). Flounder over a temperature range of 15 - 23 °C demonstrated a temperature quotient ( $Q_{10}$ ) of 3.2 for carbonate production which was higher than the  $Q_{10}$  for metabolic rate (2.6 - 2.8) observed over the same temperature range. Carbonate production rate increased by 5.3 to 5.6 fold under Cretaceous relative to modern seawater chemistries at the same temperature, which was consistent with the predicted multiplicative effects of increased salinity and seawater  $\text{Ca}^{2+}$ . The combination of increased temperature (23 °C) and Cretaceous seawater chemistry produced an almost 14-fold increase in carbonate excretion rates relative to modern seawater at 15 °C. Cretaceous carbonate precipitates exhibited a 50% increase in incorporated  $\text{Mg}^{2+}$  (8 – 11 mol%) compared to those produced in modern seawater (~ 18.5 mol%). The results of this study support the hypothesis that ocean conditions prevalent in the Cretaceous resulted in piscine carbonate production rates significantly higher than today and could have considerable implications for our understanding of the inorganic carbon cycle and carbonate records of the period.

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## Introduction

Marine teleost fish have only recently been recognised as a major source of  $\text{CaCO}_3$  in the world's oceans with conservative estimates indicating a 3 – 15% piscine contribution to the present day marine inorganic carbon cycle. (Wilson *et al.*, 2009). The intestinal excretion of carbonate precipitates is a by-product of osmoregulation whereby marine teleosts continuously drink seawater in order to prevent dehydration. Unlike the other major seawater solutes ( $\text{Na}^+$ ,  $\text{Cl}^-$ ,  $\text{K}^+$ ) which are actively absorbed by the intestine and excreted via the gills,  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  remain mostly unabsorbed. Intestinal  $\text{HCO}_3^-$  secretion leads to the alkaline precipitation of this calcium and magnesium as insoluble carbonates which are then excreted as mucus coated pellets (Walsh *et al.*, 1991; Grosell *et al.*, 2005, 2011; Wilson *et al.*, 2002, 2009). The excreted carbonates comprise of loosely aggregated crystals of mainly magnesium calcite with  $\text{MgCO}_3$  values typically ranging between 18 and 39 mol% (Perry *et al.*, 2011). The size and morphologies of these crystals vary widely across species (Salter *et al.*, 2012) and have been identified as a significant and distinctive source of fine grained carbonate sediment in shallow-water tropical marine habitats (Perry *et al.*, 2011). The high magnesium content of fish derived carbonates results in a higher solubility than the calcites and aragonites produced by the more traditionally recognised marine calcifiers such as coccolithophores and foraminifera (Feely *et al.*, 2004; Morse *et al.*, 2007). It has been suggested that piscine magnesium calcites could largely account for the significant dissolution of carbonates and increased total alkalinity at depths well above the chemical lysocline and saturation horizon of aragonite - a phenomenon that has puzzled ocean chemists for decades (Wilson *et al.*, 2009; Woolsey *et al.*, 2012).

Our recent appreciation of piscine carbonate production as a globally significant process, leads to interesting questions as to how it may have varied in the geological past. Ocean conditions have varied enormously since teleost fish first arose during the early Triassic period some 200 – 250 million years ago (MYA, Benton, 2004; Near *et al.*, 2012). In particular, aspects of seawater chemistry such as salinity, Ca/Mg ratio,  $\text{PCO}_2$  and  $\text{PO}_2$ , along with sea surface

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temperatures are known to have been very different at times throughout the Mesozoic and Cenozoic eras compared to those in the modern day oceans (De La Rocha & Paytan, 2005). Based on our understanding of the underlying physiological processes, such factors are likely to hugely influence the rate of intestinal carbonate production by teleost fish as well as the chemistry of the excreted precipitates. The Cretaceous, named after the extensive beds of  $\text{CaCO}_3$  found in the upper Cretaceous layer of continental Europe and the UK, provides an intriguing case in point.

The Cretaceous period spanned 79 million years and began 144 MYA. The climate of the Cretaceous period is generally characterized as a warm, equable greenhouse (Barron, 1983; Jenkyns *et al.*, 2004). Estimates of paleo- $\text{PCO}_2$  have been made via geochemical modelling of the long-term (multimillion year) carbon cycle and through analysis of a variety of terrestrial and marine proxies such as the stable isotope content of preserved phytoplankton and carbonate sediments as well as the stomatal density of fossil leaves (Royer *et al.*, 2001). Middle Cretaceous atmospheric  $\text{PCO}_2$  estimates range between 900 and 5500 ppm (Freeman & Hayes, 1992; Ekart *et al.*, 1999; Pagani *et al.*, 1999; Berner & Kothavala, 2001; Bice *et al.*, 2006) and it has been suggested that this wide range reflects real  $\text{PCO}_2$  extremes arising from highly active volcanism associated with high rates of tectonic movement during this period (Bice & Norris, 2002). High atmospheric  $\text{PCO}_2$  and resultant positive radiative forcing combined with the continental arrangement, which is believed to have driven the circulation of warm water to the poles, produced a warm climate that varied little from the equator to the Polar Regions (Hay, 2008).

There is now widespread consensus that ocean temperatures were considerably warmer than the present day throughout the Cretaceous period. Analysis of membrane lipid composition in fossils of the ubiquitous marine plankton Crenarchaeota provides a paleo-thermometer that is independent of initial seawater chemistry (Schouten *et al.*, 2002). This method has produced estimates of equatorial sea surface temperatures of  $> 32\text{ }^\circ\text{C}$  during the early Cretaceous (Littler *et al.*, 2011) and  $> 20\text{ }^\circ\text{C}$  and  $\sim 15\text{ }^\circ\text{C}$  in the Polar waters of

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the middle and late Cretaceous respectively (Jenkyns *et al.*, 2004). Multiple proxy studies utilising oxygen/carbon isotope composition and Ca/Mg ratios of preserved plankton and algae produce similarly high ocean temperature estimates. For example, Bice *et al.*, 2006 estimate average tropical sea surface temperatures of between 33 and 42 °C (8 – 17 °C higher than present) during the Middle Cretaceous. Over the same period, water temperatures at intermediate depths (500 – 1000 m) are estimated to have ranged between 20 and 25 °C in the tropical proto-Atlantic ocean (Friedrich *et al.*, 2008). Deep sea temperatures were an estimated average of 10 °C warmer throughout the Cretaceous compared to the modern day (Huber *et al.*, 2002).

Low temperature gradients across latitudes produced weak global winds and resulted in less upwelling and greater stagnation in the Cretaceous oceans compared to the modern day (Stanley, 1999). Indeed, black shale deposits indicate at least two mass die offs (oceanic anoxic events) occurred ~ 120 and ~ 92 MYA, each lasting for around half a million years (Erbacher *et al.*, 2001). Models based on carbon isotopic records, carbon/sulphur cycles and burial of organic matter indicate average atmospheric oxygen concentrations throughout the Cretaceous period of between 15 and 20 % (Falkowski *et al.*, 2005, Pomar & Hallock 2008) although more recent modifications to these models indicate distinctly higher O<sub>2</sub> values (Berner, 2009).

Seawater ion chemistry has fluctuated over geological time scales and is driven by numerous processes including tectonics, climate, bio-mineralization, weathering and sedimentation (Hardie, 1996; Rocha & Paytan, 2005). The Cretaceous is well recognised as a time of peak seawater calcium content (Pomar & Hallock, 2008). Indeed Cretaceous oceans are characterized as calcite seas whereby in contrast to the aragonite seas of the present day, low-magnesium calcite was the primary inorganic carbonate precipitate (Sandberg, 1983; Ries, 2011). Analysis of brines trapped within marine halites dating back to the Cretaceous period indicate a several fold enrichment in Ca<sup>2+</sup> and depletion in SO<sub>4</sub><sup>2-</sup> and Mg<sup>2+</sup> relative to modern seawater (Horita *et al.*, 2002; Timofeeff *et al.*, 2006). Similar estimates of low seawater Mg/Ca ratio during the Cretaceous are produced through examination of the trace element

146

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composition of biological carbonates such as in fossil echinoderms and the shells of rudist bivalves (Dickson *et al.*, 2002; Steuber & Rauch, 2005). According to these data, Cretaceous seawater Mg/Ca ratio averaged ~ 1.4 whereas present seawater Mg/Ca ratio is ~ 5. Salinity levels in surface waters of the Cretaceous oceans are likely to have been high due to enhanced evaporation associated with the warm climate and there is also evidence of the formation of intermediate and deep-water salines (Woo & Anderson, 1992; Friedrich *et al.*, 2008). Direct analysis of remnant seawater from the Early Cretaceous North Atlantic (thought to have been an almost fully enclosed basin) suggest a salinity twice that of modern seawater (Sanford *et al.*, 2013). Somewhat lower estimates based on stable isotope analyses of Cretaceous sediments indicate average sea surface salinity of ~41 during this period (Wagner *et al.*, 2008).

Warm temperatures, high PCO<sub>2</sub>, low PO<sub>2</sub> as well as high salinity and calcium content are all factors likely to promote intestinal carbonate production by teleost fish. Metabolism in teleost fish increases exponentially with temperature, typically demonstrating a within-species temperature quotient (Q<sub>10</sub>) of 2.4 (Clarke & Johnston, 1999) and carbonate production rates have been found to follow a similar relationship with temperature (Wilson *et al.*, 2009). Drinking rate is assumed to be the primary parameter that drives production of gut carbonates (Wilson *et al.*, 2009). Drinking rate is linked to the osmoregulatory demand associated with salinity (Genz *et al.*, 2008) as well as the ventilatory response to increased oxygen demand and possibly reduced oxygen availability (Chapter 4). The second driver of carbonate production, the intestinal supply of HCO<sub>3</sub><sup>-</sup>, is enhanced by increased ambient PCO<sub>2</sub>, increased metabolic CO<sub>2</sub> production and as a response to osmotic water loss (Grosell *et al.*, 2005, Genz *et al.*, 2008, Chapter 4). Increased concentration of imbibed Ca<sup>2+</sup> promotes carbonate precipitation in the intestinal lumen (Whittamore *et al.*, 2010) and is likely to act multiplicatively with any increase in drinking rate. Furthermore, modification of seawater chemistry (depletion in Mg<sup>2+</sup> and SO<sub>4</sub><sup>2+</sup>) are likely to alter the elemental composition and possibly morphology of the carbonate crystals excreted by fish (Salter *et al.*, 2012).

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Given the conditions thought to have prevailed in the Cretaceous oceans, it is plausible to imagine that carbonate production in teleost fish could have been substantially higher during the period compared to the modern day. If so, this would have important implications for our understanding of the Cretaceous marine inorganic carbon cycle and for our interpretation of its carbonate records. The present study aimed to experimentally quantify the effect of Cretaceous conditions on the carbonate production rate and intestinal precipitate chemistry of a modern day marine teleost. European flounder (*Platichthys flesus*) represents a useful model species for such experiments for several reasons. Firstly, compared to a typical stenohaline marine species, the euryhaline and estuary dwelling European flounder is likely to demonstrate greater tolerance to treatments involving altered seawater chemistries (Steffensen *et al.*, 1982). Secondly, their sedentary behaviour make these fish amenable to the regular collection of excreted carbonates. Finally, flounder have been used extensively in previous experimental work on gut carbonate production in fish and as such a large body of data already exists for comparative purposes (Wilson *et al.*, 2002, 2009; Cooper *et al.*, 2010; Whittamore *et al.*, 2010, Chapter 4).

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## Materials and Methods

### Experimental Animals and Temperature Acclimation

European flounder (*Platichthys flesus*, 430 ± 30 g) were caught in the estuary of the River Taw, North Devon, UK and transported to the marine aquarium facilities at the University of Exeter. Here they were maintained in two 300 l holding tanks of flowing aerated artificial seawater (Tropic Marine, Tropical Marine Centre, Bristol, UK) as part of a recirculating seawater system maintained at a salinity of 35 ± 1 and temperature of 15 ± 0.3 °C, under a 14:10 h light-dark photoperiod. The flounder were maintained on a diet of live rag worm (*Nereidae*) fed weekly and cooked mussel fed three times per week. Food was withheld for 72 hours prior to each experiment. For experiments at the upper temperature, flounder were transferred to two 180 l flow-through acclimation tanks which were warmed (Thermo-control, EHEIM, Germany) by 2 °C per day to the upper temperature (23 °C), at which they were held for a further 7 days before experiments commenced.

### Treatments

Flounder underwent a total of five treatments, the details of which are summarised in Table 11. Artificial seawater simulating modern day (Morcos, 1973) and Cretaceous seawater chemistries (Timofeeff *et al.*, 2006) were produced by dissolving individual salts in deionised water. Seawaters were thoroughly mixed and aerated for 24 hours before flounder were introduced. During each treatment, individual flounder were housed in a 20 l chamber which was in turn placed in a 90 l ambient tank (two chambers per ambient tank and 4 ambient tanks total). Seawater was fed in a closed loop to each pair of ambient tanks and associated flounder chambers from a 230 l seawater mixing tank (Figure 34). A 100 % water change of the seawater mixing tanks was performed every 72 hours. Water PO<sub>2</sub> and PCO<sub>2</sub> were controlled by bubbling with appropriate gas mixtures of O<sub>2</sub>, CO<sub>2</sub> and N<sub>2</sub> (MC Series Mass Flow Controllers, Qubit Systems Inc., Canada). Throughout each treatment, salinity, temperature (YSI 30, USA) pH (Radiometer Analytical, France), and PO<sub>2</sub> (Strath kelvin Instruments, UK) were monitored daily. Treatments lasted

7 days and were separated by a period of two weeks, during which flounder were returned to the appropriate holding / acclimation tank where they were maintained as previously described.

	Modern SW Chemistry		Cretaceous SW Chemistry		Full Cretaceous Conditions
	15 °C	23 °C	15 °C	23 °C	23 °C
Ion Concentration (mM)					
Na <sup>+</sup>	445		486		486
Cl <sup>-</sup>	525		635		635
K <sup>+</sup>	11		11		11
Ca <sup>2+</sup>	10		40		40
Mg <sup>2+</sup>	53		40		40
SO <sub>4</sub> <sup>2-</sup>	28		10		10
HCO <sub>3</sub> <sup>3-</sup>	2		2		2
Total	1077		1225		1225
Salinity	35		40		40
PO <sub>2</sub> (% Air Sat.)	> 90		> 90		75
PCO <sub>2</sub> (ppm)	400		400		3000

Table 11. Summary of the major ion concentrations and other parameters across the five artificial seawater (SW) treatments undergone by European flounder (N = 8).

### Carbonate Collection and Analysis

Carbonate precipitates were collected daily from the floor of each chamber using a plastic pipette. The precipitates were then immediately rinsed in distilled water to remove salts before being soaked for 24 hours in 5 % sodium hypochlorite (bleach) solution to remove organic material and the mucus coating. The bleach was then removed by careful pipetting and the carbonate sample was rinsed three times in deionised water to remove any remaining residues. Cleaned carbonates were dried for 48 hours in a drying oven set at 40 °C.

The dry weight of each carbonate sample was measured to provide an estimate of CaCO<sub>3</sub> excretion rate (μmol kg<sup>-1</sup> h<sup>-1</sup>). The bicarbonate equivalents (HCO<sub>3</sub><sup>-</sup> + 2CO<sub>3</sub><sup>2-</sup>) content of a sub sample (~30 %) of these precipitates was

then determined by double titration with HCl and NaOH as described by Wilson & Grosell (2003) using an autotitration system (TIM845, Radiometer Analytical) with autosampler (SAL80, Radiometer Analytical). At the completion of this titration, samples were manually acidified and diluted with deionised water based on their expected  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  content. An aliquot of this sample was then taken and immediately frozen for later analysis by ion chromatography (Dionex ICS1000) to determine the amounts of the major cations and anions incorporated into the precipitates. Crystal morphology was imaged using scanning electron microscopy (SEM, Jeol JSM 6390 LV, USA). Loose carbonate pellets were tipped onto adhesive carbon-coated tabs upon a 10 mm aluminium specimen stub. A gold-palladium coating of 20 nm thickness was applied prior to imaging.

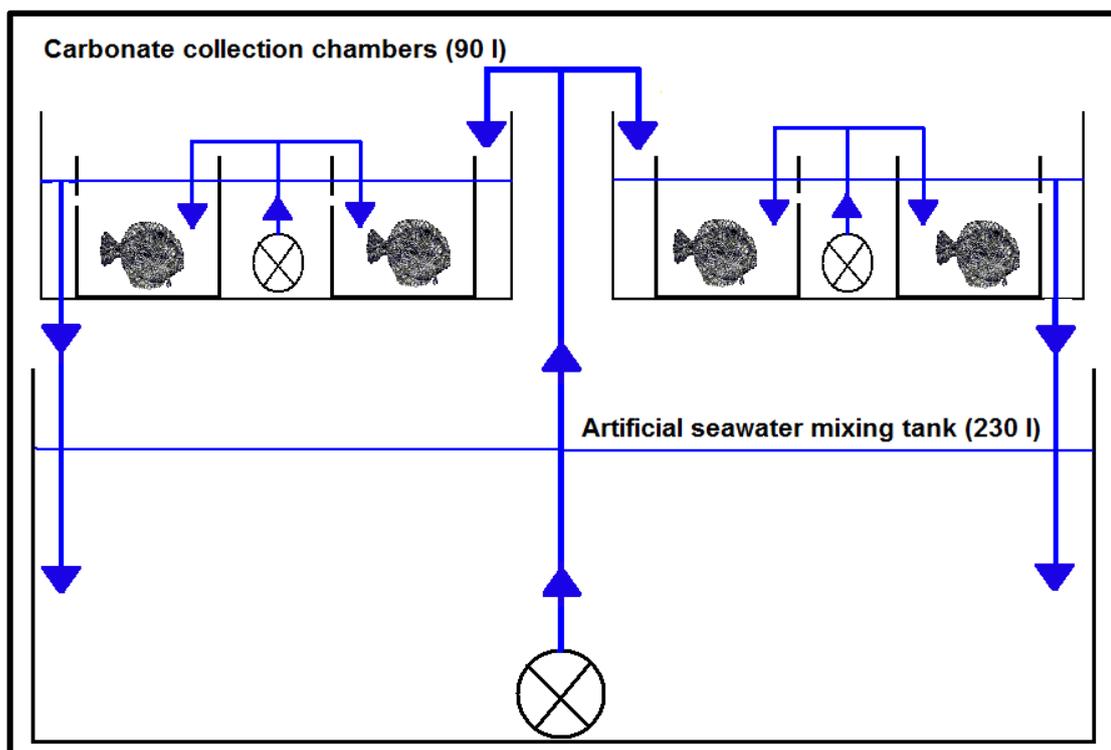


Figure 34. Diagram of the system used to facilitate daily collection of excreted calcium carbonate precipitates from individual flounder held in artificial seawater simulating modern day and Cretaceous seawater chemistries. Two identical set-ups permitted carbonate collection from a total of 8 flounder per treatment.

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## Closed Respirometry

Oxygen consumption rates ( $MO_2$ ,  $mgO_2\ kg^{-1}\ h^{-1}$ ) were determined *in situ* during treatments by closed respirometry. Water supply and aeration to the flounder chambers were ceased and the water surface was covered by two layers of bubble wrap® to minimise gas diffusion (Spicer *et al.*, 2007). Flounder were left undisturbed to consume oxygen within their chambers for a period of between 30 and 50 minutes depending on temperature treatments. Preliminary experiments showed that oxygen levels did not fall below 75 % air saturation during these time intervals. Initial and final water samples were taken and oxygen content was measured using an oxygen electrode (Strath Kelvin Instruments, UK). Partial pressure measurements of oxygen were converted to  $mgO_2\ l^{-1}$  according to solubility values reported in Green & Carrit (1967).  $MO_2$  was calculated as follows:

$$MO_2 = (Vr \times \Delta O_2) \div (\Delta t \times bw)$$

Where: oxygen consumption rate ( $MO_2$ ), respirometer volume ( $Vr$ ), time ( $t$ ), fish mass ( $bw$ ).

Blank measurements within chambers containing no fish showed minimal levels of background respiration (< 5 % of total oxygen consumption). Water flow and aeration were resumed immediately once the final water samples had been collected.

## Statistical Analysis

Data are presented as means  $\pm$  SEM unless otherwise stated. Assumptions of normality and equal variance were tested via the Shapiro-Wilk test and Levene's test respectively ( $P > 0.05$ ). Significant differences between treatments were tested for by two-way ANOVA. Tukey's test was performed *post-hoc*. Results were accepted as significant at  $P < 0.05$ . Details of the statistical analyses are summarised in the appendix to this chapter (Table 13). Statistical analyses were carried out using SPSS version 17.0, and graphs were drawn using MS Excel 2010.

## Results

### Carbonate Excretion Rate

The rate of carbonate precipitate excretion was increased by between 2.5 and 2.6 fold at 23 °C relative to 15 °C under both modern and Cretaceous seawater chemistries. Cretaceous seawater chemistry produced a 5.3 and 5.6 fold increase in precipitate excretion relative to modern seawater at 15 °C and 23 °C respectively. Increased  $\text{PCO}_2$  and reduced  $\text{PO}_2$  under the ‘full Cretaceous’ treatment (Table 11) had no significant effect on carbonate excretion rate relative to excretion rates observed under Cretaceous seawater chemistry at the same temperature (23 °C). The almost 14 fold higher excretion rates observed in flounder under Cretaceous seawater at 23 °C relative to those under modern seawater at 15 °C, was far greater than the independent additive effects of two treatment temperatures and seawater chemistries, indicating an interactive effect of these factors on carbonate excretion rates (Figure 35).

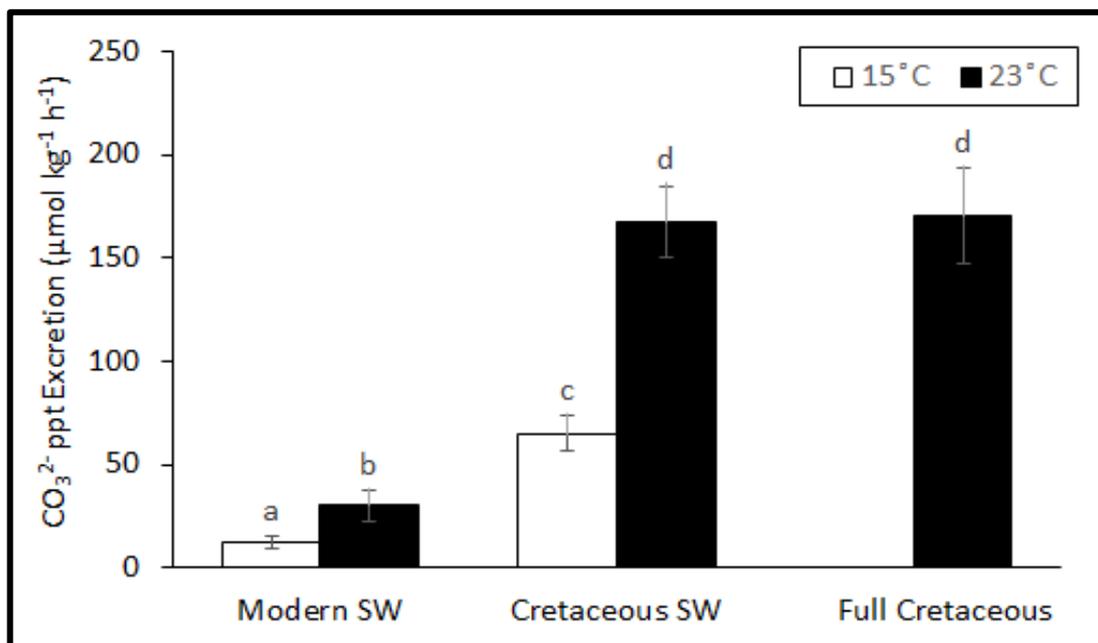


Figure 35. Mean ( $\pm$  SEM) carbonate precipitate excretion rate in European flounder (N = 8) under various seawater (SW) and temperature treatments (Table 11). Columns labelled with different letters indicate a significant difference between treatments (post-hoc Tukey test). Significant main and interactive effects of temperature and SW chemistry were detected (two-way ANOVA,  $P < 0.05$ , Table 13).

## Precipitate Ion Analysis

Within seawater treatments, both  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  precipitation rates increased by between 2 and 3 fold at 23 °C compared to 15 °C. Cretaceous seawater chemistry resulted in a 8.5 and 6.3 fold increase in  $\text{Ca}^{2+}$  precipitation rates relative to modern seawater chemistry at 15 °C and 23 °C respectively, whereas there was a smaller increase in  $\text{Mg}^{2+}$  precipitation rate (3.1 and 3.2 fold) under the same conditions (Figure 36). Carbonate precipitates produced under Cretaceous seawater chemistry demonstrated a 1.7 – 2.4 fold-lower  $\text{Mg}^{2+}$  and 2.5 – 2.6 fold-higher  $\text{SO}_4^{2-}$  mol% than those produced under modern seawater chemistry.  $\text{Mg}^{2+}$  and  $\text{SO}_4^{2-}$  mol% did not change with temperature in modern seawater but was significantly increased and decreased respectively in Cretaceous seawater at 23 °C relative to the same seawater at 15 °C. Reduced  $\text{PO}_2$  and increased  $\text{PCO}_2$  had no effect on the  $\text{Mg}^{2+}$  or  $\text{SO}_4^{2-}$  mol% of excreted carbonate precipitates (Figure 37).

	Modern SW Chemistry		Cretaceous SW Chemistry		Full Cretaceous Conditions 23 °C
	15 °C	23 °C	15 °C	23 °C	
$\text{MO}_2$ ( $\text{mg O}_2 \text{ kg}^{-1} \text{ h}^{-1}$ )	$38 \pm 3^a$	$71 \pm 7^b$	$32 \pm 4^a$	$73 \pm 6^b$	
$\text{MO}_2 Q_{10}$		2.6		2.8	
$\text{CO}_3^{2-}$ ppt Excretion Rate ( $\mu\text{mol kg}^{-1} \text{ h}^{-1}$ )	$12 \pm 3^a$	$30 \pm 8^a$	$65 \pm 9^b$	$167 \pm 17^c$	$170 \pm 23^d$
$\text{CO}_3^{2-}$ ppt Excretion $Q_{10}$		3.1		3.2	
$\text{Mg}^{2+}$ mol %	$18 \pm 0.8^a$	$18 \pm 0.7^a$	$8 \pm 0.4^b$	$11 \pm 0.7^c$	$9.6 \pm 0.4^{bc}$
$\text{SO}_4^{2-}$ mol %	$0.7 \pm 0.1^a$	$0.5 \pm 0.1^a$	$1.7 \pm 0.1^b$	$1.2 \pm 0.1^c$	$1.4 \pm 0.1^{bc}$

Table 12. Summary of oxygen consumption rate ( $\text{MO}_2$ ), carbonate excretion rate and carbonate chemistry of European flounder under various seawater and temperature treatments (Table 11). Data labelled with a different superscript indicates a significant difference of that variable between treatments.

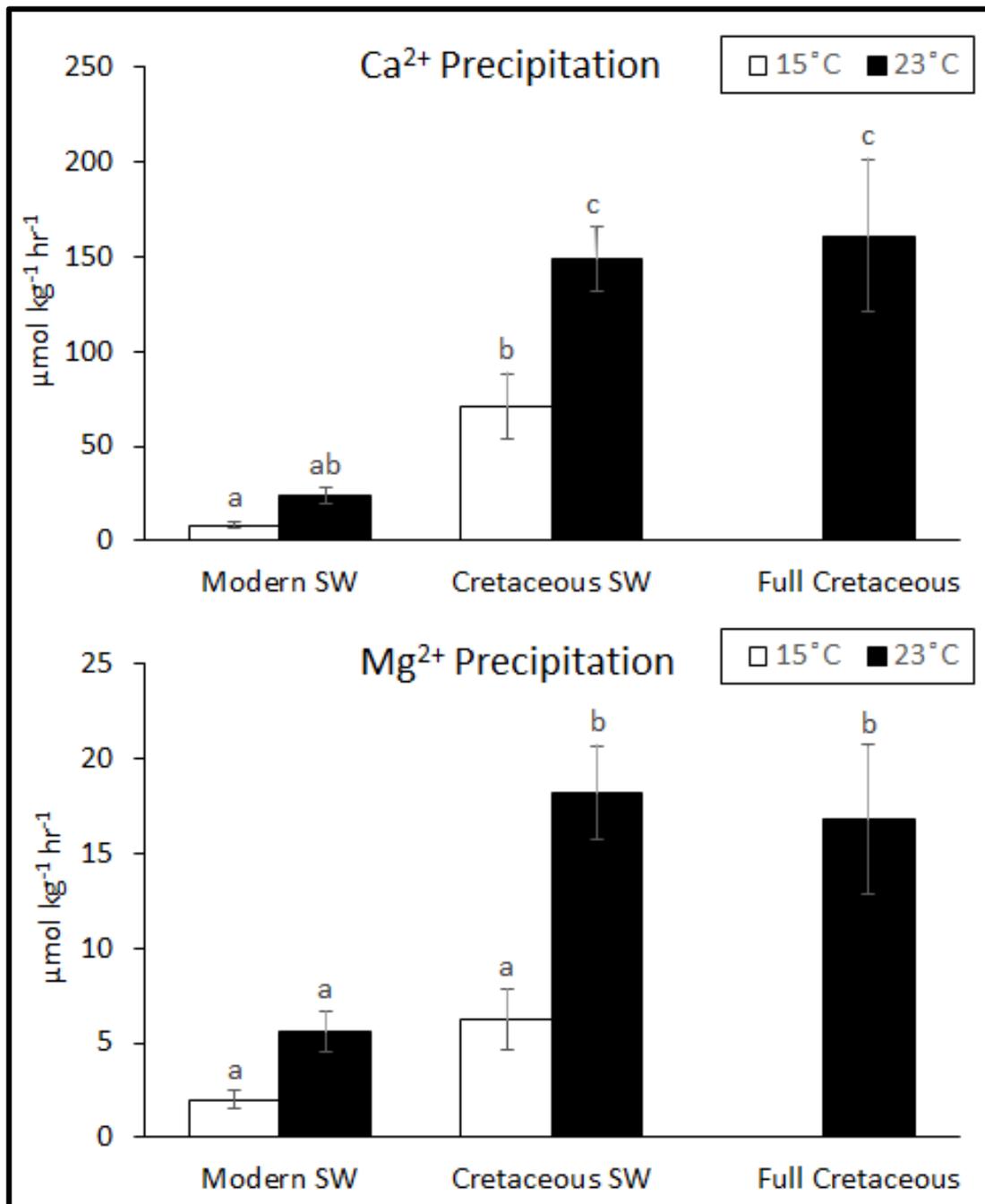


Figure 36. Mean ( $\pm$  SEM) intestinal precipitation rates of Ca<sup>2+</sup> and Mg<sup>2+</sup> ions of European flounder under various seawater (SW) and temperature treatments (Table 11). Columns labelled with different letters indicate a significant difference between treatments. Significant main effects of temperature and SW chemistry were detected (two-way ANOVA,  $P < 0.05$ , Table 13).

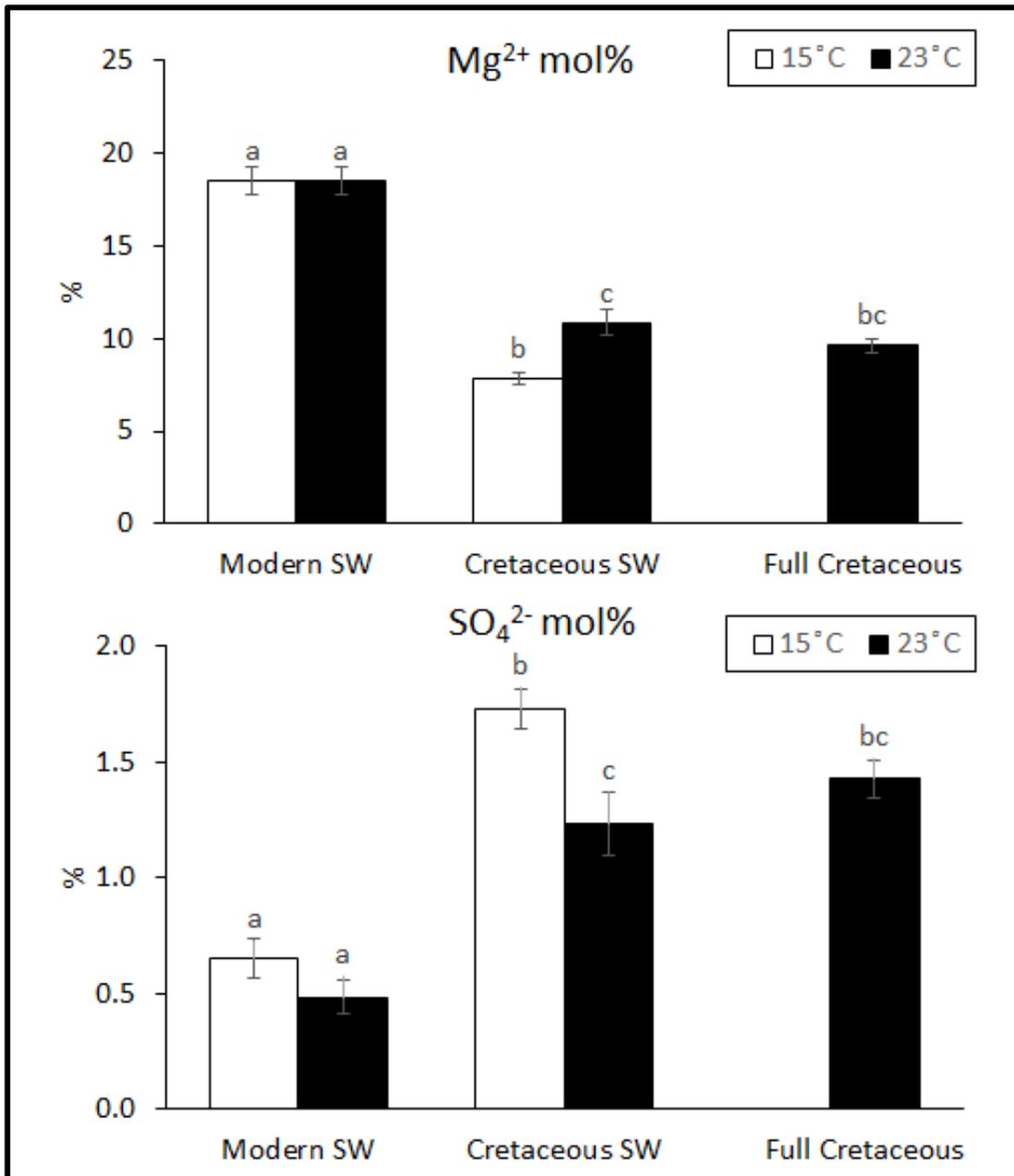


Figure 37. Mean ( $\pm$  SEM) mol% of the minor cation  $Mg^{2+}$  and anion  $SO_4^{2-}$  of the predominantly  $CaCO_3$  precipitates excreted by European flounder under various seawater chemistry (SW) and temperature treatments (Table 11). Columns labelled with different letters indicate a significant difference between treatments (post-hoc Tukey test). Significant main effects of temperature and SW chemistry were detected (two-way ANOVA,  $P < 0.05$ , Table 13).

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## Crystal Morphology

The carbonates produced by flounder under modern seawater conditions are largely dominated by monocrystalline ellipsoids with lengths ranging between 0.5 and 1  $\mu\text{m}$  (Figure 38, AB). Infrequent examples of intergrowth between two or more ellipsoids that share a common centre and perpendicularly aligned long-axis are present. Crystals appear to be consistently smooth-surfaced. Under Cretaceous conditions flounder carbonate (Figure 38, CD) appears to be dominated by larger (1 – 2  $\mu\text{m}$  in length) monocrystalline ellipsoids with more frequent occurrences of rod-shaped crystals (flat / blunt ended ellipsoids).

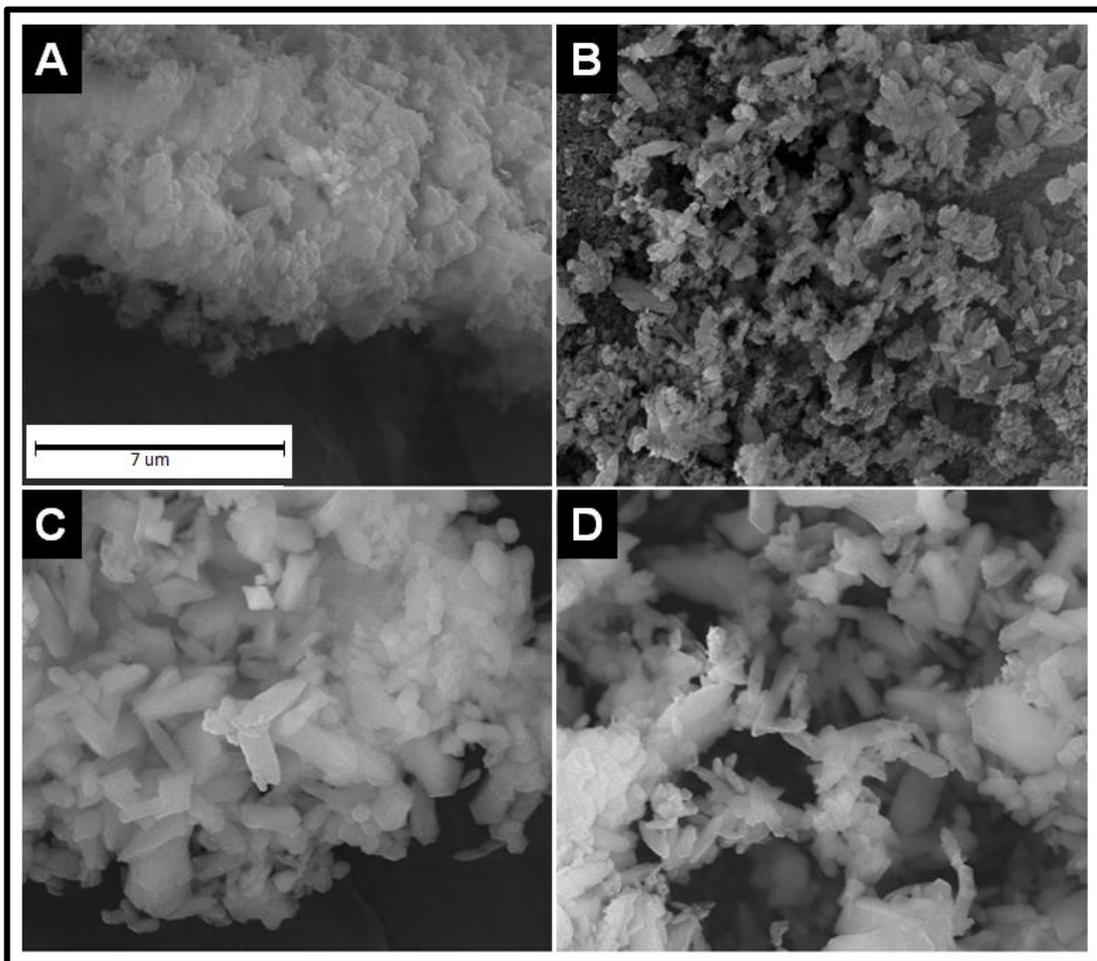


Figure 38. Secondary electron (SE) images of the crystalline structure of the carbonate precipitates produced by European flounder under artificial seawater chemistries (Table 11) simulating that of the modern day (A,B) and the Cretaceous period (C,D).

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## Discussion

### Carbonate Excretion Rate

As expected, carbonate excretion rate increased with temperature but this increase was greater than predicted by the  $Q_{10}$  for metabolic rate (Table 12). Previously an oxygen consumption  $Q_{10}$  of 2.0 has been reported for European flounder acclimated for two months at 5 and 15 °C (Duthie & Houlihan, 1982) which is lower but not too dissimilar to the  $Q_{10}$  observed in the present study (2.6 – 2.8) over a temperature range of 15 – 23 °C. This difference may be explained by the shorter (1 week) acclimation period permitted in the present study. Wilson *et al.* (2009) report a carbonate excretion  $Q_{10}$  of 2.33 in sheepshead minnow (*Cyprinodon variegatus*) when measured over 20 to 35 °C and suggest that this likely reflects the  $Q_{10}$  for drinking rate as the parameter that drives production of gut carbonates (Wilson *et al.*, 2002; Genz *et al.*, 2008), rather than metabolism. The high  $Q_{10}$  of 3.1 – 3.2 for carbonate production in the present study supports this hypothesis. The assumption that carbonate excretion follows the metabolic relationship with temperature (interspecies mean  $Q_{10}$  of 1.83, Clarke & Johnston, 1999) is incorporated into global estimates of piscine carbonate production and thus is a potentially significant source of underestimation in these models (Wilson *et al.*, 2009).

Carbonate excretion rate in Cretaceous relative to modern seawater chemistries is primarily a function of two factors: increased salinity and increased  $\text{Ca}^{2+}$  concentration. Osmoregulatory demand and hence drinking rate are likely to be directly proportional to salinity as has been demonstrated previously in the Gulf toadfish across a salinity range of between 35 and 50 (Genz *et al.*, 2008). Cretaceous seawater in the present study represents a 1.24-fold increase in osmotic gradient (assuming an isosmotic salinity of 14 in marine teleosts; Jobling, 1995) relative to modern seawater and therefore presumably entails a similar increase in drinking rate. Previously, *in vivo* perfusion of the intestine in European flounder with varying concentrations of  $\text{Ca}^{2+}$  (10 and 40 mM) was shown to produce directly proportional carbonate precipitate excretion rates (Whittamore *et al.*, 2010). The ~4-fold  $\text{Ca}^{2+}$

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enrichment in Cretaceous seawater (Table 11) combined with the assumed 1.24-fold increase in drinking volume suggests a 4.96-fold increase in total imbibed  $\text{Ca}^{2+}$  compared to flounder in modern seawater. Thus, the 5.3 to 5.6-fold increase in carbonate excretion rate observed in the present study in Cretaceous vs. modern seawater (Figure 35) is slightly higher than predicted by the differences in salinity and calcium content between these two treatments. The almost 14-fold higher rate of carbonate excretion observed in warm (23 °C) Cretaceous seawater compared to cold (15 °C) modern seawater, is also slightly higher than the predicted effect of salinity and temperature on drinking rate and their multiplicative action with seawater  $\text{Ca}^{2+}$  concentration (1.24 x 2.5 x 4 respectively). As drinking rate appears to be the key physiological variable linking temperature and salinity to carbonate excretion rate, incorporating direct measurements of drinking rate similar to those of chapter four, would be a highly informative extension of the present study.

Given the effects of reduced  $\text{PO}_2$  and increased  $\text{PCO}_2$  on carbonate excretion rate documented in chapter four, it is puzzling that no significant positive effect of these parameters was observed in the 'full Cretaceous' treatment of the present study. This may indicate that a physiological limit to intestinal carbonate production has been reached although it is unclear what the limiting factor would be. One possibility is that high oxygen demand (at 23 °C) combined with decreased oxygen availability (75 % air saturation) under full Cretaceous conditions, exceeded the capacity of flounder to further increase ventilation volume and hence no additional water loss was incurred.

Taken together, these results support the prediction that the Cretaceous was a period of substantially higher piscine carbonate production rates compared to the present day. However, two important limitations of the experimental approach should be acknowledged. Firstly, no formal tests were conducted to determine the extent of carry-over effects between treatments. Whilst a two week 'rest' period was incorporated between treatments, without a counterbalanced experimental design, the repeated stress of sequential treatments cannot be excluded as a potential confounding factor. Secondly,

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whilst flounder were progressively acclimated to each of the experimental temperatures, there was no provision for acclimation to altered seawater chemistry prior to each treatment. As a result, the high carbonate excretion rates observed in flounder under Cretaceous conditions represent an acute response, rather than a more environmentally relevant chronic response.

### Precipitate Chemistry and Morphology

The mean  $Mg^{2+}$  content ( $18.5 \pm 0.75$  mol%, Figure 37) of the carbonate precipitates excreted by flounder under modern seawater conditions falls within the middle of the wide range previously reported (0.5 - 40 mol%) for fish derived carbonates (Salter *et al.*, 2012). The high Mg-calcites (defined as >4 mol% Mg) typically excreted by fish are the product of intestinal fluid chemistry which is highly distinctive from conditions in which carbonates are formed by other marine calcifiers (Perry *et al.*, 2011). The processing of ingested seawater along the intestine results in accumulation of  $Mg^{2+}$  (from 53 mM to over 200 mM) due to the high rate of intestinal water absorption but very modest  $Mg^{2+}$  uptake (Marshall & Grosell, 2006). The intestinal accumulation of  $Mg^{2+}$  coincides with reduced  $Ca^{2+}$  concentration due to its continual precipitation and thus the  $Mg^{2+}/Ca^{2+}$  ratio in the intestinal fluid is much higher than in ambient seawater. Previously, *in vitro* experiments have shown that the amount of  $Mg^{2+}$  incorporated into calcite crystals is positively correlated with the  $Mg^{2+}/Ca^{2+}$  ratio of the precipitating solution (Meldrum & Hyde, 2001). Although there was an increase in the absolute  $Mg^{2+}$  precipitation rate, the proportion of  $MgCO_3$  incorporated into excreted carbonates was reduced to between 8 and 11 mol% under Cretaceous conditions (Figure 37), partially reflecting the 4-fold lower  $Mg^{2+}/Ca^{2+}$  ratio of Cretaceous relative to modern seawater.

High-Mg calcites are typically more soluble in seawater than other marine  $CaCO_3$  polymorphs such as aragonite, and the crystal stability of calcites has been shown to be negatively correlated with  $MgCO_3$  content (Morse *et al.*, 2007). Thus, the incorporation of  $Mg^{2+}$  into piscine carbonates is the major determinant of their preservation potential in sediments and relative dissolution

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depth in the open ocean, (Salter *et al.*, 2012; Woolsey *et al.*, 2012). The 50% reduction in Mg content of carbonates produced under Cretaceous compared to modern seawater chemistry (Figure 37) suggests that carbonates produced by fish during the Cretaceous period had greater potential for preservation in sediments and would have dissolved at greater depths compared to those produced in the present day oceans. This, combined with significantly increased carbonate production rates by fish has considerable implications for understanding carbon cycling during the Cretaceous (Wilson, 2014).

Despite the Cretaceous treatment representing an almost 3-fold depletion of seawater  $\text{SO}_4^{2-}$ , the amount of  $\text{SO}_4^{2-}$  incorporated into excreted precipitates was increased from  $< 0.65$  in modern seawater to between 1.23 and 1.73 mol% in Cretaceous seawater (Figure 37). Incorporation of  $\text{SO}_4^{2-}$  into calcites has been shown to vary as a function of crystal growth rate (Busenberg & Plummer, 1985) and therefore faster carbonate precipitation under Cretaceous conditions could account for the observed increase in  $\text{SO}_4^{2-}$  incorporation. In addition, active  $\text{SO}_4^{2-}$  secretion in exchange for luminal  $\text{Cl}^-$  has been observed in unfed winter flounder (*Pleuronectes americanus*; Pelis & Renfro, 2002). By assisting the absorption of  $\text{Cl}^-$ , this mechanism is potentially important in regulating intestinal fluid absorption (via solute-linked water transport) and may be upregulated under elevated salinity as in the Cretaceous seawater treatment. The presence of impurities such as  $\text{SO}_4^{2-}$  may significantly influence the crystal stability and hence post-excretion pathways of fish-derived carbonates (Salter *et al.*, 2012). Impurities such as  $\text{H}_2\text{O}$  and  $\text{OH}^-$  in Mg calcites typically lead to reduced stability (Bischoff *et al.*, 1987) whereas incorporation of  $\text{Sr}^{2+}$  is thought to increase stability (Mucci & Morse, 1983). As a significant and variable source of impurity in fish derived Mg calcite, the co-precipitation and influence of  $\text{SO}_4^{2-}$  is potentially an important avenue of further study.

Fish derived carbonates exhibit a diverse and highly distinctive array of crystal morphologies that fundamentally differ from all other known biogenic and abiotic sources of marine carbonates (Perry *et al.*, 2011). Five predominant crystal morphologies have been described: ellipsoids, dumb-bells, spheres, needles and euhedral rhombohedra (Salter *et al.*, 2012). In modern seawater,

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flounder produced predominantly ellipsoidal crystals of typical size (~1  $\mu\text{m}$ ). Under Cretaceous conditions carbonate crystals produced by flounder appear to be bigger (~1.5  $\mu\text{m}$ ) and largely resemble rod-shaped crystals (blunt or flat ended ellipsoids). Salter *et al.* (2012) described rod-shaped crystals as a subsidiary group formed by several tropical fish species and the same authors speculate that these may represent an intermediary step within a morphogenetic sequence whereby ellipsoidal crystals transform into dumb-bells. Hence, the greater proportion of rod shaped crystals under Cretaceous conditions may reflect faster crystal growth rates. Further imaging over a greater range of samples is required in order to fully establish the extent to which carbonates produced under Cretaceous conditions differ morphologically to those produced under modern conditions. Additionally, combining imaging with elemental analysis (e.g. energy-dispersive x-ray spectroscopy) to measure the chemistry of individual crystals would be useful in order to understand how crystal chemistry varies within a bulk carbonate sample. Ultimately, such analyses could be used to inform the identification of piscine derived carbonates preserved in Cretaceous sediments. However, further work is required to establish the long-term preservation potential and diagenesis (chemical and morphological changes over time) of fish derived calcite crystals in the sediment record.

### Implications and Future Work

Piscine carbonate production is an important consideration in terms of understating carbonate sediment budgets and records. Marine carbonate sediments, of which the mud fraction (<63  $\mu\text{m}$ ) is a major component, provide important records of ecology, ocean chemistry, biogeochemical cycling and ultimately climate shifts in the geological past (Maloof *et al.*, 2007; Hönisch *et al.*, 2012). The origin of Mg-calcite mud, has previously proved difficult to resolve (Gischler & Zingeler, 2002; Morse *et al.*, 2007) but there is now increasing evidence that marine fish are a major source (Perry *et al.*, 2011; Salter *et al.*, 2012). By combining fish biomass and carbonate excretion rate data Perry *et al.*, (2011) estimate that the current piscine contribution to total carbonate mud production around the Bahamian archipelago averages ~14 %

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and exceeds 70 % in some high fish biomass habitats. Furthermore, the identification of morphologically distinct fish derived high-Mg calcite in the fine-mud fraction of contemporary carbonate sediment samples confirm the widespread preservation of fish-derived carbonates in tropical marine environments (Perry *et al.*, 2011). Given that conditions during the Cretaceous appear to favour high rates of piscine carbonate production, the implications for the interpretation of carbonate mud records dating back to the period are considerable.

Although these experiments have demonstrated in principle the effect of Cretaceous conditions on intestinal carbonate production rates and crystal chemistry, there are clear limitations associated with extrapolating the physiological responses of flounder as a modern day teleost to be representative of fish that existed in the oceans 65 – 144 MYA. Indeed the earliest known transitional fossils of primitive flatfish (*pleuronectiforms*) date back only as far as the early Eocene (48 – 56 MYA; Friedman, 2008, 2012). However, representatives of the early chondrosteian and chondrichthyan fishes (sturgeon and sharks) excrete CaCO<sub>3</sub> when induced to drink seawater after experimental transfer to hyperosmotic conditions. This suggests that the adaptation of intestinal carbonate precipitation occurred early in the evolutionary history of fish (Taylor & Grosell, 2006). The Clupeomorph order of teleosts contains over 350 species of extant species and over 150 fossil species that date back to the early Cretaceous (Benton, 2004). As such, extant Clupeomorphs such as the Atlantic herring (*Clupea harengus*) or European Anchovy (*Engraulis encrasicolus*) represent interesting model species in which to further investigate intestinal carbonate precipitation under Cretaceous conditions.

The Cretaceous appears to have been a time of unprecedentedly rapid teleost diversification with several extant groups appearing during the period (Near *et al.*, 2012). Presumably, for teleost fish present in the high Ca<sup>2+</sup>, high salinity, and high temperature oceans of the Cretaceous, the intestinal precipitation and excretion of CaCO<sub>3</sub> as strategy for osmoregulation and maintenance of calcium homeostasis, would have been an even more critical physiological

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process than in the modern day oceans. Following the Permian-Triassic mass extinction event (~250 MYA) the ray finned fishes, the group to which teleost fish belong, took over from the almost entirely extirpated lobe-finned fishes (Randall *et al.*, 2014). Teleost fish now comprise 95% of all extant fishes (Helfman *et al.*, 2009; Hurley *et al.*, 2007) and the key to their success has been ascribed variously to modifications in swimming and feeding, genomic duplication (Ives & Randall, 2007) and hypoxia tolerance (Randall *et al.*, 2014). Given that much of their radiation coincided with a period of peak seawater  $\text{Ca}^{2+}$ , it is tempting to speculate that a well-adapted ability for intestinal  $\text{HCO}_3^-$  secretion and  $\text{CaCO}_3$  precipitation could have played a key role in the evolutionary success of teleost fish.

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## Appendix

### ANOVA Summary

Variable	Acclimation Temp.			SW Chemistry			Interaction		
	df	F	P	df	F	P	df	F	P
MO <sub>2</sub>	1,23	35.62	<0.001*	1,23	0.80	0.77	1,23	0.33	0.57
CaCO <sub>3</sub> Excretion Rate	1,30	34.57	<0.001*	1,30	85.21	<0.001*	1,30	17.22	<0.001*
Ca <sup>2+</sup> Precipitation Rate	1,73	6.82	0.011*	1,73	25.72	<0.001*	1,73	3.23	0.076
Mg <sup>2+</sup> Precipitation Rate	1,73	12.73	0.001*	1,73	15.07	<0.001*	1,73	3.35	0.071
Mg <sup>2+</sup> mol %	1,73	4.43	0.039*	1,73	259.60	<0.001*	1,73	4.33	0.041*
SO <sub>4</sub> <sup>2-</sup> mol %	1,73	10.20	0.002*	1,73	115.50	<0.001*	1,73	1.71	0.195

Table 13. Summary of two-way ANOVA results for physiological parameters and carbonate precipitate properties measured in the present study. \* indicates significant effects where  $P < 0.05$ .

### License Details

All experimental procedures within this study were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986, PIL: 30/9871, PPL: 30/2735.



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# Chapter 6



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## General Discussion and Conclusions

### Thesis Context

As a group, fish inhabit a remarkable variety of aquatic environments and exhibit a wide array of adaptive traits (Jobling, 1995; Helfman *et al.*, 2009). Many of the habitats occupied by fish are highly dynamic, naturally demonstrating substantial abiotic fluctuations over diurnal, tidal or seasonal cycles. It is also the case that, throughout their 545 million year evolutionary history, fish have existed in aquatic environments very different to those of the present day (Janvier, 1995; Pomar & Hallock, 2008). However, the past several decades have seen unprecedented rates of environmental change, at local and global scales, arising from human activities (Doney, 2010; Woodward *et al.*, 2010). Studying the interactions between fish and their environment, specifically how the physiology of fish is affected by and regulated in response to abiotic factors, is key to understanding the survival and maintenance of fish populations in changing aquatic environments (Rankin & Jensen, 1993). Not only does this provide fundamental biological insights, but such understanding, and the predictive capacity it generates, is increasingly critical given the extent of environmental change currently ongoing and the desire to conserve the ecosystem services that fish provide (Seebacher & Franklin, 2012; Cooke *et al.*, 2013).

Oxygen, carbon dioxide and temperature are major, highly interactive, abiotic variables in aquatic environments and have been the primary forms of environmental change discussed in this thesis. Hypoxia is a common natural occurrence in many aquatic habitats but is also becoming an increasingly widespread and frequent perturbation due to the effects of anthropogenic nutrient loading and climate change (Diaz & Rosenberg, 2008; Keeling *et al.*, 2009; Friedrich *et al.*, 2014). The depletion of oxygen is usually accompanied by an elevation in CO<sub>2</sub> as the waste product of respiration, hence environmental hypoxia and hypercarbia generally act in tandem (Ultsch, 1996; Burnett, 1997; Gilmour, 2001). Atmospheric CO<sub>2</sub> levels have increased by 43% since the start of the industrial revolution and there is now overwhelming

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scientific consensus that this is the dominant cause of the 0.85 °C rise in global temperatures seen over the same period (IPCC, 2014). Climate model projections indicate a further warming of 0.3 to 4.8 °C by the end of the century depending on future emission scenarios (IPCC, 2013). The oceans have absorbed nearly a third of anthropogenic CO<sub>2</sub> emissions since preindustrial times, leading to a significant acidification and alterations to the carbonate chemistry of seawater (Orr *et al.*, 2005). All three of these abiotic variables have fluctuated substantially over geological time scales (Pomar & Hallock, 2008). The Jurassic, Triassic and early Cretaceous (periods of spectacular teleost diversification) were generally characterized by high PCO<sub>2</sub>, high temperature and low PO<sub>2</sub> in comparison to the present (Randall *et al.*, 2014).

The intestinal secretion of HCO<sub>3</sub><sup>-</sup> and precipitation of CaCO<sub>3</sub> plays a major role in the osmoregulatory strategy of marine teleosts and the past two decades have seen great strides in our understanding of the physiological processes involved (Walsh *et al.*, 1991; Grosell *et al.*, 2001; Wilson *et al.*, 2002; Grosell *et al.*, 2005; Grosell & Genz, 2006; Grosell *et al.*, 2009; Cooper *et al.*, 2010; Whittamore *et al.*, 2010; Al-Jandal *et al.*, 2010; Taylor *et al.*, 2011; Ferlazzo *et al.*, 2012). Only recently however has the global significance of piscine carbonate production become clear (Wilson *et al.*, 2009; Jennings & Wilson, 2009; Perry *et al.*, 2011; Wilson, 2014). Model estimates of global marine teleost biomass and carbonate excretion rates conservatively indicate a global contribution of between 3 and 15% to total new ocean calcium carbonate (Wilson *et al.*, 2009). Applying less conservative but realistic assumptions to these models suggests that the piscine carbonate contribution could in reality be as high as 45% (Wilson, 2014). These findings have major implications for our understanding of ocean carbonate chemistry as well as carbonate sediment budgets and records (Perry *et al.*, 2011; Woosley *et al.*, 2012; Wilson, 2014). At the outset of the present work it was clear, at least from our theoretical understanding of the processes involved, that the respiratory responses of fish to environmental factors such as temperature, PO<sub>2</sub>, and PCO<sub>2</sub>, would be closely linked in terms of intestinal carbonate production and

excretion by marine teleosts. However, there existed a lack of experimental data needed to confirm and quantify these predicted effects.

## Overview of Major Findings and Implications

Broadly, this thesis can be considered to consist of two major themes. The first half (chapters two and three) examined the respiratory responses of fish to changes in environmental oxygen and temperature in order to explore intra- and inter-specific trait variation and its ecological implications. The second half (chapters four and five) predominantly focused on the effects of environmental factors ( $PO_2$ ,  $PCO_2$ , temperature and seawater chemistry) on the intestinal precipitation and excretion of  $CaCO_3$  by marine teleosts. Given that gut carbonate production is closely linked to the respiratory responses of fish to changes in environmental  $PO_2$ ,  $PCO_2$  and temperature, there is significant overlap between these two themes (Figure 39).

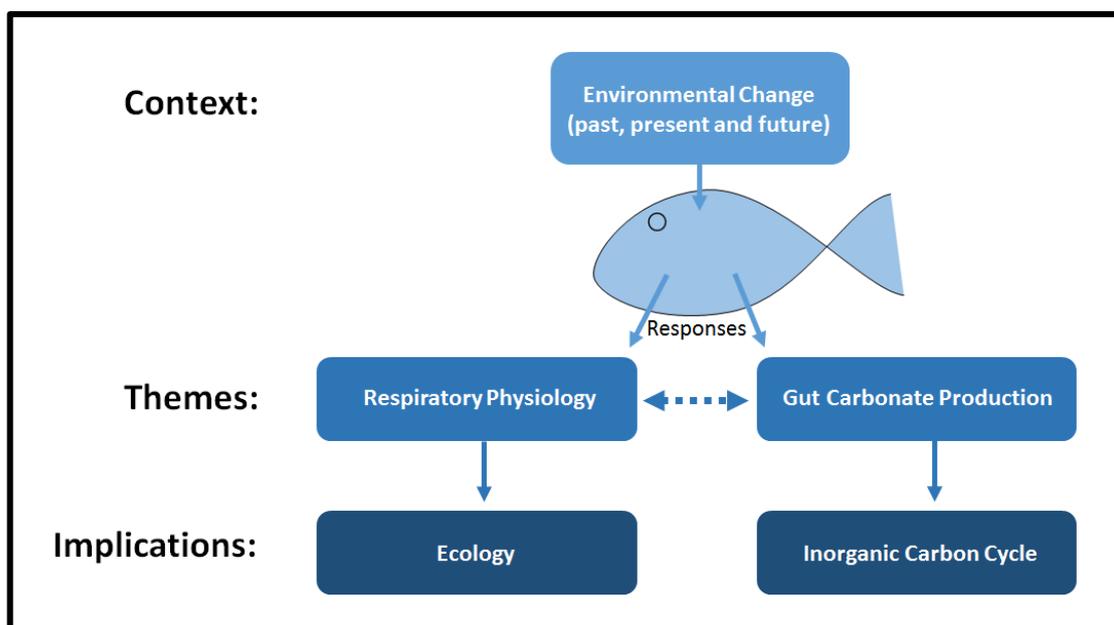


Figure 39. Diagram summarising the overarching concepts presented in this thesis.

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The first study (chapter two) involved the collation and synthesis of critical PO<sub>2</sub> (P<sub>crit</sub>) data from the published literature in order to produce a physiological trait database of hypoxia tolerance in fish. The current database consists of 331 measurements of P<sub>crit</sub> from a total of 96 published studies covering 151 fish species from 58 families. The systematic review of this literature revealed considerable variation in methodology and indeed definition of P<sub>crit</sub> as a physiological measurement between studies. Nevertheless, the collated data provides a useful representation of hypoxia tolerance across species and demonstrates the diverse range of biotic and abiotic factors that influence P<sub>crit</sub>. It was noted that whilst P<sub>crit</sub> provides a useful indicator of hypoxia tolerance, it is beneficial to consider P<sub>crit</sub> alongside other markers of overall hypoxia tolerance such as time to loss of equilibrium (LOE<sub>50</sub>), tissue specific lactate accumulation and thresholds for aquatic surface respiration. Physiological data such as P<sub>crit</sub> provides the mechanistic link between environmental change and population level effects and is therefore of great value for improving the predictive capacity of models as an aid to the management and conservation of aquatic ecosystems (Jorgensen *et al.*, 2012; Cooke *et al.*, 2013). There is considerable scope for further analyses of the present database and for its integration with similar databases of other widely measured physiological parameters in fish (e.g. SDA, aerobic scope, growth rates and critical temperature). The incorporation of these data in a widely accessible central repository of physiological trait data would likely prove to be a highly useful resources for facilitating future studies of fish ecology, conservation and management.

Chapter three focused on the contrasting biophysical environment of the coral reef flat and slope and the physiological tolerances of two reef snapper species, *Lutjanus carponotatus* and *Lutjanus adetii*. As the oxygen and temperature logger data collected over a two year period clearly demonstrates, the reef flat surrounding Heron Island (GBR) is subject to much greater fluctuations over tidal, diurnal and seasonal cycles, both in oxygen and thermal regime, compared to the more stable abiotic environment of the reef slope. Hypoxia is a daily occurrence on the reef flat with an average daily minimum dissolved oxygen concentration of 1.4 and 2.5 mgO<sub>2</sub> l<sup>-1</sup> on the inner and outer

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flat, respectively; whereas on the reef slope the daily average minimum dissolved oxygen concentration was  $6.1 \text{ mgO}_2 \text{ l}^{-1}$ . Likewise the reef flat exhibits a mean daily temperature range of  $4^\circ\text{C}$  in comparison to only  $1^\circ\text{C}$  recorded on the reef slope. *L. adetii* appears to be restricted to the reef slope whereas *L. carponotatus* can be observed in both flat and slope habitats. Previous data comparing the parasite load between *L. carponotatus* individuals caught on the flat and slope indicated that there was limited local movement between habitats leading to the suggestion that flat and slope *L. carponotatus* may form two distinct sub-populations (Cribb *et al.*, 2000).

At the outset of chapter three, it was hypothesised that *L. carponotatus* would exhibit greater thermal and hypoxia tolerance because of its ability to exploit reef flat habitats. By testing the thermal and hypoxia tolerance of *L. adetii* and *L. carponotatus*, this study sought to examine whether these metrics could be considered as functional traits underlying the variation in habitat range of these two species and therefore provide additional evidence of the benefit of considering physiology for exploring ecological patterns. Aerobic scope (AS), critical oxygen tension ( $P_{\text{crit}}$ ), critical maximum ( $\text{CT}_{\text{max}}$ ) and minimum ( $\text{CT}_{\text{min}}$ ) temperature were measured as indicators of thermal and hypoxia tolerance. *L. carponotatus* was clearly the most thermally and hypoxia tolerant of the two species, demonstrating a  $\sim 3.5^\circ\text{C}$  wider thermal tolerance zone (higher  $\text{CT}_{\text{max}}$ , lower  $\text{CT}_{\text{min}}$ ) and  $\sim 26\%$  lower  $P_{\text{crit}}$  ( $1.7 \text{ mgO}_2 \text{ l}^{-1}$ ) than *L. adetti* ( $2.35 \text{ mgO}_2 \text{ l}^{-1}$ ). These results demonstrate that inter-species variation in the distribution of these fish between flat and slope reef zones is reflected in their physiological tolerances. However, no evidence was found for intra-species variation in tolerance between flat and slope *L. carponotatus* individuals, indicating that they do not form *physiologically* distinct subpopulations between these reef zones. Understanding the physiological underpinning of inter-habitat distribution is particularly relevant given the predicted  $2 - 3^\circ\text{C}$  rise in tropical sea surface temperatures by the year 2100 (IPCC, 2013). As the present data demonstrates, both *L. adetii* and *L. carponotatus* already frequently encounter oxygen and temperature extremes close to their physiological limits. Future increases in temperature and associated fluctuations in oxygen could therefore

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lead to distributional shifts that may significantly affect key processes such as feeding, growth rates and mortality from predation. However, further study is required to assess the long term, transgenerational acclimation potential of these two species and the wider coral reef assemblage, to future climate change scenarios (Donelson *et al.*, 2011, 2012; Grenchik *et al.*, 2013).

Chapter four examined the respiratory responses of European flounder (*Platichthys flesus*) to hypoxia and hypercarbia in order to determine how these environmental factors affect gut carbonate production. Flounder demonstrated a clear hypoxic ventilatory response, increasing ventilation volume by 2.4-fold relative to normoxia when water PO<sub>2</sub> was reduced to ~ 50% air saturation. This closely corresponds to the 2.3-fold increase observed in carbonate excretion rate over the same PO<sub>2</sub> range and thus supports the initial hypothesis that increased branchial water loss associated with hyperventilation requires compensatory increases in drinking rate - ultimately resulting in increased intestinal carbonate precipitation and excretion. However, measurements of drinking rate using <sup>51</sup>Cr-EDTA labelled seawater detected no significant increase in drinking rate in response to hypoxia. It is unclear why this was the case but the limitations of the experimental method, in particular the constraints placed on incubation time, cannot be discounted. The relationship between water loss, drinking rate and carbonate excretion has previously been demonstrated in the Gulf toadfish (*Opsanus beta*) exposed to varying environmental salinities (Genz *et al.*, 2008). Therefore, it appears to be premature at this stage to dismiss elevated drinking rate as the mechanism behind increased carbonate excretion rate under hypoxia, as originally hypothesised. Further drinking rate measurements are required to fully elucidate the degree and indeed presence of a drinking response to hypoxia in marine teleosts. In particular, it would be ideal to use rectal catheterized fish to facilitate longer term (>24 hours) collection of rectal fluid, and avoid the potential for radiolabelled marker to pass through the entire intestine within the experimental period.

European flounder exposed to hypercarbia for 7 days (~3000 µatm) demonstrated an almost fully compensated respiratory acidosis as indicated

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by a slight decline in blood pH (0.14) and a ~3-fold increase in blood PCO<sub>2</sub> and HCO<sub>3</sub><sup>-</sup> concentration. The same level of hypercarbia produced a 1.5-fold increase in carbonate excretion rate, presumably due to increased intestinal HCO<sub>3</sub><sup>-</sup> secretion fuelled by elevated intracellular and blood PCO<sub>2</sub> / HCO<sub>3</sub><sup>-</sup>. In combination, hypoxia and hypercarbia acted synergistically, increasing carbonate excretion rate by 4.3-fold. Interestingly, the hypoxia tolerance of European flounder was significantly reduced during simultaneous hypercarbia, as indicated by an 18.5% increase in P<sub>crit</sub> relative to that determined under normocarbic hypoxia. This finding is significant given the differences, as discussed in chapter two, between previous studies in the use of closed (hypercarbic) or flow-through (normocarbic) respirometry techniques to determine P<sub>crit</sub>. Overall, the results presented in chapter four suggest that hypoxia and hypercarbia have a significant positive effect on gut carbonate excretion rates by marine teleosts. Thus, these results have significant implications for updating global model estimates of piscine carbonate production which do not currently account for variation in environmental PO<sub>2</sub> or PCO<sub>2</sub>. The inclusion of these environmental variables into global estimates is especially important given the prevalence of hypoxia and hypercarbia in the marine environment, particularly within coastal systems where fish biomass tends to be concentrated (Jennings & Wilson, 2009). However, further experimental work is required to establish these effects over a greater range of environmentally relevant PO<sub>2</sub> and PCO<sub>2</sub> as well as in a representative range of marine teleost species.

The final study of this thesis (chapter five) investigated calcium carbonate excretion rate and the precipitate chemistries of the European flounder undergoing environmental conditions simulating those of the Cretaceous oceans (high salinity, temperature and PCO<sub>2</sub>; and low Mg/Ca ratio and PO<sub>2</sub>). Relative to modern seawater chemistry, flounder demonstrated a 5.3 – 5.6-fold increase in carbonate excretion rate in seawater chemistry mimicking the ‘calcite seas’ of the Cretaceous (4-fold higher Ca<sup>2+</sup> concentration and 1.14-fold higher salinity). This increase in excretion rate was largely consistent with the predicted multiplicative effects of increased drinking rate and imbibed calcium

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concentration. When Cretaceous seawater chemistry was combined with elevated temperature (+8 °C warmer, typical of mid-Cretaceous hot-house conditions), carbonate excretion rate increased by almost 14-fold relative to modern seawater at 15 °C (which roughly coincides with present day global average sea surface temperature; Smith & Reynolds, 2005). These results support the hypothesis that ocean conditions prevalent in the Cretaceous would have resulted in piscine carbonate production rates substantially higher than the present day. Furthermore, the lower Mg/Ca ratio of Cretaceous seawater was reflected in the carbonate precipitate chemistry which showed a 50% reduction in incorporated magnesium compared to the precipitates produced by flounder in modern seawater. This low magnesium content suggests that carbonates produced by fish during the Cretaceous period had greater potential for preservation in sediments and would have dissolved at greater depths compared to those produced in the present day oceans. Combined, these results have considerable implications for our understanding of the inorganic carbon cycle and carbonate records of the Cretaceous oceans. However, the long-term fate of fish derived carbonates in the sedimentary record is currently unclear with further work required to assess the likely chemical transformations of piscine carbonates within sediments.

### General Limitations

In addition to the specific experimental limitations previously discussed within each study chapter, there are important wider limitations associated with extrapolating the results of these laboratory-based experiments to the 'real-world' environment. Generalisations regarding the responses of fish to environmental change that are based on measurements at the individual level, are likely to become decreasingly robust when applied at increasing biological, spatial and temporal scales. Therefore, it is necessary to acknowledge these limitations when considering implications relating to ecology and carbon cycling (Figure 39).

A significant limitation of laboratory-based animal physiology studies are those imposed by the laboratory environment itself. Captivity in artificial habitats (e.g.

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stock tanks, acclimation tanks, respirometers) may expose fish to a multitude of stressors that they are unlikely to face in their natural range. These include environmental sources of stress such as aversive noise or artificial lighting, as well as confinement-specific stressors such as restricted movement, reduced retreat space or repeated handling (Morgan & Tromborg, 2007). In fish, the primary physiological response to stress (chronic or acute) is the release of cortisol, an adrenally-derived glucocorticoid hormone. The circulation of cortisol has been shown to induce a suite of secondary responses in fish including metabolic, cellular, osmoregulatory, haematological and immunological changes (Barton, 2002). Thus, physiological measurements made in individuals that are acutely or chronically stressed by the conditions of their captivity, may not be entirely representative of populations in their natural environment. However, chronic and acute stress is by no means unique to captive fish and indeed factors such as the lack of predation pressure, abundance of food and high water quality, may result in lower levels of stress in captivity compared to the wild (Plante *et al.*, 2003). This again may cause a differentiation between the physiology observed in laboratory fish and the natural physiological state of individuals in the wild.

Given the huge diversity of fish species and the environments they occupy, single-species studies are of obviously limited value in drawing conclusions that generalise for fish as an entire group. Likewise, a single population is unlikely to be entirely representative of the species as a whole. Practical considerations such as ease of collection, survivorship in captivity and tolerance to the experimental protocol, often constrain the range of species used in physiological studies. For example, research conducted into piscine carbonate production has so far been largely concentrated on two species, the Gulf toadfish (*Opsanus beta*) and European flounder (Walsh *et al.*, 1991; Grosell *et al.*, 2001, 2005, 2009; Wilson *et al.*, 2002, 2009; Whittamore *et al.*, 2010). Whilst both these species provide convenient models for such studies, as euryhaline species the extent to which they are illustrative of a typical marine teleost is questionable. In contrast, carbonate production in the mesopelagic fishes (*Myctophidae*) remains largely unstudied. This group of

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fishes dominate global biomass and as such are arguably the most relevant to study in terms of understanding the total contribution of fish to the marine inorganic carbon cycle (Irigoien *et al.*, 2014, Wilson, 2014). However, successful collection and maintenance of live mesopelagic fish specimens for physiological study is likely to be extremely challenging.

The experiments presented in this thesis are limited in their temporal scale (treatment durations < 10 days). Whilst these short-term studies may be very relevant to understanding physiological responses to acute environmental changes (e.g. over diurnal or tidal cycles) their applicability to more long-term environmental shifts is questionable. This is because short-term studies cannot account for the potential effects of long-term acclimation, developmental plasticity or trans-generational adaptation. These factors have previously been shown in a variety of fish species to significantly limit the physiological impacts of environmental challenges such as PO<sub>2</sub> and temperature (Reardon & Chapman, 2010; Fu *et al.*, 2011; Donelson *et al.*, 2011, 2012; Grenchik *et al.*, 2013; Dan *et al.*, 2014). Hence, issues of temporal scale represent a significant caveat to predictions made in the present thesis regarding the responses of fish to environmental change in the future and geological past.

## Future Perspectives

At the heart of this thesis has been the recognition that physiology provides the mechanistic basis for understanding the interactions between fish and their environment. Thus, physiological studies are key to predicting the impacts of environmental change (past, present and future) on fish populations and the ecosystem services that they generate. Specifically, this thesis has attempted to demonstrate, within the limitations discussed above, how physiological responses at the individual level to changes in environmental factors such as oxygen, carbon dioxide and temperature, have wider implications that scale from local ecological patterns all the way up to global carbon cycles.

As the rate of environmental change gathers pace, environmental managers and policy makers are increasingly called upon to mitigate threats, reverse

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species declines, restore degraded ecosystems and manage the sustainability of natural resources. Traditionally, conservation decisions have been derived from studying trends and correlations observed at population, community and ecosystem levels. However, examining the physiological mechanisms underlying conservation problems allows for the establishment of cause-and-effect within these relationships (Cooke *et al.*, 2013). Thus, the incorporation of physiological measurements into ecological models will greatly improve the power of these models as tools for predicting and managing the effects of environmental change. The emerging field of conservation physiology therefore represents a vital direction for future fish physiology research.

There is also significant scope to further develop our understanding of the physiology and global significance of piscine carbonate production. To date, great strides have been made in elucidating the fundamental physiological mechanisms, as well as the inherent abiotic and biotic interactions involved. However, further experimental work across an environmentally relevant range of conditions and species is required in order to generate data that better reflects piscine carbonate production in the 'real-world' oceans. Ultimately, the consolidation of such data could be used to improve the robustness and resolution of piscine carbonate modelling over local and global scales. In combination with a more detailed appreciation of the environmental fate of piscine derived carbonates, this work is likely to lead to fascinating further insights into the role played by fish in the marine organic carbon cycle of the past, present and future.



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# Chapter 7



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