# A deadly trio of climate change? Interactive effects of temperature, $O_2$, and $CO_2$ on the physiology of fish

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Submitted by Daniel Wenban Montgomery, to the University of Exeter as a thesis for the degree of Doctor of Philosophy in Biological Sciences, December 2020.



I certify that all material in this thesis which is not my own work has been identified and that any material that has previously been submitted and approved for the award of a degree by this or any other University has been acknowledged.

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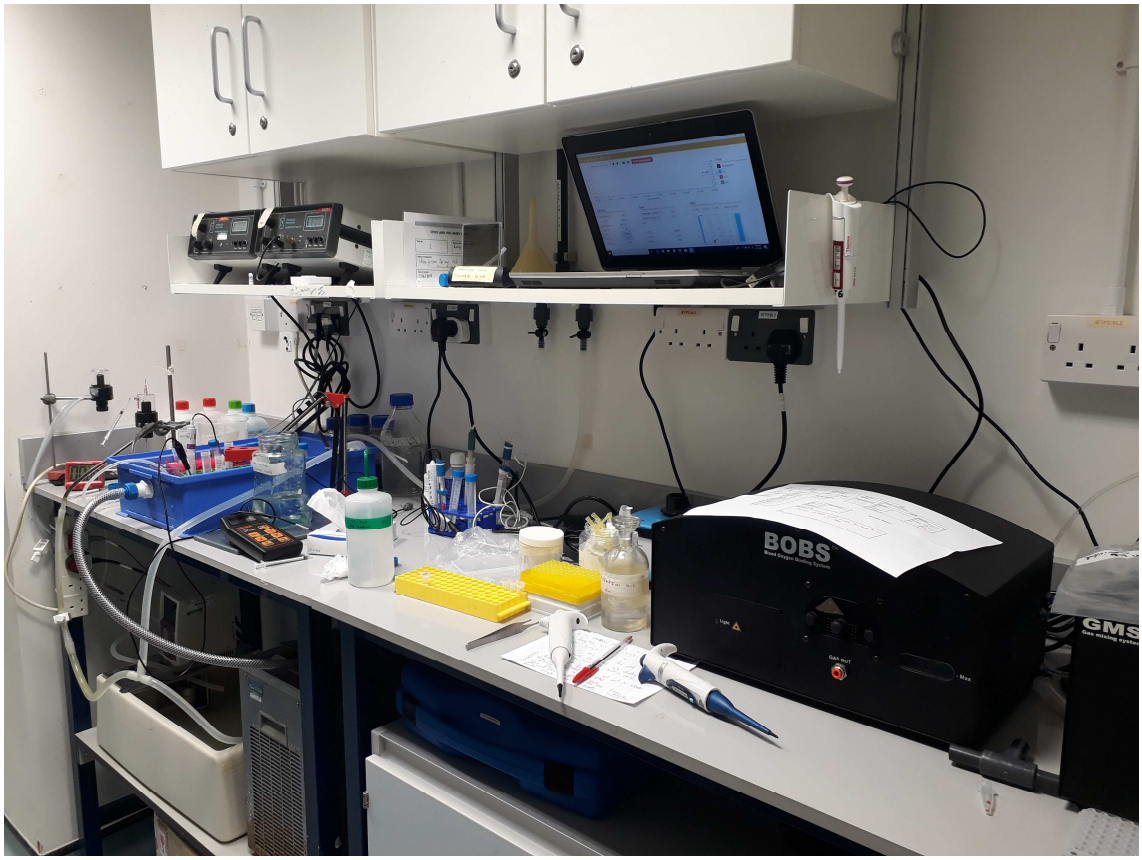
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# Thesis Abstract

Global climate change is causing warming of the world's oceans as well as reductions in $O_2$ levels and increases in $CO_2$. Concern has been raised that interactions between these three factors may be non-linear, meaning that their combined impacts cannot be predicted from responses to an individual factor. Therefore, multi-factor studies are needed to quantitatively assess the interactive effects of these three variables to improve predictions of how species will respond to climate change. This thesis investigates the physiological impacts of combined changes in temperature, $O_2$, and $CO_2$ on acid-base regulation, aerobic respiration, and tolerance limits of fish.

In **chapter II** a review of the primary literature is conducted to identify studies in which interactions between combinations of at least two of temperature, $O_2$, and $CO_2$ on the biology (physiology and behaviour) of marine fish were investigated. This review highlights where interactive effects are most commonly observed, considers possible explanations for the high variability between studies in the types of interactions observed, and proposes future work to fill key knowledge gaps and enhance prediction of population level responses. **Chapter III** examines the time scale of acid-base regulatory responses in European sea bass (*Dicentrarchus labrax*) acutely exposed to high $CO_2$. Sea bass showed one of the fastest acid-base regulatory responses observed in any fish species enabling it to rapidly restore blood $O_2$ carrying capacity (within 40 minutes at 10,000 µatm) after initial blood acidosis caused a ~3-fold decrease in haemoglobin's affinity for oxygen. Rapid acid-base regulation capacity in sea bass may be a result of adaptations to natural environmental variability in ecosystems they inhabit or to physiological challenges related to their ecology. In nature $CO_2$ increases when $O_2$ declines – as such **chapter IV** investigates the impacts of environmentally realistic rises in $CO_2$ on responses to hypoxia in sea bass. Sea bass exhibited greater hypoxia tolerance (~20% reduced $O_{2crit}$), associated with increased haemoglobin-$O_2$ affinity (~32% fall in $P_{50}$) of red blood cells, when exposed to reciprocal changes in $O_2$ and $CO_2$. This result suggests that increasing environmental $CO_2$ during hypoxia facilitates increased $O_2$ uptake by sea bass in hypoxic conditions. Consequently, considering environmental $CO_2$ may be vital to accurately assess impacts of hypoxia on fish species more generally.

**Chapter V** examines the potential for acutely increased $CO_2$ to affect critical thermal maximum ($CT_{max}$) of rainbow trout (*Oncorhynchus mykiss*) via either respiratory acidosis or by limiting $O_2$ supply capacity. Trout were exposed to combinations of high $CO_2$ (~10,000 µatm) and hyperoxia (~42 kPa = 2x atmospheric) before blood acid-base chemistry, Hb-$O_2$ affinity, aerobic scope, and finally $CT_{max}$ were measured. Despite large changes in acid-base chemistry, pH, and aerobic scope between treatments we saw no impacts of any treatments on $CT_{max}$ of trout. These results suggest that mechanisms determining $CT_{max}$ of trout are independent of blood pH or $O_2$ and that combined changes in environmental $O_2$ and $CO_2$ are unlikely to affect critical thermal tolerance limits of fish. Furthermore, it provides evidence that processes such as disruption of lipid membranes may be the true mechanism by which upper critical thermal limits are set.

Lastly, **chapter VI** investigates interactive effects between combined changes in temperature, $O_2$, and $CO_2$ on aerobic performance of European sea bass. Fish were exposed to combinations of temperature (14, 18, or 22°C) and $CO_2$ (~400 or ~1000 µatm) for at least two weeks before we measured aerobic scope at four $O_2$ levels. Combined impacts of temperature and $O_2$ resulted in a negative synergistic interaction on aerobic scope so that hypoxia had greater effects on aerobic scope at warmer temperatures. In contrast $CO_2$ showed no interactive effects with either temperature or $O_2$, either individually or in combination. This result suggests that responses of fish to climate change will primarily be a result of interactive effects of changes in environmental temperature and $O_2$, not $CO_2$.

This thesis demonstrates the complexity of multi-factor interactions between temperature, $O_2$, and $CO_2$ on the physiology of fish species. Results from each study improve knowledge of when and how interactive effects of combined environmental changes on respiratory physiology might occur. Ultimately, these findings will contribute to the growing evidence base which will enable more accurate predictions of the impacts of climate change on fish species.

# Outputs, Training, and Development

## Journal Publications

Gordon, T.A.C., Harding, H.R., Clever, F.K., Davidson, I.K., Davison, W., **Montgomery, D.W.**, Weatherhead, R.C., Windsor, F.M., Armstrong, J.D., Bardonnet, A., Bergman, E., Britton, J.R., Côté, I.M., D'agostino, D., Greenberg, L.A., Harborne, A.R., Kahilainen, K.K., Metcalfe, N.B., Mills, S.C., Milner, N.J., Mittermayer, F.H., Montorio, L., Nedelec, S.L., Prokkola, J.M., Rutterford, L.A., Salvanes, A.G.V., Simpson, S.D., Vainikka, A., Pinnegar, J.K. and Santos, E.M. (2018), Fishes in a changing world: learning from the past to promote sustainability of fish populations. J Fish Biol, 92: 804-827. https://doi.org/10.1111/jfb.13546

**Montgomery, D.W.**, Simpson, S.D., Engelhard, G.H., Birchenough S.N.R. and Wilson R.W. (2019) Rising $CO_2$ enhances hypoxia tolerance in a marine fish. *Sci Rep* **9,** 15152. https://doi.org/10.1038/s41598-019-51572-4

## Conference Presentations

Fisheries Society of the British Isles 50th Anniversary Conference, Exeter, UK. *Multi-stressor impacts of the 'deadly trio' on hypoxia tolerance of a commercial fish species*. July, 2017.

Marine Biological Association of the UK 15th Postgraduate Conference, Plymouth, UK. *Multi-stressor impacts of the 'deadly trio' on hypoxia tolerance and aerobic performance of the European sea bass, Dicentrarchus labrax*. May 2018.

Society of Experimental Biology Annual Meeting, Florence, Italy. *Do bold fish flee first? Effects of personality on escape performance of schooling shiner perch, Cymatogaster aggregata*. July, 2018.

Society of Experimental Biology Annual Meeting, Florence, Italy. *A deadly trio of marine change: multi-stressor impacts on the European sea bass, Dicentrarchus labrax*. July, 2018.

University of Exeter BioCon, Exeter, UK. *Rising $CO_2$ improves hypoxia tolerance in a marine fish*. April, 2019.

10th International Congress of Comparative Physiology and Biochemistry, Ottawa, Canada. *Rising $CO_2$ improves hypoxia tolerance of a marine fish*. August, 2019.

10th International Congress of Comparative Physiology and Biochemistry, Ottawa, Canada. *Multi-stressor impacts of the 'deadly trio' on aerobic performance of the European sea bass, Dicentrarchus labrax*. August, 2019.

**Training Courses**

Red Kite Veterinary Consultants Ltd., 2016. *E1L, PIL A/K, and PIL B.*

University of Exeter, 2017. *Learning and Teaching in Higher Education (Stage 1).*

Falmouth Training Solutions, 2019. *Personal Survival Techniques.*

College of Aquaculture and Fisheries, Can Tho University, Vietnam, 2018. *Acid Base and Ion Regulation in a Hypercapnic World.*

Friday Harbor Laboratories, University of Washington, USA, 2017. *Fish Swimming: Kinematics, Ecomorphology, Behaviour and Environmental Physiology*.

PR Statistics, UK, 2019. *Environmental Niche Modelling*.

Cardiff University, UK, 2017. *Statistical analysis with R.*

**Professional Development**

Isles of Scilly Inshore Fisheries Conservation Authority, 2019. Assisted with monitoring of inshore potting fleet to assess status of lobster, crab, and crawfish stock.

Cefas, 2019. Assisted with Irish Sea Demersal Fish Survey on board R/V Cefas Endeavour.

Lizard Island Research Station, 2019. Research assistant for Tim Gordon (PhD Candidate at the University of Exeter) investigating recruitment of coral reef fish.

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# Glossary

**Acid-base regulation** = regulation of the pH of body fluids via transfer of acids or bases between the environment and an organism

**Adenine triphosphate (ATP)** = an organic compound produced during respiration which is used to provide chemical energy for cellular processes

**Adrenergic response** = responses triggered by adrenaline or noradrenaline.

**Aerobic scope** = the difference between SMR and MMR, considered to represent the maximum possible energy available to an organism to be used for activity, growth, reproduction etc. once minimum energy needs of survival are met.

**Allosteric modulators** = substances that bind to receptors on a molecule and changes the response of the molecule to a stimulus.

**Analysis of Variance (ANOVA)** = collection of statistical test designed to compare differences among group means in a sample

**Anoxic/anoxia** = conditions of zero $O_2$, usually fatal for vertebrate life

**Anthropocene** = a proposed geological epoch beginning from the start of significant human impact on the Earth's geology and ecosystems.

**Aquatic surface respiration** = use of the top few centimetres of the water column by an organism to increase $O_2$ uptake during hypoxic conditions

**Bohr effect** = shifts in the oxygen equilibrium curve of haemoglobin as a result of changes in concentration of $CO_2$ or the pH of the environment. Increased $CO_2$/decreased pH causes a right shift of the OEC resulting in an increase in $P_{50}$. This means that $O_2$ levels have to be higher in order for haemoglobin to become 50 % saturated, indicating that Hb-$O_2$ binding affinity has reduced.

**Bradycardia** = slower than usual heart rate.

**Cardiorespiratory system** = the organs involved in transporting $O_2$ from the environment to the tissues and the removal of waste products including $CO_2$, major components of the cardiorespiratory system of fish are the heart, gills, and blood vessels.

**Catecholamines** = hormones made by the adrenal gland, including epinephrine (adrenalin) and norepinephrine (noradrenaline)

**Critical $O_2$ tension ($O_{2crit}$)** = the partial pressure of environmental $O_2$ at which a fish can no longer maintain SMR.

**Critical temperature ($T_{crit}$)** = the upper or lower temperature tolerance limit of a fish.

**Critical thermal maximum ($CT_{max}$)** = the upper critical temperature tolerance of a fish, usually defined as the temperature at which a loss of equilibrium is observed when fish are exposed to a constant increase in temperature.

**Dissolved Inorganic Carbon (DIC)** = the sum of the dissolved inorganic carbon molecules in a solution

**Ecophysiology** = the study of how the environment interacts with the physiology of an organism.

**Ectotherms** = animals whose body temperature varies with the environment

**Erythrocyte** = a red blood cell (RBC), they contain haemoglobin and transport $O_2$ and $CO_2$ to and from the tissues.

**Generalised Linear Mixed-effects Model (GLMM)** = a type of linear regression model that allows both fized and random effects to be included

**Guanine triphosphate (GTP)** = an organic compound that is required to form RNA, it also acts as the main allosteric modulator of haemoglobin in fish

**Haematocrit (Hct)** = the proportion of blood volume that is comprised of erythrocytes (red blood cells).

**Haemoglobin (Hb)** = the respiratory pigment found in erythrocytes of mammals and fish, it is responsible for the majority of $O_2$ transport to the tissues.

**Hb-$O_2$ binding affinity ($P_{50}$)** = how readily haemoglobin acquires and releases $O_2$ molecules. This is usually defined using $P_{50}$ which is the $O_2$ level required to saturate Hb by 50 %. Increases in $P_{50}$ represent decreases in Hb-$O_2$ affinity and vice versa.

**$HCO_3^-$** = bicarbonate

**Hills' number** = measure of cooperativity of haemoglobin. If the number is above 1 it indicates that each molecule of $O_2$ that binds to haemoglobin increases the affinity of haemoglobin for $O_2$. If the number is below 1 this indicates that each $O_2$ molecule that binds to haemoglobin decreases the affinity of haemoglobin for $O_2$.

**Hypercapnia** = conditions of elevated $CO_2$ – this can occur environmentally or in the body fluids of an organism

**Hyperoxia** = $O_2$ conditions higher than normal atmospheric concentrations

**Hypoxic/hypoxia** = conditions of low or depleted $O_2$.

**Intermittent-flow respirometry** = system used to measure oxygen consumption by an organism. It involves placing an organism into a sealed chamber and measuring the decline in $O_2$. The chamber is then flushed with oxygenated medium (either water or air depending on species measured) before being resealed to repeat the measurement of $O_2$ consumption. This cycle can be continued for long periods to allow multiple measurements of an organism $O_2$ consumption and calculation of metabolic rates.

**IPCC** = intergovernmental Panel on Climate Change.

**Loss of equilibrium** = loss of righting response of an organism

**Marine heatwave (MHW)** = periods of extremely high temperatures in the oceans. Defined as occurring when temperatures exceed the 95 th percentile of monthly recorded temperatures for more than 5 days in a row.

**Maximum Metabolic Rate (MMR)** = the maximum possible energy production of a fish in a given environment.

**Metabolic acidosis** = decrease in pH of body fluids as a result of increased acid production (i.e. from anaerobic respiration), a loss of bicarbonate, or an inability to excrete excess acids

**Metabolic alkalosis** = increase in pH of body fluids as a result of increased $HCO_3^-$ levels (i.e. produced as a by-product of digestion) or decreased $H^+$ levels

**Mitochondrial Rich Cell (MRC)** = cells in the gills of fish responsible for acid-base and ion regulation, also called ionocytes.

**NHE** = $Na^+/H^+$ exchanger

**Non-bicarbonate buffering** = refers to all other relevant buffers than the carbonic acid-bicarbonate buffer system, primarily comprised of proteins.

**Normocapnia** = normal $CO_2$ conditions

**Normoxia** = normal $O_2$ conditions

**Optimum temperature ($T_{opt}$)** = the temperature at which the performance of an organism, or a physiological system, is highest.

**Oxconformer** = an organism whose $O_2$ consumption rate varies with changes in environmental $O_2$

**Oxygen consumption rate ($\dot{M}O2$)** = the amount of $O_2$ consumed by an organism in a given time period, measured as a proxy for aerobic metabolic rate

**Oxygen equilibrium curve (OEC)** = a sigmoidal curve used to describe the saturation status of haemoglobin as $O_2$ levels change. The $O_2$ at which haemoglobin would be 50 % saturated with $O_2$ is termed the $P_{50}$.

**Oxygen Limiting Zone (OLZ)** = areas of the world's oceans where $O_2$ levels are lower than typical and could have negative impacts on marine organisms. Usually considered to occur when $O_2$ levels fall below 100 μM.

**Oxygen Minimum Zone (OMZ)** = areas in the open ocean in which saturation of $O_2$ in the water column is at its lowest, typically defined as having $O_2$ levels < 63 μM and occurring between 200-1000 m.

**Oxyregulator** = an organism with the ability to maintain constant $O_2$ uptake despite declines in environmental $O_2$ level.

**$pH_e$** = pH of extracellular fluid (usually blood)

**$pH_i$** = pH of intracellular fluid.

***$pO_2$*** = partial pressure of $O_2$.

**Recirculating Aquaculture System (RAS)** = aquarium systems where water exchange is limited and instead waste water is treated by a series of filters (to remove organic matter and ammonia) before being recirculated to fish tanks.

Enables production of fish on land without the need for nearby natural water sources.

**Representative Concentration Pathway (RCP)** = a greenhouse gas concentration pathway used by the IPCC to describe possible future climate scenarios.

**Respiratory acidosis** = a decrease in the pH of body fluids as a result of increased environmental $CO_2$

**Respiratory quotient** = ratio of $CO_2$ produced to $O_2$ consumed while food is metabolized.

**Root effect** = increased $CO_2$ or decreased pH cause a right shift of the OEC and reduced carrying capacity of fish haemoglobin. This effect differs from the Bohr effect as sufficiently large increases in $CO_2$ or decreases in pH not only cause a change in Hb-$O_2$ binding affinity but also lower co-operativity of haemoglobin. This results in a downward shift of the OEC so that Hb never becomes 100 % saturated regardless of the $O_2$ level.

**Routine Metabolic Rate (RMR)** = the energy used by a fish during normal behaviour.

**Standard Metabolic Rate (SMR)** = the minimum energy needed by an animal to meet maintenance requirements of cells in a given environment.

**Total Alkalinity (TA)** = measurements of the concentration of all alkaline substances dissolved in water, primarily bicarbonate ($HCO_3^-$), carbonate ($CO_3$), and hydroxide ($OH^-$).

**Total $CO_2$ ($TCO_2$)** = measure of carbon dioxide including $CO_2$ in physical solution or loosely bound to proteins, bicarbonate ($HCO_3^-$) or carbonate ($CO_3$) anions, and carbonic acid ($H_2CO_3$)

# Chapter I

## General Introduction

### 1. The Anthropocene: an era of global change

Human activities are causing rapid and widespread changes to environmental conditions on Earth. This has led to the declaration of a new geological epoch, the Anthropocene (Crutzen, 2002). Since the beginning of the industrial revolution (~1860) there has been a rise in emissions of greenhouse gases, primarily carbon dioxide ($CO_2$), produced via the burning of fossil fuels, changes in land use, and cement production (Andres *et al.*, 1999). In 2019 annual global $CO_2$ emissions were estimated to reach record levels of 36.8 billion tonnes (Friedlingstein *et al.*, 2019) and atmospheric $CO_2$ concentrations have risen from ~280 ppm in the pre-industrial era to a mean of 417 ppm in May 2020 (Keeling and Keeling, 2020). This is higher than any point since the evolution of modern humans (*Homo sapiens*) and represents the highest atmospheric $CO_2$ level for at least 800,000 years (Lüthi *et al.*, 2008). Approximately 30 % of total anthropogenic $CO_2$ emissions have been absorbed by the world's oceans (Sabine *et al.*, 2004; Gruber *et al.*, 2019), this has mitigated the increase in atmospheric $CO_2$ but resulted in a parallel increase in ocean $pCO_2$ (Caldeira and Wickett, 2003; Orr *et al.*, 2005).

Increasing atmospheric $CO_2$ is causing warming of global mean surface air temperatures (Stips *et al.*, 2016) with 2019 being the 2$^{nd}$ warmest year on record, 0.95 °C higher than the 1901-2000 average (NOAA, 2020). To date >90 % of the excess heat generated by global warming has been stored in the world's oceans (Abram *et al.*, 2019) and this has led to the highest level of ocean heat content in the upper ocean (0-2000 m) recorded in human history (Cheng *et al.*, 2020). As a result ocean sea surface temperature (SST) increased by 0.11 °C per decade between 1971 and 2010 (IPCC, 2014) and the pace of warming has increased in recent years (Gleckler *et al.*, 2016; Bates and Johnson, 2020). Ocean temperatures are expected to continue increasing, with mean SST 1-3 °C warmer by the end of the century (IPCC, 2014).

As temperatures rise the oxygen ($O_2$) content of the world's oceans is also expected to decrease. This is because of reducing $O_2$ solubility in water as temperature increases, higher biological demand for $O_2$ as a result of increased

respiration rates, and increased stratification of the water column slowing replenishment of $O_2$ from the atmosphere into subsurface water (Diaz and Breitburg, 2009; Oschlies *et al.*, 2018). Since 1960 global ocean $O_2$ content has decreased by ~2 % (Schmidtko *et al.*, 2017) and it is projected to continue to decline, by 1-7 %, through the 21st Century (Keeling *et al.*, 2009).

In addition to changes in the global environment there is growing recognition that climate change does not affect all areas of the globe equally. Warming of SST between 1982 and 2006 varied from >1 °C in areas such as the North Sea, East China Sea, and Newfoundland-Labrador Shelf to < 0.1°C in the SW Australian shelf, Patagonian Shelf and Antarctic Sea (Belkin, 2009). Extreme warming events such as marine heatwaves are also becoming more common and more severe (Oliver *et al.*, 2018). Oxygen decline also does not occur uniformly. Faster rates of decline have been observed in coastal seas (Gilbert *et al.*, 2010). This is probably a result of the combined effects of changes in ocean circulation (Claret *et al.*, 2018) and increasing prevalence of low $O_2$ (hypoxic) events (Rabalais *et al.*, 2009; Altieri and Gedan, 2015). Away from coastal waters $O_2$ declines also vary, for example in the subtropical North Atlantic the rate of $O_2$ decline is ~4-fold higher than the global average (Bates and Johnson, 2020). Oxygen declines in the open ocean include the expansion of $O_2$ minimum zones (OMZs) which create large areas where $O_2$ levels are severely depleted (Stramma *et al.*, 2008; Paulmier and Ruiz-Pino, 2009). These large OMZs also contain $CO_2$ levels much higher than are expected in surface oceans in 2100 (Paulmier *et al.*, 2011). This link between decreased $O_2$ and increased $CO_2$ is because they result from the same process; respiration (Robinson, 2019). As such, in areas where $O_2$ is depleted $CO_2$ is expected to rise – this includes during hypoxic events (Melzner *et al.*, 2013) as well as over seasonal or diurnal cycles (Gobler and Baumann, 2016).

How global climate change will impact upon the species and ecosystems of the planet is now of key concern to researchers, conservationists and governments (IPCC, 2014). It has already been documented that species extinction rates are increasing as a result of human activity, potentially leading us towards the 6th mass extinction event in the planet's history (Barnosky *et al.*, 2011). In marine systems, global climate change is predicted to reduce the complexity of ecosystem structures (Nagelkerken and Connell, 2015) and impacts of environmental change are already causing widespread poleward

shifts in the distribution and abundance of marine organisms (Simpson *et al.*, 2011a; Hastings *et al.*, 2020).

One of the key groups of organisms affected by environmental change is fish. Fish are the most diverse vertebrate group on earth with >34,000 species identified (Froese and Pauly, 2019). Fish occupy almost all aquatic ecosystems on Earth and are highly abundant – recent estimates suggest that total biomass of fish is ~0.7 Gt C, over 1/3 of the biomass of all animal groups (Bar-On *et al.*, 2018). As a result fishes play a key role in ecosystem functioning (Holmlund and Hammer, 1999). For example, they may exert top down control on food-webs (Baum and Worm, 2009; Eriksson *et al.*, 2009), or via bottom-up processes fish populations can control the abundance of large predators (Cury *et al.*, 2011; Engelhard *et al.*, 2014). Fish also contribute to nutrient cycling and transport (Deegan, 1993; McIntyre *et al.*, 2007), are involved in physical processes such as sedimentation and the inorganic carbon cycle (Wilson *et al.*, 2009; Salter *et al.*, 2017), and improve overall ecosystem resilience (Daskalov *et al.*, 2007; Hughes *et al.*, 2007). Additionally, fish are of enormous importance to people. Worldwide fish production was recorded at 210.9 million tonnes in 2018, directly employing an estimated 59.5 million people, and accounting for ~17 % of total animal protein intake (FAO, 2020). As such, understanding impacts of a changing environment on fish is vital to enable conservation of aquatic ecosystems, maintenance of ecosystem services, and increased food security.

## 2. Physiology as a means to predict the future?

For an individual organism, responses to changes in environmental conditions will be a result of impacts upon that individual's physiology. Therefore, understanding how environmental factors affect physiological performance of fish (*fish ecophysiology*; Rankin and Jensen, 2012) provides a means to predict impacts of anthropogenic climate change on fish species (Rijnsdorp *et al.*, 2009). By focussing on physiology we can gain insight into the mechanisms through which environmental conditions affect performance, providing fundamental understanding of how organism's 'work', increasing the power and accuracy of predicted effects of continued anthropogenic influences, including climate change (Jørgensen *et al.*, 2012; McKenzie *et al.*, 2016).

Investigations into impacts of environmental conditions on the physiology of fish have been ongoing for over a century (for example see the pioneering work of Ege and Krogh, 1914). As such there is a wealth of literature detailing impacts of temperature, $O_2$, and $CO_2$ on fish physiology (Evans and Claiborne, 2006). Of particular focus has been metabolism – as production of ATP (adenosine triphosphate, an organic compound used as a source of energy by living cells) is essential for life. Aerobic respiration is the most efficient means of producing ATP (~15 times more efficient than anaerobic glycolysis). Therefore, it can be argued that effects of temperature, $O_2$, and $CO_2$ on cardiorespiratory systems may hold the key for predicting impacts of climate change on fish species.

## 2.1. Temperature

The vast majority of fish species are ectotherms, and therefore their body temperature is dependent on the temperature of their environment. As such temperature is often termed the 'abiotic master factor' for fish as it impacts almost all biochemical and physiological processes (Fry, 1971). Higher temperatures provide more free energy needed for chemical reactions, resulting in increased rates of reactions (Reynolds and Casterlin, 1980). As a result, ATP production to provide the energy required for cellular maintenance (referred to as standard metabolic rate, SMR) increases as temperatures rise. This leads to an increase in $O_2$ consumption (typically measured as a proxy for aerobic metabolic rate; Clark *et al.*, 2013) by fish in order to supply increased demand to mitochondria. Increased $O_2$ consumption at higher temperatures is exacerbated by impacts of temperature on the efficiency of mitochondrial ATP production (Salin *et al.*, 2015, 2016). For example, increased temperatures lead to higher proton leak from mitochondria and reduced mitochondrial efficiency – i.e. greater $O_2$ consumption in order to produce the same amount of ATP (Salin *et al.*, 2019; Little *et al.*, 2020). The temperature quotient ($Q_{10}$ - the relative rate of change given a 10 °C increase in temperature) for SMR has been estimated for teleost fish as ~1.83 (Clarke and Johnston, 1999). This indicates $O_2$ consumption to meet base energy requirements of fish will exponentially increase as temperature rises.

Temperature also affects the maximum aerobic metabolic rate (MMR – the maximum possible rate of ATP production) of fish. In contrast to SMR,

temperature does not lead to exponential increases in MMR. Instead it is commonly thought that MMR of fish initially increases with temperature to a certain point before plateauing or declining (Fry, 1971). This alteration in response presumably occurs because MMR is limited by the rate of $O_2$ transport from the environment to the mitochondria. For example, increasing temperatures can impair cardiac performance, limiting $O_2$ supply capacity at high temperatures and leading to reduced MMR (Eliason *et al.*, 2011). As such, at higher temperatures the increase in SMR outpaces MMR leading to a drop in the aerobic scope (the difference between SMR and MMR) of the animal. Aerobic scope indicates the amount of energy potentially available to an organism once basal energy demands are met. A high aerobic scope is generally considered to increase fitness of an organism by allowing increased energy allocation to processes such as growth, reproduction, or activity (Clark *et al.*, 2013). As a result of impacts of temperature on SMR and MMR, aerobic scope is thought to follow a thermal performance curve (Figure 1) with the temperature at which peak aerobic scope occurs indicating the optimum temperature ($T_{opt}$) of aerobic performance of the animal (Clark *et al.*, 2013; Norin and Clark, 2016). Using this concept, it has then been proposed that the point at which aerobic scope reaches zero (i.e. SMR = MMR) represents the critical temperature limit ($T_{crit}$) of the animal as further increases/decreases in temperature would result in energy requirements exceeding the maximum capacity for energy production (Pörtner and Knust, 2007).

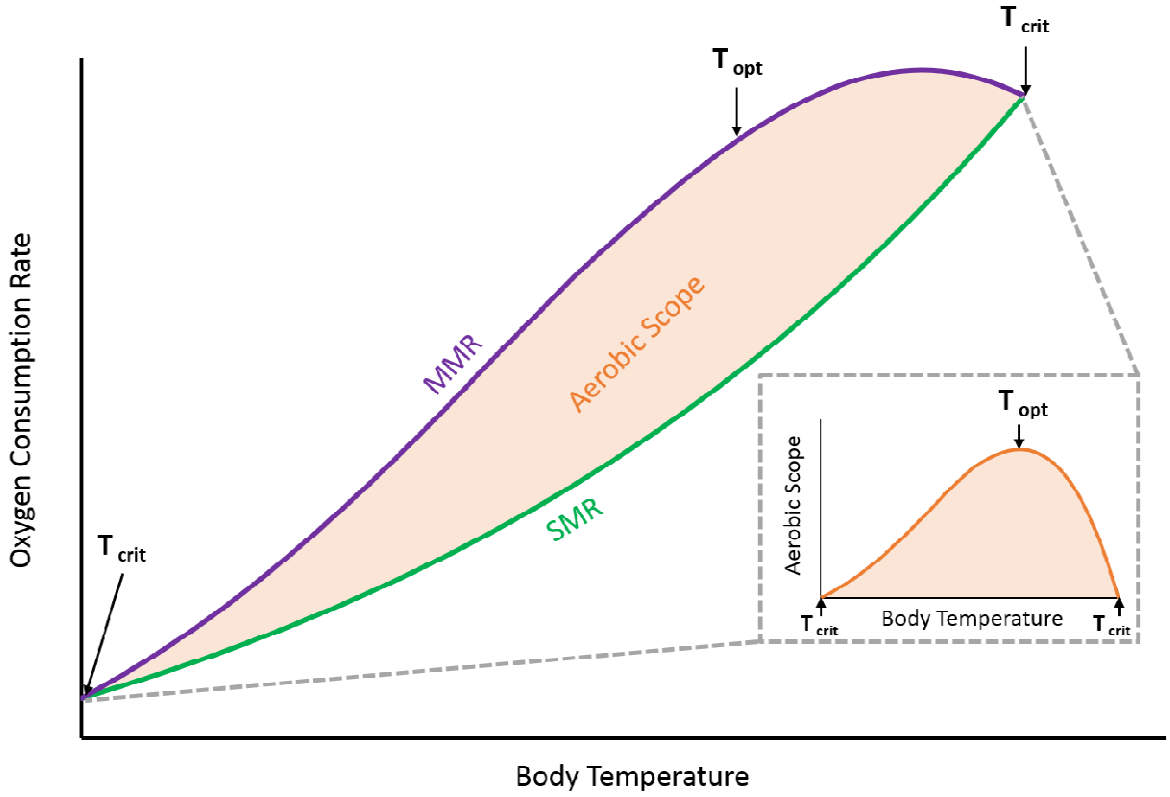


**Figure 1:** Conceptual illustration of the impact of temperature on standard metabolic rate (SMR, minimum $O_2$ consumption required to provide energy via aerobic metabolism for cellular maintenance), maximum metabolic rate (MMR, maximum possible $O_2$ consumption and aerobic energy production), and aerobic scope (i.e. potential energy available for non-essential processes such as growth). The optimum temperature ($T_{opt}$) of the organism is hypothesised to occur when aerobic scope is maximised and the critical temperature limit when aerobic scope equals zero ($T_{crit}$).

## 2.2. Oxygen

Changes in environmental $O_2$ affect metabolic rates of fish by directly impacting upon the capacity to deliver $O_2$ to the cell. When environmental $O_2$ falls fish adopt compensatory strategies in order to maintain oxygen uptake (known as 'oxyregulating') and therefore aerobic metabolism (Richards *et al.*, 2009). Initially fish maintain $O_2$ uptake by increasing ventilation (hyperventilation) of the gills – this allows for the same extraction of $O_2$ from water (despite reduced $O_2$ availability) into the blood and maintenance of arterial $pO_2$ (Perry *et al.*, 2009). Increased ventilation offers one immediate

solution for maintaining $O_2$ uptake however, if environmental $O_2$ continues to fall, is insufficient on its own to maintain $O_2$ delivery to tissues.

In contrast to ventilation, fish show a decreased heart rate (bradycardia) when exposed to hypoxia (Farrell, 2007; Gamperl and Driedzic, 2009). It is hypothesised that bradycardia may protect the heart from impacts of reduced $O_2$ by lowering ATP demand and increasing the diastolic period (thus increasing time for $O_2$ to diffuse into the spongy myocardium) (Farrell, 2007). On its own bradycardia would appear to be self-defeating for fish – the reduced heart rate may lower demand and increase supply of $O_2$ for the heart but would correspondingly reduce overall $O_2$ supply capacity for the rest of the fish. However, fish protect against potential negative effects of bradycardia by increasing stroke volume to maintain overall cardiac output (Gamperl and Driedzic, 2009). Bradycardia appears to be an adaptation to acute but not chronic hypoxia. Fish exposed to chronic hypoxia show no evidence of reduced heart rate, although this occurs at the cost of reduced stroke volume and cardiac output (Petersen and Gamperl, 2010; Motyka *et al.*, 2017). Thus, cardiac responses to hypoxia appear to have a function of protecting $O_2$ supply to the heart as opposed to benefiting overall organism performance.

Blood is the tissue responsible for $O_2$ transport from the gills to the cells. Oxygen in blood is primarily bound to haemoglobin (Hb) in erythrocytes with only a small percentage (~1-2 %) of total $O_2$ content dissolved in plasma (Takeda, 1990). Therefore, increasing haematocrit is one mechanism to increase $O_2$ transport capacity. Increased haematocrit enhances $O_2$ transport capacity during periods of increased $O_2$ demand (Brijs *et al.*, 2020) and can also maintain $O_2$ supply when environmental $O_2$ falls. Haematocrit increases as a result of splenic contraction in response to low environmental $O_2$ and is triggered by catecholamines stimulating α-adrenergic receptors (Nilsson and Grove, 1974; Yamamoto *et al.*, 1985). A second mechanism to increase $O_2$ supply when environmental $O_2$ falls is to increase the amount of $O_2$ bound to each Hb molecule. Haemoglobin is a tetramer and each of the four haem subunits binds to a single $O_2$ molecule. The relationship between plasma $O_2$ level and the amount of $O_2$ bound to Hb is described by the oxygen equilibrium curve (OEC) which is usually sigmoidal in nature (Wells, 2009). As the partial pressure of $O_2$ ($pO_2$) decreases the proportion of Hb that is bound to $O_2$

decreases. To counter this the Hb-$O_2$ affinity can be increased by allosteric modulators including increased pH (Wells, 2009). The primary allosteric modulators of fish Hb are organic phosphates, particularly ATP and GTP (Val, 2000). These molecules stabilise Hb in a low affinity state (Jensen *et al.*, 1998), and so decreased intracellular concentration of ATP and GTP increases Hb-$O_2$ affinity, allowing greater uptake of $O_2$ at the gills. It is not surprising then that decreases in ATP and GTP levels occur in response to reduced environmental $O_2$ (Val *et al.*, 1995). Changes in pH also affect Hb-$O_2$ affinity via the Bohr effect. Catecholamines released in response to hypoxia bind to β-adrenergic receptors on erythrocytes which stimulates a $Na^+/H^+$ exchanger (NHE) (Nikinmaa, 1983). This results in an efflux of $H^+$ and a raising of erythrocyte pH, increasing Hb-$O_2$ affinity.

If environmental $O_2$ falls sufficiently fish are no longer able to maintain $O_2$ uptake rates required for SMR, despite compensatory mechanisms described above. This point, referred to as the critical $O_2$ tension ($O_{2crit}$), is commonly considered to represent the hypoxia tolerance of the fish (Figure 2). Beyond it fish become ‘oxyconformers’ (i.e. $O_2$ uptake rates are dependent on environmental $pO_2$) and rely on unsustainable anaerobic respiration to survive (Rogers *et al.*, 2016).

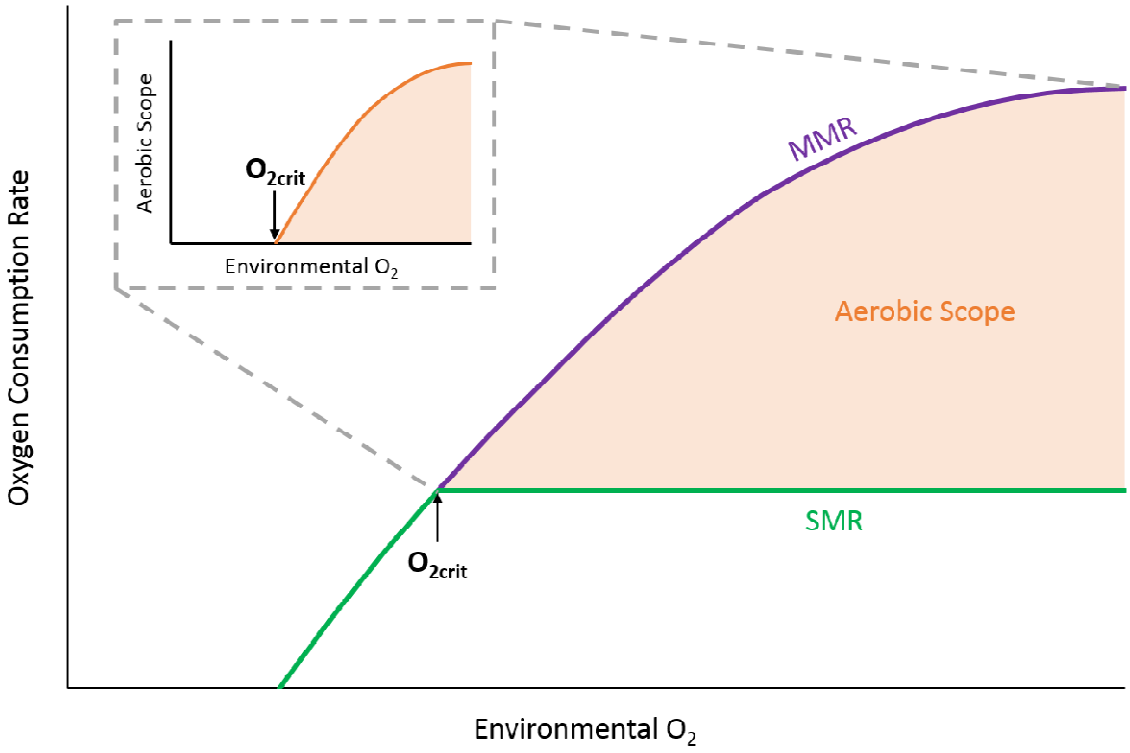


**Figure 2**: Conceptual illustration of the impact of environmental $O_2$ on standard metabolic rate (SMR, minimum $O_2$ consumption required to provide energy via aerobic metabolism for cellular maintenance), maximum metabolic rate (MMR, maximum possible $O_2$ consumption and aerobic energy production), and aerobic scope (i.e. potential energy available for non-essential processes such as growth). The $O_2$ level at which aerobic scope becomes zero is referred to as the critical $O_2$ tension ($O_{2crit}$) and represents the hypoxia tolerance threshold of the fish.

As can be seen in Figure 2, environmental $O_2$ declines have to be relatively large before effects on SMR are observed. In contrast, even moderate hypoxia, which would be more likely to occur over large geographical areas, results in the reduction of MMR and therefore aerobic scope (Claireaux and Chabot, 2016; Seibel and Deutsch, 2020). The reduced energy available for costly processes such as digestion limits food intake (Pichavant *et al.*, 2001) which, over longer time periods, will result in reduced growth rates (Wang *et al.*, 2009). As such, it is clear that the effects of hypoxia on metabolism can lead to similar consequences as those proposed for temperature.

## 2.3. Carbon dioxide

In contrast to temperature and $O_2$, the influence of $CO_2$ on aerobic metabolism is less clear. Initially increased environmental $CO_2$ (hypercapnia) leads to a respiratory acidosis in fish (Brauner *et al.*, 2019). As a result of the pH sensitivity of Hb this then leads to decreases in Hb-$O_2$ affinity and, theoretically, decreased $O_2$ transport capacity (Heuer and Grosell, 2014). As changes in pH disrupt cellular functioning acid-base balance is one of the most tightly controlled biological processes – therefore the majority of fish have highly developed regulatory mechanisms to compensate for $CO_2$ induced respiratory acidosis (Perry and Gilmour, 2006; Esbaugh, 2017). As such the initial acidosis caused by increased environmental $CO_2$ is regulated within hours to days (depending on magnitude of $CO_2$ change and species of fish). While this restores Hb-$O_2$ affinity the associated changes in acid-base balance, ion regulation and cardiorespiratory processes have been hypothesised to cause a loading stress (i.e. increased energetic cost) and increase the SMR of fish (Farrell, 2016; Heuer and Grosell, 2016a). While this has been documented in some species (Munday *et al.*, 2009) meta-analysis has indicated that the majority of studies indicate no impact of elevated $CO_2$ on SMR (Lefevre, 2016, 2019).

A second possible mechanism through which hypercapnia could affect metabolism is via limiting stress on MMR (Heuer and Grosell, 2014). However, this has been hypothesised to be a result of the acidification of blood and subsequent reduction of $O_2$ uptake. As fish generally regulate blood pH when exposed to hypercapnia there would appear to be limited support for decreased MMR in response to hypercapnia after the initial respiratory acidosis is regulated (Lefevre, 2019). However, $CO_2$ may impact on other aspects of the cardiorespiratory system that may also limit MMR. For example, increased $CO_2$ can affect cardiac performance (Crespel *et al.*, 2019) and mitochondrial respiration (Strobel *et al.*, 2013a; Leo *et al.*, 2017a) To date there has been limited investigation of the impacts of increased $CO_2$ on MMR of fish (Hannan and Rummer, 2018). However, in the studies that have been conducted (26 identified by Hannan and Rummer, 25 identified by Lefevre) the majority (~70 %) report no effects of hypercapnia on MMR with the remaining studies (~30 %) providing approximately equal evidence for both increased and decreased MMR

(Hannan and Rummer, 2018; Lefevre, 2019). The lack of a clear effect of hypercapnia on SMR and MMR predictably result in little evidence for generalised effects on aerobic scope (Lefevre, 2019). Consequently, it would appear that hypercapnia is unlikely to affect whole organism fitness via impacts on aerobic scope in the same manner as temperature or $O_2$.

## 2.4. Combined impacts of temperature, $O_2$, and $CO_2$?

Whilst most experiments have studied these variables independently, the responses of fish to climate change will be a result of the combined impacts of temperature, $O_2$, and $CO_2$. Combined effects of multiple stressors can act in an additive (1 + 1 = 2), synergistic (1 + 1 > 2), or antagonistic (1 + 1 < 2) manner (Crain *et al.*, 2008). The potential for warming, decreased $O_2$, and increased $CO_2$ to cause non-linear (i.e. synergistic or antagonistic) interactive effects on organisms has been highlighted as a key issue by marine scientists (Rudd, 2014). If non-linear interactions occur this will prevent accurate predictions of impacts of climate change on organisms from studies investigating each factor separately. It is predicted that non-linear effects are most likely to occur where factors act on different, but dependent, mechanisms (Kneitel and Chase, 2004). Accordingly, combined changes in temperature, $O_2$, and $CO_2$ (which can individually impact metabolic rate via effects on different physiological processes) may result in non-linear impacts on aerobic metabolism which may result in unexpected impacts on organism fitness. Indeed, this concept has been developed into the well-known hypothesis of Oxygen- and Capacity-Limited Thermal Tolerance (OCLTT), proposed by Pörtner (2001), as a single unifying theory to predict combined impacts of changes in temperature, $O_2$, and $CO_2$ on aerobic metabolism of organisms.

According to the OCLTT hypothesis, organismal fitness is maximised at the temperature where aerobic scope is optimised as this represents the temperature where there is the maximum amount of available energy to invest in processes such as growth and reproduction (Pörtner and Knust, 2007). Therefore, any factors which result in a lowering of aerobic scope will reduce organism fitness and changes in aerobic scope can be used as a simple metric for predicting impacts of climate change on organism populations. The simplicity of this hypothesis has made it broadly appealing and there has been widespread adoption and discussion of the OCLTT since it was first proposed.

Building on the initial hypothesis, Pörtner and Farrell (2008) suggested that decreased $O_2$ and increased $CO_2$ in the environment will synergistically interact to lower aerobic scope across its temperature performance curve (Figure 3). However, experimental work which has sought to quantify interactive effects of temperature and $CO_2$ on metabolism has shown that many fish species do not conform to predictions from the OCLTT hypothesis (Lefevre, 2016) and this has led to general questioning of the applicability of the hypothesis (Clark *et al.*, 2013; Schulte, 2015; Nilsson and Lefevre, 2016; Jutfelt *et al.*, 2018). However, to date there has been little investigation of combined impacts of changes in all three of temperature, $O_2$, and $CO_2$ on cardiorespiratory physiology and metabolism of fish. This is concerning as it has been predicted that occurrence of non-linear interactions is twice as likely when combined changes in three, rather than two, factors occur (Crain *et al.*, 2008) Therefore, this thesis aimed to investigate the interactive effects of combined changes in temperature, $O_2$, and $CO_2$ on the cardiorespiratory physiology and metabolism of fish.

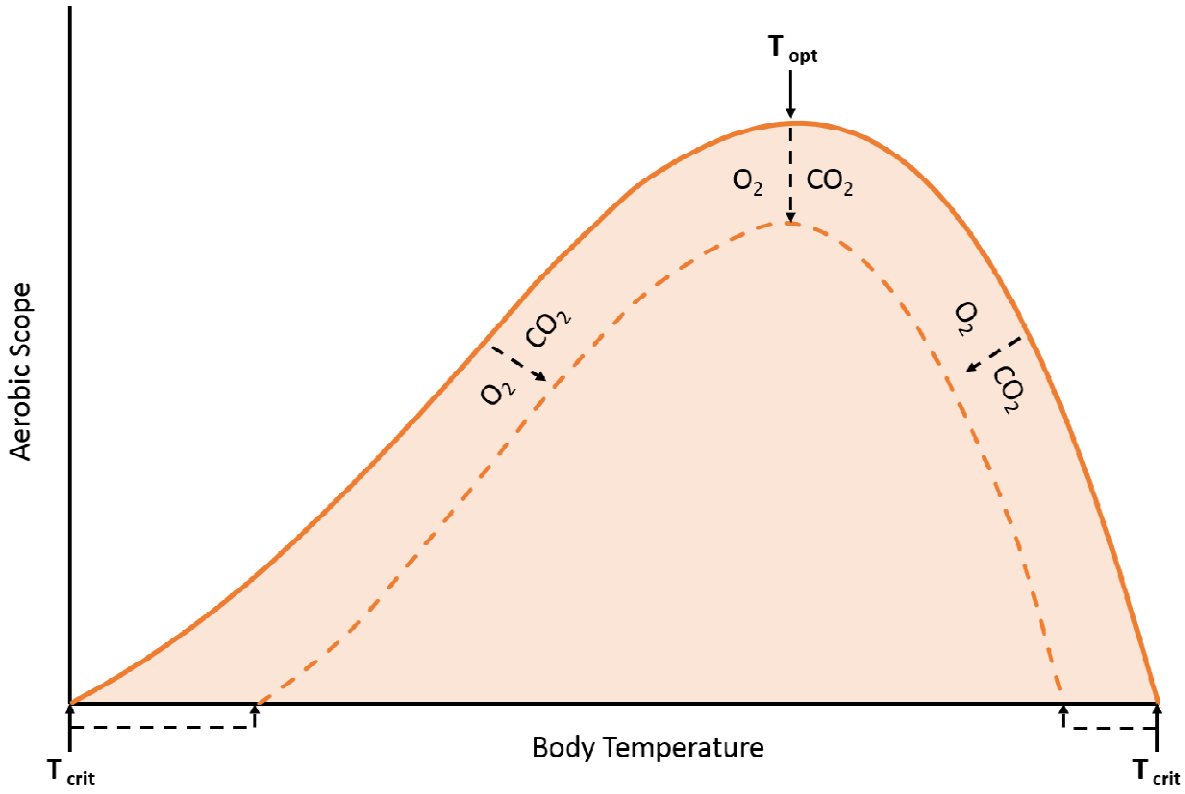


**Figure 3:** Conceptual illustration of the synergistic impact of decreased $O_2$ and increased $CO_2$ on the thermal performance curve (solid line) of aerobic scope. The dotted line indicates the hypothesised thermal performance curve of aerobic scope when fish are subjected to additional impacts of reduced

environmental $O_2$ and increased $CO_2$ (adapted from Pörtner and Farrell, 2008). The change in the thermal performance curve leads to reduced aerobic scope at the organism's optimum temperature ($T_{opt}$) and a narrowing of the available temperature range (increased low temperature $T_{crit}$ and decreased high temperature $T_{crit}$).

## 3. Thesis Outline

This thesis presents four years of my work investigating physiological responses of fish to combined changes in environmental temperature, $O_2$, and $CO_2$. In **chapter II** I conducted a review of the literature to identify the context in which fish will experience combined changes in temperature, $O_2$, and $CO_2$, where non-linear interactive effects on physiology of fish are most likely to occur, provide potential explanations for variation in results, and suggest possible pathways to improve future research. The remaining four chapters present the results of experimental work to investigate how combined changes in temperature, $O_2$, and $CO_2$ may affect physiology of fish within different environmental contexts (Figure 4). Although climate change is causing a steady gradual increase in $CO_2$ levels, fish can also be exposed to rapid and acute fluctuations of $CO_2$ which naturally occur in many aquatic environments (Baumann *et al.*, 2015; Helmuth *et al.*, 2016). To date interactions between acute, large increases in $CO_2$ and other environmental challenges such as increased temperature or reduced $O_2$ have received less attention than interactions involving ocean acidification. As such, in **chapter III** and **IV** I focus on responses of fish to environmentally relevant rapid changes in $CO_2$ and the potential for interactive effects between rapid $CO_2$ changes and other environmental variables on physiological performance of fish.

Fish species that live in environments where acute fluctuations of $CO_2$ occur will face physiological challenges. For example, rapid changes in environmental $CO_2$ levels disrupt internal acid-base balance of fish, resulting in altered pH of body fluids, which will impact on enzyme functioning and, crucially, $O_2$ transport by haemoglobin. Consequently, fish species that live in habitats that experience large natural fluctuations in $CO_2$ may have adaptations to mitigate impacts of acute $CO_2$ change on physiological performance. For example, most marine fish species possess the ability to rapidly regulate acid-base disturbances in

order to prevent negative impacts of changes in environmental $CO_2$ on physiological performance (Brauner *et al.*, 2019). It may be that species which inhabit habitats where $CO_2$ levels naturally fluctuate have developed acid-base regulatory mechanisms which act more rapidly than those of species that inhabit environments where $CO_2$ levels are more stable. Specifically, I would predict that fish species which experience acute natural $CO_2$ fluctuations should be able to regulate acid-base disturbances in a shorter time frame than disturbances caused by $CO_2$ changes in the environment usually occur. Additionally, regulation of acid-base disturbances should prevent impacts of fluctuating $CO_2$ on key functions such as $O_2$ transport. To test this hypothesis, in **Chapter III** I investigate the time course of acid-base regulatory responses of European sea bass (*Dicentrarchus labrax*, a species which inhabits coastal estuaries and salt marshes during summer months when large natural variations in $CO_2$ can occur) after acute exposure to elevated $CO_2$ (hypercapnia). I aim to identify impacts of acute $CO_2$ exposure and subsequent acid-base regulation on $O_2$ transport capacity and what mechanisms sea bass use to regulate respiratory acidosis and blood $O_2$ transport.

Impacts of acute changes in $CO_2$ on fish may be most important during hypoxia, as biological respiration which causes reduced $O_2$ also produces $CO_2$ (Melzner *et al.*, 2013; Wallace *et al.*, 2014). As a result, when fish are exposed to hypoxia in the environment they are almost always simultaneously exposed to hypercapnia. Previous research has shown that simultaneous exposure to elevated $CO_2$ during hypoxia can result in interactive effects which increase the impacts of reduced $O_2$ on fish (DePasquale et al., 2015; Gobler and Baumann, 2016). I hypothesised that simultaneous increases in $CO_2$ as $O_2$ declines may lower hypoxia tolerance of fish by reducing haemoglobin-$O_2$ affinity and decreasing $O_2$ uptake from water into the blood. Therefore, in **chapter IV** I investigate whether including environmentally realistic increases in $CO_2$ during $O_2$ declines affects the critical $O_2$ tension (a proxy for hypoxia tolerance) of European sea bass. In addition, I determine whether any changes in hypoxia tolerance of sea bass are linked to blood chemistry parameters and haemoglobin-$O_2$ affinity of sea bass.

In the second two experimental chapters I focus on investigating interactions between temperature, $O_2$, and $CO_2$ to determine if they correspond with

predictions from the OCLTT hypothesis. One of the predictions of the OCLTT hypothesis is that reductions in aerobic scope as a result of decreased $O_2$ or increased $CO_2$ will reduce upper temperature tolerances of fish (Pörtner and Farrell, 2008). Studies testing these predictions have so far focussed on longer term exposures to $CO_2$ however impacts of $CO_2$ on aerobic scope are likely greatest immediately after acute exposure when respiratory acidosis reduces haemoglobin-$O_2$ affinity (Mairbäurl and Weber, 2012). Furthermore, existing evidence suggests that some fish species do not show reduced $CT_{max}$ when aerobic scope is decreased (e.g. Ern *et al.* 2016), as proposed by the OCLTT. This raises questions as to what the underlying mechanisms behind $CT_{max}$ are. Several alternate mechanisms have been raised including mechanisms which may be pH sensitive. As a result acute exposures to $CO_2$ may impact $CT_{max}$ via direct effects of respiratory acidosis rather than the associated impacts on $O_2$ transport capacity. Therefore, I hypothesised that acute exposures to elevated $CO_2$ could impact $CT_{max}$ either via reduced $O_2$ transport capacity (as predicted by the OCLTT hypothesis) or via direct effects of reduced pH of body fluids (which could provide evidence to support alternate underlying mechanisms of $CT_{max}$). Consequently, in **chapter V** I investigate whether critical thermal maximum ($CT_{max}$) of rainbow trout is reduced after acute exposure to increased $CO_2$ in combination with either normal or increased environmental $O_2$. By doing so I sought to isolate impacts of acutely increased $CO_2$ on $CT_{max}$ as either a result of decreased pH or reduced $O_2$ transport capacity and so improve understanding of the mechanisms which determine critical thermal limits of fish.

The OCLTT hypothesis also predicts that decreased $O_2$ and increased $CO_2$ will reduce the aerobic scope of fish across its temperature performance curve (Figure 3). Although research has investigated combined impacts of temperature change and decreased $O_2$ or increased $CO_2$ on aerobic scope, to date no studies have investigated interactive effects between all three environmental variables upon aerobic scope of a fish species. Therefore, in **Chapter VI** I expose European sea bass to combinations of warming, hypoxia, and hypercapnia to determine if interactive effects occur on aerobic scope as would be predicted by the OCLTT hypothesis. I also aim to determine if impacts of increased temperature or $CO_2$ on metabolic rate affect hypoxia tolerance

($O_{2crit}$) of sea bass and if changes in aerobic performance are linked to blood chemistry or $O_2$ transport capacity of blood.

Finally, in **chapter VII** I provide a general discussion about the findings of the work presented in the thesis, including the limitations of experimental approaches used, with personal suggestions about the implications for the impacts of climate change on fish and directions for future research.

Each chapter is presented as a standalone paper and so are written to be understood outside the context of this thesis, with their own specific introduction and discussion sections. **Chapter IV** has already been published (Montgomery *et al.*, 2019) with the remaining chapters either being under review or currently being prepared for submission to peer reviewed journals.

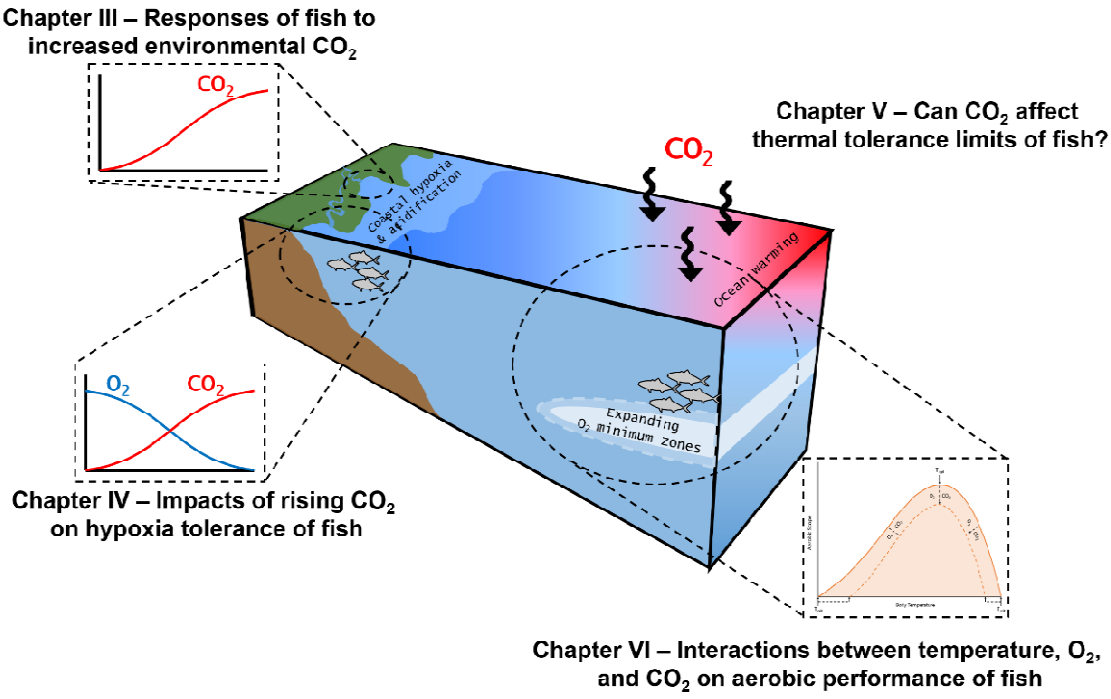


**Figure 4:** Graphical summary of the four experimental chapters presented in this thesis. Each is designed to investigate interactive effects between combined changes in temperature, $O_2$, and $CO_2$ to improve our understanding of how fish may be physiologically affected by climate change.

# Chapter II

## A ‘deadly trio’ of marine environmental change? Past progress and future recommendations for investigating interactive effects of warming, rising $CO_2$ and hypoxia on fish

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**Author Contributions:** D.W.M. was primarily responsible for the concept of the review, conducting the literature search, analysing the literature, and writing the chapter. S.N.R.B., G.H.E., S.D.S., and R.W.W. commented on drafts of the chapter.

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## Abstract

Anthropogenic climate change is associated with warming, reduced $O_2$, and increasing $CO_2$ in the world's oceans. These combined factors, referred to as a "deadly trio" of marine environmental change, interact to cause biological effects on marine fish which may have population-level consequences. Interactions can be non-linear which presents a challenge when attempting to predict impacts of climate change using data from single factor studies. The potential for combinations of warming, reduced $O_2$, and rising $CO_2$ to affect fish will depend on regionally and locally specific patterns of change as well as the type of habitats which species live in. We identified 68 primary research articles that investigated interactive effects of climate change factors on marine fish. There are large discrepancies in research effort with ~56 % of studies focussing exclusively on combinations of temperature and $CO_2$. Only one study included manipulations of all three of these factors simultaneously. Most studies focused on physiological measures of impact, and there was large variability in the prevalence of non-linear interactions between measurement types and species. Further studies are needed to address critical gaps in knowledge related to ecologically important processes, such as growth and reproduction. Experiments also need to give greater consideration to the magnitude and duration of treatments used in order to increase the relevance of results. We believe that a combination of scenario-based (i.e. specific documented or predicted environmental conditions for a particular location or species) and mechanistic approaches (fully factorial studies across a wide range of environmental conditions) can lead to an improvement in the predictive power of multi-factor experiments. Ultimately, providing much-needed evidence to develop management strategies to mitigate impacts of climate change on key fish species.


## 1. Introduction

The oceans are facing major changes as a result of increasing concentrations of greenhouse gases in the atmosphere. Increasing emissions of carbon dioxide ($CO_2$) from human activities is resulting in warming of the surface ocean as a result of global atmospheric temperature rise (Lyman *et al.*, 2010; Bopp *et al.*, 2013). As $CO_2$ levels increase in the atmosphere there is a

parallel increase in the $CO_2$ content of the oceans. This causes a decrease in pH and is referred to as ocean acidification (Caldeira and Wickett, 2003).

While research has focussed on warming and acidification as the main physical impacts of the changing climate on marine systems, oxygen ($O_2$) in the world's oceans has also been declining (Bopp *et al.*, 2013). Increasingly areas of the oceans are experiencing hypoxia, conditions where $O_2$ levels fall so low that animals struggle to survive (usually defined as areas where oxygen content is below 63 μmol $L^{-1}$/~2 mg $L^{-1}$). In the most extreme cases anoxia occurs (when $O_2$ is absent) which results in fish kill events (Thronson and Quigg, 2008; Becker *et al.*, 2009; La and Cooke, 2011). The prevalence and severity of these hypoxic events is predicted to increase as a result of climate change (Meire *et al.*, 2013; Altieri and Gedan, 2015; Breitburg *et al.*, 2018). In the open oceans low $O_2$ conditions are expanding as the planet warms and the area of ocean with $O_2$ contents below 70 μmol $L^{-1}$ has increased by ~15 % (4.5 million $km^2$) in the last 40 years (Diaz and Rosenberg, 2008; Paulmier and Ruiz-Pino, 2009; Stramma *et al.*, 2010). In shelf seas, hypoxic zones were reported to cover a global area roughly the size of the United Kingdom in 2008, and the number of reported hypoxic zones has been doubling every decade since 1960 (Diaz and Rosenberg, 2008). This means there will be increasing impacts of hypoxic zones/events on marine fish (Schmidtko *et al.*, 2017).

We know that warming, reduced $O_2$, and increased $CO_2$ individually impact on marine organisms and these three factors have been identified as a 'deadly trio' of marine climate change in recognition of their co-occurrence during previous major ocean extinction events (Bijma *et al.*, 2013). As ectotherms, fish are particularly sensitive to warming seas, with body temperature and therefore their metabolism closely linked to ambient conditions. Additionally, changes in both environmental $CO_2$ and $O_2$ influence respiratory gas exchange across the gills and blood gas content (Randall and Daxboeck, 1984). Thus, environmental changes in temperature, $CO_2$ and $O_2$ can lead to direct impacts on the physiological state of individual fish. For example, warming increases the resting energetic demands of fish, and when fish are exposed to temperatures above their physiological optimum reduces their exercise performance (Fry and Hart, 1948; Fry, 1971; Nilsson *et al.*, 2009). In addition, warming affects growth and reproduction of individuals as well as population distributions (Neuheimer *et*

*al.*, 2011; Pankhurst and Munday, 2011; Hastings *et al.*, 2020). Elevated $CO_2$ conditions can impact upon the sensory abilities of fish (Simpson *et al.*, 2011b; Porteus *et al.*, 2018; Heuer *et al.*, 2019), and also modify physiological processes, such as acid–base regulation (Heuer and Grosell, 2014; Brauner *et al.*, 2019). Declining $O_2$ levels can constrict the habitat available to commercially and ecologically valuable species (Stramma *et al.*, 2012), whilst also reducing growth and reproduction (Pichavant *et al.*, 2001; Wu, 2009). Hypoxic events lead to both sub-lethal effects and the potential for increased mortality in fish and other organisms (Thronson and Quigg, 2008; Altieri *et al.*, 2017). As fish are affected by individual changes in temperature, $CO_2$, and $O_2$ levels, concurrent changes of all three are highly likely to result in interactive effects (Pörtner *et al.*, 2014), so it is pertinent to ask how fish will cope with the combined challenges of a lack of $O_2$ in the warmer, acidified oceans we are creating?

Improving our understanding of interactive effects of these environmental factors is vital to support models that can predict effects of climate change (Pörtner and Peck, 2010; Doney *et al.*, 2011; Wernberg *et al.*, 2012; Hollowed *et al.*, 2013; Gordon *et al.*, 2018), but until recently few experimental studies have investigated impacts of combined environmental changes on marine fish. Here we seek to a) summarise the complicated inter-relationships between changes in temperature, $O_2$, and $CO_2$ that fish will experience, b) review evidence of their interactive effects on marine fish, and c) provide recommendations to improve the accuracy, relevance and predictive power of future studies.

## 2. When will fish be exposed to combined warming, hypoxia, and high $CO_2$?

Anthropogenic changes in aquatic temperature, $O_2$, and $CO_2$ are often considered in isolation (Diaz and Rosenberg, 2008; Doney *et al.*, 2009; Feeley *et al.*, 2009). Yet these factors regularly occur together in marine systems (Keeling *et al.*, 2009; Melzner *et al.*, 2013; Boyd *et al.*, 2015), and in most instances in nature responses of fish to climatic change will be the result of exposure to two or more of these factors changing simultaneously. The extent to which individual fish species and populations are exposed to combinations of

these three factors will largely depend on the geographic regions they inhabit, local oceanographic conditions and the ecological traits of a given species. As such, the first step to identifying how fish will be affected is to recognise when and where these combined effects will take place.

Rising average global temperature and atmospheric $CO_2$ content means that fish species living in the surface oceans will experience ~1-3 $^oC$ of warming and a rise of up to 600 µatm $CO_2$ over the next 80 years (IPCC, 2014). This will be accompanied by a reduction of ~1-7 % in the total $O_2$ content of the world's oceans (Keeling *et al.,* 2009). However, average global changes in these environmental variables are not a result of uniform changes across the planet but result from regionally specific patterns of change.

Instances of reduced $O_2$ content include oxygen limiting zones (OLZ = $O_2$ < 100 µmol $L^{-1}$) and oxygen minimum zones (OMZ = $O_2$ < 20 µmol $L^{-1}$) in the open ocean (Gilly *et al.*, 2013; Breitburg *et al.*, 2018). Globally, ocean warming is contributing to de-oxygenation by the expansion of widespread OMZs in the tropical open oceans (Stramma *et al.*, 2008). Oxygen limiting zones are most widespread in the thermoclines of the Eastern Tropical North & South Pacific, Eastern Tropical South Atlantic and Arabian Sea where oxygen content regularly falls below 100 µmol $L^{-1}$ (Doney, 2010; Breitburg *et al.*, 2018). The expansion of these OLZs and OMZs will increase the prevalence of hypoxic conditions in adjacent shelf seas as a result of seasonal upwelling events (Pierce *et al.*, 2012) . Additionally, shoaling of the OLZ boundary to a shallower depth has the potential to spread permanent low $O_2$ zones into shelf seas (Gilly *et al.*, 2013). In coastal regions not associated with oceanic OMZs changes in oceanographic currents have been linked to predictions of long term declines in $O_2$ content to ~75 % of present levels by 2100 (Claret *et al.*, 2018). Regional low $O_2$ can also occur as a result of large-scale eutrophication driven hypoxic zones. In extreme cases these large hypoxic zones can become established over many years and can result in $O_2$ conditions continuously below 2 mg $L^{-1}$ for many months each year (Carstensen and Conley, 2019; Rabalais and Turner, 2019). Globally, hypoxic zones are predicted to experience average temperature increases of 2 °C by the end of the century and this warming will cause increased stratification in the water column which reduces mixing, preventing replenishment of deeper waters with $O_2$ from surface waters. Rising

temperatures will also increase bacterial $O_2$ demand thus driving even greater declines in $O_2$ content, while also exacerbating the effects of hypoxia by increasing $O_2$ requirements of ectotherms (Altieri and Gedan, 2015).

Coastal hypoxic events are primarily driven by eutrophication (enrichment with chemical nutrients). This results in high primary productivity and the export of large amounts of organic matter down the water column. Subsequent breakdown of organic matter by bacteria has a large $O_2$ demand, leading to hypoxic conditions in subsurface waters (Rabalais *et al.*, 2009). By definition, these low $O_2$ areas also experience elevated $CO_2$ (and consequently reduced pH) through the same process, i.e. high rates of bacterial respiration (Breitburg *et al.*, 2018). The high $CO_2$ associated with hypoxia has been recently highlighted in marine systems, such as the Bohai Sea in China, the northern Gulf of Mexico (Cai *et al.*, 2011; Zhai *et al.*, 2012), Puget Sound in Canada (Reum *et al.*, 2014) and Long Island Sound in eastern USA (Wallace *et al.*, 2014). Thus, regional patterns of $O_2$ decline will frequently be mirrored by areas of increased $CO_2$ as a result of elevated bacterial respiration during hypoxic events.

Reciprocal changes in $O_2$ and $CO_2$ can be predicted, both for present day climatic conditions as well as for ocean conditions representing different future carbon emission scenarios (Representative Concentration Pathways [RCP] scenarios; IPCC, 2014). When $O_2$ concentrations fall below approximately 30 % air saturation (~63 µmol $L^{-1}$, i.e. the threshold commonly used for defining hypoxic systems), the resulting $CO_2$ level is at least 4 times the current open ocean surface $CO_2$ levels (which are approximately 413 µatm) in all atmospheric $CO_2$ scenarios and between 1.5 and 4 times higher than the end of century predictions (approximately 1000 µatm) commonly used for ocean acidification experiments (Figure 1). Furthermore, in anoxic systems $CO_2$ levels will increase to between 4,000 and 6,000 µatm. This is equivalent to $CO_2$ levels which, under conditions of normal $O_2$ availability (i.e., under normoxic conditions), are known to cause a range of deleterious physiological impacts on fish and invertebrates, including reduced growth and impaired immune function (Ellis *et al.*, 2016; Mota *et al.*, 2019, 2020).

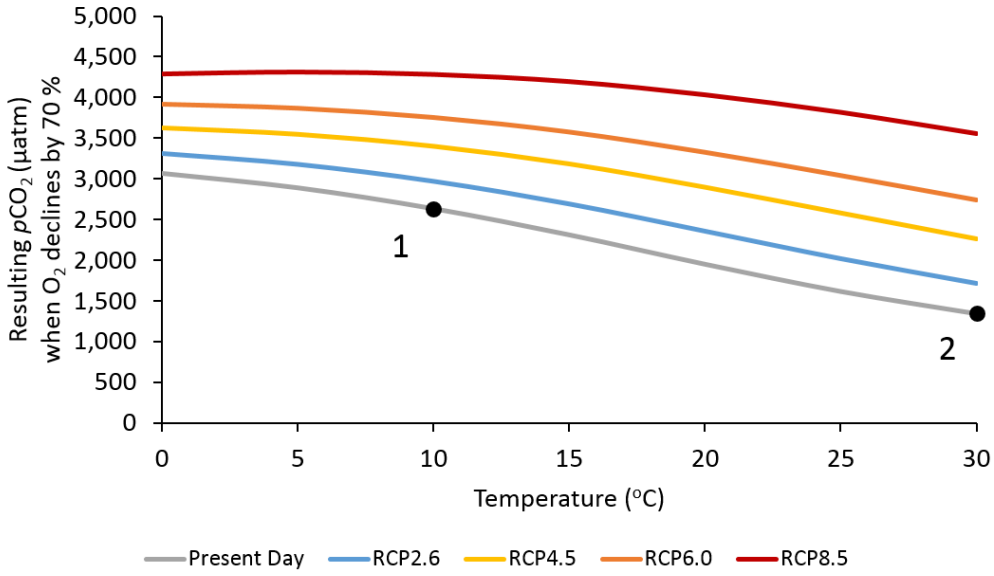


**Figure 1:** Predicted increases in $CO_2$ level caused by bacterial respiration at different temperatures when $O_2$ content is depleted by 70% from air saturated levels. Highest resulting $pCO_2$ levels occur in colder temperatures. For example, under current ambient $CO_2$ levels (400 µatm) a decrease in $O_2$ level by 70 % resulted in ~2-fold higher $CO_2$ levels at 10°C (Point 1) than 30 °C (Point 2). This is mostly a result of higher $O_2$ solubility of seawater resulting in a greater change in $O_2$ content (and therefore production of $CO_2$) at the colder temperature when oxygen is depleted to 30% air saturation. Production of $CO_2$ by bacteria is based on an assumed respiratory quotient of 1 (based on Del Giorgio and Duarte, 2002). Differences in resultant $pCO_2$ are indicated across a range of initial $pCO_2$ (different coloured lines) based on present day atmospheric $CO_2$ (~400 µatm) and four IPCC RCP scenarios (from IPCC, 2014). Calculations of the increase in $pCO_2$ during respiration-driven hypoxia events were performed using the CO2SYS program (Pierrot *et al.*, 2006) using a salinity of 35, initial average $pCO_2$ and pH values of oceanic seawater predicted under the various RCP scenarios (IPCC, 2014), and assuming no change in total alkalinity with increasing $pCO_2$ (Gattuso *et al.*, 2010).

Projected increases in average global sea surface temperatures also result from regionally specific patterns. While temperatures in open ocean areas are currently increasing at approximately 0.1 °C per decade, coastal seas in Europe, East Asia and Eastern Canada have shown rapid warming of ~0.5 °C per decade (Belkin, 2009). In these regions temperatures may increase by 2-3 °C by the mid-21st century (Schrum *et al.*, 2016). On top of changes in average

ocean temperatures, large marine heatwave (MHW) events can result in rapid warming of between 2 and 5 $^{o}$C above ambient temperatures over a time period of days to weeks (Hobday *et al.*, 2018) . The frequency and duration of MHWs are predicted to increase as a result of climate change (Frölicher *et al.*, 2018), with the number of days each year that MHWs occur increasing by 54 % between 1925 and 2016 (Oliver *et al.*, 2018). Marine heatwaves are most likely to occur in western and eastern boundary current regions (Holbrook *et al.*, 2019) with areas of the Arctic Ocean, North-West Atlantic, North Pacific and tropical West Pacific predicted to see greatest increases in MHWs as a result of climate change (Frölicher *et al.*, 2018; Oliver *et al.*, 2019).

Overall, regional changes in these three environmental factors may not impact upon an entire species' range (that may occupy a large geographic area) but will have large effects on individual populations and sub-populations at a scale which is relevant for resource management and policy (e.g. at a Large Marine Ecosystem or FAO/ICES Fishing Areas scale) (Mills *et al.*, 2013). However, fish are not only affected by large scale changes in temperature, $O_2$ and $CO_2$. Individual organisms will also experience changes in localised environmental conditions (Bates *et al.*, 2018) over time scales of minutes to days (e.g. diel and tidal fluctuations which influence temperature as well as $O_2$/$CO_2$) and over spatial scales as small as centimetres to metres (Helmuth *et al.*, 2016). On these low scales environmental variations can be many times greater than even the most extreme events at larger scales (e.g. daily temperature variations of > 10 $^{o}$C and pH change of 0.5-1.0 units) (Hofmann *et al.*, 2011; Helmuth *et al.*, 2016). Understanding how climate influences variability at the local level will be key to determining how individual fish will respond to combined changes in the environment. This is because the mortality, growth and reproduction of individual organisms will be determined by the interactions between temperature, $CO_2$ and $O_2$ that the individual experiences. As population level responses are ultimately driven by the combined performance of the individuals within that population, the interactive effects at a local scale will contribute to responses of fish at a wider level (Helmuth *et al.*, 2014).

As well as considering how changes in temperature, $O_2$, and $CO_2$ individually could combine to affect fish species there is growing evidence that

combinations of warming, hypoxia, and rising $CO_2$ lead to interactions which can themselves be non-linear in nature (Oschlies *et al.*, 2008; Melzner *et al.*, 2013; Shaw *et al.*, 2013). This can lead to an exacerbation of each of these three individual environmental conditions and increase impacts on fish. Hypoxic events provide a good case study system to demonstrate biogeochemical interactions between the 'deadly trio'. For example, the increase in $CO_2$ during a respiration-induced hypoxic event becomes greater as global ambient ocean $CO_2$ rises (i.e. at higher global $CO_2$ values according to the RCP scenarios, Figure 2).

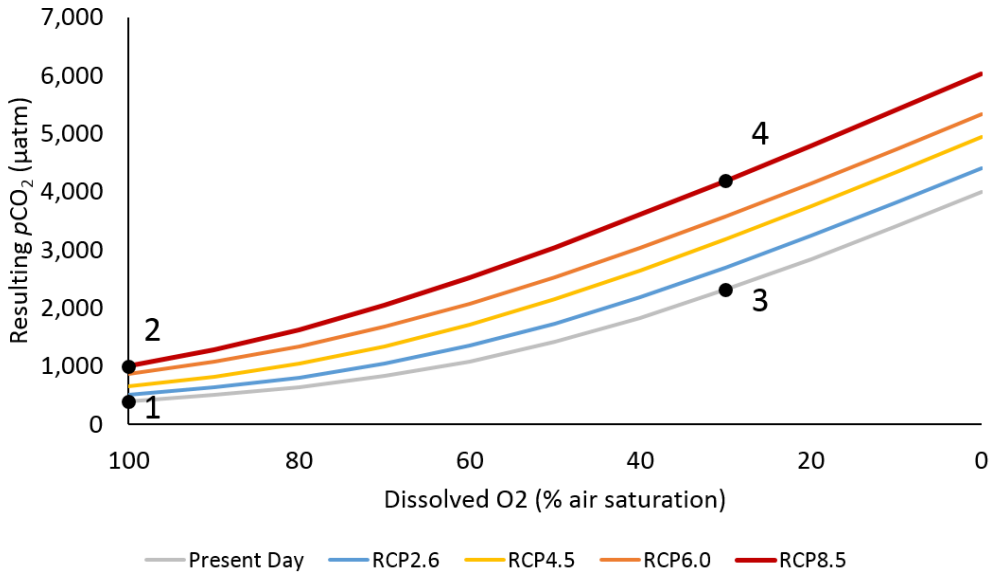


**Figure 2:** Increasing $pCO_2$ as a result of respiratory driven decrease in $O_2$ content during hypoxic events at a temperature of 15 $^{o}$C. Differences in resultant $pCO_2$ are indicated across a range of normoxic $pCO_2$ (different coloured lines) based on current atmospheric $CO_2$ and four IPCC RCP scenarios for the year 2100 (from IPCC, 2014). There is a greater increase in $CO_2$ as $O_2$ declines for higher atmospheric $CO_2$ conditions. For example, in normoxic conditions $CO_2$ levels in present day conditions are ~600 µatm lower than expected $CO_2$ levels under RCP 8.5 in 2100 (point 2). However, when $O_2$ content decreases by 70 % the difference between $CO_2$ levels in present day conditions (point 3) and under RCP 8.5 conditions in 2100 (point 4) are ~3 fold greater than in normoxia. Production of $CO_2$ by bacteria is based on an assumed respiratory quotient of 1 (Del Giorgio and Duarte, 2002). Calculations of the increase in $pCO_2$ during respiration-driven hypoxia events were performed using the CO2SYS program (Pierrot *et al.*, 2006) using a salinity of

35, and initial average $pCO_2$ and pH values of oceanic seawater predicted under the various RCP scenarios (IPCC, 2014), and assuming no change in total alkalinity with increasing $pCO_2$ (Gattuso *et al.*, 2010).

The greater increase in $CO_2$ during hypoxia for higher atmospheric $CO_2$ conditions is exacerbated at higher temperatures (Figure 1). This suggests a synergistic interaction such that both warmer and higher $CO_2$ starting points result in ever increasing $CO_2$ values being reached for a given low $O_2$ level (% air saturation) during hypoxic events. The synergistic interaction between climate change induced high $CO_2$ and eutrophication driven high $CO_2$ has been modelled for hypoxic zones in the northern Gulf of Mexico and the Baltic Sea, where $pCO_2$ levels have been predicted to reach 3,400 – 4,500 µatm during hypoxic events (in comparison to maximum values of ~1700 – 3200 µatm today) when ambient $CO_2$ levels equal 1000 µatm (Sunda and Cai, 2012; Melzner *et al.*, 2013).

Oxygen limiting zones in the open ocean will also be affected by biogeochemical interactions between $CO_2$ and $O_2$ (Paulmier *et al.*, 2011). Occurrence of high surface $pCO_2$ (as a result of rising atmospheric $CO_2$) can further reduce dissolved $O_2$ levels in OMZs by increasing the C:N ratio of particulate organic matter exported from surface waters. This is a departure from standard Redfield stoichiometry (Oschlies *et al.*, 2008). Bacteria require more $O_2$ to process this organic matter, as the majority of $O_2$ demand during organic material re-mineralization is related to oxidation of carbon rather than nitrogen (Oschlies *et al.*, 2008). It has been suggested that this increase in $O_2$ requirement could lead to a 50 % increase in volume of oceanic OMZs by the end of this century (Oschlies *et al.*, 2008; Gruber, 2011). The fundamental connections between this trio of environmental change (both in terms of their chemical interactions in seawater and the biological responses to them) highlights the need to identify whether their cumulative effects on fish are additive, antagonistic or synergistic.

## 3. Review of past research

Ichthyologists have increasingly used physiological and behavioural experiments in order to understand and, ideally, predict how fish species will react to environmental change (reviewed by McKenzie *et al.*, 2016). Most

research has focussed on the effects of warming, high $CO_2$ or low $O_2$ in isolation (Lefevre, 2016; Nilsson and Lefevre, 2016). This has provided vital information on the individual mechanistic responses to these factors. However, it is unlikely to be sufficient to predict future effects if this trio of change has non-linear interactions. Multiple stressors can interact in either an additive (i.e. 1 + 1 = 2), antagonistic (i.e. 1 + 1 <2) or synergistic manner (i.e. 1 + 1 >2). Synergistic and antagonistic interactions are especially important for us to determine, as they could result in so called 'ecological surprises' (Crain *et al.*, 2008; McBryan *et al.*, 2013; Todgham and Stillman, 2013). To identify research in which interactive effects of warming, high $CO_2$ and low $O_2$ have been investigated in marine fish, we conducted several searches of the literature (in May 2019) using combinations of the key terms in a Boolean string in Thomson Reuters Web of Science (all databases): (Marine AND (Climat* OR Warm* OR Acidif* OR Anthropogenic OR Impacts) AND (Multi* OR Stress* OR Interact* OR Synerg* OR Non-linear) AND (Environment* OR (CO2 OR Carbon dioxide OR Acidifica* OR Hypercapn* OR Hypercarb*) OR (Temperat* OR Warm* OR Therm*) OR (Hypoxi* OR Oxygen)) AND (Fish OR Teleost* OR Shark OR Elasmobranch*) AND Experiment). We also searched a combination of the same terms in Google Scholar and checked the first 10 pages for each search. Studies were only included in which an interactive effect was investigated on a marine species in a laboratory setting. We also limited our search to studies from 1990 onwards, to coincide with the increased scientific research into effects of climate change following the publication of the 1st IPCC report (IPCC, 1990). This resulted in 68 primary research articles being identified in which a marine fish species was exposed to a combination of at least two of the three climate change stressors (Figure 3). The full details of all papers identified in the literature search can be found in the supplementary materials.

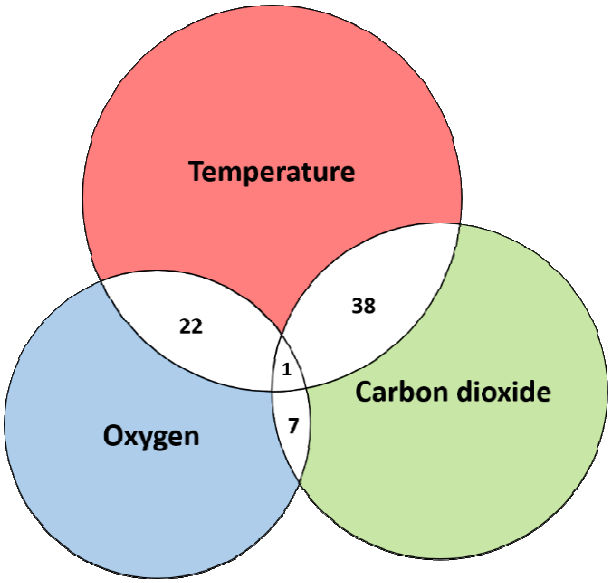


**Figure 3**: Number of primary research articles published which incorporate experiments testing the effects of at least two of warming, high $CO_2$ and low $O_2$ upon marine fish. Total number of studies = 68. Studies in which a diadromous fish species was exposed to stressors in fresh water were excluded as salinity has complicating influences on climate change stressors (Rogers *et al.*, 2016).

The need for more research on potential interactive effects has clearly been heeded with >70 % of the publications identified being published after 2015. This growth has been accompanied by an increasingly diverse range of biological responses measured in an expanding number of species. We further investigated each of the 68 studies to identify areas where non-linear interactions are most common. Where possible we determined the life stage and source of animals used, and duration and magnitude of exposure. We also classified the type of end measurements recorded into 14 categories: metabolism/aerobic performance, development and growth, mortality/survival, enzyme activity, environmental tolerance, blood chemistry, feeding, escape response, swimming performance/activity, reproduction, gene/protein expression, cellular stress response, cardiac/ventilatory response, and behaviour (e.g. boldness, laterality).

### 3.1. Evidence of non-linear interactions?

It has been proposed that non-linear interactions are most likely either when exposure to one factor induces tolerance to the other (antagonistic) or when factors act on different, but dependent, mechanisms (synergistic) (Blanck, 2002;

Kneitel and Chase, 2004; Harvey *et al.*, 2013). By contrast, additive effects may occur when both factors act via the same mechanism (Crain *et al.*, 2008; Harvey *et al.*, 2013). As such, knowledge of the mechanisms through which the trio individually affect marine fish may allow us to determine which categories of measurements would be most likely to display non-linear interactions.

Twenty out of the 68 studies included experiments where a species tolerance to one stressor was tested after exposure to a second stressor (e.g. hypoxia tolerance across a range of temperatures). Such studies, unless a second metric was measured, do not allow for a characterisation of the interaction between stressors but can give an indication of whether stressor combinations have positive or negative impacts on species. Within these 20 studies, 13 investigated impacts of warming on hypoxia tolerance, with all studies finding reduced hypoxia tolerance when temperatures were increased., Six studies have considered impacts of increased $CO_2$ on hypoxia tolerance, with half reporting negative effects of $CO_2$. One study has researched how thermal tolerance is affected by hypoxia or high $CO_2$. Severe hypoxia caused a drop in thermal tolerance but high $CO_2$ had no impact (Ern *et al.*, 2017).

Of the remaining 48 studies, 81 % (39) reported at least one instance of a non-linear interaction. Twenty-seven of these 39 studies reported antagonistic interactions and the same number reported synergistic interactions. This proportion of studies reporting non-linear interactions is similar to that identified in previous meta-analyses of the relationship of impacts caused by a range of other marine multi-stressors of 74 – 75 % reported by Crain et al. (2008) and Darling and Côté (2008). However, most studies incorporated multiple end measurements, with a total of 412 individual end measurements observed within the 68 studies. Sixty-two measurements did not allow an interactive effect to be determined, and of the remaining 350 measurements approximately 67 % (234) reported an additive (i.e. non-linear) interaction, ~16 % reporting an antagonistic effect (57) and ~17 % reporting a synergistic interaction (59).

### 3.1.1. Temperature x $CO_2$

To date the majority (56 %) of multi-factor studies have focussed on the temperature–$CO_2$ interaction, and the number of end measurements produced in these studies is ~6 x higher than the combined number of end measures for

interactions between temperature and $O_2$, or $CO_2$ and $O_2$ (Table 1). The larger research effort focussed on interactions between warming and rising $CO_2$ likely reflects a greater historical emphasis on these two factors than on hypoxia in IPCC reports and scientific publications. For example, temperature/warming are mentioned 206 times and carbon dioxide/$CO_2$ 108 times, compared to only two mentions of oxygen/$O_2$ in the IPCC 4$^{th}$ assessment synthesis report (IPCC, 2007). Non-linear interactions were reported from 96 end measurements, giving an overall rate of ~30 % (when discounting measurements from which interactive effects were not quantifiable).

Interactive effects of temperature and $CO_2$ have been theorised to occur for respiration and metabolism of fish as both temperature (Ege and Krogh, 1914; Clarke and Johnston, 1999; Clarke and Fraser, 2004; Steffensen, 2005) and $CO_2$ (Rummer *et al.*, 2013b; Heuer and Grosell, 2014, 2016) have been observed individually to impact on these processes. Non-linear interactive effects between these two have been observed in ~42 % of measurements of metabolism/aerobic scope, which include both synergistic and antagonistic effects on resting aerobic metabolic rates (oxygen consumption) (Ferrari *et al.*, 2015; Kreiss *et al.*, 2015; Dahlke *et al.*, 2016; Di Santo, 2016) as well as antagonistic effects on maximum metabolic rate (Di Santo, 2016) and antagonistic effects on aerobic scope (the difference between maximal and resting oxygen consumption rate – i.e. the potential for an animal to increase energy production above basal demands ) (Pope *et al.*, 2014; Di Santo, 2016).

Changes in aerobic scope have been proposed as a whole organism metric which can be used to evaluate impacts of environmental change on fish (Pörtner and Knust, 2007). Specifically, increased $CO_2$ and reduced $O_2$ are theorised to interact synergistically to reduce aerobic scope across an organism's thermal performance curve, which will subsequently impair ecologically important performance traits such as growth and reproduction (Pörtner, 2010; Bozinovic and Pörtner, 2015). As such, observations of non-linear interactions between warming and increased $CO_2$ upon metabolism and aerobic scope would be expected to lead to similar levels of non-linear interactions in measurements of ecologically important processes such as development/growth, reproduction, and survival chances. Over a quarter of total end measurements in studies investigating combined warming and increased

$CO_2$ have been of metrics of development and growth (Table 1), but the majority of these studies did not demonstrate non-linear interactions. Unfortunately, few studies make direct measurements of both metabolism/aerobic scope and ecologically important processes. Some evidence shows non-linear interactive effects which occur on aerobic scope but not on growth (Grans *et al.*, 2014). In other measures of ecologically important performance traits there has been a lack of investigations e.g. reproduction (Table 1), this is clearly an area where greater research effort is required in order to confirm whether interactive effects of environmental factors on metabolism/aerobic scope are reflected in ecologically important end measurements.

Non-linear interactive effects were most common from measurements of cellular stress responses (~73 % of measured responses were non-linear), which perhaps indicates that fish require increased use of cellular protective mechanisms to avoid negative effects of combined exposure to warming and increased $CO_2$ (Huth and Place, 2016). Such non-linear interactive effects may lead to reduced ability to acclimate to rapid environmental changes (Strobel *et al.*, 2012, 2013b; Enzor and Place, 2014) or to cope with additional stress caused by other factors (such as reduced $O_2$, chemical pollution or disease) (Lapointe *et al.*, 2014; Feidantsis *et al.*, 2015; Sampaio *et al.*, 2016). However, interactive effects upon cellular processes were often not linked to whole organism level processes. As such it is currently uncertain how the high proportion of non-linear interactions noted in these measurements will impact upon whole organism fitness.

Perhaps the most concerning interactive effects of temperature and $CO_2$ are those recorded for measurements of mortality/survival with approximately 50 % of measurements displaying non-linear effects (Table 1). These measurements were not just made in experiments using extreme treatments but in many investigations using changes that reflect predicted global average temperature and $CO_2$. The effects documented include synergistic decreases in embryo survival and hatching success in 5 species (Rosa *et al.*, 2014; Flynn *et al.*, 2015; Dahlke *et al.*, 2016; Pimentel *et al.*, 2016) and decreases in post-hatch larval survival in one species (Gobler *et al.*, 2018). Non-linear interactive effects on mortality/survival are not limited to early life stages, synergistic increases in

mortality have been noted for juveniles of two species (Munday *et al.*, 2009; Feidantsis *et al.*, 2015). However, other species show no interactive effects on mortality or survival (e.g. Pope *et al.*, 2014). While it is not clear why some species are more affected than others it may relate to the differences in treatment magnitude used in studies – with interactive effects between $CO_2$ and temperature potentially only occurring when fish are exposed to temperatures above their thermal optimum.

### 3.1.2. Temperature x $O_2$

Almost all studies investigating interactions between temperature and $O_2$ have focussed on measurements of environmental tolerances (which did not allow a quantification of the interactive effect in ~90 % of cases) or metabolism/aerobic performance. Focus on these two groups of end measurements is unsurprising given that warming and reduced $O_2$ may result in a 'double whammy' where temperature increases fishes' $O_2$ demand while at the same time hypoxia reduces $O_2$ availability in the environment. Accordingly, increased temperatures were found to cause significant declines in hypoxia tolerance, in the form of the critical oxygen limit, in all species for which this was investigated (Schurmann and Steffensen, 1997; Claireaux *et al.*, 2000; Cerezo Valverde *et al.*, 2006; Hilton *et al.*, 2008; Corkum and Gamperl, 2009; Nilsson *et al.*, 2010; Remen *et al.*, 2013, 2015; Lapointe *et al.*, 2014; Sørensen *et al.*, 2014). Combinations of increased temperature and reduced $O_2$ caused non-linear impacts in ~50 % of measurements of metabolism/aerobic performance (Table 1). Non-linear interactions primarily affect active metabolic rates as opposed to resting metabolism (Crocker and Cech, 1997; Claireaux and Lagardère, 1999; Claireaux *et al.*, 2000; Lefrancois and Claireaux, 2003; Lapointe *et al.*, 2014) which leads to non-linear reductions in aerobic scope. However, there have been almost no studies which link combined effects of elevated temperature and reduced $O_2$ on metabolism with ecologically relevant metrics of whole organism performance. This is an area where further research is vitally required and is unexpected given the need for evidence to confirm whether changes in aerobic scope lead to impacts on measures such as growth and reproduction.

### 3.1.3. $CO_2$ x $O_2$

The comparatively small number of studies focussing on the interaction between elevated $CO_2$ and hypoxia in marine fish is surprising given the known link between respiratory $O_2$ consumption and $CO_2$ excretion. Twenty-three of the 31 measurements we identified allowed an interactive effect to be determined. Of these ~48 % reported a non-linear interaction. Measurements of environmental tolerance (~36 %) and development/growth (~32 %) were most common, followed by measurements of mortality/survival (~19 %). Non-linear responses were common in measures of environmental tolerance (~86 %) and mortality/survival (~60 %) but not observed in measures of development and growth (Table 1). As high $CO_2$ levels are ubiquitous in low $O_2$ areas, the high proportion of non-linear responses noted in measures of tolerance and mortality/survival is concerning. Almost all experiments focussed on the effects of severe hypoxia (which would simultaneously imply a large rise in $CO_2$ in nature) and these experiments usually took place over short time periods. This may help explain the lack of interactive effects noted for measures of development/growth as differential impacts on these processes would likely be measurable only over somewhat longer time scales.

### 3.1.4. Temperature x $CO_2$ x $O_2$

Only one of the studies identified, by Ern *et al.* (2017), investigated a biological response involving all three of the factors (impact of hypoxia and elevated $CO_2$ upon temperature tolerance). As many fish species will inevitably be challenged by combined warming, rising $CO_2$ and hypoxia in future oceans the dearth of studies investigating all three is concerning. Determining interactive effects between all three factors is especially important as the addition of a third stressor has been predicted to result in a doubling in the number of synergistic interactions (Crain *et al.*, 2008).

**Table 1:** The number of end measurements and proportion of non-linear interactions (from end measurements in which a non-linear interaction could be identified) for 8 of the 14 measurement categories identified from experiments investigating interactive effects of temperature, $CO_2$ and $O_2$ on marine fish. Details on the remaining 6 measures can be found in the supplementary materials.

| | **Temperature x CO2** | | **Temperature x O2** | | **O2 x CO2** | |
|---|---|---|---|---|---|---|
| Measurement type | Number of end measurements | % Non-linear | Number of end measurements | % Non-linear | Number of end measurements | % Non-linear |
| Metabolism/aerobic performance | 48 | 41.7% | 21 | 47.1% | 2 | 0.0% |
| Development & Growth | 81 | 19.8% | 1 | 0.0% | 10 | 0.0% |
| Mortality/survival | 19 | 47.4% | 0 | n/a | 6 | 60.0% |
| Environmental tolerance | 0 | n/a | 21 | n/a | 11 | 85.7% |
| Feeding | 20 | 65.0% | 1 | 0.0% | 0 | n/a |
| Reproduction | 9 | 11.1% | 0 | n/a | 0 | n/a |
| Cellular stress response | 30 | 72.7% | 0 | n/a | 0 | n/a |

## 3.2. Factors which may affect the prevalence of non-linear interactions

### 3.2.1. Source of fish

Across all experiments identified, wild caught fish were most commonly used: between 64 and 70 % of all end measurements were recorded from wild-caught fish (Table 2). The use of wild-caught fish for climate change research is generally encouraged, as fish reared in aquaculture or research facilities often show physiological differences to wild counterparts (Sundell *et al.*, 1998), that can result in different responses and tolerances to environmental change (Morgan *et al.*, 2019). This is often considered to be especially important when focusing on responses to warming and rising $CO_2$ as fish from non-wild sources are generally reared at constant, warmer temperatures and exposed to increased $CO_2$ levels that can exceed treatment levels commonly used for climate change research (Ellis *et al.*, 2016). Little difference, however, was found between wild-sourced and aquaculture-reared fish in the proportion of non-linear responses to the combined effects of temperature and $CO_2$

In contrast, the use of wild-caught fish results in higher rates of non-linear interactions than seen in cultured fish when investigating combined effects of temperature and $O_2$, and $CO_2$ and $O_2$ (Table 2). It is not immediately apparent why non-linear responses would be more common in wild fish for these combinations of environmental change. It may be that the increased proportion of non-linear effects is an artefact of the relatively low sample size of measurements made in wild vs. non-wild fish for these combinations of environmental factors. We would recommend that continued use of wild-caught fish is preferential to account for the effects of prior environmental experiences on the responses of organisms (Allan *et al.*, 2014; Murray *et al.*, 2014; Shama *et al.*, 2014).

**Table 2:** The number of measurements and proportion of non-linear interactions (from end measurements in which a non-linear interaction could be identified) found in fish from different sources in experiments investigating interactive effects of temperature, $CO_2$ and $O_2$.

| | **Temperature x $CO_2$** | | **Temperature x $O_2$** | | **$O_2$ x $CO_2$** | |
|---|---|---|---|---|---|---|
| **Source of fish** | Number of measurements | % Non-linear | Number of measurements | % Non-linear | Number of measurements | % Non-linear |
| Wild Caught | 198 | 29.5% | 34 | 58.3% | 22 | 73.3 % |
| Commercial aquaculture | 95 | 30.5% | 13 | 33.3% | 9 | 0.0% |
| Research facility | 17 | 23.5% | 2 | 100.0% | 0 | n/a |

### 3.2.2. Species and treatments used

In total 53 species of fish were included in the 68 studies identified in our literature search. Over half of these species (31/53) were used to investigate interactions between temperature and $CO_2$, with 20 species used for temperature and $O_2$ interactions and only 8 species used for $O_2$ and $CO_2$ interactions. However, only 22 of these species have been included in more than one study, and only 7 species have had the effects of more than one combination of factors studied. This emphasises a lack of replication both to build on findings of individual studies for particular species and also to investigate responses of species to more than one combination of

environmental conditions. The problem of a lack of replication in wider climate change studies has recently been highlighted by the inability to repeat high-profile findings that have indicated that exposure to elevated $CO_2$ impairs behaviour in a range of tropical fish species (Clark *et al.*, 2020). In addition repeated experiments with Atlantic silversides have demonstrated substantial intra- and inter-experiment variability, highlighting the need for increased replication of multi-factor experiments to improve the robustness of findings (Murray and Baumann, 2018).

Interrogating the geographic range of species used, and the location of study laboratories (Figure 4) reveals that almost all species examined are exclusively found in coastal and continental shelf environments, in particular in the North West and North East Atlantic as well as the tropical Indo-Pacific (Figure 4A). Research effort has principally been focussed in Antarctica (14 datasets), Australia (22 datasets), the east-coast of North America (18 datasets), and Western Europe (30 datasets) with only one dataset identified from Asia and none from the continents of South America and Africa (Figure 4B).

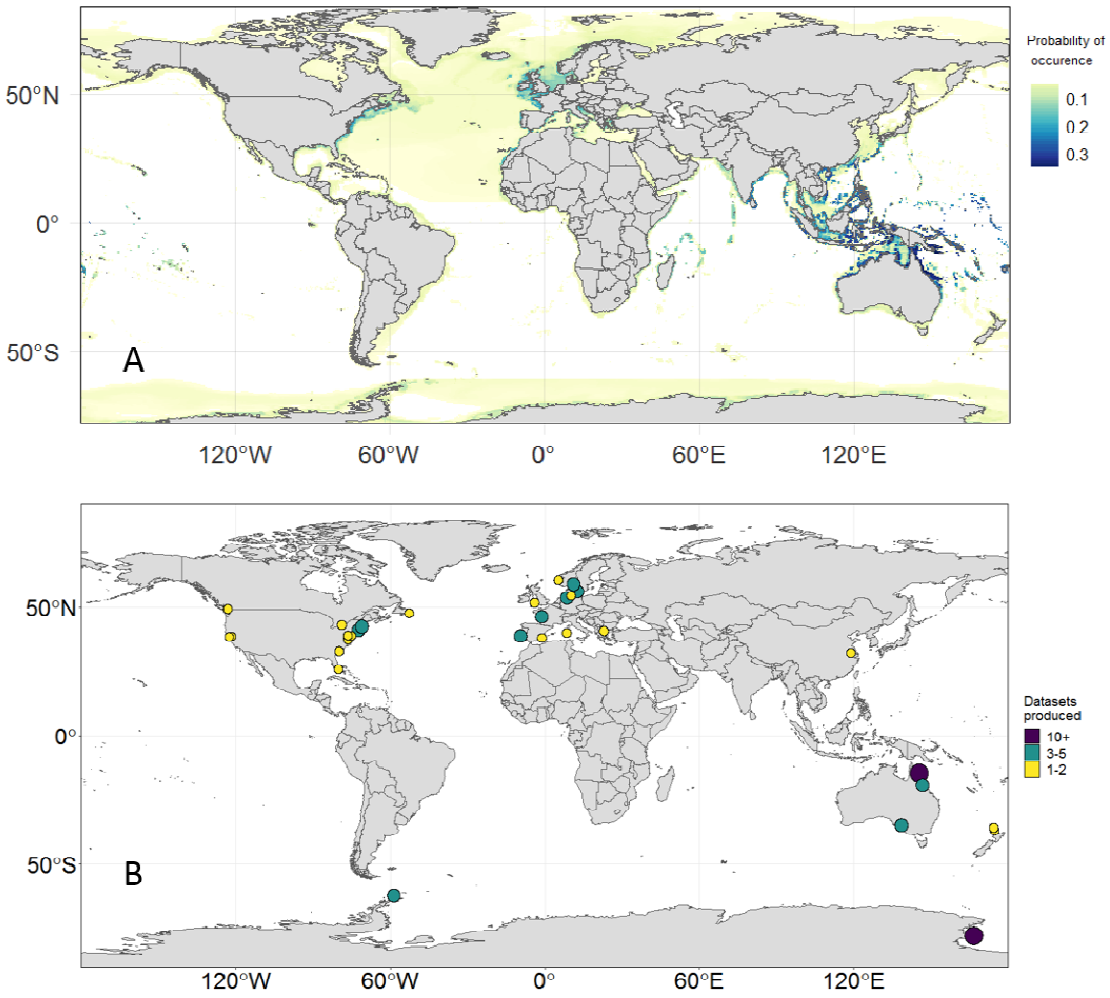


**Figure 4:** Map highlighting **A.** the cumulative probability of any of the 53 species used in multi-factor experiments occurring across the globe. The probability of individual species occurrence were obtained from AquaMaps (Kaschner *et al.*, 2019) **B.** The location of experiments which have reported datasets focussing on multi-factor interactions.

The use of coastal and continental shelf fish species in multi-factor research should make us consider how relevant treatments used in experiments investigating interactive effects are. For example, 58 % of studies investigating $CO_2$ in multi-factor interactions used an average $CO_2$ increase of between 501 and 1000 µatm per treatment level (Figure 5) and >80 % of datasets produced involved only 2 treatment levels. While this may accurately reflect changes in $CO_2$ in the surface layer of open oceans over the next 80 years (IPCC, 2014), it is unlikely to represent $CO_2$ conditions fish will already experience in coastal habitats and nearshore habitats where fluctuations in $CO_2$ occur over hours to

months. These fluctuations in $CO_2$ can be 4-5 times larger than the changes expected over the next 80 years in open ocean habitats (Baumann, 2019). By using exposure levels of a smaller magnitude than coastal fish species will naturally experience today, studies likely under-estimated the potential for interactive effects between $CO_2$ and temperature or $O_2$. Instead, we would recommend that biologists could 'match' treatment conditions to the habitats and environments that individual species occupy in order to make ecologically relevant predictions (Gunderson *et al.*, 2016). This approach is more intuitive for factors such as temperature. For example, temperature treatments are often based on average increases in temperature above the maximum temperatures that the study species may experience during peak summer temperatures in their environment (e.g. see Faleiro *et al.*, 2015; Miller *et al.*, 2015).

The timescales that fish are exposed to treatments for are also important to consider. For both $CO_2$ and temperature treatments durations of 2 weeks to 6 months are used in ~60 % of studies. A treatment duration of two weeks is commonly considered to be the minimum length of time a fish will need to acclimate to a new temperature exposure. However, such short treatment durations are often referred to as 'future shock' experiments as they involve exposing fish to future ocean conditions without taking into account the adaptive potential for species to adjust to gradual changes in ocean conditions (Foo and Byrne, 2016). As such, the duration and magnitudes of temperature treatments that have been used in many multi-factor experiments may be more relevant to predicting impacts of present day extreme events such as marine heatwave events.

In contrast to temperature and $CO_2$, hypoxia treatments were almost exclusively conducted over short time periods of 0-6 hours. This is primarily because a.) this is the length of time over which continuous $O_2$ declines (the most common $O_2$ treatment identified) are typically administered when measuring tolerances to hypoxia (Rogers *et al.*, 2016), and b.) measures of environmental tolerance were the most common end measurement made in studies involving interactive effects of $O_2$ with temperature or $CO_2$ (Table 1). Only ~12 % of studies have included low $O_2$ exposures which lasted for more than 6 hours. Incorporating moderate declines in $O_2$ (which will occur over larger areas than hypoxic events and may be seasonally present throughout

coastal seas) over longer time scales into multi-factor research is required in order to assess whether sub-lethal effects of reduced $O_2$ (such as reduced growth) will result in non-linear interactions when fish are exposed to combined warming and increased $CO_2$.

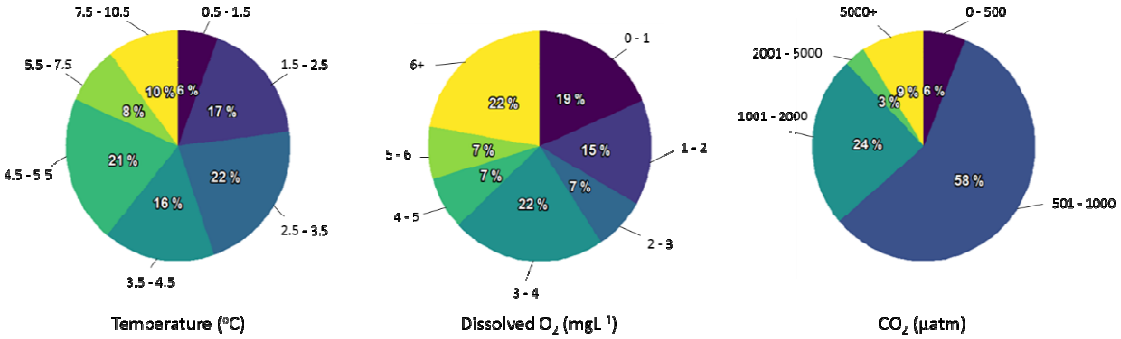


**Figure 5:** Mean magnitude of temperature, $O_2$ and $CO_2$ change per treatment level across all 68 multi-factor climate change studies identified.

### 3.2.3. Life stages

Across all experiments juvenile fish have been most frequently used when investigating interactive effects of warming, rising $CO_2$ and hypoxia (Table 2). Juvenile fish are commonly used for laboratory studies due to logistical constraints involved with using adult fish (i.e. the requirement of larger facilities to accommodate the increased size and biomass of adult fish) and their reduced husbandry requirements when compared to embryo and larval fish. In addition, juvenile fish are often easier to source when using wild-caught fish for experiments. However, use of juvenile fish provides limitations for multi-factor research. For example, changes in reproduction or recruitment, both of which are impossible to investigate if only using juvenile life-stages, have large impacts on population dynamics (Victor, 1983; Doherty and Fowler, 1994; Kidd *et al.*, 2007) and are a key part of fisheries stock management (Morgan, 2008; Kjesbu, 2009). Individually temperature, hypoxia and $CO_2$ have all been shown to affect reproduction and recruitment in fish species (Wu *et al.*, 2003; Wu, 2009; Pankhurst and Munday, 2011; Forsgren *et al.*, 2013). As such, the lack of research investigating potential interactive effects between this trio of environmental change on factors affecting reproduction and recruitment is a critical area where more research is required. This has been recently highlighted by Dahlke *et al.* (2020) who showed that embryos and spawning adults have reduced thermal tolerance (and therefore lower thermal safety

margins) when compared to larvae, juveniles and non-spawning adults, potentially creating thermal bottlenecks for populations to adapt to environmental change.

The relatively low number of studies investigating interactive effects in early life stages is also of concern. For example only one study has included measurement of interactive effects of warming and reduced $O_2$ on larvae or eggs/embryos. Early life stages are generally considered more vulnerable to environmental fluctuations and climate change than juveniles and adults (Pankhurst and Munday, 2011; Dahlke *et al.*, 2020). Despite this, non-linear interactions were most common from measurements using juvenile fish, for interactions between temperature and $CO_2$ and between $O_2$ and $CO_2$ (Table 3). However, the differences in prevalence of non-linear interactions between life-history stages may be dependent on the types of end measurements made as opposed to inherent differences between life-history stages. For example, non-linear interactions were extremely prevalent in measurements of feeding and cellular stress response when fish were exposed to combined temperature and $CO_2$ treatments (Table 1) and these measurements were predominantly made with juvenile fish.

**Table 3:** The number of measurements and proportion of non-linear interactions (from end measurements in which a non-linear interaction could be identified) found across life stages of fish used in experiments investigating interactive effects of temperature, $CO_2$ and $O_2$.

| | **Temperature x CO2** | | **Temperature x O2** | | **O2 x CO2** | |
|---|---|---|---|---|---|---|
| **Life Stage used** | Number of measurements | % Non-linear | Number of measurements | % Non-linear | Number of measurements | % Non-linear |
| Eggs/Embryos | 125 | 23.6% | 0 | 0.0% | 12 | 8.3% |
| Larvae | 96 | 15.8% | 1 | 0.0% | 12 | 8.3% |
| Juveniles | 202 | 35.2% | 26 | 50.0% | 16 | 90.9% |
| Adults | 31 | 22.6% | 21 | 58.3% | 0 | n/a |

## 4. A two-pronged approach to future multi-factor research?

Despite numerous non-linear interactions being documented for aspects of fish physiology and behaviour there is still much uncertainty about when and

why interactive effects between temperature, $O_2$, and $CO_2$ will occur. Although there is growing focus on the issue of multi-stressor interactions in fish biology (for example see Toft *et al.*, 2018), to date there has not been a concerted effort by scientists to investigate the combined effects of warming, low $O_2$, and high $CO_2$ on marine fish. This is probably a result of the logistical, financial and temporal challenges of such experiments. In addition, where multi-factor research has been conducted there has been little overall coordination. For example the choices of species used, end measurements made, and treatment magnitudes and duration used in individual experiments are often disparate to experiments conducted in other research groups. This results in barriers to general comparisons between studies. In addition, research effort is unevenly distributed which means that evidence of interactive effects of warming, rising $CO_2$ and reduced $O_2$ on many end measurements are confined to a small subset of species that are often not suitable to be used to draw general conclusions. Finally, most studies fail to link documented interactive effects to ecologically relevant direct measures of organism fitness (i.e. growth, survival, reproduction). This results in a reliance on theoretical frameworks, many of which are not universally supported, to infer the wider population importance of interactive effects noted. This relatively uncoordinated approach to multi-factor experimental work with fish likely contributes to the continued uncertainty in the potential for interactive effects. We believe that future multi-factor research should be designed to take either a scenario-based or mechanistic approach. Each would provide information about non-linear interactions between warming, low $O_2$, and rising $CO_2$ in different contexts.

### 4.1. Scenario-based approaches

Scenario-based approaches are most relevant to use when specific, targeted knowledge of combined effects are needed. This may include an understanding of how specific, predicted changes in a region may impact on crucial species (for example important fisheries species or keystone species for vulnerable or valuable ecosystems). When scenario-based experiments are conducted it is critical that the treatments used are relevant to the specific environment that the species investigated will experience. This will require accurate projections of local environmental changes from climate modellers as well as a detailed understanding of environmental variability. It will also require

experimental biologists to have a good appreciation of the inter-relationships between environmental factors, some of which we highlighted in section 2 (i.e. specifics of when and where fish are exposed to the trio). For example, recent research has highlighted that increased $CO_2$ which is ubiquitous with low $O_2$ can alter the hypoxia tolerance of fish (Montgomery *et al.*, 2019). By doing this, experimental research can give relevant and targeted information about how specific environmental changes will affect individual fish species researched.

Situations where scenario-based approaches are most suitable should be identified in collaboration with stakeholders such as fisheries scientists, ecologists, environmental managers and policy makers. This is important in order to ensure that experimental work includes end measurements which are relevant for these stakeholders and give direct information about organism performance which can be used to predict population level effects. This is one of the key strengths of scenario-based approaches, they can give direct evidence for how individual fish species will respond to specific combinations of warming, rising $CO_2$ and reduced $O_2$. There are also benefits to experimental biologists in taking a scenario-based approach. By focussing on whole organism measures of performance we can avoid the requirement for specialised equipment and complex techniques needed for more detailed measurements. This would reduce the logistical complexities inherent in multi-factor research.

One example of how we can use scenario-based approaches to predict combined effects of environmental changes is by integration with species distribution models (SDMs). Traditionally these models correlate present day population distributions and abundances with environmental factors before predicting how populations may alter alongside future changes in these environmental factors (Peck *et al.*, 2016). To incorporate non-linear interactions between the present trio of environmental stressors it has been proposed that physiological metrics be used to improve species distribution models and translate multi-stressor environmental impacts to a population response (Koenigstein *et al.*, 2016). Aerobic scope has been particularly suggested by physiologists as an individual metric linked to whole organism performance, including processes such as growth and reproduction (Jørgensen *et al.*, 2012), although as we noted earlier there is surprisingly limited experimental evidence

that directly confirms this assumption. Nevertheless aerobic scope can be incorporated into a species distribution model approach, and this has been demonstrated to successfully predict timings of changes in population distribution with seasonal changes in multiple environmental factors across small spatial scales (Cucco *et al.*, 2012). As such, by quantifying interactive effects on aerobic scope of organisms in scenario-based laboratory experiments, non-linear interactions could be incorporated into aerobic scope based species distribution models (Koenigstein *et al.*, 2016).

However, scenario-based approaches do have several limitations which do not make them suitable for all contexts. Most importantly they do not reveal why interactive effects occur and so are of limited use when trying to predict combined effects of the trio outside of the specific scenario investigated (e.g. not within the variable range investigated or to different species). As it is impossible to investigate all possible combinations of environmental changes on every relevant species the sole use of scenario-based experiments is not practical to predict wider scale impacts of the trio. Therefore, to complement scenario-based approaches it has been proposed that a more mechanistic approach to multi-stressor studies can provide wider frameworks to make predictions of future effects.

## 4.2. Mechanistic Approaches

A mechanistic approach to multi-factor experiments aims to provide detailed knowledge, not only of whole organism performance but also on the mechanistic basis behind observed responses (Griffen *et al.*, 2016). This approach instinctively appeals to physiologists and there are several frameworks that have been proposed to determine interactive effects of environmental change on fish. The most prominent is undoubtedly the oxygen-and-capacity limited thermal tolerance (OCLTT) hypothesis first proposed by Pörtner (2001). The use of this framework has led to the development of a so-called metabolic constraint index to predict how combined changes in temperature and $O_2$ will impact the habitat suitability of marine environments (Deutsch *et al.*, 2015). Recent experimental work has provided support for this approach by demonstrating that changes in metabolic constraints are highly correlated with observed shifts in populations of black seabass, *Centropristus striata*, in the North-West Atlantic (Slesinger *et al.*, 2019). However, it has been

shown that many species studied do not conform to responses predicted by the OCLTT hypothesis (Lefevre, 2016) and the general applicability of the OCLTT as a single unifying framework for all fish species has been repeatedly questioned (Clark *et al.*, 2013; Schulte, 2015; Nilsson and Lefevre, 2016; Jutfelt *et al.*, 2018). Recently the OCLTT hypothesis has been further developed to produce a mechanistic oxygen- and temperature-limited metabolic niche framework (Ern, 2019). This builds upon theories in the OCLTT hypothesis and provides empirical testable hypothesis for many physiological metrics. This therefore provides future research with a starting point to investigate the mechanistic interactions between temperature and $O_2$ on fish.

An alternative to using physiological frameworks based on proposed mechanistic responses is to integrate mechanistic impacts of environmental change directly into forecasts of changes in fish populations using modelling techniques such as Individual-Based Models (IBMs) or Dynamic Energy Budgets (DEB). These models allow integration of environmental and individual variation by incorporating individual physiological and behavioural measures, allowing a bottom-up, mechanistic approach to modelling (Sibly *et al.*, 2013; van der Vaart *et al.*, 2016; Teal *et al.*, 2018). However, these approaches rely on parameterization of a large range of biological measures and, unless the mechanism of interactive effects in these measures is known, this may create major barriers to the accurate modelling of future impacts of climate change on fish populations (Del Raye and Weng, 2015).

In order to improve experiments investigating the mechanism of interactions between multiple factors, it has been recommended that studies increase the number of levels tested for each factor. One problem in current multi-factor research is the tendency to use only two levels for each factor (i.e. presence/absence or low/high levels of a factor). While this reduces the logistical complexity of experiments, and so reduces the research costs, examination of interactions between a broader range of levels for each factor is necessary to truly determine mechanistic impacts of multi-stressors. This means that mechanistic studies should also look to use larger variations in their treatments rather than basing treatments on specific environmental projections (this may be particularly relevant for mechanistic studies incorporating $CO_2$). While this will create greater logistical and statistical challenges a recent review

by Boyd *et al.* (2018) provides recommendations to reduce the logistical challenges of conducting such research.

## 5. Conclusion

More targeted research by experimental biologists on non-linear interactions of warming, low $O_2$, and high $CO_2$ are needed as these are likely to co-occur and have widespread effects on marine fish. Despite the recent increase in research in this area there are many problems to be overcome to improve the accuracy and predictive power of experimental studies. These include increasing the relevance of treatment levels to study organisms, matching treatment magnitude and duration to likely environmental changes to be experienced in their habitat, and growing the breadth of species and life stages investigated.

Combined effects of warming and low $O_2$ are likely to have the most severe impacts on individual organisms as environmentally relevant changes in these factors can result in mortality of fish. However, combined effects of temperature x $CO_2$ and $CO_2$ x $O_2$ have shown the greatest occurrence of non-linear interactions. Researching interactive effects of $O_2$ x $CO_2$, temperature x $O_2$ and all three stressors combined have been severely understudied when compared to interactions between temperature x $CO_2$. In addition, research effort has often focussed on a limited number of end measurements. Of particular concern are the lack of studies investigating ecologically relevant parameters of whole organism performance such as growth and reproduction which are vital to understanding the resilience of future fish populations to environmental change. We believe that developing multi-factor research along two pathways, scenario-based and mechanistic approaches, will help to address several issues which have been identified here and ultimately enhance our ability to predict interactive effects of warming, falling $O_2$, and rising $CO_2$ in future oceans.

## 6. Supplementary Materials

The database of literature identified in this review is available to access at: **https://doi.org/10.24378/exe.3043**

# Chapter III

## Rapid acid-base regulation by European sea bass (*Dicentrarchus labrax*) in response to acute environmental hypercapnia

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**Author contributions:** D.W.M. was primarily responsible for designing the research, conducting experimental work, data analysis and writing the chapter. R.W.W. assisted with designing the research, conducting experimental work, data analysis, and commented on drafts of the paper. S.D.S., G.H.E., and S.N.R.B. assisted with statistical analysis and commented on drafts of the paper.

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## Abstract

Globally rising aquatic $CO_2$ has renewed interest in acid-base regulatory responses to hypercapnia in fish which has been well established in various freshwater species, but fewer studies have focussed on the speed of responses in saltwater fish. We observed that following sudden exposure to ~1 % $CO_2$ (~10,000 µatm – 10 times higher than end-of-century predictions due to climate change) European sea bass (*Dicentrarchus labrax*) exhibit complete regulation of extracellular/blood pH ($pH_e$) within ~2 hours and erythrocyte intracellular pH ($pH_i$) within ~40 minutes. To our knowledge this is one of the fastest acid-base recoveries documented in any fish. Recovery from respiratory acidosis was achieved via a 5-fold accumulation of blood $HCO_3^-$ to ~22 mM after ~2 hours at 10,000 µatm. This was accompanied by a transition from a small whole body net base excretion to a large net acid excretion, primarily driven by changes in apparent bicarbonate uptake. Rapid restoration of erythrocyte $pH_i$ within 40 minutes also returned haemoglobin's affinity for $O_2$ to pre-exposure levels. Hypercapnia increased haemoglobin content of blood by ~10 % regardless of duration of exposure (10, 40 and 120 min). Sea bass were unable to acid-base regulate after 2 hours of hypercapnia when they were simultaneously exposed to low $HCO_3^-$ seawater (~200 µM compared to ~2,800 µM in normal seawater). However, this was likely a result of lower pH in the sea water that prevented $H^+$ excretion via $Na^+/H^+$ exchangers (NHE). This extremely rapid acid-base regulation may enable sea bass to maintain physiological performance while inhabiting shallow water environments during summer where $CO_2$ variation is naturally high. Understanding why species like sea bass show faster acid-base regulation than other saltwater species may help to predict which species will be vulnerable to acute changes in $CO_2$, particularly where this occurs in combination with other environmental stressors.


## 1. Introduction

The build-up of $CO_2$ in aquatic environments, known as environmental hypercapnia, causes significant physiological challenges for water breathing animals, including fish. As environmental $CO_2$ increases this leads to a corresponding rise in the $CO_2$ content of fish blood, either by reducing the concentration gradient between $CO_2$ in venous blood and the environment or, if

environmental $CO_2$ becomes high enough, by direct diffusion of $CO_2$ from the environment into the blood (Tresguerres and Hamilton, 2017). This increase in blood $CO_2$ content leads to a transient decrease in blood pH (referred to as a respiratory acidosis) which can cause significant disruption to respiratory proteins, enzyme activity, protein synthesis and sensory performance (Langenbuch and Pörtner, 2003; Rummer *et al.*, 2013a; Heuer and Grosell, 2014; Pimentel *et al.*, 2015; Tresguerres and Hamilton, 2017; Porteus *et al.*, 2018). As such, maintenance of acid-base status is crucial and there has been much interest in acid-base regulation mechanisms in fish. The majority of research has focussed on freshwater fish species and has established well developed mechanisms of acid-base regulation which typically occur over periods of 24 – 72 hours (Perry and Gilmour, 2006; Brauner *et al.*, 2019).

Globally, aquatic $CO_2$ levels are increasing as a result of anthropogenic climate change (Orr *et al.*, 2005; Doney *et al.*, 2011; IPCC, 2014), and this has renewed interest in acid-base regulatory mechanisms of, particularly saltwater, fish. Climate change research typically includes relatively small changes in $CO_2$ from ambient conditions of ~400 µatm $CO_2$ to maximum exposures of ~1000-2000 µatm and focusses on impacts of long-term $CO_2$ exposure on physiological performance measures, such as metabolic rate (Lefevre, 2019), or behaviour of fish (Munday *et al.*, 2014). While this research is crucial for understanding chronic effects of living at higher ambient $CO_2$ levels it does not give information about the initial acid-base response upon exposure to hypercapnia. A focus on climate change has turned attention away from large acute changes in environmental $CO_2$. However, there is growing recognition that variations in $CO_2$ levels many times greater than expected as a result of climate change can occur over short time periods in some saltwater environments (Cai *et al.*, 2011; Sunda and Cai, 2012; Melzner *et al.*, 2013; Wallace *et al.*, 2014). In addition, mobile saltwater organisms, such as fish, may experience large changes in $CO_2$ when moving through the environment (Paulmier *et al.*, 2011). Such changes in $CO_2$ can be rapid (occurring over minutes to hours) as well as large in magnitude, for example changes of ~4000 µatm $CO_2$ have been recorded in some coastal ecosystems (Melzner *et al.*, 2013; Baumann *et al.*, 2015). This would be expected to cause large impacts on

the physiology of saltwater fish if the established time course of acid-base regulation in freshwater fish is representative of saltwater fish.

To date only a few studies have investigated the time course of acid-base regulation after acute exposure to hypercapnia in saltwater fish (Perry, 1982; Toews *et al.*, 1983; Larsen *et al.*, 1997; Hayashi *et al.*, 2004; Esbaugh *et al.*, 2012; Allmon and Esbaugh, 2017). This has demonstrated that saltwater fish species can acid-base regulate at a greater rate than freshwater species (e.g. hours for saltwater species and days for freshwater) (Brauner *et al.*, 2019). In the majority of fish species respiratory acidosis is compensated for by accumulation of bicarbonate ($HCO_3^-$) in blood plasma. Current models from freshwater fish suggest increased $HCO_3^-$ is obtained via the carbonic anhydrase (CA) catalysed hydration of $CO_2$ in mitochondrial rich cells (MRCs) of fish gills (Claiborne *et al.*, 2002; Perry and Gilmour, 2006; Esbaugh, 2017; Brauner *et al.*, 2019). Alternatively, recent research has suggested that fish may also utilise an as yet undescribed mechanism of direct $HCO_3^-$ uptake from the environment (Esbaugh *et al.*, 2012) and increased environmental $HCO_3^-$ has been shown to alter the rate of acid-base regulation in several fish species (Larsen and Jensen, 1997; Weakley *et al.*, 2012; Tovey and Brauner, 2018). As saltwater environments commonly have much higher environmental concentration of $HCO_3^-$ than present in freshwater, active $HCO_3^-$ uptake may help explain increased rates of acid-base regulation observed in saltwater species and allow them to avoid adverse physiological impacts of acute $CO_2$ changes.

In this experiment we sought to investigate the acid-base response of European sea bass, *Dicentrarchus labrax*, an active predatory species which, during summer months, inhabits shallow coastal, estuarine and saltmarsh environments where large fluctuations in $CO_2$ levels can occur over short time periods. Our aims were to characterise the time needed to restore blood pH after acute exposure to environmental hypercapnia, determine how acid-base regulation affects $O_2$ transport, and investigate potential mechanisms responsible for $HCO_3^-$ accumulation.

# 2. Materials and Methods

## 2.1. Fish

Juvenile European sea bass, *Dicentrarchus labrax*, were obtained by seine netting in estuaries and salt marshes in Dorset and the Isle of Wight on the south coast of the United Kingdom. Once collected sea bass were transferred to the Aquatic Resources Centre of the University of Exeter where they were held in ~500 L tanks on a recirculating aquaculture system (RAS, total volume ~ 2500 L) at temperatures between 14 and 22 $^{o}$C. Sea bass were fed three times a week with commercial pellet with a supplement of frozen mussel once a week. For ~ 6 months before experiments sea bass were maintained at a temperature of 14 $^{o}$C and ambient atmospheric $CO_2$ levels of ~500-600 µatm. Prior to all experimental procedures sea bass were withheld food for a minimum of 72 hours. Animal collections were conducted under appropriate permits (Marine Management Organisation permit #030/17 & Natural England permit #OLD1002654) and all experimental procedures were carried out under home office licence P88687E07 and approved by the University of Exeter's Animal Welfare and Ethical Review Board.

## 2.2. Hypercapnia exposure system

Sea bass were exposed to hypercapnia in a ~250 L experimental system comprised of a ~150 L header tank which fed water to five individual ~20 L isolation tanks at a rate of ~4 L min$^{-1}$ (with overflowing water recirculating back to the header tank). Fish were moved to individual isolation tanks and left to acclimate overnight (minimum of 12 hours) before exposure to hypercapnia. During the acclimation period the experimental system was fed by water from the RAS at a rate of ~10 L min$^{-1}$ (with overflowing water recirculating back to the RAS). The experimental system was temperature controlled using a heater/chiller unit (Grant TX150 R2, Grant Instruments, Cambridge, UK) attached to a temperature exchange coil in the header tank to maintain a temperature of 14 °C.

Prior to hypercapnia exposure isolation boxes were switched from being fed by the experimental system to being directly supplied from the RAS (at the same flow rate of 4 L min$^{-1}$). The header tank was isolated from the RAS before $pCO_2$ levels were adjusted by altering pH to ~6.9 which corresponds to a $pCO_2$

level of ~1 % using an Aqua Medic pH computer. A treatment dose of ~1 % $CO_2$ (~10,000 µatm) was used to allow direct comparisons with many previous studies. The pH computer controlled an electronic solenoid valve, which was opened to deliver pure $CO_2$ to a diffuser in the header tank when pH levels rose above pH 6.92 and closed when pH levels dropped below pH 6.88. Once pH levels in the header tank stabilised individual isolation boxes could be exposed to hypercapnia by swapping the inflow of water to the box from the RAS to the experimental (now high $CO_2$) system. To reduce fluctuations in the $CO_2$ levels that sea bass experienced during exposures the gas aerating each individual tank was switched from ambient air to a gas mix of 1 % $CO_2$, 21 % $O_2$ and 78 % $N_2$ (G400 Gas mixing system, Qubit Biology Inc.).

To characterise the time course of acid-base regulation sea bass were exposed to hypercapnia treatments for either ~10 minutes, ~40 minutes or ~135 minutes before measurements were taken. We monitored the pH of the overflowing water from each isolation tank using a separate pH probe periodically during exposures. This revealed that hypercapnia treatments in isolation tanks matched the pH level of the header tank ~5 minutes after initial exposure. Measurements of an additional group of sea bass were obtained at ambient $CO_2$ levels (~0.05 % $CO_2$) to act as a pre-exposure control (hereafter this group is referred to as time = 0).

### 2.3. Blood sampling and analysis

Following exposure to hypercapnia we took blood samples in order to measure changes in blood-acid base chemistry. Blood samples were obtained following the methodology outlined by Montgomery *et al.* (2019). The gill irrigation tank used was filled with water from the header tank and maintained at an appropriate $CO_2$ level by aeration with the same gas mix feeding the isolation tanks. Whilst blood samples were obtained measurements of seawater pH (NBS scale), temperature and salinity as well as samples of water to measure total $CO_2$ ($TCO_2$)/Dissolved Inorganic Carbon (DIC) were taken from each isolation tank (Table 1). We conducted seawater DIC analysis using a custom built system described in detail by Lewis *et al.* (2013). Measurements of pH, salinity, temperature and DIC were then input into the seawater carbon calculator programme, CO2SYS (Pierrot *et al.*, 2006) to calculate *p*CO2 and total alkalinity (TA) based on the equilibration constants refitted by Dickson and

Millero (1987). We measured the water chemistry of the gill irrigation chamber following the same process as for isolation chambers, with one DIC sample taken at the end of blood sampling (supplementary Table S1).

**Table 1:** Mean ± S.E. of water chemistry parameters in individual isolation tanks.

| | Exposure Length | | | |
|---|---|---|---|---|
| | 0 min (Control) | ~10 min (10.8 ± 0.26 min) | ~40 min (41.0 ± 2.82 min) | ~135 min (133.9 ± 2.27 min) |
| Temperature (°C) | 13.94 ± 0.04 | 13.90 ± 0.03 | 13.94 ± 0.02 | 13.89 ± 0.04 |
| pH (NBS) | 8.15 ± 0.01 | 6.98 ± 0.01 | 6.96 ± 0.01 | 6.95 ± 0.01 |
| Salinity | 34.1 ± 0.1 | 34.1 ± 0.1 | 34.7 ± 0.2 | 34.0 ± 0.1 |
| $pCO_2$ (µatm) | 583.4 ± 9.9 | 8,866.9 ± 220.2 | 9,316.3 ± 711.1 | 9,322.1 ± 163.7 |
| TA (µM) | 3,251.4 ± 19.5 | 2,804.0 ± 15.5 | 2,832.8 ± 11.0 | 2,776.9 ± 29.2 |

Immediately after sampling, we measured extracellular pH ($pH_e$) on 30 µL of whole blood using an Accumet micro pH electrode and Hanna HI8314 pH meter calibrated to 14 °C using $pH_{NBS}$ 7.04 and 10.01 specific buffers. Measurements of blood $pH_e$ were made in a temperature-controlled water bath. Three 75 µL micro capillary tubes were then filled with whole blood and sealed with Critoseal capillary tube sealant (Fisher) and paraffin oil and centrifuged for 2 minutes at 10,000 rpm. Haematocrit was measured using a Hawksley micro-haematocrit reader. Plasma was extracted from these tubes for analysis of $TCO_2$ using a Mettler Toledo 965D carbon dioxide analyser. Plasma $pCO_2$ and $HCO_3^-$ were then calculated from $TCO_2$, temperature and blood pH using the Henderson-Hasselbalch equation with values for solubility and $pK^1_{app}$ based on Boutilier *et al.* (1984, 1985). Haemoglobin (Hb) content of the blood was also assessed by the cyanmethemoglobin method (using Drabkin's reagent, Sigma). We then centrifuged half the remaining whole blood at 10,000 rpm for 2 minutes at 4 °C. The resulting plasma was separated and 10 µL was diluted in ultrapure water, snap frozen in liquid nitrogen, and stored at -80 °C before later being used to measure plasma cation and anion concentrations using ion chromatography (Dionex ICS 1000 & 1100, Thermo-Scientific, UK). The remaining plasma was also snap frozen in liquid nitrogen and stored at -80 °C before we measured plasma glucose and lactate levels using a YSI 2900D Biochemistry Analyzer (Xylem Analytics, UK). After separating the plasma we blotted the surface of the

leftover red blood cell (RBC) pellet to remove the white blood cell layer. We then followed the freeze-and-thaw method (by snap freezing the RBC pellet in liquid nitrogen for 10 seconds and thawing the pellet for 1 minute in a water bath set to ~ 37 °C) to measure intracellular pH of RBCs ($pH_i$) as described by Zeidler and Kim (1977), and validated by Baker *et al.* (2009b). All measurements or storage of blood occurred within 10 minutes of blood sampling. Finally, we then measured Hb-$O_2$ affinity ($P_{50}$) following the same methods outlined in Montgomery *et* al. ( 2019) using a Blood Oxygen Binding System (BOBS, Loligo systems), as detailed by Oellermann *et al.* (2014).

## 2.4. Flux measurements

We measured the flux of acid-base relevant ions between sea bass and seawater over a two hour time period for fish in ambient conditions and immediately following exposure to hypercapnia (Table 2). Fish were transferred to the individual isolation tanks ~24 hours before we made flux measurements. Flow to the isolation tanks was stopped at the start of flux measurements and water chemistry maintained at the desired $pCO_2$ by gassing the tanks with either ambient air (control) or a 1 % $CO_2$ gas mix (hypercapnia). Seawater samples were taken at the beginning and end of the 2 hour flux period. Samples for measuring titratable alkalinity were preserved by adding 4 µL of 4 % (w/v) mercuric chloride per 10 mL of seawater and stored at 4 °C (Dickson *et al.*, 2007) prior to analysis by double titration using a Metrohm autotitrator (815 Robotic USB Sample Processor XL, Switzerland). Measurements of alkalinity were made using a double titration method modified from Cooper *et al.* (2010) as detailed by Middlemiss *et al.* (2016). Briefly, measurements were made using a 20 mL sample titrated to pH 3.89 using 0.02 M HCl whilst gassing with $CO_2$-free $N_2$, pH was then returned to starting values by titrating with 0.02 M NaOH. Samples for measuring total ammonia were frozen at -20 °C before ammonia concentration was measured using a modified version of the colourimetric method of Verdouw *et al.* (1978). A calibration curve was constructed using $NH_4Cl$ standards in seawater.

Acid-base relevant fluxes (µmol $kg^{-1}$ $h^{-1}$) were then calculated using the following equation:

$$J_x = [([X]_i - [X]_f) \times V]/(M \times t)$$

as described by Wilson and Grosell (2003), where V is the volume of water (L) in the isolation tank (after the initial sample is taken), M is the mass of the sea bass (kg), t is the duration of the flux period (h) and $[X]_i$ and $[X]_f$ are the ion concentrations in the chamber water (µmol $L^{-1}$) at the beginning and end of the flux period. By reversing the initial and final values titratable acid, instead of base, fluxes can be calculated so that a positive value equals acid uptake (i.e. $HCO_3^-$ excretion) and a negative value equals acid excretion (i.e. $HCO_3^-$ uptake). We then calculated net acid-base fluxes (µeq $kg^{-1}$ $h^{-1}$) as the sum of titratable acid and total ammonia ($T_{amm}$) flux (McDonald and Wood, 1981).

## 2.5. Low Alkalinity exposure

To test the influence of environmental $HCO_3^-$ concentration on acid-base regulation capability we exposed a group of sea bass to hypercapnia in low total alkalinity seawater. We created low alkalinity water by adding sufficient 1 M hydrochloric acid to an isolated volume of ~250 L seawater to reduce total alkalinity from “normal” levels of ~2800 µM to a low level of ~200 µM. Following addition of 1 M HCl this isolated header tank was aerated overnight to equilibrate $CO_2$ with atmospheric levels. We then adjusted the $pCO_2$ in the header tank to the desired level of ~10,000 µatm using the methods detailed in section 2.2 and a pH set point of 5.75. Fish were placed in the individual isolation boxes as part of the semi-recirculating experimental system and left to acclimate overnight. We then exposed fish to the combined low alkalinity and hypercapnia treatment. Flow from the header tank of the experimental system to individual isolation boxes was stopped. We then drained ~75 % of the water from the isolation box and refilled it using the low alkalinity, hypercapnic water from the previously prepared ~250 L seawater volume. This process was repeated 3 times over a period of ~5 minutes. The gas mix aerating each isolation box was also switched from ambient air to a 1 % gas mix as described in section 2.2 in order to maintain $pCO_2$ levels during the treatment exposure. Following an exposure length of ~135 minutes we anaesthetised each fish and took blood samples to measure acid-base chemistry parameters as detailed in section 2.3. We measured water chemistry of isolation boxes (Table 2) and gill

irrigation chambers (Supplementary material, Table S1) at the time of blood sampling using the methods detailed in section 2.2.

**Table 2:** Mean ± S.E. of water chemistry parameters in individual isolation tanks for low alkalinity hypercapnia exposure

| Exposure length (min) | pH (NBS) | Temperature (°C) | Salinity | $pCO_2$ (µatm) | TA (µM) |
|---|---|---|---|---|---|
| 135.0 ± 4.6 | 5.70 ± 0.03 | 13.78 ± 0.07 | 34.0 ± 0.1 | 11,258 ± 192 | 188 ± 12 |

## 2.6. Tissue sampling and immunohistochemistry

We sampled tissue from seabass exposed to ambient $CO_2$ conditions and to hypercapnia for ~120 minutes in normal alkalinity seawater (Table 3). Exposure to hypercapnia followed details set out in section 2.2 and 2.3. Fish were then terminated using an overdose of benzocaine (~1 g $L^{-1}$) before samples of gill tissues (n = 5), stomach tissue (n = 3), and posterior and anterior intestinal tissue (n = 3) were taken. A sample of each of these tissues was flash frozen in liquid $N_2$ and stored at -80 °C for western blotting. The remaining tissue was rinsed with phosphate buffered saline (PBS) before being fixed in 4 % (w/v) paraformaldehyde (PFA) overnight (~10 hours) at 4 °C. Following overnight fixation we transferred tissue samples to a 50 % ethanol solution for ~10 hours at 4 °C before finally transferring them to a 70 % ethanol solution for storage at 4 $^{o}$C prior to immunohistochemistry.

We also took blood samples from terminated fish. These blood samples were centrifuged (2 min at 1000 g) at 4 °C before the supernatant and white blood cell layer was removed. We then washed the RBC pellet three times with Cortland's saline before the RBC pellet was fixed for 20 minutes using equal parts 3 % PFA in 0.6X PBS and Electron Microscopy Sciences buffer EMS #15949-60 (Electron Microscopy Sciences, USA). After fixation, we washed the RBC pellet three times with PBS and stored pellets at 4 °C before immunofluorescence analysis.

These samples are currently being processed at Scripps Institution of Oceanography in California and at the time of writing this chapter the full results from this analysis are not available. Instead, some preliminary indications of acid-base regulatory mechanisms from the analysis are included in the discussion.

### 2.7. Statistical analysis

All statistical analysis was performed using R v3.6.3 (R Core Team, 2020). Changes in blood chemistry parameters over time in response to hypercapnia exposure were analysed using one-way ANOVA before assumptions of equal variances of data and normality of model residuals were checked. Post hoc-tests were conducted on least-square means generated by package 'emmeans' (Lenth, 2020), with Tukey adjusted p-values for multiple comparisons. Some data did not meet required assumptions for one-way ANOVA. Unequal variances were observed in measurements of $pCO_2$ between treatments, as such we used Welch's ANOVA with Tukey's pairwise comparisons using Benjamini-Hochberg corrections for post-hoc testing. If violations of normality occurred we first attempted to transform data to meet assumptions using box-cox transformations from package 'MASS' (Venables and Ripley, 2002). Transformed data were then analysed using one-way ANOVA before model residuals were checked. This method allowed measurements of lactate to be analysed using one-way ANOVA after data had been box-cox transformed ($\lambda = 0.2$). However, box-cox transformation did not prevent violations of model assumptions for measurements of blood pH and $P_{50}$. As such, these measurements were analysed using the Kruskal-wallis test with post-hoc comparisons made with Dunn's test from package 'FSA' (Ogle *et al.*, 2020), using Benjamini-Hochberg corrections for multiple comparisons. Flux measurements were analysed using Student's t-test after checking data met assumptions of normality and equal variance.

## 3. Results

### 3.1. Blood chemistry

Exposure to environmental hypercapnia caused significant changes in blood pH of seabass across exposure time (Kruskal-wallis test, $\chi^2 = 25.0$, df = 3, $p < 0.001$). We observed a pronounced acidosis of the blood from 7.84 (± 0.02) in control fish (time = 0) to 7.50 (± 0.03) after exposure to hypercapnia for ~10 minutes (Figure 1A & D). Following this initial acidosis sea bass were able to regulate blood pH so that blood pH was not significantly different from control fish after a time of ~135 minutes (Figure 1A & D).

We observed significant changes in plasma $pCO_2$ during exposure to hypercapnia (Figure 1B & D, Welch's ANOVA, F = 202.5, df = 3, p < 0.001). The initial decrease in blood pH of sea bass was driven by a rapid and large (~4-fold) increase in plasma $pCO_2$ within the first 10 minutes of exposure. There was a small but significant decline in plasma $pCO_2$ between fish sampled at ~10 minutes after exposure and fish sampled ~135 minutes after exposure (Figure 1B) despite there being no significant differences in the environmental $CO_2$ level between sample times (Table 1). Blood pH regulation was achieved via a ~5-fold increase in plasma $HCO_3^-$ (95 % CI 15.99-18.77 mM) over the ~135 minute exposure (Figure 1C & D, One-way ANOVA, F = 203.3, df = 3, p < 0.001).

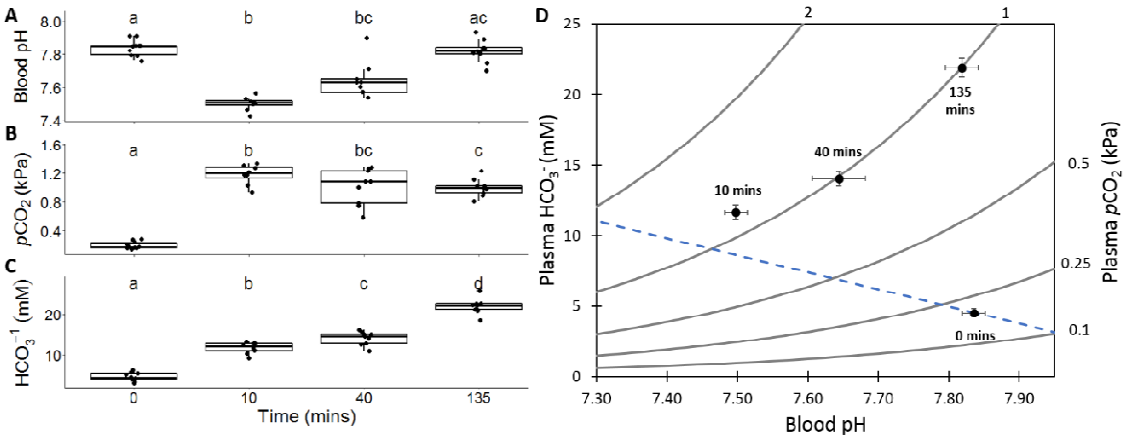


**Figure 1:** Changes in **A.** blood pH, **B.** plasma $pCO_2$, and **C.** plasma $HCO_3^-$ of European sea bass, *Dicentrarchus labrax*, between control fish (~0.05 % $CO_2$, Time = 0, n= 10) and fish exposed to ~0.9 % $CO_2$ for ~10 minutes (n = 8), ~40 minutes (n = 9) and ~135 minutes (n = 9). Significant differences between parameters at each time point are indicated by different lower case letters (**A.** Dunn's test, p < 0.05; **B.** Pairwise comparison using Benjamini-Hochberg correction, P < 0.05 **C.** Pairwise comparison of least square means, p < 0.05) **D.** Combined changes of all three acid-base parameters are expressed as a pH/$HCO_3^-$/$pCO_2$ diagram (blue dashed line indicates estimated non-bicarbonate blood buffer line based on equations from Wood *et al.* (1982)), values represent mean ± S.E.M.

## 3.2. Oxygen transport capacity

Changes in whole blood pH during hypercapnia exposure were reflected in changes in RBC $pH_i$ (Figure 2A, one-way ANOVA, $F = 12.34$, $df = 3$, $p < 0.001$). However, following initial intracellular acidosis after 10 minutes of exposure, RBC $pH_i$ was not significantly different from control levels after ~40 minutes of exposure to hypercapnia (Figure 2A). As expected, the significant changes in RBC $pH_i$ affected $P_{50}$ (Kruskall-wallis test, $\chi^2 = 17.4$, $df = 3$, $p < 0.001$); leading to a ~3-fold increase in $P_{50}$ after 10 minutes, from 1.78 kPa $O_2$ (± 0.30 kPa $O_2$) in control fish to 5.60 kPa $O_2$ (± 0.36 kPa $O_2$). The rapid recovery of RBC $pH_i$ after ~40 minutes led to $P_{50}$ returning to pre-exposure levels (Figure 2B).

Aside from changes in blood $O_2$ binding affinity we also observed other changes which would affect $O_2$ transport capacity. Fish exposed to hypercapnia exhibited an ~8-10 % increase in haematocrit (Figure 2C), although this result was marginally non-significant (One-way ANOVA, $F = 2.40$, $df = 3$, 0.086). Fish exposed to hypercapnia displayed increased haemoglobin levels (Figure 2D), with haemoglobin levels increasing by ~25 % in fish exposed for ~10 minutes and ~ 135 minutes when compared to control fish (One-way ANOVA, $F = 4.60$, $df = 3$, $p = 0.009$)

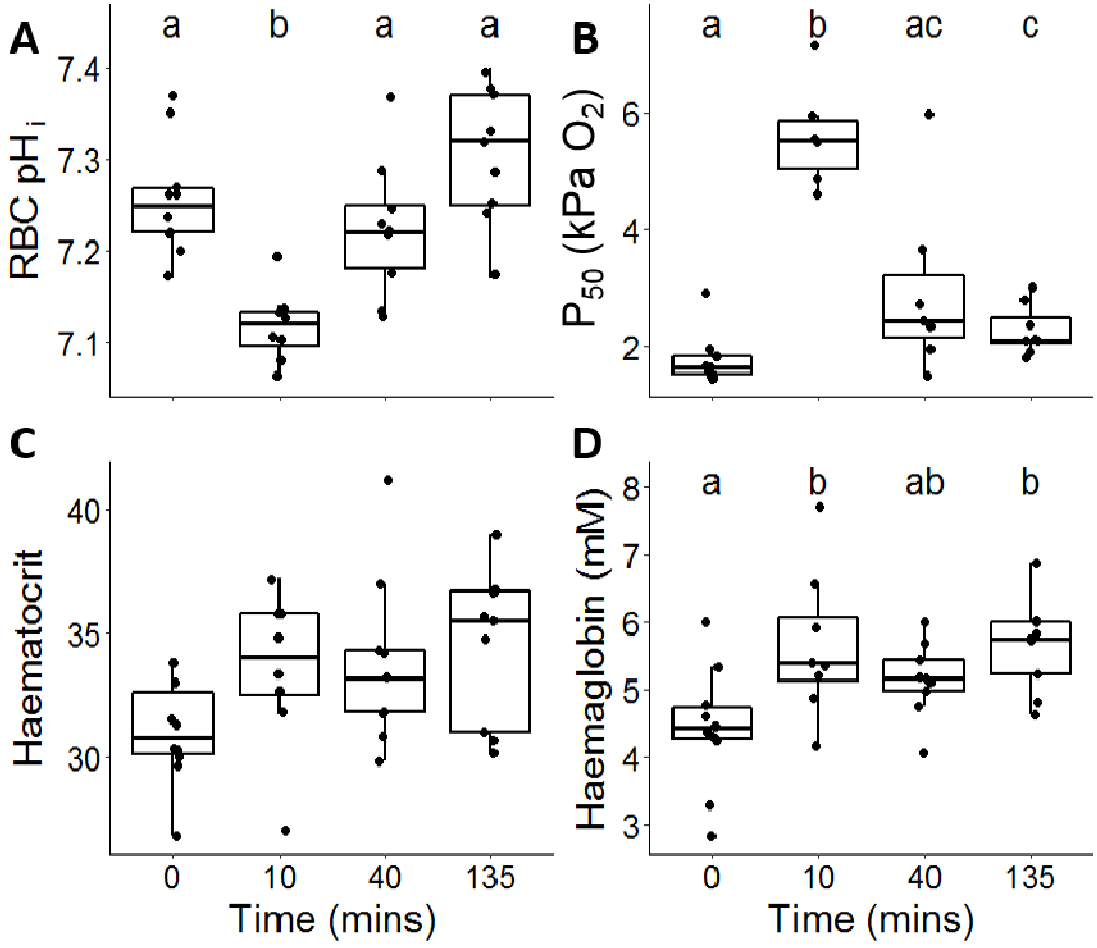


**Figure 2:** Changes in **A.** red blood cell intracellular pH (RBC $pH_i$), **B.** blood-$O_2$ binding affinity ($P_{50}$), **C.** haematocrit and **D.** haemoglobin level between control fish (~0.05 % $CO_2$, Time = 0) and fish exposed to ~0.9 % $CO_2$ for ~10 minutes, ~40 minutes and ~135 minutes. Significant differences between parameters at each time point are indicated by different lower case letters (**A.**, **C.** and **D.** Pairwise comparisons of least square means, $p < 0.05$, **B.** Dunn's test, $p < 0.05$).

### 3.3. Flux measurements

Fish exposed to hypercapnia for ~120 minutes (~0.84 % $CO_2$) exhibited a switch from a small net base excretion (in control fish exposed to ~0.07 % $CO_2$) to an ~2.5-fold larger magnitude net acid excretion (Figure 3C, Student's t-test, $t = -2.25$, $df = 13$, $p = 0.042$). This was mainly driven by a switch from a small apparent $HCO_3^-$ excretion to a large apparent $HCO_3^-$ uptake (Figure 3A), but was contributed to by a much smaller increase in $T_{amm}$ excretion (Figure 3B).

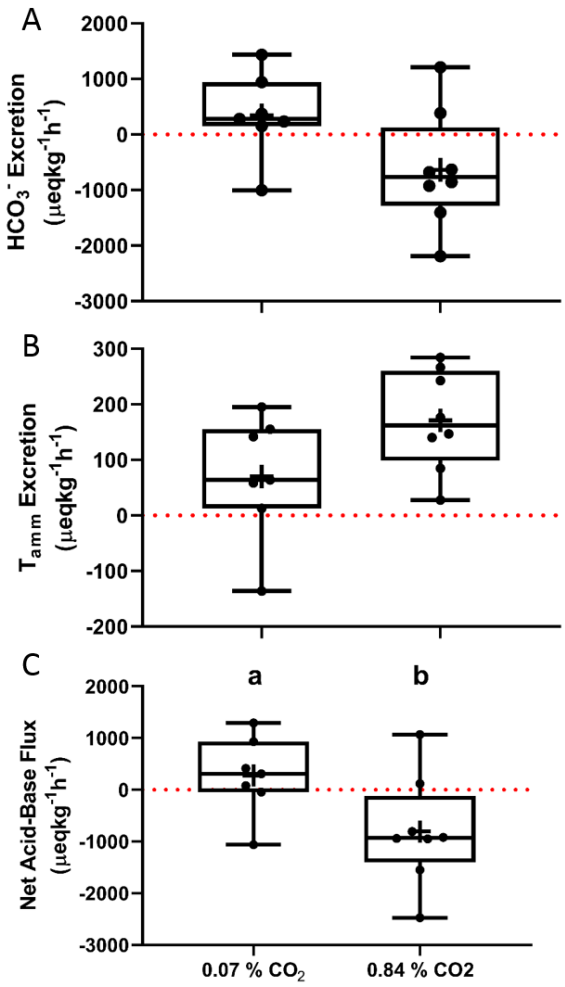


**Figure 3:** Changes in **A.** excretion of $HCO_3^-$, **B.** excretion of total ammonia ($T_{amm}$) and **C.** net acid-base flux for European sea bass in control conditions (n = 7, ~0.07 % $CO_2$) and during an ~120 minute exposure to hypercapnia (n = 8, ~0.84 % $CO_2$). Significant difference in parameters are indicated by different lower case letters (Student's t-test, $p < 0.05$).

### 3.4. Response to Hypercapnia in Seawater with Low Total Alkalinity

Hypercapnia exposure caused significant changes in blood pH over time, with fish exposed to hypercapnia in low total alkalinity seawater showing a different response to fish exposed in normal total alkalinity sea water (One-way ANOVA, $F = 136.8$, $df = 3$, $p < 0.05$). In low total alkalinity seawater sea bass were unable to compensate for a respiratory acidosis when exposed to acute hypercapnia for ~135 minutes (Figure 4A). Blood pH was 0.37 (95 % CI = 0.33-0.41) lower than fish exposed to hypercapnia in normal alkalinity seawater for the same length of time and the same as we recorded in fish ~10 minutes after

exposure to hypercapnia in normal seawater. Plasma $pCO_2$ was also significantly affected during hypercapnia exposure at different alkalinity treatments (One-way ANOVA, F = 141.2, df = 3, p < 0.001). Fish exposed to hypercapnia in low total alkalinity seawater showed significantly higher plasma $pCO_2$ levels than fish exposed to hypercapnia in normal alkalinity seawater for the same length of time (Figure 4B). While this plasma $pCO_2$ level was not higher than observed in fish exposed to hypercapnia in normal alkalinity for ~10 minutes, the increase in plasma $pCO_2$ of 0.17 kPa (95 % CI = 0.06-0.27) after ~135 minutes of exposure is in line with an ~0.2 kPa higher level of environmental $CO_2$ that these fish were exposed to (Table 1 & 2).

Hypercapnia and total alkalinity also had significant effects on plasma $HCO_3^-$ (One-way ANOVA, F = 269.3, df = 3, p < 0.001). Sea bass in low total alkalinity seawater could not actively accumulate $HCO_3^-$ when exposed to environmental hypercapnia (Figure 4C & D). Plasma $HCO_3^-$ level in sea bass exposed to hypercapnia in low total alkalinity seawater after ~135 minutes was 11.8 mM (95 % CI = 10.6-12.9 mM), similar to that seen in the first 10 minutes of exposure to hypercapnia in normal alkalinity seawater (Figure 4C), and close to the increase in $HCO_3^-$ expected as a result of non-bicarbonate buffering of the increased plasma $pCO_2$ (Figure 4D).

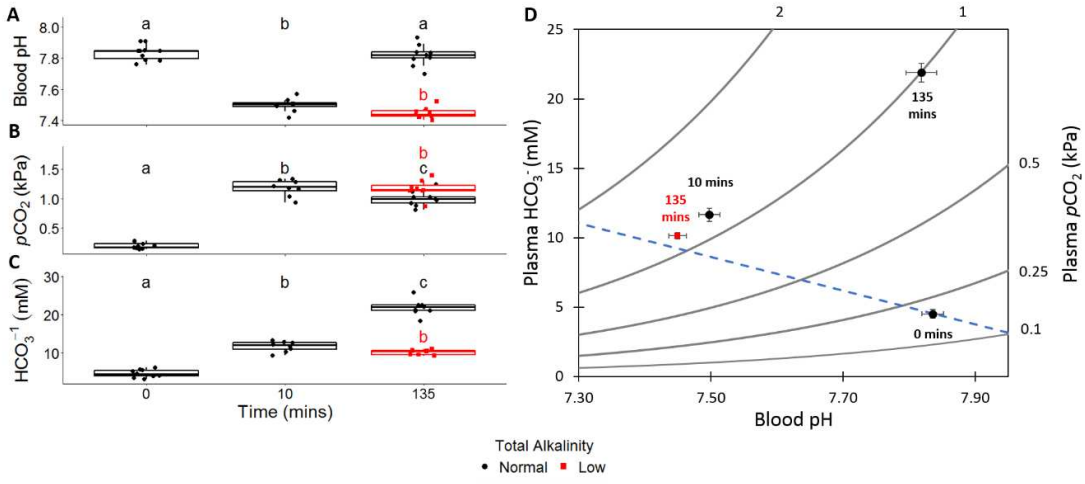


**Figure 4:** Comparison of **A.** blood pH, **B.** plasma $pCO_2$ and **C.** plasma $HCO_3^-$ between control fish (n = 10, Time = 0), fish exposed to hypercapnia for ~10 minutes in normal (~2800 µM) total alkalinity seawater (n = 8), fish exposed to hypercapnia for ~135 minutes in normal (~2800 µM) total alkalinity seawater (n = 9), and fish exposed to hypercapnia for ~135 minutes in low (~200 µM) total

alkalinity seawater (n = 8). Significant differences between parameters at each time point are indicated by different lower case letters (Pairwise comparison of least squares means, $p < 0.05$). For measurements taken after ~135 minutes of exposure to hypercapnia the colour of the letter is matched to the total alkalinity treatment. **D.** Combined changes of all three acid-base parameters are expressed as a pH/$HCO_3^-$/$pCO_2$ diagram (blue dashed line indicates estimated non-bicarbonate blood buffer line based on equations from Wood *et al.* (1982)), values represent mean ± S.E.M.

### 3.5. Plasma Ion Levels

Plasma chloride levels decreased by 13.1 mM (95% CI = 10.1-16.2 mM) from 134.9 mM (± 5.1) in fish in normocapnia to 121.7 mM (± 1.3) in fish exposed to hypercapnia for ~135 minutes (Kruskall-wallis test, $\chi^2 = 11.1$, df = 4, $p = 0.025$). This decrease in plasma chloride was not seen in fish exposed to hypercapnia in low alkalinity water (Figure 5A). Decreases in plasma chloride showed a correlation with increasing bicarbonate (Figure 5A inset, Kendall's tau correlation, $\tau = -0.32$, $p = 0.005$). Plasma sodium levels showed no significant changes over the time course of hypercapnia exposure (One-way ANOVA, $F = 1.063$, $p = 0.391$), and there were no differences in sodium levels after ~135 minutes of exposure to hypercapnia between fish in normal and low alkalinity seawater (Figure 5B). As such, there was no correlation between plasma sodium levels and $H^+$ concentration (Figure 5B inset, Kendall's tau correlation, $\tau = 0.08$, $p = 0.471$).

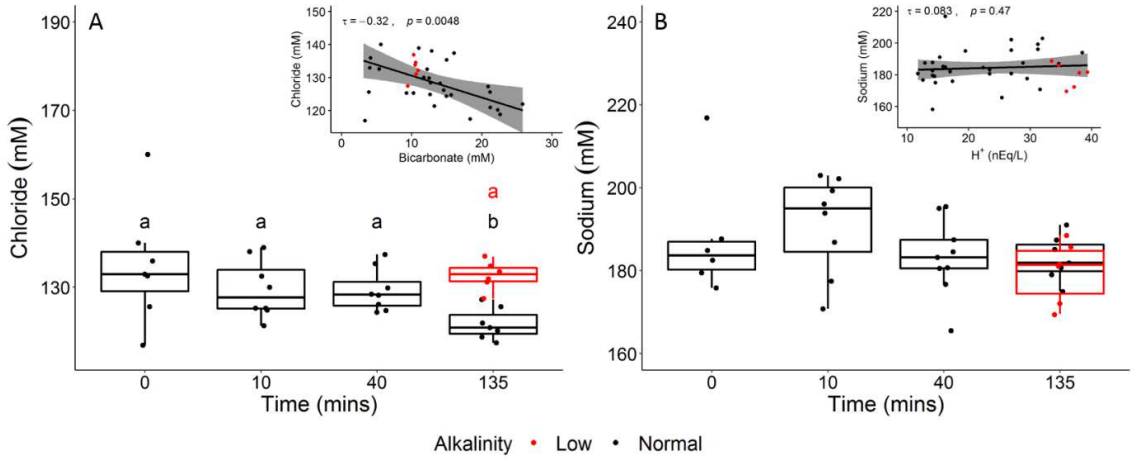


**Figure 5:** Comparison of **A.** plasma chloride and **B.** plasma sodium between control fish (n = 7, Time = 0), fish exposed to hypercapnia for ~10 minutes in normal (~2800 µM) total alkalinity seawater (n = 8), fish exposed to hypercapnia for ~40 minutes in normal (~2800 µM) total alkalinity seawater (n = 9), fish exposed to hypercapnia for ~135 minutes in normal (~2800 µM) total alkalinity seawater (n = 7) and fish exposed to hypercapnia in low (~200 µM) total alkalinity seawater (n = 6). Significant differences between chloride levels at each time point are indicated by different lower case letters (Pairwise comparison of least squares means, $p < 0.05$). For measurements taken after ~135 minutes of exposure to hypercapnia the colour of the letter is matched to the total alkalinity treatment. Insets show correlation between **A.** plasma chloride and bicarbonate levels and **B.** plasma sodium and $H^+$ levels. T and p values shown represent results of Kendall's tau correlation. Shaded area represents 95 % CI of linear regression between measures.

## 4. Discussion

Our results indicate that European sea bass have a highly developed acid-base regulatory system with the capacity to rapidly regulate hypercapnia-induced blood acidosis. Regulation of blood pH after exposure to ~1 % $CO_2$ was achieved via a switch from net base excretion to net acid excretion and a subsequent ~5-fold increase in plasma $HCO_3^-$, as typical of the acid-base regulatory response of saltwater teleost fish (Brauner *et al.*, 2019). Rapid extracellular pH regulation was reflected in an even faster restoration of RBC intracellular pH. As expected the initial RBC intracellular acidosis resulted in a large decrease in Hb-$O_2$ affinity which subsequently returned to pre-exposure

levels with the recovery of RBC $pH_i$. The increased speed of RBC $pH_i$ restoration when compared to extracellular pH likely indicates that sea bass RBCs possess β-adrenergic stimulated NHE which functions to restore $pH_i$ and Hb-$O_2$ affinity (Nikinmaa, 2012). Hypercapnia also caused a 20 % increase in the blood haemoglobin concentration which was correlated with a non-significant trend for ~10 % increase in haematocrit after exposure to hypercapnia. Increases in blood haemoglobin content and haematocrit in response to hypercapnia induced acidosis have been observed in teleost fish previously (Vermette and Perry, 1988; Perry and Kinkead, 1989; Crocker and Cech, 1997; Lee *et al.*, 2003). This is thought to be a result of α-adrenergic stimulated spleen contraction to release RBCs in order to boost $O_2$ transport capacity of blood whilst hypercapnia-induced acidosis reduces Hb-$O_2$ affinity (Perry and Kinkead, 1989).

The speed of acid-base regulation that we observed in European sea bass is the fastest recorded for $pH_i$, and one of the fastest recorded for $pH_e$ in any fish species. To date the majority of work conducted on the acid-base regulatory response in fish has been conducted with freshwater teleosts which typically take >24 hours to regulate blood pH after exposure to 1 % $CO_2$ (Perry *et al.*, 1981; Perry, 1982; Smatresk and Cameron, 1982; Claiborne and Heisler, 1984, 1986; Baker *et al.*, 2009a; Damsgaard *et al.*, 2015). Despite relatively little research, it is generally accepted that saltwater teleosts can regulate acid-base disturbances at a faster rate than freshwater teleosts (Brauner *et al.*, 2019). We identified five saltwater teleost species in which the time course of the acid-base regulatory response was characterised after exposure to 1 % $CO_2$ (Figure 6). Of these species only one, the Japanese amberjack (*Seriola quinqueradiata*), has been observed to restore blood $pH_e$ faster than European sea bass (~1 h v. ~2 h 15 min; Figure 6E). However, recovery from respiratory acidosis in this species was measured at 20 $^oC$ (Hayashi *et al.*, 2004) compared to at 14 $^oC$ in the present study. As warmer temperatures are known to increase the rate of acid-base regulation (Kieffer *et al.*, 1994) sea bass at 20 $^oC$ may regulate pH at a similar rate to Japanese amberjack. The remaining four species regulated blood pH between 3 and 24 hours post $CO_2$ exposure (Figure 6B, C, D, and F).

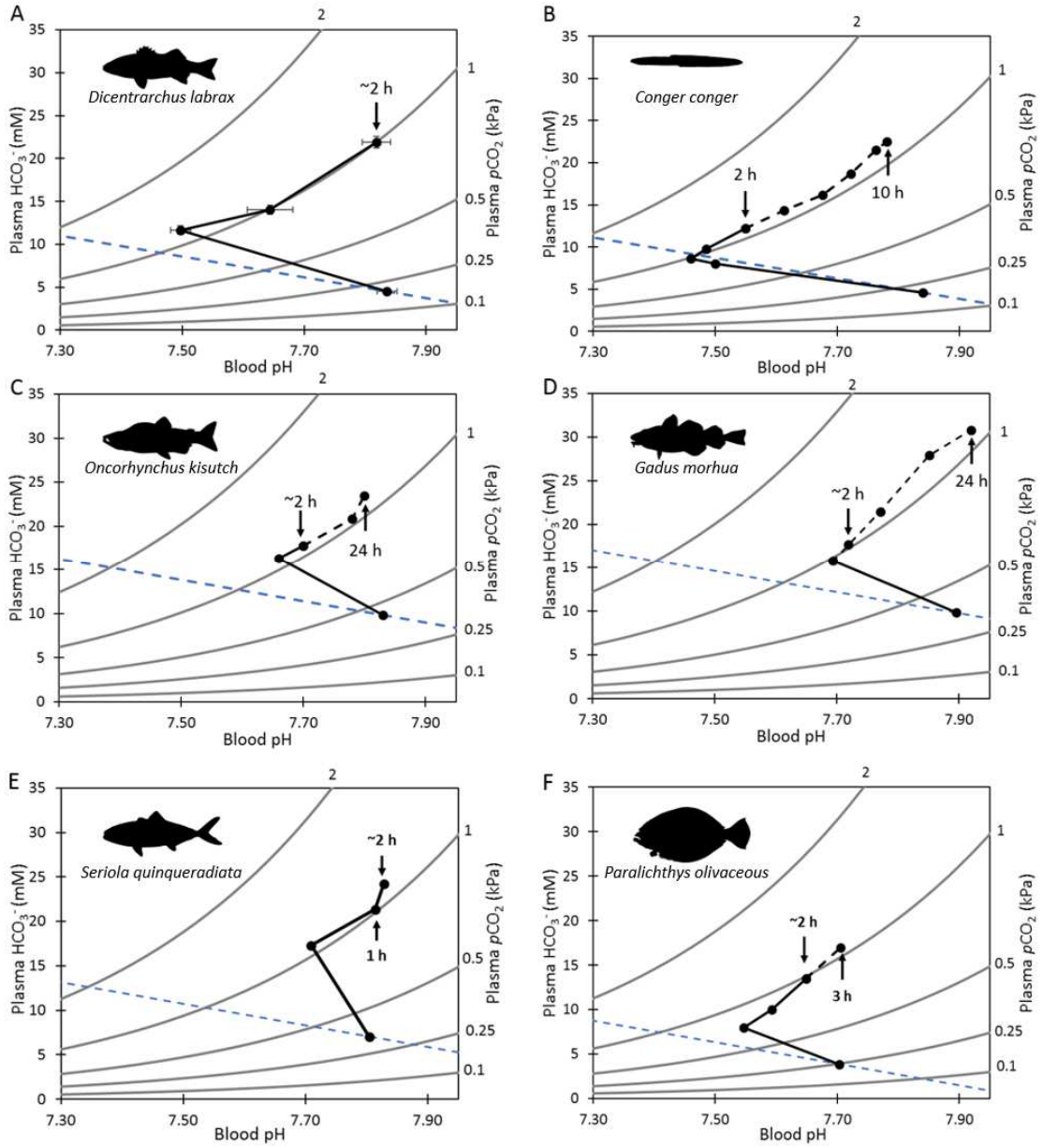


**Figure 6:** Blood pH/$HCO_3^-$/$pCO_2$ plots for **A.** European sea bass, *Dicentrarchus labrax* (Present study), **B.** Conger eel, *Conger conger* (Toews *et al.*, 1983), **C.** Coho salmon, *Oncorhynchus kisutch* (Perry, 1982), **D.** Atlantic cod, *Gadus morhua* (Larsen *et al.*, 1997), **E.** Japanese amberjack, *Seriola quinqeradiata* (Hayashi *et al.*, 2004, raw data provided by pers. comm. with Atsushi Ishimatsu), **F.** Japanese flounder, *Paralichthys olivaceus* (Hayashi *et al.*, 2004). The corresponding blood pH and $HCO_3^-$ of each species at a time ~2 hours after 1 % $CO_2$ exposure is indicated to allow direct comparisons with European sea bass. Times below the relevant point indicate when blood pH was not statistically different from pre-exposure pH for each species. The time course of the acid-base response after 2 hours is indicated by a dashed black line. The

dashed blue line is the approximated non-$HCO_3^-$ buffer line based on the mean haematocrit of sea bass from the present study and calculated using the equation for rainbow trout from Wood *et al.* (1982).

The greater rate at which sea bass, and other saltwater teleosts, acid-base regulate in comparison to freshwater teleosts may be a result of the higher ion concentrations present in salt water (Brauner *et al.*, 2019). In particular, increased concentrations of $HCO_3^-$, $Na^+$, and $Cl^-$ have been shown to increase the rate and capacity for acid-base regulation in response to hypercapnia (Larsen and Jensen, 1997; Weakley *et al.*, 2012; Tovey and Brauner, 2018). In sea bass the accumulation of plasma $HCO_3^-$ to regulate blood pH was correlated with decreases in plasma $Cl^-$. In freshwater fishes $HCO_3^-/Cl^-$ exchange between MR cells in the gills and blood during acid-base regulation occurs via the basolateral anion exchanger (AE) (Claiborne *et al.*, 2002; Brauner *et al.*, 2019). Similar mechanisms for $HCO_3^-/Cl^-$ exchange have been documented in medaka, *Oryzias latipes*, acclimated to saltwater (Liu *et al.*, 2016). However, Esbaugh (2017) suggests that $HCO_3^-$ uptake into the blood of saltwater fish is achieved via a $Na^+$-$HCO_3^-$ cotransporter (NBC) in the basolateral membrane of MR cells. This then stimulates the osmoregulatory transporter NKCC resulting in movement of $Cl^-$ out of the blood into the MR cell alongside $Na^+$ and $K^+$. It is generally thought that the $HCO_3^-$ which is accumulated as part of acid-base regulation is produced via carbonic anhydrase mediated hydration of $CO_2$, which can be provided either by the MR cell's own metabolism or by diffusion from the blood into MR cells. Protons produced by this reaction are then excreted primarily via apical NHE which leads to an intake of $Na^+$ (Claiborne *et al.*, 2002). Although we did see a slight transient rise in plasma $Na^+$ on initial exposure to acidosis there was no significant increase in plasma $Na^+$ in sea bass exposed to ~1 % $CO_2$ and no correlation between plasma $Na^+$ and $H^+$ levels. This matches findings in other saltwater teleosts (Toews *et al.*, 1983; Larsen *et al.*, 1997; Hayashi *et al.*, 2004; Ishimatsu *et al.*, 2008) and could provide evidence that $Cl^-$ not $Na^+$ is the primary counter-ion important for acid-base regulation in saltwater fish. This contrasts with typical models of acid-base regulation in freshwater fish (Claiborne *et al.*, 2002) and the recent model of acid-base regulation for lower levels of hypercapnia for saltwater fish proposed by Esbaugh (2017). As a result there has been

speculation as to whether direct $HCO_3^-$ uptake in exchange for $Cl^-$ may play a role in acid-base regulation (Esbaugh *et al.*, 2012; Tovey and Brauner, 2018). This could help explain the effect of changes in environmental $HCO_3^-$ and $Cl^-$ on rate of acid-base regulation. However, for saltwater fish where environmental $Cl^-$ levels are much higher than internal $Cl^-$ it would appear that this mechanism would not be beneficial. Instead the lack of increase in $Na^+$ may simply indicate that the osmoregulatory mechanism for $Na^+$ excretion, diffusion via paracellular 'leaky tight' pathways (Edwards and Marshall, 2012), has sufficient capacity to prevent increases in $Na^+$ levels in the blood during acid-base regulation.

Sea bass exposed to hypercapnia in low alkalinity (~200 µM) saltwater lost their ability to accumulate $HCO_3^-$ and compensate for respiratory acidosis in the same time period as bass in normal alkalinity seawater. Bass in low alkalinity seawater also did not show decreased plasma $Cl^-$ levels when exposed to hypercapnia. While these results may initially seem to support a role of $HCO_3^-$ /$Cl^-$ exchange in acid-base regulation of sea bass we instead believe that these results occur because of an inhibition of NHE. This inhibition occurred because hypercapnia in low alkalinity treatments created much lower environmental pH than hypercapnia in normal seawater (ΔpH = ~1.2), this drop in environmental pH would likely have resulted in $H^+$ excretion via NHE becoming thermodynamically unviable given the expected $pH_i$ and intracellular $Na^+$ in MR cells (based on calculations in Parks *et al.*, 2008). The inability of sea bass to excrete $H^+$ via NHE also explains the lack of decrease in plasma $Cl^-$; if $H^+$ excretion from MR cells does not take place then hydration of $CO_2$ to create $H^+$ and $HCO_3^-$ would be inhibited which would prevent accumulation of $HCO_3^-$ by sea bass and subsequent reduction in plasma $Cl^-$ (Esbaugh, 2017). Therefore, despite some evidence for environmental $HCO_3^-$ uptake mechanisms in one species of saltwater fish (Esbaugh *et al.*, 2012) our results more likely indicate that rapid acid-base regulation in response to hypercapnia is achieved via NHE mediated $H^+$ excretion. It has been shown that other species of saltwater fish maintain sufficiently high levels of NHE to enable rapid responses to environmentally realistic increases in $CO_2$ (Michael *et al.*, 2016; Allmon and Esbaugh, 2017). Sea bass in control conditions have been demonstrated to possess apically localised NHE3b transporters in MR cells (Kwan and Tresguerres, pers. comm.) and so it may be that sea bass also possess

sufficient NHE levels for acid-base regulation without needing to upregulate expression of this transporter.

The enhanced ability of sea bass to rapidly acid-base regulate in response to a ~1 % $CO_2$ exposure when compared to many other salt water fish may be an adaptation to changes in environmental $CO_2$ that they naturally encounter. During summer months sea bass inhabit shallow coastal estuaries and salt marsh habitats in which they feed (Doyle *et al.*, 2017). In these habitats sea bass may experience large changes in $CO_2$ over short time periods (Hofmann *et al.*, 2011; Melzner *et al.*, 2013; Wallace *et al.*, 2014), for example in salt marshes on the US coast $CO_2$ fluctuations of ~0.4 % $CO_2$ occurred during tidal periods in summer months (Baumann *et al.*, 2015). The high speed of acid-base regulation of sea bass that we observed indicate that sea bass would be able to regulate the large natural fluctuations of $CO_2$ in <1 hour. This would minimize impacts of environment $CO_2$ fluctuations on $O_2$ transport capacity which may be crucial for active predatory teleosts to maintain energetically costly activities such as foraging. However, it is clear that this cannot be the sole driver for enhanced acid-base regulatory capacities in some species. For example, Japanese amberjack show a similarly fast acid-base regulatory response (Hayashi *et al.*, 2004), but they primarily inhabit pelagic, offshore ecosystems in which large variation in environmental $CO_2$ may be less likely to occur. An alternative may be that active, predatory species have developed higher capacities for acid-base regulation to deal with large metabolic acidosis (as a result of anaerobic respiration used during intense exercise involved in prey capture) or metabolic alkalosis (as a result of digestion of large protein-rich meals; Bucking and Wood, 2008; Cooper and Wilson, 2008). Determining what causes differences in acid-base regulatory capacity between species may be important to understand how acute changes in $CO_2$ might impact upon species (particularly when they occur in combination with other stressors such as hypoxia). This is an avenue where we believe further research effort is necessary.

## 5. Conclusion

Overall, our study highlights the capacity of European sea bass to rapidly regulate large environmental $CO_2$ disturbances. This also indicates that the

relatively small increases in $CO_2$ caused by anthropogenic emissions of greenhouse gases (IPCC, 2014) are unlikely to cause significant physiological effects for European sea bass as a result of impacts on acid-base regulation. Rapid pH regulation appears to occur via established pathways of acid excretion (i.e. NHE). Importantly for their functionality, this ability of sea bass enables them to quickly restore $O_2$ transport capacity of blood. As sea bass likely experience rapid changes in $CO_2$ in nature, this likely minimises physiological impacts of fluctuating $CO_2$ and may prevent disruption of energetically expensive activities such as foraging or digestion.

# 6. Supplementary materials

**Table S1:** Water chemistry of gill irrigation chambers used while blood sampling fish

| | Exposure length | | | |
|---|---|---|---|---|
| | 0 min | ~10 min | ~40 min | ~135 min |
| Temperature (°C) | 14.00 ± 0.00 | 14.05 ± 0.15 | 14.00 ± 0.30 | 13.95 ± 0 .15 |
| pH (NBS) | 8.14 ± 0.02 | 6.95 ± 0.02 | 6.91 ± 0.01 | 6.91 ± 0.04 |
| Salinity | 34.6 ± 0.4 | 35.6 ± 0.5 | 34.8 ± 0.7 | 34.6 ± 0. 7 |
| $pCO_2$ (µatm) | 588.4 ± 97.1 | 9518.9 ± 443.4 | 12109.0 ± 1616.5 | 10101.6 ± 867.9 |
| TA (µM) | 3120.0 ± 371.9 | 2833.8 ± 36.2 | 3300.9 ± 360.5 | 2749.7 ± 26.5 |

**Table S2:** Water chemistry of gill irrigation chambers used while blood sampling fish in low alkalinity water

| pH (NBS) | Temperature (°C) | Salinity | $pCO_2$ (µatm) | TA (µM) |
|---|---|---|---|---|
| 5.53 ± 0.31 | 14.05 ± 0.05 | 35.05 ± 0.25 | 12709.3 ± 1025.7 | 187.5 ± 129.1 |

# Chapter IV

## Rising $CO_2$ enhances hypoxia tolerance in a marine fish

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## Abstract

Global environmental change is increasing hypoxia in aquatic ecosystems. During hypoxic events, bacterial respiration causes an increase in carbon dioxide ($CO_2$) while oxygen ($O_2$) declines. This is rarely accounted for when assessing hypoxia tolerances of aquatic organisms. We investigated the impact of environmentally realistic increases in $CO_2$ on responses to hypoxia in European sea bass (*Dicentrarchus labrax*). We conducted a critical oxygen ($O_{2crit}$) test, a common measure of hypoxia tolerance, using two treatments in which $O_2$ levels were reduced with constant ambient $CO_2$ levels (~530 µatm), or with reciprocal increases in $CO_2$ (rising to ~2,500 µatm). We also assessed blood acid-base chemistry and haemoglobin-$O_2$ binding affinity of sea bass in hypoxic conditions with ambient (~650 µatm) or raised $CO_2$ (~1770 µatm) levels. Sea bass exhibited greater hypoxia tolerance (~20 % reduced $O_{2crit}$), associated with increased haemoglobin-$O_2$ affinity (~32 % fall in $P_{50}$) of red blood cells, when exposed to reciprocal changes in $O_2$ and $CO_2$. This indicates that rising $CO_2$ which accompanies environmental hypoxia facilitates increased $O_2$ uptake by the blood in low $O_2$ conditions, enhancing hypoxia tolerance. We recommend that when impacts of hypoxia on aquatic organisms are assessed, due consideration is given to associated environmental increases in $CO_2$.


## 1. Introduction

A lack of $O_2$ is one of the greatest challenges that most life can face. In terrestrial ecosystems conditions of low $O_2$ are rare. In contrast low $O_2$, referred to as hypoxia, is much more common in freshwater and marine ecosystems (Diaz, 2001; Diaz and Rosenberg, 2008; Townhill *et al.*, 2017; Breitburg *et al.*, 2018). Hypoxia occurs because high biological demand for $O_2$ can exceed the rate of $O_2$ supply to the ecosystem, leading to a reduction in environmental $O_2$ levels (Rabalais *et al.*, 2009; Robinson, 2019). However, the challenges of hypoxia are not solely a result of reduced $O_2$. Organisms must also contend with simultaneous but reciprocal changes in the other respiratory gas, $CO_2$.

When $O_2$ decreases in aquatic systems there is a corresponding increase in $CO_2$ (Sunda and Cai, 2012; Wallace *et al.*, 2014). This is a by-product of respiration, the same process that causes depletion of $O_2$. As such high $CO_2$ during hypoxia is ubiquitous and unavoidable. This coupling of $O_2$ and $CO_2$ has

been highlighted numerous times in oceanographic sciences, most recently by Robinson (2019). Yet unaccountably, despite the known link between decreasing $O_2$ and increasing $CO_2$ during hypoxia (Wallace *et al.*, 2014; Gobler and Baumann, 2016), the issue of increased environmental $CO_2$ during periods of low $O_2$ has been relatively overlooked by biologists.

Implications of rising $CO_2$ during hypoxia on aquatic organisms are particularly important to address in the face of human driven climate change. Hypoxic areas are predicted to become more common and more severe, particularly in marine systems, with the de-oxygenation of the world's oceans recently highlighted as a major component of climate change (Diaz and Rosenberg, 2008; Oschlies *et al.*, 2008, 2018; Altieri and Gedan, 2015; Breitburg *et al.*, 2018). In addition, there will be an increase in ambient $CO_2$ as rising atmospheric $CO_2$ is absorbed by the world's oceans (Doney *et al.*, 2009). Non-linear interactive effects between higher atmospheric $CO_2$ and $CO_2$ accumulation during hypoxia will lead to increased $CO_2$ levels during hypoxia in future oceans (Melzner *et al.*, 2013). This means that effects of rising $CO_2$ during hypoxia in marine systems will be amplified by climate change.

Typically, experiments which test responses to hypoxia or impacts from hypoxia on aquatic organisms create hypoxic conditions by off-gassing oxygen from water by gassing with pure nitrogen or a mix of nitrogen ($N_2$) and $O_2$ (for examples see Chabot and Dutil, 1999; Domenici *et al.*, 2000; Brandt *et al.*, 2009; Almeida *et al.*, 2017). This creates low $O_2$ conditions without the concurrent $CO_2$ increase that would be expected in the environment. The lack of studies in which an environmentally realistic simultaneous decrease in $O_2$ and increase of $CO_2$ have been conducted may lead to mismeasurement of responses to hypoxia. Recently, several studies on marine fish and invertebrates have demonstrated interactive effects of low oxygen and increased $CO_2$ (Gobler *et al.*, 2014; Steckbauer *et al.*, 2015; Gobler and Baumann, 2016), with some species exhibiting loss of equilibrium (LoE) and death at higher $O_2$ concentrations when $CO_2$ is simultaneously elevated (Gobler *et al.*, 2014; Miller *et al.*, 2016). However, these experiments do not give insight into the physiological mechanisms underlying the influence of $CO_2$ on hypoxic responses of fish.

Previously observed impacts of hypoxia-associated rises in $CO_2$ on hypoxia tolerance of fish could be a result of changes in $O_2$ uptake, as $CO_2$ has been shown to impact upon several aspects of organismal biology that are involved in $O_2$ uptake and transport (Nikinmaa, 1997; Rummer and Brauner, 2015). We aimed to assess whether concurrent increases in $CO_2$ during decreases in $O_2$ affect $O_2$ uptake in a marine fish, the European sea bass (*Dicentrarchus labrax*) by conducting a standard critical $O_2$ level test ($O_{2crit}$). Under normal $O_2$ (normoxic) conditions fish maintain a minimum level of $O_2$ consumption rate ($\dot{M}O_2$), referred to as the standard metabolic rate (SMR), in order to meet maintenance energetic demands of essential processes through aerobic respiration (Chabot *et al.*, 2016). As the level of $O_2$ in water drops fish deploy a number of responses (i.e. increased ventilatory water flow and cardiac output, increased haematocrit, functional changes in gill morphology, changes in Hb-$O_2$ affinity) in order to maintain and regulate this minimum level of $\dot{M}O_2$ (Gamperl and Driedzic, 2009; Perry *et al.*, 2009; Wells, 2009). If environmental $O_2$ continues to drop there comes a point at which fish are unable to regulate $\dot{M}O_2$ to meet minimal energy demands, referred to as the critical $O_2$ level ($O_{2crit}$). At $O_2$ levels below $O_{2crit}$ fish become oxy-conformers (where $\dot{M}O_2$ is directly proportional to environmental $O_2$ availability) and fish become increasingly reliant on anaerobic metabolism which is unsustainable in the medium to long term. In the past the measure of $O_{2crit}$ has been used as a proxy for overall hypoxia tolerance but recently this approach has been questioned (Speers-Roesch *et al.*, 2013; Wood, 2018). Nevertheless $O_{2crit}$ does provide information related to the ability of fish to maintain $O_2$ uptake and supply during hypoxia, and its prevalence in the literature allows comparison of responses between species (Regan *et al.*, 2019). Furthermore, we investigated whether any changes in $O_{2crit}$ could be linked to changes in blood acid-base chemistry and blood gas transport via alteration of Hb-$O_2$ binding caused by rising environmental $CO_2$. Our hypothesis was that the simultaneous increase in $CO_2$ during a progressive decrease in $O_2$ would decrease hypoxia tolerance (increase $O_{2crit}$) and that this response may be a result of blood acid-base disturbance decreasing Hb-$O_2$ affinity and $O_2$ transport.

# 2. Materials and Methods

## 2.1. Fish Collection and Husbandry

We collected juvenile sea bass from estuaries and coastal lagoons on the south Dorset coast and Isle of Wight in June 2017 (Marine Management Organisation permit #030/17 & Natural England permit #OLD1002654). Prior to experimentation, these fish were held in the University of Exeter's Aquatic Resource Centre in an aerated recirculating aquaculture system and fed a commercial pellet at a ration of ~1-2 % body weight per day three times a week (for system water chemistry see Table 1). All fish were starved for a minimum of 72 hours prior to the start of all measurements to ensure the metabolism was not affected by digestion (i.e. specific dynamic action Chabot *et al.*, 2016). All experimental procedures were carried out under home office licence P88687E07 and approved by the University of Exeter's Animal Welfare and Ethical Review Board.

**Table 1:** Water chemistry parameters of the recirculating aquaculture system in which sea bass were held prior to experimental work (mean ± S.E. shown).

| **Time held in system (days)** | **Temperature (ºC)** | **pH (NBS scale)** | **Salinity** | **Total Alkalinity (µM/kgSW)** | ***p*$CO_2$ (µatm)** |
|---|---|---|---|---|---|
| 318 | 18.01 ± 0.03 | 8.04 ± 0.04 | 33.25 ± 0.70 | 2064.6 ± 136.0 | 516.9 ± 41.1 |

## 2.2. Measuring hypoxia tolerance

We determined oxygen consumption rates ($\dot{M}O_2$) of sea bass using an intermittent-flow respirometer system. The respirometer system set up followed recommendations set out by Svendsen *et al.* (2016). Briefly, the system comprised of a sealed 4.515 L respirometer chamber connected to a recirculating loop, including an in-line recirculating pump (Eheim universal 600, Deizisau, Germany), and a measurement chamber into which a temperature-compensated fibre optic oxygen optode (Firesting $O_2$ oxygen meter, Pyroscience GmBH, Germany) was placed. Oxygen optodes were calibrated in water at the start of experiments at 100% air saturation and 0% air saturation according to manual instructions. Respirometry was conducted in a semi-closed system consisting of three 100 L experimental tanks fed by a 100 L sump, with

overflowing water from the experimental tanks recirculating back to the sump. A second pump was used to periodically flush the respirometer system with water from the surrounding tank. This pump was controlled by an automated computer program (AquaResp 3, AquaResp®) to intermittently flush the respirometer. Five respirometer chambers were distributed between the three experimental tanks (maximum of 2 chambers per experimental 100 L tank). The sump was temperature controlled (18.27 ± 0.02 $^{o}$C, mean ± S.E.) using a heater/chiller unit (Grant TX150 R2, Grant Instruments, Cambridge, UK) attached to a temperature exchange coil. Together these tanks formed a 400 L system with the same temperature, oxygen and water chemistry parameters for all respirometers.

Individual sea bass (average mass = 131.2 ± 7.5 g), chosen at random, were placed inside the respirometers and allowed an overnight recovery period, for a minimum of 13 hours, before $O_{2crit}$ tests began. While sea bass were in the respirometers measurements of $\dot{M}O_2$ were conducted every 10 minutes, including a flush period of 300 s, a wait period of 60 s and a measurement period of 240 s. During the wait and measurement period the chamber was sealed by switching off the flush pump and the decline in dissolved $O_2$ within the chamber was continuously measured by the fibre optic $O_2$ electrode.

Following the overnight recovery period a standard $O_{2crit}$ test was conducted. Oxygen levels in the respirometer system were reduced from ~ 100 % air saturation to ~ 15 % air saturation over the course of 6 hours (decline in $O_2$ was ~20 % air saturation per hour between ~100 % air saturation and ~40 % air saturation and ~10 % air saturation per hour from ~40 % air saturation until the end of trials). Oxygen was regulated by gassing the sump and experimental tanks with a mix of $N_2$ and $O_2$ (G400 Gas mixing system, Qubit Biology Inc.) at a rate of 10 L $min^{-1}$ following a pre-set automated protocol (Flowvision, Alicat software). Levels of $CO_2$ within the system were controlled under one of two treatments (with 8 fish exposed to Treatment 1 and 8 separate fish exposed Treatment 2):

- Treatment 1 (Constant $CO_2$) - ambient levels of $CO_2$ were maintained by including 0.04 % $CO_2$ as part of the gas mix delivered to the respirometer system.

- Treatment 2 (Rising $CO_2$) - the proportion of $CO_2$ in the gas mix was gradually increased as $O_2$ was decreased. This increase in $CO_2$ was designed to reflect environmentally realistic increases in $CO_2$ predicted as a result of depletion of $O_2$ by bacterial respiration (assumed respiratory quotient of 1; Del Giorgio and Duarte, 2002), using the seawater carbonate chemistry calculator CO2sys (see supplementary material for predictions of increased $CO_2$ during hypoxia).

Water chemistry of treatment 2 was monitored once per hour by measuring $pH_{NBS}$, temperature and salinity as well as taking a 12 mL water sample to measure total $CO_2$ ($TCO_2$)/Dissolved Inorganic Carbon (DIC). Water chemistry of treatment 1 was monitored at the start and end of the treatment to ensure no change in water $pCO_2$ occurred over the time course of the $O_{2crit}$ trial. Seawater DIC analysis was conducted using a custom built system described in detail by Lewis *et al.* (2013). These four parameters were then input into the seawater carbon calculator programme, CO2SYS to calculate $pCO_2$ based on the NBS pH scale, equilibration constants from Mehrbach *et al*. refitted by Dickson and Millero, and $KSO_4$ dissociation constants from Dickson. The reciprocal changes in $O_2$ and $CO_2$ during $O_{2crit}$ tests for each treatment are illustrated in Figure 1. $O_{2crit}$ tests were stopped once a minimum of 3 $\dot{M}O_2$ measurements showed a transition from an oxy-regulating to oxy-conforming state for each fish or fish showed a large drop in $\dot{M}O_2$ and signs of distress in the respirometer. No fish exhibited LoE during trials. Following completion of $O_{2crit}$ trials experimental tanks were aerated with ambient air to swiftly restore $O_2$ and $CO_2$ levels.

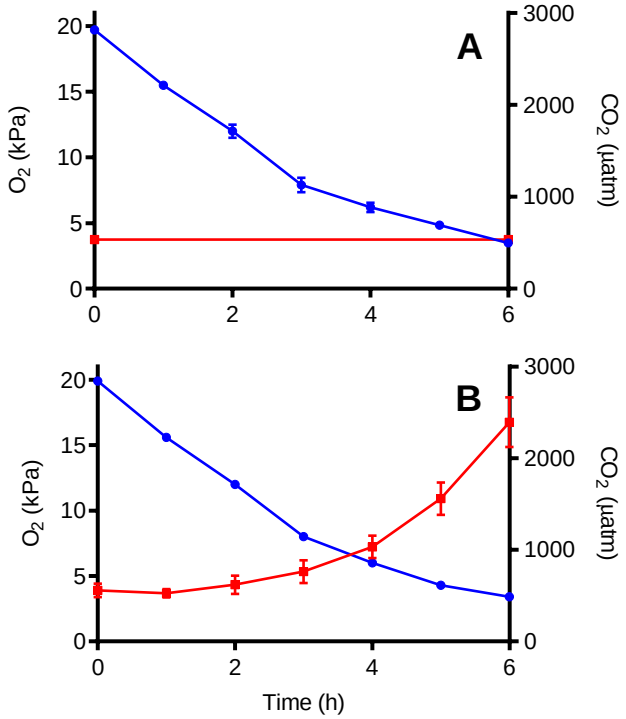


**Figure 1:** Changes in partial pressure of $O_2$ (expressed as kPa $O_2$) and $CO_2$ (µatm) during (**A**) two $O_{2crit}$ trials representing treatment 1 in which $O_2$ was reduced with no change in $CO_2$; and (**B**) two trials representing treatment 2 where $O_2$ was reduced with a corresponding rise in $CO_2$. Data presented are means ± S.D.

Sea bass were left to recover in respirometers, for a minimum of 1 hour post-trial, until $O_2$ levels reached ~ 21 kPa $O_2$ (~100 % air saturation). Fish were then removed from respirometers and background respiration was measured for a minimum of 1 hour (6 measurement cycles) for all respirometers immediately post trial.

### 2.2.1. Oxygen consumption rate ($\dot{M}O_2$) analysis

Following each 240 s measurement period $\dot{M}O_2$ was automatically calculated by the AquaResp3 software. A linear regression was fitted to the $O_2$ versus time data for each measurement period. The slope of this regression (s, kPa $O_2$ $h^{-1}$) was then used to calculate $\dot{M}O_2$ (mg $O_2$ $kg^{-1}$ $h^{-1}$) using the equation outlined by Svendsen *et al.* (2016):

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

where $V_{resp}$ is the respirometer volume minus the volume of the fish (L), α is the solubility of $O_2$ in water ($mgO_2$ $L^{-1}$ $kPa^{-1}$) for the relevant salinity and temperature, and m is the mass of the fish (kg). Calculations of $\dot{M}O_2$ where s had a $R^2$ of < 0.98 were removed from subsequent analysis. For the purpose of establishing $O_{2crit}$ values from a plot of $\dot{M}O_2$ versus ambient $O_2$ level, the oxygen saturation of each measurement period was defined as the average of the dissolved $O_2$ measurement over the measurement period. The average background respiration, over the 1 hour post-trial measuring period, for each respirometer (average background respiration was < 2 % of fish $\dot{M}O_2$) was subtracted from $\dot{M}O_2$ measurements to correct fish $\dot{M}O_2$ for background respiration.

We calculated the standard metabolic rate (SMR) in R v3.5.3 (R Core Team, 2020) using function calcSMR in package fishMO2 (Chabot *et al.*, 2016). All $\dot{M}O_2$ values from the overnight recovery period and beginning of the $O_{2crit}$ trial in which average dissolved $O_2$ saturation was >80 % air saturation were included for SMR calculations. This resulted in approximately 14-16 hours of $\dot{M}O_2$ data to calculate SMR from for each fish. We estimated SMR for all fish using the mean of the lowest 10 $\dot{M}O_2$ measurements during the overnight period. Although Chabot *et al.* (2016) recommend use of the mean of the lowest normal distribution (MLND) or quantile (q = 0.2) methods to calculate SMR we chose the mean of the 10 lowest values as it produced a value of SMR that most accurately matched the consistent low $\dot{M}O_2$ measurements of oxy-regulating fish at values of $pO_2$ above the $O_{2crit}$ point. Additionally, for each fish the coefficient of variation in the mean lowest normal distribution (MLND) was assessed using a ROUT test (Q = 1 %) to check whether variation in low $\dot{M}O_2$ measurements was consistent between fish (to account for potential differences in activity in the respirometer prior to $O_{2crit}$ trials). The ROUT test removed one fish from the rising $CO_2$ treatment which displayed a CoV of 34.6. Mean CoV of the remaining 15 fish was 11.85 ± 1.26 (± S.E.M).

We then used function calcO2crit from the package to calculate the $O_{2crit}$ for each individual fish, using the estimated SMR of each individual, as detailed in the supplementary material of Claireaux & Chabot (2016). This function identifies the portion of the $O_{2crit}$ test where metabolic rate data follows an oxygen conforming relationship and fits a linear regression line through this

data, $O_{2crit}$ is then calculated as the oxygen level at which this regression line crosses the calculated SMR of the individual fish. Plots of $\dot{M}O_2$ against $O_2$ during $O_{2crit}$ trials showing calculated $O_{2crit}$ for each individual can be seen in the supplementary material (Supplementary material Figure 1-16). Calculations were conducted using a gap limit of 0.83 kPa $O_2$ (4 % air saturation) and a maximum number of 7 $\dot{M}O_2$ points to fit the regression line through the oxygen conforming component of the data used to estimate $O_{2crit}$.

2.3. Measuring Blood Chemistry

Following $O_{2crit}$ trials sea bass were moved to individual 7 L chambers, which were aerated and supplied with seawater from the aquarium re-circulating system at a rate of about 4 L $min^{-1}$. After an overnight acclimation period we then exposed the fish to a decrease in $O_2$ levels to ~6.4 kPa $O_2$ (30 % air saturation) over a period of 4 hours (equivalent to the rate of decrease in $O_2$ used in the previous $O_{2crit}$ tests). We chose to blood sample fish at an $O_2$ level above $O_{2crit}$ to ensure that anaerobic metabolism did not influence blood chemistry. This was combined with the same $CO_2$ regime each fish experienced during the $O_{2crit}$ test (i.e. either constant or reciprocally rising $CO_2$). Once an $O_2$ level of ~6.4 kPa $O_2$ (30 % air saturation) was achieved the fish were allowed to acclimate for 1 hour before being individually anaesthetised in situ using a dose of 100 $mgL^{-1}$ of benzocaine. Once fish were judged to be sufficiently anaesthetised (cessation of gill ventilation and lack of response to a pinch of the anal fin) they were immediately transferred within 5 seconds to a gill irrigation table (with the same $pO_2$ and $pCO_2$ levels of the respective treatment), where anaesthesia was maintained with a dose of 37.5 $mgL^{-1}$ of benzocaine. Gill ventilation was artificially maintained by a micro-pump, so that the operculum were just open and exhalant water flow could just be visualised. Once a stable gill water flow was established blood was sampled by caudal vessel puncture using a 1 ml heparinised syringe. This method has been demonstrated to obtain accurate measurements of blood chemistry parameters comparable to those achieved using cannulation (Davison & Wilson, University of Exeter, personal communication). At the time of blood sampling water $pCO_2$ was 656 ± 44 µatm (mean ± S.E.) for fish in the constant ambient $CO_2$ regime and 1763 ± 43 µatm (mean ± S.E.) for fish in the progressively rising $CO_2$ regime. Water $pO_2$ was 8.1 ± 0.2 kPa (mean ± S.E., ~ 38 % air saturation) for fish in the constant ambient

$CO_2$ regime and 8.7 ± 0.2 kPa (mean ± S.E., ~ 41 % air saturation) for fish in the progressively rising $CO_2$ regime. Following blood sampling fish were transferred to seawater isolation tanks containing ~20.8 kPa $O_2$ (100 % air-saturated) to recover from the anaesthetic. They were then monitored over a period of 24 hours before we returned them to their original holding tanks.

Immediately after sampling, whole blood *p*O2 was measured at 18 $^oC$ in a temperature-controlled system (Strathkelvin 1302 electrode and 781 meter; Strathkelvin Instruments Ltd, Glasgow, UK). We measured extracellular pH on 30 µL of whole blood using an Accumet Micro pH electrode and Hanna HI8314 pH meter at 18 °C calibrated to $pH_{NBS}$ 7.04 and 9.21 specific buffers. Three 75 µL micro capillary tubes were then filled with whole blood and sealed with Critoseal capillary tube sealant (Fisher) and paraffin oil and centrifuged for 2 minutes at 10,000 rpm. Haematocrit was measured using a Hawksley micro-haematocrit reader. Plasma was extracted from these tubes for analysis of $TCO_2$ using a Mettler Toledo 965D carbon dioxide analyser. Plasma $pCO_2$ and $HCO_3^-$ were then calculated from $TCO_2$, temperature and blood pH using the Henderson-Hasselbalch equation with values for solubility and $pK^1_{app}$ based on Boutilier *et al.* (1984, 1985). Haemoglobin content of the blood was also assessed by the cyanmethemoglobin method (using Drabkin's reagent, Sigma). Half the remaining whole blood was then centrifuged at 10,000 rpm for 2 minutes at 4 $^oC$. The resulting plasma was separated and snap frozen in liquid nitrogen and stored at -80 $^oC$ before later being used to measure plasma glucose and lactate using a YSI 2900D Biochemistry Analyzer (Xylem Analytics, UK). All measurements or storage of blood occurred within 10 minutes of blood sampling.

2.4. Measuring Hb-oxygen binding

We measured Hb-$O_2$ affinity ($P_{50}$) using a Blood Oxygen Binding System (BOBS, Loligo systems), detailed in Oellermann *et al.* (2014). A sample of the same whole blood used for blood chemistry measurements was diluted at a ratio of 1:4 in its own plasma. 1 µL of this blood was then used for measurements. The BOBS exposed this blood sample to gas mixes with a progressive increase in $O_2$ whilst measuring absorbance of light across a spectrum ranging from 200 to 800 nm. For each individual fish the gas mix that blood was exposed to matched the calculated $pCO_2$ of the blood sample. The

change in absorption of light at a wavelength of 435 nm was used to assess changes in oxygenation of Hb, as previously used by Verhille & Farrell (2012). Background changes in absorption of the blood sample were corrected using the isosbestic wavelength of 390 nm (Verhille and Farrell, 2012). Following measurements the BOBS calculated the oxygen equilibrium curve of the sample using Hill's formula before estimating $P_{50}$ (Oellermann *et al.*, 2014).

### 2.5. Statistical analysis

We conducted all statistical analysis in R v3.5.3 (R Core Team, 2020). There was no significant difference in mass of fish between the treatment groups (One-Way ANOVA, $F_{1,13}$ = 2.821, p = 0.117) or of estimated SMR of fish between treatment groups (Unpaired t-test, t = 0.8455, d.f. = 13, p = 0.413). There was, however, a significant correlation between estimated SMR and calculated $O_{2crit}$ for all fish (Pearson's correlation, df = 13, t = 3.32, R = 0.68, p = 0.0056). As such the effect of $CO_2$ treatment on $O2_{crit}$ was assessed using an ANCOVA with SMR as a covariate. All other analyses were conducted using general linear modelling (GLM). All values in the text are reported as mean ± standard error (S.E.)

## 3. Results

### 3.1. $O_{2crit}$ tests

There was evidence of enhanced tolerance to hypoxia in sea bass exposed to rising compared to constant $CO_2$ conditions. This was indicated by measurements of $O_{2crit}$ in European sea bass being significantly different between fish exposed to either constant, or rising $CO_2$ levels during $O_{2crit}$ tests when accounting for variation in SMR (Figure 2; ANCOVA, $F_{1,12}$ = 7.525, p = 0.0178). A $CO_2$ increase during $O_{2crit}$ tests resulted in a 20 % reduction of $O_{2crit}$ (3.88 ± 0.19 kPa $O_2$, 18.7 ± 0.9 % air saturation, mean ± S.E.) when compared to tests in which $CO_2$ levels were maintained at ambient levels (4.87 ± 0.22 kPa $O_2$, 23.4 ± 1.1 % air saturation, mean ± S.E.).

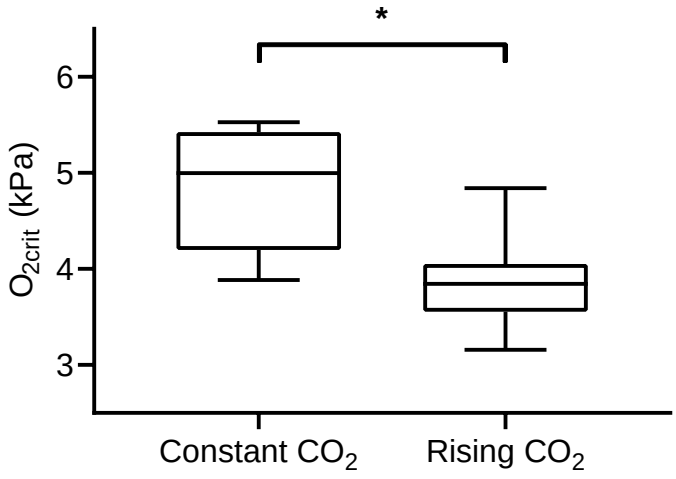


**Figure 2:** Calculated critical oxygen level ($O_{2crit}$) of European sea bass, *Dicentrarchus labrax*, when $O_{2crit}$ tests include a constant ambient $CO_2$ level (~500 µatm, N = 8) or an ecologically realistic rise in $CO_2$ (~500-2500 µatm, N = 7) during the test. * indicates significant difference between $CO_2$ regimes ($p<0.05$). Boxes represent median value and inter-quartile range, whiskers represent minimum and maximum values.

3.2. Blood chemistry analysis

A comparison of blood chemistry parameters between the two treatment groups indicated that sea bass fully compensated for the rise in $CO_2$ during hypoxia within 5 hours (the period of exposure prior to blood sampling). Blood pH ($pH_e$) was not different between fish exposed to constant ambient $CO_2$ (7.87 ± 0.03) and fish exposed to progressively rising $CO_2$ (7.88 ± 0.02) (Figure 3A, GLM, $F_{1,14} = 0.23$, $p = 0.64$). The acidifying effect of the ~79 % rise in blood $pCO_2$ levels in the rising $CO_2$ regime (0.272 ± 0.019 kPa $CO_2$) compared to the constant ambient $CO_2$ regime (0.152 ± 0.015 kPa $CO_2$) (Figure 3C, GLM, $F_{1,13} = 23.9$, $p < 0.001$) was fully compensated by elevating blood $HCO_3^-$ (Figure 3D, GLM, $F_{1,13} = 40$, $p < 0.001$). Plasma $HCO_3^-$ was 88 % higher under the rising $CO_2$ regime (6.76 ± 0.29 mM) when compared to the constant ambient $CO_2$ (3.60 ± 0.43 mM). There were no differences in haematocrit (Figure 3B, general linear model, $F_{1,13} = 0.69$, $p = 0.42$) or plasma lactate (Figure 3E, general linear model, $F_{1,14} = 1.48$, $p = 0.24$) between fish sampled under a constant ambient $CO_2$ regime (haematocrit = 39.4 ± 0.8 %, lactate = 0.88 ± 0.26 mM) or rising $CO_2$ regime (haematocrit = 38.2 ± 1.1 %, lactate = 0.53 ± 0.12 mM). Blood glucose levels were ~26 % lower in fish exposed to a progressively

rising $CO_2$ regime (4.50 ± 0.53 mM) when compared to a constant ambient $CO_2$ regime (6.09 ± 0.31 mM) (Figure 3F, GLM, $F_{1,14} = 6.74$, $p = 0.021$).

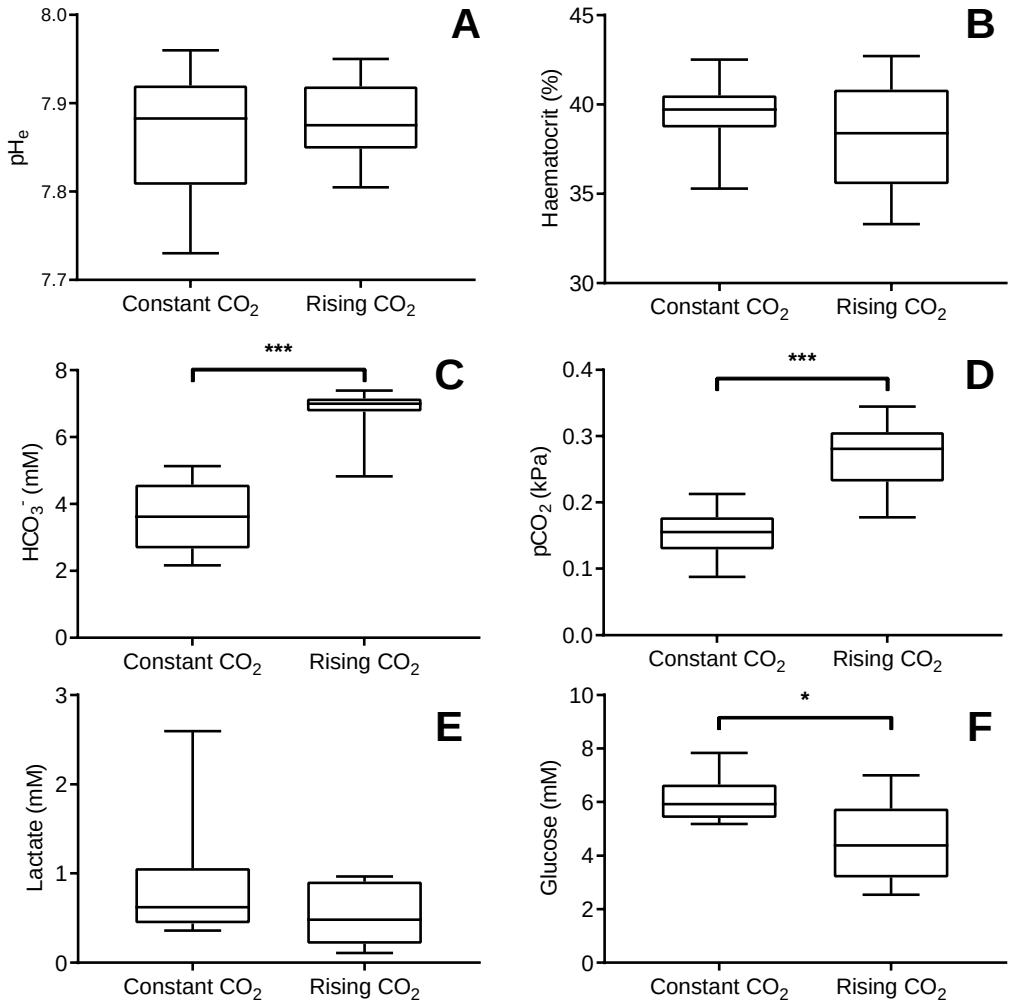


**Figure 3:** Blood chemistry characteristics of European sea bass sampled at ~8.4 kPa $O_2$ (~40 % air saturation) following a progressive $O_2$ decline accompanied by either constant $CO_2$ (~ 650 µatm $CO_2$, N = 8) or a progressive increase in $CO_2$ (sampled at ~1770 µatm $CO_2$, N = 8). Blood pH (**A**), haematocrit (**B**), plasma lactate (**E**) and plasma glucose (**F**) were directly measured whilst blood $pCO_2$ (**C**) and plasma $HCO_3^-$ (**D**) were calculated (see Methods for details). * indicates statistical significance with $p < 0.05$ and *** indicates statistical significance with $p < 0.001$. Boxes represent median value and inter-quartile range, whiskers represent minimum and maximum values.

### 3.3. Haemoglobin affinity for oxygen

Oxygen affinity of haemoglobin was increased in fish sampled under the progressively rising $CO_2$ regime (Figure 4A, GLM, $F_{1,12} = 10.42$, $p = 0.0073$). Haemoglobin $P_{50}$ was decreased by ~32.5 % in fish under the progressively rising $CO_2$ regime (1.64 ± 0.15 kPa $O_2$, 7.9 ± 0.7 % air saturation) compared to fish sampled with a constant ambient $CO_2$ (2.43 ± 0.20 kPa $O_2$, 11.7 ± 1 % air saturation). There was no significant change in Hills number between treatments (Figure 4B, GLM, $F_{1,12} = 0.50$, $p = 0.494$).

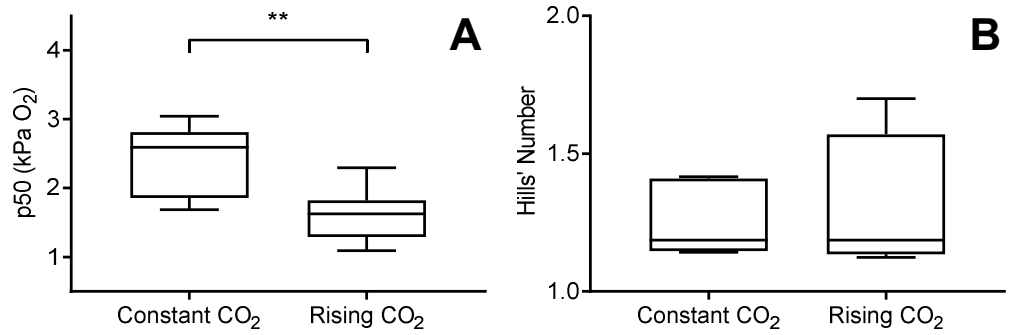


**Figure 4:** Haemoglobin $P_{50}$ (**A**) and Hills number (**B**) for fish sampled at ~8.4 kPa $O_2$ (~40 % air saturation) following a progressive $O_2$ decline accompanied by either constant $CO_2$ (~ 650 µatm $CO_2$, N = 7) or a progressive increase in $CO_2$ (sampled at ~1770 µatm $CO_2$, N = 7). Measurements were made using a gas mix which matched the calculated blood $pCO_2$ of each individual fish blood sample. ** indicates statistical significance with $p < 0.01$. Boxes represent median value and inter-quartile range, whiskers represent minimum and maximum values.

## 4. Discussion

Our results highlight the biological importance of simultaneously rising $CO_2$ under conditions where $O_2$ levels in water are depleted – a scenario that reflects the natural conditions during hypoxia which will be exacerbated by climate change – by demonstrating that ecologically relevant changes of $CO_2$ impact physiological performance of a marine fish at both the molecular and whole organism level. We hypothesised that rising $CO_2$ during progressive $O_2$ decreases would lead to an increase in $O_{2crit}$ as a result of increased blood $CO_2$, decreased blood pH and the associated Bohr/Root effect of fish haemoglobin

(Hb), in which Hb-$O_2$ affinity (Bohr effect) and the total capacity of Hb for $O_2$ (Root effect) are reduced when pH falls. In contrast, we show that increasing $CO_2$ as $O_2$ declined led to enhanced hypoxia tolerance of sea bass with a 20 % lower critical oxygen level (Figure 2). This change in whole organism hypoxic response was accompanied by an increase in Hb-$O_2$ affinity of blood cells in fish exposed to concurrent $CO_2$ rises (Figure 4). The change in Hb-$O_2$ affinity was not accompanied by a change in blood pH (although the one hour acclimation to treatment $pCO_2$ prior to blood sampling may have contributed to this result). This provides a potential mechanistic basis to explain improved $O_{2crit}$, enabling sea bass to enhance $O_2$ uptake during hypoxia and thus maintain normal aerobic metabolism to lower environmental $O_2$.

The driver of increased Hb-$O_2$ affinity in sea bass exposed to concurrent $O_2$ decline and $CO_2$ rise is not clear from our results. Several allosteric factors that can modulate the affinity of haemoglobin for $O_2$ could be involved, including pH, organic phosphates and inorganic ions. Fish haemoglobin is highly sensitive to pH, which modulates Hb-$O_2$ affinity and carrying capacity via the Bohr and Root effects (Pelster and Weber, 1991), but we found no differences in blood pH of sea bass between treatment groups. In addition, the in-vivo increase in $pCO_2$ in fish exposed to concurrent $CO_2$ rises during hypoxia led to an opposite response of Hb-$O_2$ affinity than would be expected by an in-vitro rise in $pCO_2$ which would result in pH induced Bohr/Root effects. Increased Hb-$O_2$ affinity could result from increased intracellular pH of erythrocytes (Nikinmaa, 1983), as acute hypoxic exposure has been shown to stimulate a β-adrenergic stimulated increase in intracellular erythrocyte pH in rainbow trout (Tetens and Christensen, 1987). Alternatively, increased Hb-$O_2$ affinity could be due to decreased red cell nucleoside triphosphates (NTPs) (Weber and Lykkeboe, 1978; Pelster and Weber, 1991), a known hypoxia adaptation, but this can take more than 6 days to complete (Soivio *et al.*, 1980). Sea bass may also have a particularly strong β-adrenergic response and/or fast NTP response, although there is little evidence to suggest this because $P_{50}$ measurements from fish exposed to hypoxia at ambient $CO_2$ levels do not differ from $P_{50}$ measurements in normoxic fish from the same population (see **chapter III**). It is possible that rising $CO_2$ during hypoxia may modulate the β-adrenergic response and/or the red cell NTP response within the time frame (~4-6 hours) of our treatments.

However, direct measurements of red cell $pH_i$ and NTP content of sea bass in each treatment group would be needed to confirm this.

A third possible driver of increased Hb-$O_2$ affinity in sea bass exposed to concurrent $CO_2$ rise during hypoxia could be decreased erythrocyte chloride ($Cl^-$) (Mairbäurl and Weber, 2012). Although erythrocyte $Cl^-$ was not directly measured in our study, plasma $HCO_3^-$ was approximately 3 mM higher in fish exposed to concurrent $CO_2$ rises during hypoxia than fish which experienced constant ambient $CO_2$ during hypoxia (Figure 2D). The higher $HCO_3^-$ in fish exposed to rising ambient $CO_2$ during hypoxia is likely a result of rapid compensation for a respiratory acidosis due to rising blood $pCO_2$. This change in plasma $HCO_3^-$ is typically mirrored by a reciprocal change in plasma $Cl^-$ (Perry *et al.*, 1992; Esbaugh *et al.*, 2012) which is likely to be followed by a similar decline in erythrocyte $Cl^-$.

Analysis of $O_{2crit}$ is a common measure of hypoxia tolerance in fish but concurrent $CO_2$ increases during hypoxia have been generally unaccounted for. A recent meta-analysis by Rogers *et al.* (2016) constructed a database of $O_{2crit}$ research of fish (both freshwater and marine). This analysis identified two broad methods employed in $O_{2crit}$ measurements:

1. Closed respirometry where $O_2$ is usually reduced by the $O_2$ consumption of the fish (52 identified studies) or;
2. Intermittent or flow-through respirometry in which $O_2$ is usually reduced via gassing with pure $N_2$ or combined $N_2$ and $O_2$ mixes (32 identified studies).

The use of closed respirometry in the majority of studies would result in concurrent $CO_2$ rises as $O_2$ is depleted by fish $O_2$ consumption. The increase in ambient $CO_2$ during closed respirometry is well known and often used as a criticism of this respirometry technique (Steffensen, 1989). In contrast, use of intermittent-flow respirometry in $O_{2crit}$ trials normally necessitates the reduction of $O_2$ in the water by aeration with $N_2$ or a mix of $N_2$ & $O_2$. As a result $CO_2$ would likely decrease during the time course of hypoxia induction (as the gas mixture would contain zero $CO_2$, rather than ~400 µatm present in atmospheric air). Such a change in $CO_2$ during the $O_{2crit}$ trial would be the opposite of that seen in nature. Therefore it may be considered that closed respirometry provides

conditions which give a more environmentally relevant measure of $O_{2crit}$ (Wood, 2018).

As our results indicate that rising $CO_2$ during hypoxia directly affects the ability of sea bass to maintain $O_2$ uptake, it could be expected that the use of closed respirometry methods would result in lower measurements of $O_{2crit}$ than intermittent-flow methods for the same species. This effect has not been documented for species in which a direct comparison has been made – with either there being no effect of respirometry method on $O_{2crit}$ (Rogers *et al.*, 2016; Regan and Richards, 2017) or higher $O_{2crit}$ measurements when closed respirometry is used (Regan and Richards, 2017). However, such comparisons are complicated by differences in the rate of hypoxia induction (RHI) by different studies, which in turn will influence how much time fish have to regulate blood pH when ambient $CO_2$ is rising. For example, Regan and Richards (2017) have demonstrated that the faster rates of hypoxia induction (RHI) typical of closed respirometry $O_{2crit}$ trials lead to higher values of $O_{2crit}$ (i.e. lower hypoxia tolerance) when compared with longer trials using slower RHI's typical of the intermittent-flow method. . The effect of RHI on $O_{2crit}$ was proposed by Regan and Richards (2017) as a potential explanation of the results of Snyder *et al.* (2016) (i.e. higher $O_{2crit}$ in closed respirometry compared to intermittent-flow respirometry). The speed of RHI during closed respirometry will also effect the speed of $CO_2$ rise. Almost all studies using closed respirometry to measure $O_{2crit}$ do not report changes in $CO_2$ over the course of measurement period. When accumulation of $CO_2$ during a closed respirometry $O_{2crit}$ trial was measured by Regan and Richards (2017), $CO_2$ levels were ~8,000 µatm after ~90 minutes. However, we should note that this $pCO_2$ level was measured after the $O_{2crit}$ point, when anaerobic metabolism continues to produce $CO_2$ in the absence of $O_2$ consumption, but also metabolic acid production and excretion further drives up water $pCO_2$ in the respirometer due to excess $H^+$ ions titrating ambient $HCO_3^-$ to $CO_2$. Regardless, at $O_2$ levels above $O_{2crit}$ the rate of $CO_2$ onset will be faster than those used in our current study as a result of the faster RHI. Increased speed of $CO_2$ onset in closed respirometry trials may 'outstrip' the ability of fish to acid-base regulate, causing an uncompensated respiratory acidosis during the time of the trial, which in turn would decrease Hb-$O_2$ affinity via the Bohr & Root effects and potentially increase $O_{2crit}$. Similarly, fish species

which have reduced ability to acid-base regulate may have an increased $O_{2crit}$ when rising $CO_2$ is included in trials.

Our results indicate improved hypoxia tolerance during rising $CO_2$ in European sea bass. This contrasts with previous research investigating interactive effects of $CO_2$ and hypoxia on $O_{2crit}$ of fish. Woolly sculpin, *Clinocottus analis* (an intertidal species that can breathe air), exposed to ~1100 µatm $CO_2$ showed no impact on $O_{2crit}$ after 7 days acclimation but after 28 days had $O_{2crit}$ measurements ~34 % higher than fish held in ambient (~400 µatm) conditions (Hancock and Place, 2016). Higher $O_{2crit}$ after 28 days corresponded with higher RMR and $Na^+$, $K^+$, ATPase activity. This contrast in results could indicate that the beneficial effect of acute rises in $CO_2$ associated with natural hypoxia documented in our study are potentially reversed when fish are exposed to long term constantly high $CO_2$ associated with anthropogenic climate change. Moreover, acute changes in $CO_2$ had no effect on $O_{2crit}$ of the estuarine fish species mummichog, *Fundulus heteroclitus*, and Norfolk spot, *Leiostomus xanthurus* when they were exposed to ~8,000-10,000 µatm $CO_2$ immediately prior to an $O_{2crit}$ trial (Cochran and Burnett, 1996). As such the effect of $CO_2$ on $O_{2crit}$ will likely depend on differences in physiological responses to $CO_2$ and $O_2$ between species.

Simultaneously rising $CO_2$ also shows variable impacts on non-metabolic responses to hypoxia of several species. Cycling $CO_2$ had no effect on aquatic surface respiration (ASR), the use of the thin surface layer of water for aquatic respiration (Kramer and McClure, 1982), or survival in juvenile *Menidia menidia*, *Fundulus majalis*, *Fundulus heteroclitus* or *Morone saxatalis* exposed to short term cycles of $O_2$ (Dixon *et al.*, 2017). In contrast, combined hypoxia and acidification resulted in an increase in the $O_2$ level at which *Menidia menidia* and *Menidia beryllina* first performed ASR, consistently performed ASR, exhibited LoE, and finally died (Miller *et al.*, 2016). Additionally, combined high $CO_2$ (~2,000 µatm) and hypoxia had no effect on survival of larval *Cyprinodon variegatus*, an additive negative effect on larval *Menidia beryllina*, and a synergistic negative effect on larval *Menidia menidia* (DePasquale *et al.*, 2015). This variation in effect of $CO_2$ on hypoxia responses could be a result of methodological differences (e.g. constant high $CO_2$ in Dixon *et al.* (2017), cycling DO/pH in DePasquale *et al.* (2015), and concurrent $CO_2$ rise/$O_2$

decrease in Miller *et al.* (2016)), the level of $CO_2$ used in studies (e.g. $CO_2$ levels used by Miller *et al.* ( 2016) were ~23,000 µatm which is much higher than levels likely to be commonly found in the environment during hypoxia and may have contributed to the negative effects of rising $CO_2$ noted in the study), differences in species and life stages used (changes in physiological tolerance across life stages have been noted for thermal tolerance by Komoroske *et al.* (2014)), or possibly variability in response as a result of differences in previous environmental experience (Murray *et al.*, 2014). The role of environmental variability in species sensitivities to $CO_2$ has recently been outlined in the proposed Ocean Variability Hypothesis (OVH) (Baumann, 2019) and warrants testing on various species in the future.

## 5. Conclusion

Overall, our results indicate that the environmentally realistic, simultaneous rises in $CO_2$ during a hypoxic event increased the hypoxia tolerance (i.e. reduces $O_{2crit}$) of European sea bass which is at least partly explained by an enhanced ability of fish to uptake $O_2$ via increased Hb-$O_2$ affinity. Miller *et al.* (2016) also demonstrated impacts of concurrent $CO_2$ rise on measurements of hypoxia tolerance, although in an opposite direction to that noted in our study. As concurrent $CO_2$ rises during hypoxia are the norm in nature, evidence that this affects physiology of organisms exposed to hypoxia highlights an important shortcoming of research to predict tolerances to hypoxia of fish. More research on this issue is needed to clarify how common this modifying effect of $CO_2$ on the response to hypoxia is and whether such measurements in the lab are ecologically relevant. A greater understanding of this issue may allow more accurate assessments of the impacts of hypoxic events on marine fish in nature, aiding management and conservation of fish species. With specific regard to measurements of $O_{2crit}$ we believe future studies should include concurrent rising $CO_2$ in the following ways:

1. Intermittent-flow respirometer studies should include increases of $CO_2$ relevant to hypoxic events that organisms may experience,
2. Closed respirometry studies should report the start and end $CO_2$ levels in the respirometer

In addition, both methods should aim to create an environmentally relevant rate of hypoxia induction/$CO_2$ increase for the species studied, and consistently report $CO_2$ levels measured. By incorporating these recommendations we believe that future studies of $O_{2crit}$ will give more representative estimations of species hypoxia tolerance.

## 6. Supplementary Materials

Supplementary materials can be found online at: https://static-content.springer.com/esm/art%3A10.1038%2Fs41598-019-51572-4/MediaObjects/41598_2019_51572_MOESM1_ESM.pdf

# Chapter V

## Critical thermal maximum ($CT_{max}$) of rainbow trout (*Oncorhynchus mykiss*) is resistant to respiratory acidosis and changes in $O_2$ supply capacity

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**Author contributions:** D.W.M. was primarily responsible for designing the research, conducting experimental work, data analysis and writing the chapter. R.W.W. assisted with designing the research, conducting experimental work, and commenting on drafts of the paper. S.D.S., G.H.E., and S.N.R.B. assisted with designing the research and commented on drafts of the paper.

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## Abstract

With climate change, rising levels of environmental $CO_2$ may heighten the sensitivity of fish to warming by impacting upon their thermal tolerance limits. However, potential mechanisms by which $CO_2$ may affect thermal tolerances of fish have yet to be determined. In this study we explored whether acute exposure to increased $CO_2$ can impact upon critical thermal tolerance ($CT_{max}$) limits. We sought to isolate potential effects of acute $CO_2$ exposure on thermal tolerance via either respiratory acidosis or $O_2$ supply capacity by exposing rainbow trout, *Oncorhynchus mykiss*, to ~1 % $CO_2$ (10,000 µatm) in combination with normoxia (~21 kPa $O_2$) or hyperoxia (~42 kPa $O_2$). Acute (~30 minute) exposure to ~1 % $CO_2$ caused a large respiratory acidosis in trout exposed in both normoxia and hyperoxia (blood pH decreased by ~0.43 pH units). Exposure to hyperoxia in combination with ~1 % $CO_2$ increased aerobic scope of trout by ~30% when compared to fish exposed to $CO_2$ in normoxia and ~20 % when compared to trout in ambient $O_2$ and $CO_2$ conditions. Despite large changes in pH and aerobic scope we saw no impacts of any treatments on $CT_{max}$ of trout which was tightly regulated across all individuals (S.D. = ±0.23 °C). Additionally, longer-term changes in blood chemistry associated with regulating pH disturbances also did not impact $CT_{max}$. These findings provide further evidence that changes in aerobic scope of fish do not impact on their critical thermal tolerance limits in contrast to suggestions of the oxygen- and capacity-limited thermal tolerance hypothesis. Our results also indicate that the true mechanisms that determine $CT_{max}$ of trout are pH independent - this may rule out factors such as protein disruption, cardiac failure, or neural impairment which have all shown sensitivity to intracellular acidosis. Instead the resistance of $CT_{max}$ to short term exposure of increased $CO_2$ may support proposals that disruption of cellular or mitochondrial membrane functions are the primary cause of physiological failure during acute warming.


## 1. Introduction

Rising ocean temperatures, and increasing frequency and severity of marine heatwaves, are leading to shifts in the distribution and abundances of species (Hastings *et al.*, 2020). For the majority of ectotherms changes in environmental temperatures are reflected in body temperatures. As a result, shifting

distributions and abundances are likely direct results of warming temperatures exceeding thermal tolerance limits of an organism's physiological performance (Sunday *et al.*, 2012). With global climate change, both atmospheric and aquatic $CO_2$ levels are also rising exponentially (causing decreased pH) (Orr *et al.*, 2005). Moreover, average dissolved $O_2$ levels are declining (Breitburg *et al.*, 2018), and the prevalence of hypoxic (low $O_2$) events is increasing (Diaz and Rosenberg, 2008). Therefore, it is important to understand the interactive effects of all three variables, and specifically to determine if changes in environmental $CO_2$ and $O_2$ can impact upon the thermal tolerance of organisms.

A prominent hypothesis recently put forward to explain how warming temperatures, rising CO2 and declining O2 levels may together impact fish, is the 'oxygen- and capacity-limited thermal tolerance' (OCLTT) hypothesis (Pörtner and Knust, 2007). This hypothesis predicts that upper temperature tolerance limits of organisms occur because of an inability of the cardio-respiratory system to increase $O_2$ supply to meet rising tissue $O_2$ demands. The difference between whole organism $O_2$ supply capacity and tissue $O_2$ demand can be measured as the aerobic scope (i.e. the difference between resting or "standard" metabolic rate (SMR) and maximum metabolic rate (MMR)) (Clark *et al.*, 2013). As such, environmental factors which result in changes in aerobic scope would be expected to lead to alterations in the thermal tolerance limits of ectotherms. This has led to predictions that decreased environmental $O_2$ and increased $CO_2$ will reduce the thermal tolerance of aquatic organisms, exacerbating the effects of global warming, as they can have direct impacts on the aerobic scope of aquatic ectotherms (Pörtner and Peck, 2010; Pörtner, 2012).

One metric for assessing thermal tolerance limits of ectotherms is the critical thermal maximum ($CT_{max}$) which is the temperature at which physiological failure occurs (e.g. onset of muscle spasms, loss of righting response) when an organism is exposed to an acute, constant linear increase in temperature (Beitinger *et al.*, 2000). This metric provides a relatively quick, repeatable method for quantifying an organism's acute thermal tolerance (Morgan *et al.*, 2018; O'Donnell *et al.*, 2020) and is also correlated to thermal tolerance over longer time frames, which may suggest that similar physiological mechanisms underlie tolerance to both acute and chronic warming (Åsheim *et al.*, 2020).

To date some studies have shown that environmental $O_2$ can affect $CT_{max}$ of aquatic ectotherms (Ellis *et al.*, 2013; Zanuzzo *et al.*, 2019; Islam *et al.*, 2020). Even so, the majority of research has shown limited, or no, evidence that $O_2$ availability alters $CT_{max}$ of fish as predicted by the OCLTT hypothesis (for example see Brijs *et al.*, 2015; Devor *et al.*, 2015; Ern *et al.*, 2016; Motyka *et al.*, 2017). Similarly, while studies have shown that increased environmental $CO_2$ can decrease $CT_{max}$ of some ectotherms (Metzger *et al.*, 2007; Walther *et al.*, 2009) results from fish species show no, or little, effects of increased $CO_2$ on $CT_{max}$ (Clark *et al.*, 2017; Ern *et al.*, 2017). As there is limited evidence that $O_2$ supply capacity effects thermal tolerance of many species there must be alternative mechanisms which can determine critical thermal limits. Some possible alternatives include disruption of neural function (Ern *et al.*, 2015; Jutfelt *et al.*, 2019), impairment of cell membranes (Bowler, 2018), enzyme denaturation (Somero and Dahlhoff, 2008), ion imbalances (particularly of potassium) (O'Sullivan *et al.*, 2017), or failure of critical organs (e.g. heart failure) (Sidhu *et al.*, 2014; Jørgensen *et al.*, 2017).

So far studies investigating impacts of increased $CO_2$ on thermal tolerance limits of fish have exclusively investigated the potential for chronic impacts of $CO_2$. This is because increases in average levels of aquatic $CO_2$ resulting from global climate change will take place over many decades. Despite this, over shorter time spans, fish species can experience changes in $CO_2$ that can be many times greater than those expected to occur as a result of long-term climate change, even within time periods as short as minutes or hours (Helmuth *et al.*, 2014). Studies on impacts of acute respiratory acidosis are often focussed on its effects on $O_2$ transport by haemoglobin (Hannan and Rummer, 2018); however, respiratory acidosis can also have other direct physiological effects which could impact whole organism thermal tolerance. For example, even small reductions in intracellular pH caused by respiratory acidosis can have large effects on tissue function, including cardiac and brain tissue (Gesser and Poupa, 1983; Farrell and Milligan, 1986; Siesjö *et al.*, 1993), and it has long been known that protein function and metabolic activity of enzymes is highly dependent on pH (Yang and Honig, 1993). Therefore, if an acute $CO_2$ increase has impacts on $CT_{max}$ of fish this may give insights into the mechanisms which determine acute physiological thermal tolerance limits.

Rainbow trout, *Oncorhynchus mykiss*, are a salmonid species commonly used by physiologists to investigate mechanisms by which environmental factors affect physiological performance. Previous research has shown that thermal tolerance of rainbow trout is not $O_2$ dependent (Motyka *et al.*, 2017) making this species suitable to investigate whether $CO_2$ can influence thermal tolerance via alternative mechanisms to $O_2$ limitation. As such, we aimed to investigate whether acute $CO_2$ exposures can impact $CT_{max}$ of rainbow trout independently of changes in $O_2$ supply capacity, and specifically address the issue of disturbed acid-base balance.

## 2. Materials and Methods

### 2.1. Animal husbandry and acclimation

Freshwater rainbow trout (*Oncorhynchus mykiss*) were obtained from a commercial supplier (Houghton Springs Hatchery, Dorset, UK) in three batches between January and September 2020 (Table 1). Trout were held in ~500 L holding tanks (stocking density ~10 kg $m^{-3}$) supplied by a freshwater recirculating aquaculture system (RAS, pH = 7.69 ± 0.07, temperature = 15.0 ± 0.3 $^{o}$C, salinity = 0.13 ± 0.04, mean ± S.D.) and fed with a commercial pellet (Horizon 40, Skretting) at a ration of ~1 % body mass 3 times a week. Each batch of fish was held in the RAS for at least 4 weeks to acclimate to laboratory conditions and ensure normal feeding behaviour had resumed prior to experimental work starting. Temperature was maintained for acclimation and experiments at 15 $^{o}$C by maintaining both water and air temperature within the temperature-controlled room at this set point. All experimental procedures were carried out under UK Home Office licence (P88687E07) and were approved by the University of Exeter's Animal Welfare and Ethical Review Board.

**Table 1:** Measurement type, number of fish, and mean body mass of the three batches of trout used for experiments.

| Batch ID | Group ID | Measurement type | Number of fish | Body mass ± S.E. (g) |
|---|---|---|---|---|
| #1 | A | Respirometry | 30 | 205.0 ± 13.6 |
| #2 | B | Respirometry | 14 | 189.7 ± 19.6 |
| | C | Blood Sampling | 33 | 368.5 ± 10.9 |
| #3 | D | $CT_{max}$ | 48 | 273.6 ± 9.9 |

## 2.2. Experimental treatments

Prior to treatment exposure food was withheld for a minimum of 5 days to ensure physiological responses to treatments were not affected by the specific dynamic action of digestion (Chabot *et al.*, 2016). Trout were then moved from stock tanks to a semi-recirculating experimental system. The semi-recirculating system comprised a ~150 L header tank that fed four individual isolation tanks (~20 L) at a rate of 4 L $min^{-1}$ with overflowing water returning to the header tank. The header tank was fed from the stock RAS at a rate of ~10 L $min^{-1}$ with overflowing water returning to the RAS. The semi-recirculating experimental system was temperature controlled using a heater/chiller unit (Grant TX150 R2, Grant Instruments, Cambridge, UK) attached to a temperature exchange coil in the header tank to maintain a temperature of ~15 $^{o}$C. Trout were then exposed to one of four treatments:

1. control $CO_2$ in normoxia (~0.08 % $CO_2$ & ~21 kPa $O_2$)
2. acute high $CO_2$ in normoxia (~30 minutes at ~1 % $CO_2$ & ~21 kPa $O_2$)
3. acute high $CO_2$ in hyperoxia (~30 minutes at ~1 % $CO_2$ & ~42 kPa $O_2$)
4. chronic high $CO_2$ in normoxia (~72 hours at ~1 % $CO_2$ & ~21 kPa $O_2$)

For control $CO_2$ treatments isolation tanks were gassed with ambient air. For ~1 % $CO_2$ treatments (~10,000 µatm) in normoxia the experimental header tank was dosed with $CO_2$ by an Aqua Medic pH computer which controlled gassing of $CO_2$ via an electronic solenoid to maintain a pH level at a set-point (~6.7) necessary to achieve ~1 % $CO_2$. In addition, isolation tanks were gassed with a gas mix of 1% $CO_2$, 21 % $O_2$, and 78 % $N_2$ (G400 Gas mixing system, Qubit Biology Inc.). For ~1 % $CO_2$ treatments in hyperoxia, isolation boxes and the experimental header tank were gassed with a gas mix including ~50 % $O_2$ in order to produce an environmental $pO_2$ of ~42 % (~42 kPa $O_2$).

## 2.3. Blood chemistry and $O_2$ transport

After treatment exposure, fish used for blood sampling were anaesthetised in-situ using a dose of 70 mg $L^{-1}$ of benzocaine. Blood samples were then obtained from trout, using methods detailed in Montgomery *et al.* (2019), via caudal puncture of blood vessels while gills were irrigated by water with the same $CO_2$ and $O_2$ as the respective treatment and containing ~30 mg $L^{-1}$ benzocaine to maintain anaesthesia. Whilst blood samples were obtained,

measurements of water pH (NBS scale), temperature, $pO_2$, and salinity, as well as water samples to measure total $CO_2$ ($TCO_2$)/Dissolved Inorganic Carbon (DIC), were taken from each isolation tank (Table 2). We conducted seawater DIC analysis using a custom built system described in detail by Lewis *et al.* (2013). Measurements of pH, salinity, temperature and DIC were then input into the seawater carbon calculator programme, CO2SYS (Pierrot *et al.*, 2006) to calculate $pCO_2$ and total alkalinity (TA) based on the equilibration constants refitted by Dickson and Millero (1987). We measured the water chemistry of the gill irrigation chamber following the same process as for isolation chambers, with one DIC sample taken at the end of blood sampling (supplementary Table S1).

**Table 2:** Mean ± S.E. of water chemistry parameters and $O_2$ level in individual isolation tanks at the time of blood sampling.

| Treatment | Temperature ($^oC$) | pH (NBS) | Salinity | $pO_2$ (kPa) | $pCO_2$ (µatm) | TA (µM) |
|---|---|---|---|---|---|---|
| Control $CO_2$ | 14.7 ± 0.1 | 7.70 ± 0.02 | 0.12 ± 0.00 | 20.03 ± 0.11 | 795 ± 34 | 827 ± 22 |
| Acute $CO_2$ | 15.0 ± 0.0 | 6.69 ± 0.01 | 0.12 ± 0.00 | 20.08 ± 0.16 | 7755 ± 226 | 762 ± 19 |
| Acute $CO_2$ + Hyperoxia | 14.9 ± 0.0 | 6.69 ± 0.01 | 0.13 ± 0.00 | 38.35 ± 0.41 | 8228 ± 167 | 813 ± 8 |
| Chronic $CO_2$ | 15.0 ± 0.0 | 6.68 ± 0.01 | 0.12 ± 0.00 | 20.28 ± 0.13 | 8078 ± 306 | 789 ± 24 |

Immediately after sampling we measured extracellular pH, haematocrit (Hct) and plasma $TCO_2$ following details set out in Montgomery *et al.* (2019). Plasma $pCO_2$ and $HCO_3^-$ were then calculated from $TCO_2$, temperature and blood pH measurements using the Henderson-Hasselbalch equation with values for solubility and $pK^1_{app}$ based on Boutilier *et al.* (1984, 1985). Measurements of haemoglobin (Hb) content, plasma glucose and lactate were also completed following analysis methods from Montgomery *et al.* (2019). We centrifuged whole blood (10,000 g) for 2 minutes at 4 $^oC$, before removing the supernatant and blotting the surface of the leftover red blood cell (RBC) pellet to remove the white blood cell layer. We then followed the freeze-and-thaw method (by snap freezing the RBC pellet in liquid nitrogen for 10 seconds and thawing the pellet for 1 minute in a water bath set to ~ 37 $^oC$) to measure intracellular pH of RBCs ($pH_i$) as described by Zeidler and Kim (1977), and validated by Baker *et al.* (2009). Next, 10 µL of supernatant was diluted in ultrapure water, snap frozen in liquid nitrogen, and stored at -80 $^oC$ before later being used to measure plasma

anion and cation concentrations using ion chromatography (Dionex ICS 1000 & 1100, Thermo-Scientific, UK). All measurements or storage of blood occurred within 10 minutes of blood sampling. Finally, we measured Hb-$O_2$ affinity and Hills number using a Blood Oxygen Binding System (BOBS, Loligo systems), detailed in general in Oellermann *et al.* (2014) and specifically for fish blood in Montgomery et al. (2019) using a gas mix with the same $pCO_2$ level as calculated for the individual fish.

## 2.4. Respirometry

Metabolic rate of trout was estimated by measuring oxygen consumption rates ($\dot{M}O_2$) using temperature compensated fiber-optic $O_2$ sensors (Robust $O_2$ probe + Firesting $O_2$ meter, Pyro Science, GmbH, Germany) in an intermittent-flow respirometer system, details of which can be found in Montgomery *et al.* (2019), set-up following recommendations by Svendsen *et al.* (2016). A linear regression was fitted to the $pO_2$ versus time data from respirometry measurements. The slope (s) of this regression (kPa $O_2$ $h^{-1}$) was then used to calculate $\dot{M}O_2$ (mg $O_2$ $kg^{-1}$ $h^{-1}$) using the equation outlined by Svendsen *et al.* (2016):

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

where $V_{resp}$ is the respirometer volume minus the volume of the fish (L), α is the solubility of $O_2$ in water ($mgO_2$ $L^{-1}$ $kPa^{-1}$) for the relevant salinity and temperature, and m is the mass of the fish (kg).

In preparation for respirometry prior to $CT_{max}$ tests, trout were moved to respirometers the day before $\dot{M}O_2$ measurements were made to acclimate to respirometers. For trout to be used in subsequent $CT_{max}$ tests in acute high $CO_2$ in normoxia and acute high $CO_2$ in hyperoxia treatments, water chemistry in the respirometers was maintained at control $CO_2$ and $O_2$ levels. Trout in the chronic high $CO_2$ treatment were moved to respirometers after ~54 hours exposure to increased $CO_2$ to complete the remainder of their 72 hour exposure in the respirometer chambers with water chemistry maintained at ~1 % $CO_2$. Measurements of $\dot{M}O_2$ were then made for a minimum of ~16 hours to calculate standard metabolic rate (SMR). Fish were then removed from respirometers and returned to isolation chambers and left to recover from handling for 1 hour. Trout from chronic $CO_2$ treatments were moved to isolation boxes with ~1 %

$CO_2$ whereas fish from all other treatments were moved to isolation boxes with ambient levels of $CO_2$ and $O_2$. Fish used for acute high $CO_2$/normoxia and acute high $CO_2$/hyperoxia treatments were then exposed to ~1 % $CO_2$ with either ~21 kPa or ~42 kPa $O_2$ for ~30 minutes. Trout were then transferred to a circular chase tank (60 cm diameter with ~15 cm water depth with the appropriate $CO_2$ and $O_2$ level for the treatment). Fish were then chased to exhaustion (cessation of burst swimming and lack of response to a light pinch of the caudal fin) before immediately transferring fish back to respirometer chambers (which now had the correct $CO_2$ and $O_2$ levels appropriate to the treatment) and measuring $\dot{M}O_2$ for one hour to calculate maximum metabolic rate (MMR, average time from end of chase to beginning to measurements was ~1-2 minutes). For all measurements background respiration of empty respirometer chambers was measured before and after SMR and MMR measurements for a minimum of 1 hour. Measurements of pH, temperature, $pO_2$, and salinity, as well as samples of water for DIC measurement, were taken for each isolation tank, the chase tank (after all fish were chased) and the respirometer system (during $\dot{M}O_2$ measurements pre- and post-exhaustive exercise). These parameters were then used to calculate water carbonate chemistry conditions (see Table 3 for water chemistry and $O_2$ levels during respirometry measurements and supplementary materials Table S2 for water chemistry and $O_2$ levels in isolation tanks and the chase chamber) as detailed in section 2.3.

**Table 3:** Mean ± S.E. of water chemistry parameters and $O_2$ level in respirometry tanks at the time of blood sampling.

| Treatment | Measurement | Temperature (°C) | pH (NBS) | Salinity | $pO_2$ (kPa) | $pCO_2$ (µatm) | TA (µM) |
|---|---|---|---|---|---|---|---|
| Control | SMR | 15.1 ± 0.1 | 7.77 ± 0.02 | 0.18 ± 0.02 | 19.53 ± 0.16 | 707 ± 36 | 854 ± 10 |
| | MMR | 15.1 ± 0.1 | 7.68 ± 0.01 | 0.19 ± 0.02 | 18.61 ± 0.21 | 836 ± 21 | 831 ± 7 |
| Acute $CO_2$ | SMR | 15.0 ± 0.0 | 7.75 ± 0.02 | 0.17 ± 0.02 | 19.70 ± 0.09 | 720 ± 29 | 835 ± 13 |
| | MMR | 15.0 ± 0.0 | 6.73 ± 0.02 | 0.17 ± 0.02 | 18.76 ± 0.15 | 7334 ± 460 | 790 ± 15 |
| Acute $CO_2$ | SMR | 15.1 ± 0.1 | 7.77 ± 0.01 | 0.12 ± 0.00 | 19.72 ± 0.19 | 666 ± 26 | 806 ± 16 |
| + hyperoxia | MMR | 15.0 ± 0.1 | 6.73 ± 0.03 | 0.12 ± 0.01 | 40.81 ± 0.39 | 6837 ± 227 | 739 ± 20 |
| Chronic $CO_2$ | SMR | 15.1 ± 0.1 | 6.72 ± 0.03 | 0.15 ± 0.01 | 19.88 ± 0.10 | 7662 ± 549 | 800 ± 18 |
| | MMR | 15.0 ± 0.2 | 6.72 ± 0.06 | 0.14 ± 0.03 | 18.76 ± 0.19 | 7650 ± 1143 | 803 ± 52 |

## 2.5. Critical thermal maximum ($CT_{max}$)

After treatment exposures, $CT_{max}$ trials were conducted with two trout simultaneously. To do this, flow to isolation boxes from the experimental system header tank was stopped and a closed system was created with isolation boxes being fed by a heater/chiller unit (Grant TX150 R2, Grant Instruments, Cambridge, UK) at a rate of ~3 L $min^{-1}$ (90 % replacement time ~15 min) and overflowing water returning to the heater/chiller unit. Temperature was then continuously increased by the heater/chiller at a rate of ~0.05 $^{o}C$ $min^{-1}$ (3 $^{o}C$ $min^{-1}$) until $CT_{max}$ was reached (determined as the point at which trout showed loss of equilibrium for ~5 seconds). Throughout $CT_{max}$ trials $CO_2$ and $O_2$ levels appropriate for each treatment were maintained by gassing the sump chamber in the heater chiller with the appropriate gas mix (Table 4). Throughout the trial the temperature and $pO_2$ of each isolation chamber was monitored (Firesting $O_2$ meter, Pyro Science, Gmbh, Germany). Measurements of pH and salinity, as well as a water sample for DIC measurement, were taken at the start and end of each $CT_{max}$ trial and water chemistry calculated as described in section 2.3.

**Table 4:** Mean ± S.E. of starting temperatures, heating rates, water chemistry parameters, and $O_2$ levels in isolation tanks during $CT_{max}$ trials.

| Treatment | Start temperature ($^{o}C$) | Heating rate ($^{o}C$ $min^{-1}$) | pH (NBS) | Salinity | $pO_2$ (kPa) | $pCO_2$ (µatm) | TA (µM) |
|---|---|---|---|---|---|---|---|
| Control $CO_2$ | 15.48 ± 0.04 | 0.048 ± 0.000 | 7.61 ± 0.02 | 0.10 ± 0.00 | 18.07 ± 0.13 | 1031 ± 47 | 790 ± 17 |
| Acute $CO_2$ | 15.65 ± 0.05 | 0.047 ± 0.000 | 6.59 ± 0.02 | 0.11 ± 0.00 | 18.24 ± 0.16 | 10215 ± 351 | 740 ± 14 |
| Acute $CO_2$ + Hyperoxia | 15.66 ± 0.02 | 0.047 ± 0.000 | 6.58 ± 0.02 | 0.10 ± 0.00 | 38.60 ± 0.31 | 10181 ± 416 | 715 ± 15 |
| Chronic $CO_2$ | 15.65 ± 0.05 | 0.047 ± 0.000 | 6.61 ± 0.02 | 0.11 ± 0.00 | 18.35 ± 0.15 | 10934 ± 293 | 833 ± 10 |

## 2.6. Data analysis

All statistical analysis was conducted in R v3.6.3 (R Core Team, 2020). Changes in blood chemistry ($pH_e$, $pH_i$, $pCO_2$, $HCO_3^-$) and $O_2$ transport (Hct, Hb, $P_{50}$, Hills' number) across treatments were analysed using one-way ANOVA before assumptions of normality and equal variance of model residuals were checked by visual inspection of residual plots. If one-way ANOVA indicated significant differences between treatments post-hoc tests were then conducted using Tukey's Honest Significant Differences (Tukey's HSD). Measurements of blood $pO_2$ did not meet statistical assumptions required for one-way ANOVA

and so were analysed using a non-parametric Kruskal-wallis test with post-hoc tests conducted using Dunn's test from package 'FSA' (Ogle *et al.*, 2020).

Calculations of oxygen consumption rates ($\dot{M}O_2$) where the linear regression between $O_2$ and time had a $R^2$ of < 0.95 were removed from subsequent analysis of metabolic rate to ensure accuracy of $\dot{M}O_2$ measurements. Before calculating metabolic rates, background respiration was subtracted from the measured $\dot{M}O_2$ values by assuming a linear change in background respiration from the measurement taken at the beginning and end of respirometry trials. Mean background respiration was ~2 % of trout SMR across all trials. $\dot{M}O_2$ measurements were then mass corrected by scaling background corrected $\dot{M}O_2$ measurements to an average individual mass of 200 g (this corresponded closely with the mean mass, 200.2 g, of all fish used for $\dot{M}O_2$ measurements) using the following equation:

$$\dot{M}O_2\ (200\ g) = \dot{M}O_2 \times \left(\frac{M}{200}\right)^{(1-A)}$$

where $\dot{M}O_2$ (200 g) is the predicted oxygen consumption ($mgO_2\ kg^{-1}\ h^{-1}$) of an individual scaled to a body mass of 200 g, $\dot{M}O_2$ is the measured oxygen consumption of a trout with mass of M, and A is the mass exponent describing the relationship between metabolic rate and body mass. We chose to use a mass exponent of 0.89 because it best describes the most recent analysis of the relationship between standard metabolic rate (SMR) and mass in fish (Jerde *et al.*, 2019). We then estimated SMR as the mean of the lowest ten $\dot{M}O_2$ measurements from pre-chase respirometer measurements. Maximum metabolic rates were estimated as the highest $\dot{M}O_2$ measurement calculated using an iterative 30 second measurement period, as described by Zhang *et al.* (2019), from respirometer measurements immediately post-exhaustive exercise of trout. Aerobic scope for each trout was then calculated as the difference between estimates of SMR and MMR.

The effect of treatments on SMR, MMR, and aerobic scope was then analysed using general linear mixed models (GLMM) in order to account for the potential random effect caused by using fish from two separate batches (i.e. batches #1 and #2, Table 1) for respirometry measurements. For each metabolic rate measurement the GLMM used included treatment as a categorical explanatory variable and batch number as a random intercept term.

Residual diagnostic plots of each GLMM were then assessed using package 'DHARMa' to confirm validity of model fit (Hartig, 2020). If treatment had a significant effect on metabolic rate measurements, post-hoc tests were conducted between each of the four treatment groups, using pairwise comparisons of least-squares means generated from package 'emmeans' (Lenth, 2020).

Finally, the effect of treatment on $CT_{max}$ of trout was analysed using a one-way ANOVA with post-hoc tests conducted using Tukey's HSD.

# 3. Results

## 3.1. Blood acid-base chemistry

As expected, exposure to ~1 % $CO_2$ caused significant changes to blood $pH_e$ (one-way ANOVA, F = 49.88, df = 3, p << 0.001), plasma $pCO_2$ (one-way ANOVA, F = 41.12, df = 3, p << 0.001), and plasma $HCO_3^-$ (one-way ANOVA, F = 64.39, df = 3, p << 0.001). Trout acutely exposed to high $CO_2$, in both normoxia and hyperoxia, experienced a significant respiratory acidosis with whole blood $pH_e$ decreasing by 0.46 (Tukey HSD, p << 0.001, 95 % CI = 0.36 – 0.53; Figure 1A) and 0.40 (Tukey HSD, p << 0.001, 95 % CI = 0.30 – 0.48; Figure 1A) respectively in comparison to control fish. Reduced $pH_e$ was a result of increased plasma $pCO_2$ (Figure 1B). Acutely exposed trout in normoxia and hyperoxia had plasma $pCO_2$ levels 1.11 kPa (Tukey HSD, p << 0.001, 95 % CI = 0.91 – 1.24) and 0.86 kPa (Tukey HSD, p << 0.001, 95 % CI = 0.67 – 1.06) greater than control fish. Trout chronically exposed to high $CO_2$ also showed significantly higher plasma $pCO_2$ when compared to control fish (Tukey HSD, p << 0.001, mean difference = 0.79 kPa, 95 % CI = 0.62 – 0.98) but had mean blood $pH_e$ levels 0.29 (Tukey HSD, p << 0.001, 95 % CI = 0.21 – 0.35) and 0.23 (Tukey HSD, p << 0.001, 95 % CI = 0.14 – 0.30) higher than trout exposed to high $CO_2$ acutely in normoxia and hyperoxia. The increase in blood $pH_e$ in fish exposed to chronic high $CO_2$ was a result of increases in mean plasma $HCO_3^-$ levels of 6.2 mM (Tukey HSD, p << 0.001, 95 % CI = 5.2 – 7.4) and 6.7 mM (Tukey HSD, p << 0.001, 95 % CI = 5.4 – 8.3) versus acutely exposed fish in normoxia and hyperoxia respectively (Figure 1C). Although chronically exposed trout had plasma $HCO_3^-$ that was ~10 mM higher than control fish, they still had mean blood $pH_e$ levels 0.18 (Tukey HSD, p = 0.002, 95 % CI = 0.09 – 0.26)

lower than control fish (Figure 1A). This indicates that the accumulation of $HCO_3^-$ by chronically exposed fish only resulted in a partial compensation of the respiratory acidosis caused by increased plasma $pCO_2$ (Figure 1D).

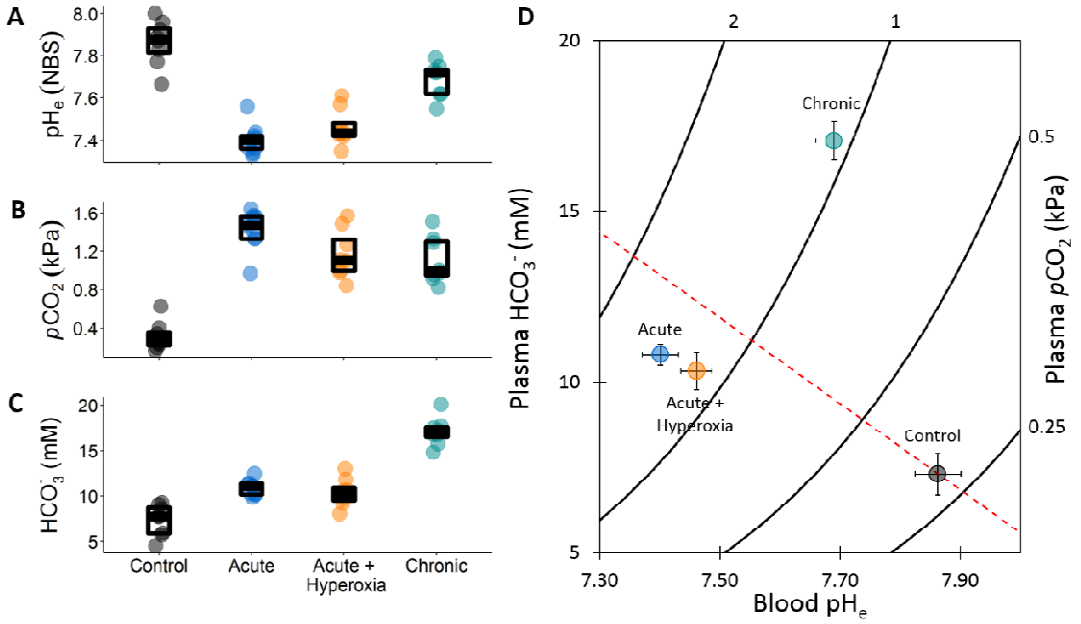


**Figure 1:** Changes in **A.** blood $pH_e$, **B.** plasma $pCO_2$, and **C.** plasma $HCO_3^-$ of rainbow trout between control fish (~0.1 % $CO_2$, ~21 % $O_2$, n = 8), fish exposed to ~1 % $CO_2$ acutely (~30 minutes) in normoxia (~21 % $O_2$, n = 9) and hyperoxia (~42 % $O_2$, n = 8), and chronically (~72 hours) in normoxia (~21 % $O_2$, n = 8). Boxes show inter-quartile range and median values while points indicate raw values. **D.** Combined changes of all three acid-base parameters are expressed as a pH/$HCO_3^-$/$pCO_2$ diagram (red dashed line indicates estimated non-bicarbonate blood buffer line based on equations from Wood *et al.* (1982)), values represent mean ± S.E.M.

As well as impacts on blood $pH_e$, exposure to high $CO_2$ also caused significant changes in RBC $pH_i$ (one-way ANOVA, F = 20.53, df = 3, p << 0.001; Figure 2). In trout acutely exposed to high $CO_2$ we measured an intracellular acidosis of RBCs in concurrence with the observed extracellular acidosis. Mean RBC $pH_i$ was 0.18 lower than measured in control trout for both fish exposed in normoxia (Tukey HSD, p << 0.001, 95 % CI = 0.12 – 0.23; Figure 2) and hyperoxia (Tukey HSD, p << 0.001, 95 % CI = 0.13 – 0.24; Figure 2). In contrast to blood $pH_e$, trout chronically exposed to high $CO_2$ did not have significantly lower RBC $pH_i$ when compared with control fish (Tukey HSD, p =

0.757, mean difference = -0.01, 95 % CI = -0.08 – 0.06; Figure 2) indicating complete RBC pH regulation.

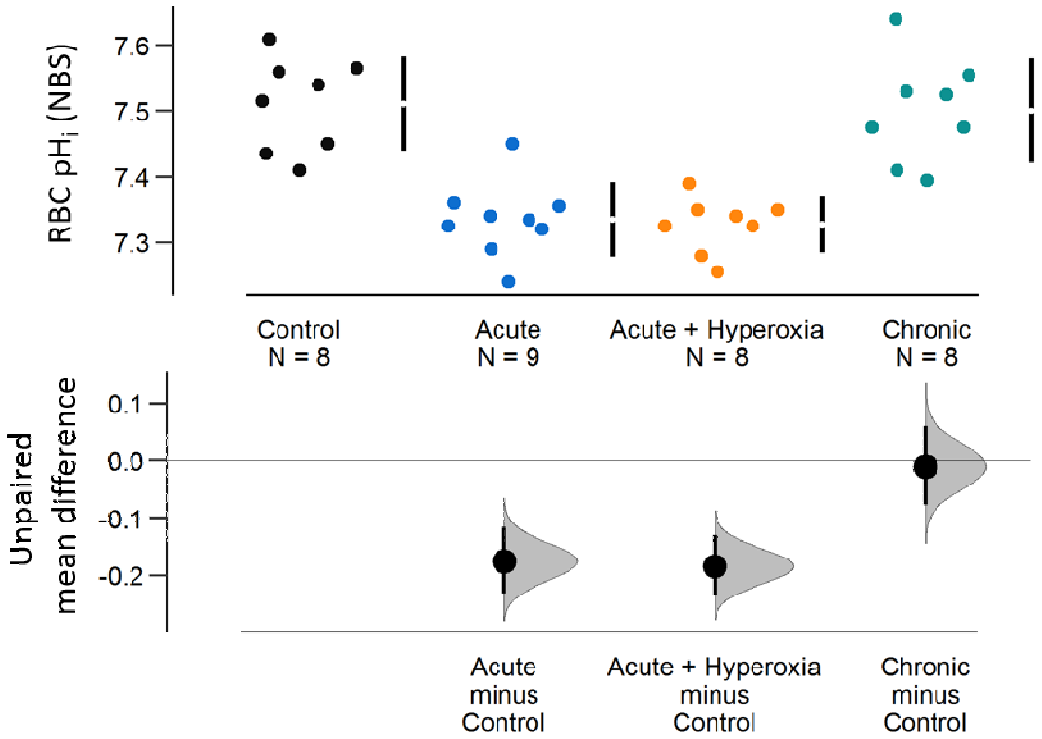


**Figure 2:** Red blood cell (RBC) $pH_i$ of trout exposed to either control (~0.1 % $CO_2$, ~21 % $O_2$, n = 8), acute (~30 minutes, ~1 % $CO_2$, ~21 % $O_2$, n = 9), acute + hyperoxia (~30 minutes, ~1 % $CO_2$, ~42 % $O_2$, n = 8), or chronic (~72 hours, ~1 % $CO_2$, ~21 % $O_2$, n = 8) treatments. The top panel shows raw data points with standard deviation indicated by the vertical black line (the central point of the line indicates the mean). The bottom panel shows mean difference effect size between fish exposed to acute, acute + hyperoxia, or chronic treatments when compared to fish in control treatments, with bootstrapped 95 % confidence interval (black bar) and the resampled distribution of the mean difference. Estimation plots, mean difference effect sizes and bootstrapped confidence intervals were created using package 'dabestr' (Ho *et al.*, 2019).

### 3.2. Blood $O_2$ transport

There were no differences in Hct (one-way ANOVA, F = 1.10, p = 0.367) or Hb concentration (one-way ANOVA, F = 0.91, p = 0.447) of rainbow trout across all treatments. Blood $pO_2$ levels did show significant differences between treatments (Kruskal-Wallis test, $\chi^2$ = 16.39, p = 0.001, Figure 3A) which was a result of significantly higher mean $pO_2$ in trout exposed to acute $CO_2$ with hyperoxia when compared to trout exposed to normoxia in control $CO_2$ (Dunn's

test, p = 0.010; mean difference = 15.6 kPa $O_2$, 95 % CI = 9.9 – 20.8), acute $CO_2$ (Dunn's test, p < 0.001; mean difference = 18.0, 95 % CI = 12.8 – 22.0), and chronic $CO_2$ treatments (Dunn's test, p = 0.011, mean difference = 15.9, 95 % CI = 10.4 – 21.3).

Measurements of Hb-$O_2$ binding affinity ($P_{50}$) indicated that high $CO_2$ exposure caused significant changes in $P_{50}$ (one-way ANOVA, F = 14.41, df = 3, p << 0.001). Trout acutely exposed to high $CO_2$ in both normoxia and hyperoxia showed significantly higher $P_{50}$ values than control fish, mean difference = 1.90 (95 % CI = 1.16 – 2.51) and 1.94 kPa $O_2$ (95 % CI = 1.32 – 2.65) respectively, or chronic $CO_2$ treatments, mean difference = 1.35 (95 % CI = 0.61 – 1.92) and 1.39 kPa $O_2$ (95 % CI = 0.80 – 2.07) respectively (Figure 3B). Changes in $P_{50}$ were accompanied by changes in Hills' number (one-way ANOVA, F = 25.27, df = 3, p << 0.001).

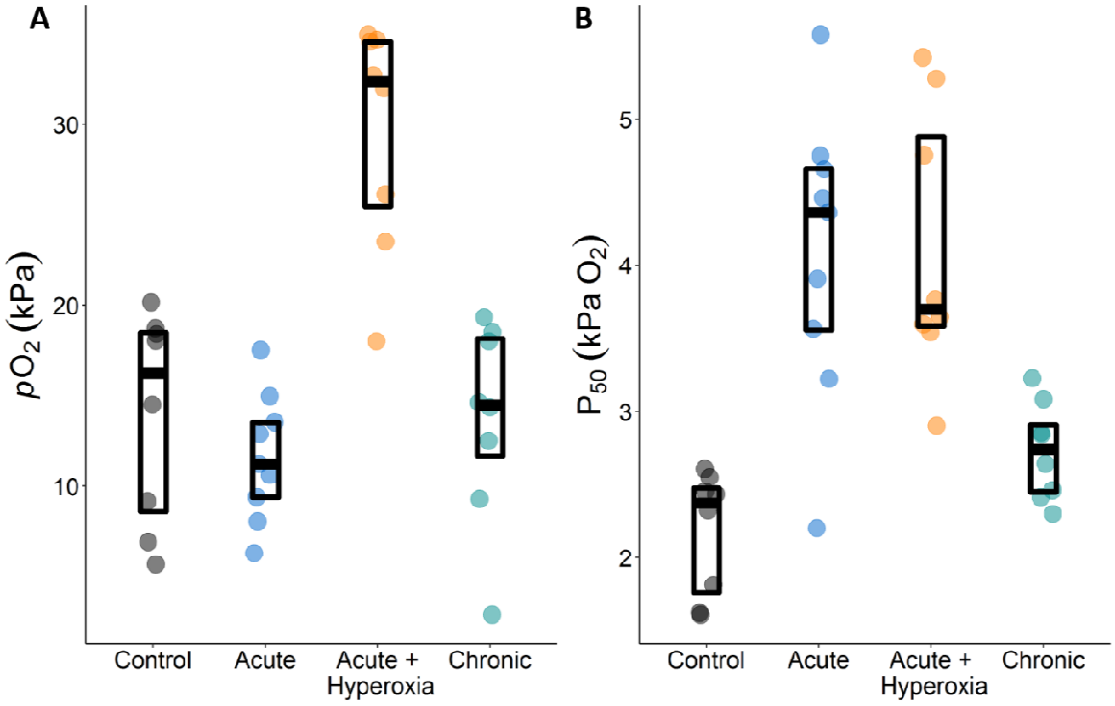


**Figure 3: A.** Plasma *p*$O_2$ and **B.** Hb-$O_2$ binding affinity ($P_{50}$) of trout exposed to control (~0.1 % $CO_2$, ~21 % $O_2$, n = 8), acute (~30 mins, ~1 % $CO_2$, ~21 % $O_2$, n = 9), acute + hyperoxia (~30 mins, ~1 % $CO_2$, ~42 % $O_2$, n = 8), or chronic (~72 hours, ~1 % $CO_2$, ~21 % $O_2$, n = 8) treatments. Boxes show inter-quartile range and median values while points represent raw values.

### 3.3. Respirometry

General linear mixed models were used to account for potential batch effects, which were non-significant, on metabolic rates of trout (Supplementary materials, Table S1-3). Standard metabolic rate of trout was not affected by treatment (GLMM, $\chi^2$ = 5.20, df = 3, p = 0.158, marginal $R^2$ = 0.10, conditional $R^2$ = 0.15, Figure 4A) but MMR was (GLMM, $\chi^2$ = 33.59, df = 3, p << 0.001, marginal $R^2$ = 0.52, conditional $R^2$ = 0.52, Figure 4B). There was a trend for an ~12 % reduction in MMR in trout exposed to acute high $CO_2$ in normoxia compared to control fish (pairwise comparisons of least-square means, p = 0.118; mean difference = 85.3 $mgO_2\ kg^{-1}\ h^{-1}$, 95 % CI = 30.3 – 143.0). Somewhat surprisingly, fish chronically exposed (~72 hours) to high $CO_2$ showed a similar MMR as trout acutely exposed to high $CO_2$ in normoxia (mean difference = 0.18 $mgO_2\ kg^{-1}\ h^{-1}$, 95 % CI = -61.6 – 57.2). Trout exposed to acute high $CO_2$ in hyperoxia showed a significant 21 % increase in MMR when compared to control fish (pairwise comparisons of least-square means, p = 0.003; mean difference = 144.0 $mgO_2\ kg^{-1}\ h^{-1}$, 95 % CI = 62.7 – 229.0), and ~32 % higher when compared to trout exposed to acute high $CO_2$ in normoxia (pairwise comparisons of least-square means, p << 0.001; mean difference = 229.0 $mgO_2\ kg^{-1}\ h^{-1}$, 95 % CI = 148.0 – 307.0) or chronic $CO_2$ in normoxia (pairwise comparisons of least-square means, p << 0.001; mean difference = 229.0 $mgO_2\ kg^{-1}\ h^{-1}$, 95 % CI = 144.0 – 316.0).

Changes in MMR between treatments were reflected in measurements of aerobic scope (GLMM, $\chi^2$ = 33.16, df = 3, p << 0.001, marginal $R^2$ = 0.51, conditional $R^2$ = 0.51, Figure 4C). There was a trend for trout exposed to acute high $CO_2$ and chronic high $CO_2$ treatments in normoxia to have aerobic scope measurements ~14 % lower (pairwise comparisons of least-square means, p = 0.138; mean difference = 81.0 $mgO_2\ kg^{-1}\ h^{-1}$, 95 % CI = 25.2 – 138.0) and ~12 % lower (pairwise comparisons of least-square means, p = 0.204; mean difference = 73.1 $mgO_2\ kg^{-1}\ h^{-1}$, 95 % CI = 14.3 – 138.0) than trout in control conditions. In contrast, trout exposed to acute high $CO_2$ in hyperoxia had aerobic scopes ~24 % higher than trout in control conditions (pairwise comparisons of least-square means, p = 0.002; mean difference = 146.0 $mgO_2\ kg^{-1}\ h^{-1}$, 95 % CI = 69.8 – 231.0).

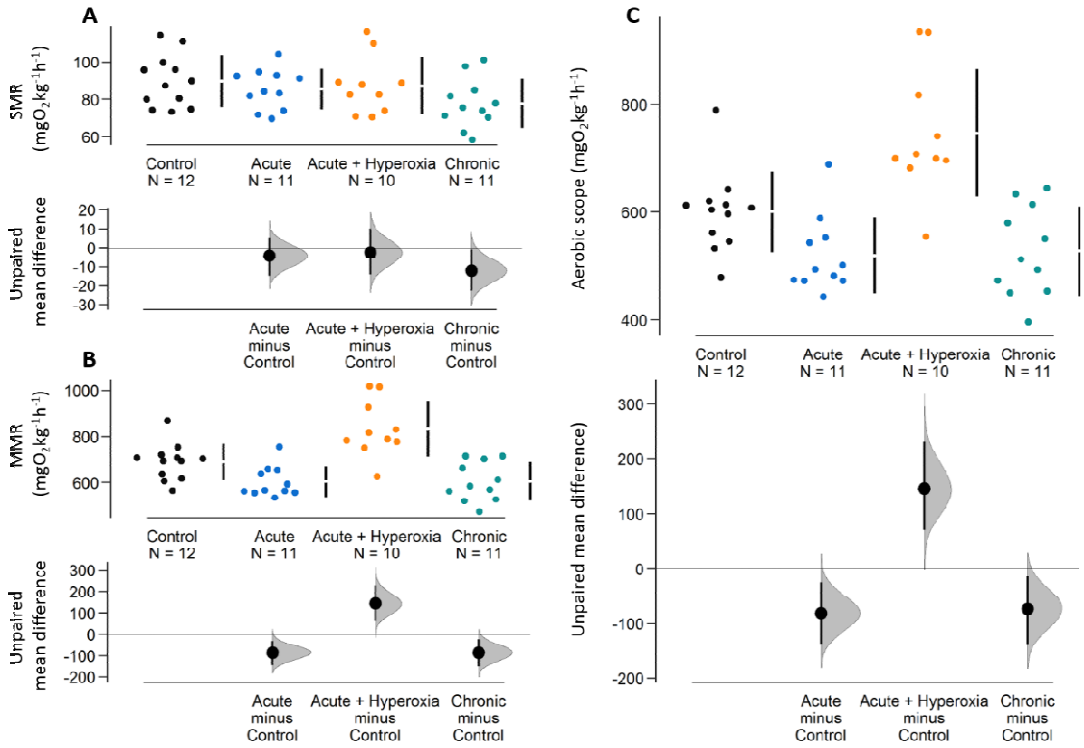


**Figure 4: A.** Standard metabolic rate (SMR), **B.** maximum metabolic rate (MMR), **C.** and aerobic scope of trout exposed to control (~0.1 % $CO_2$, ~21 % $O_2$, n = 8), acute high $CO_2$ (~30 mins, ~1 % $CO_2$, ~21 % $O_2$, n = 9), acute high $CO_2$ + hyperoxia (~30 mins, ~1 % $CO_2$, ~42 % $O_2$, n = 8), or chronic high $CO_2$ (~72 hours, ~1 % $CO_2$, ~21 % $O_2$, n = 8) treatments. The top panel shows raw data points with standard deviation indicated by the vertical black line (the central point of the line indicates the mean). The bottom panel shows mean difference effect size between fish exposed to acute, acute + hyperoxia, or chronic treatments and fish in control treatments with bootstrapped 95 % confidence interval (black bar) and the resampled distribution of the mean difference. Estimation plots, mean difference effect sizes and bootstrapped confidence intervals were created using package 'dabestr' (Ho *et al.*, 2019).

### 3.4. Critical thermal maximum ($CT_{max}$)

There were no differences in $CT_{max}$ between trout across all treatments with means ranging from 29.04 to 29.22 °C across treatments and very small variance (S.E. ranging from ± 0.04 to ± 0.09 °C) within each treatment (one-way ANOVA, F = 1.42, p = 0.251, Figure 5).

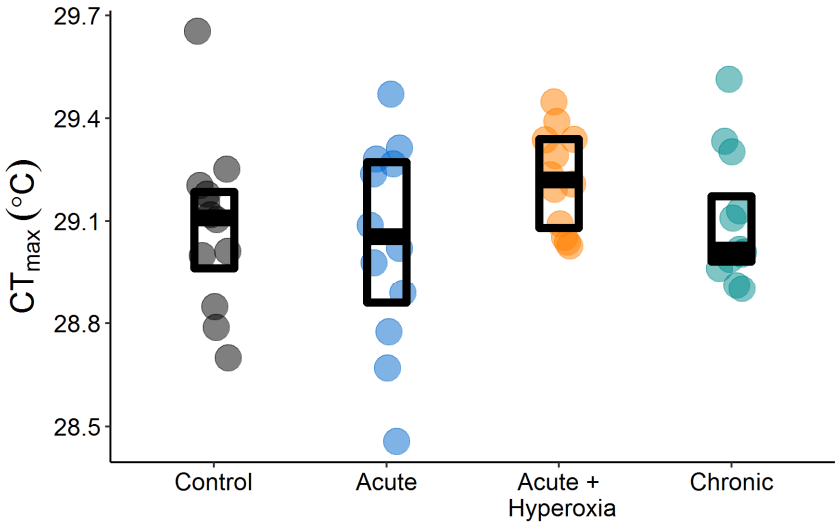


**Figure 5:** Critical thermal tolerance of trout exposed to control (~0.1 % $CO_2$, ~21 % $O_2$, n = 12), acute high $CO_2$ (~30 mins, ~1 % $CO_2$, ~21 % $O_2$, n = 12), acute high $CO_2$ + hyperoxia (~30 mins, ~1 % $CO_2$, ~42 % $O_2$, n = 12), or chronic high $CO_2$ (~72 hours, ~1 % $CO_2$, ~21 % $O_2$, n = 12) treatments. Boxes show inter-quartile range and median values while points represent raw values.

## 4. Discussion

Our aim was to investigate whether acute exposures to high $CO_2$ would reduce thermal tolerances of rainbow trout via direct effects of blood acidosis or indirect effects of $O_2$ supply capacity. We show that acute exposure to increased $CO_2$ causes a large respiratory acidosis in rainbow trout but that this does not affect $CT_{max}$. We also show that fish exposed to high $CO_2$ levels for 72 hours, and which show partial acid-base regulation, have unaffected $CT_{max}$. Combined, these results provide further empirical evidence that changes in environmental $CO_2$ do not affect thermal tolerances of fish. Furthermore, we confirm results of previous research that upper thermal tolerance limits of trout are not affected by changes in aerobic scope and so do not conform to predictions of the OCLTT hypothesis (Pörtner and Farrell, 2008).

### 4.1. Impacts of $CO_2$ and Hyperoxia on $O_2$ supply capacity

As expected, acute exposures to high $CO_2$ led to a pronounced respiratory acidosis in blood and red blood cells of trout exposed to normoxia or hyperoxia (Figure 1 & Figure 2). As fish haemoglobin is highly pH sensitive (Pelster and Weber, 1991) this acidosis led to a pronounced decrease in the haemoglobin-

$O_2$ binding affinity (i.e. an increase in $P_{50}$, Figure 3B). We originally hypothesised that an acute acidosis would cause decreases in MMR precisely for this reason, i.e. reduced Hb-$O_2$ binding affinity causing a reduction in the capacity for $O_2$ uptake from the environment. Instead, our results show only marginal impacts of acute $CO_2$ exposure on MMR (12 % reduction) when compared to control fish (Figure 4B). In addition, fish from the chronic high $CO_2$ exposure (~72 hours) show similar MMR to fish from the acute high $CO_2$ treatment (Figure 4B) despite large differences in Hb-$O_2$ affinity (Figure 3B). This perhaps indicates that Hb-$O_2$ saturation does not have an important role in determining MMR when fish are in normoxic conditions. Based on the measured $P_{50}$, Hills coefficient, and mean blood $pO_2$ from our treatments, the haemoglobin of fish exposed to acute high $CO_2$ would have ~12 % lower $O_2$ saturation than fish in control conditions (calculations based on equations in Pörtner, 1990). While this does match the ~12 % drop in mean MMR between control and acute high $CO_2$ treatments (Figure 4B), it does not explain the lack of difference in mean MMR between fish in acute high $CO_2$ and chronic high $CO_2$ treatments.

Fish exposed to acute high $CO_2$ and hyperoxia exhibited higher MMR than any other treatment group, despite having a much lower Hb-$O_2$ affinity than either control fish, or fish chronically exposed to high $CO_2$. Similar increases of MMR when exposed to hyperoxia have previously been shown in other species (Brijs *et al.*, 2015; McArley *et al.*, 2018) although not in rainbow trout (Duthie and Hughes, 1987). These results are surprising as the majority of blood $O_2$ content is transported by haemoglobin, which is almost fully saturated at normoxia, with only a small percent dissolved in plasma (Takeda, 1990). As such, hyperoxia should not cause large increases in blood $O_2$ transport capacity. These results also disagree with the proposal by Seibel and Deutsch (2020) that maximum $O_2$ uptake capacity occurs at normoxia for most fish species. However, it should be noted that our study, as well as the work by Brijs *et al.* (2015) and McArley *et al.* (2018), measured MMR after chasing fish to exhaustive exercise, whereas previous research with rainbow trout measured MMR in a swimming respirometer (Duthie and Hughes, 1987). It has recently been shown that using chase protocols may underestimate the true MMR of fish (Raby *et al.*, 2020; Zhang *et al.*, 2020). As a result, the increased MMR that we

measured in trout exposed to hyperoxia may be a reflection of limitations in using chase protocols to elicit MMR when compared to methods where MMR is measured using swim tunnel protocols. For example, chase protocols primarily stimulate anaerobic burst swimming and it may be that the metabolic acidosis that occurs as a result of anaerobic respiration has impacts on the $O_2$ transport pathway which are compensated for when environmental $O_2$ is increased. One example of this could be decreased Hb-$O_2$ affinity if there was a further intracellular RBC acidosis after exercise, although adrenergic responses are usually thought to maintain arterial RBC $pH_i$ during exercise to maintain Hb-$O_2$ affinity (Milligan and Wood, 1986; Primmett *et al.*, 1986). Alternatively, hyperoxia may allow maintenance of high diffusion gradients between the blood and tissues when increased $O_2$ demand increases arteriovenous differences and decreases venous $pO_2$ (Farrell and Clutterham, 2003; Claësson *et al.*, 2016). Hyperoxia may also increase cardiac performance by increasing $O_2$ supply to the heart from venous blood (Takeda, 1990; Ekström *et al.*, 2016), although this may not be of benefit to rainbow trout as they possess a coronary blood supply (Ekström *et al.*, 2017). As such, the precise mechanisms behind increased MMR measurements after exhaustive exercise in fish exposed to hyperoxia are still to be determined.

### 4.2. Oxygen supply capacity and respiratory acidosis do not affect thermal tolerance ($CT_{max}$)

Despite large differences in aerobic scope between fish exposed to hyperoxia and fish exposed to normoxia, across all treatments, we did not observe any significant changes in thermal tolerance (Figure 5). Our results agree with several previous studies which show little or no effect of changes in aerobic scope on $CT_{max}$ of fish (Brijs *et al.*, 2015; Devor *et al.*, 2015; Ern *et al.*, 2016, 2017; Motyka *et al.*, 2017). This indicates that critical thermal limits of rainbow trout are determined by an alternative mechanism than $O_2$ supply capacity. We had hypothesised that acute high $CO_2$ exposure may reduce $CT_{max}$ of trout if the mechanisms underlying thermal tolerance are also impacted by respiratory acidosis. Conversely, we saw no impacts of acute or chronic high $CO_2$ exposures on $CT_{max}$ of trout (irrespective of changes in aerobic scope). This result may suggest that the mechanisms which determine critical thermal tolerance are not sensitive to pH, and so may provide evidence that critical

thermal tolerance is not a result of protein disruption, cardiac failure, or neural impairment – all of which are affected by intracellular acidosis (Gesser and Poupa, 1983; Farrell and Milligan, 1986; Siesjö *et al.*, 1993; Hiromasa *et al.*, 1994; Vaughan-Jones *et al.*, 2009). Instead, processes which are pH insensitive, such as breakdown of membrane structures (both cellular and mitochondrial) as a result of increased fluidity of the phospholipid membrane (Bowler, 2018; Chung and Schulte, 2020) may be the primary mechanism behind acute thermal tolerance limits. Alternatively, it may be that the acidosis caused by the acute high $CO_2$ exposure used in our study was of insufficient magnitude to cause sufficient impairment of pH sensitive functions which affect $CT_{max}$. We used a $CO_2$ treatment of ~1 % (equivalent to ~10,000 µatm $CO_2$) as this is sufficient to cause a prolonged respiratory acidosis in rainbow trout (Perry, 1982) and is of a similar order of magnitude to that which fish may experience in the environment (Demars and Trémolières, 2009; Brauner *et al.*, 2019). However, the $CO_2$ level used in our treatments was ~5 times lower than lethal limits of some marine species (Hayashi *et al.*, 2004). Additionally, the expected intracellular acidosis from our $CO_2$ treatment was ~3-6 times lower than the changes in intracellular pH observed to affect neural function of fish (Hiromasa *et al.*, 1994). Despite this our high $CO_2$ treatment is of a similar level to those used to investigate impacts on cardiac function (Farrell and Milligan, 1986) and causes an acidosis of similar magnitude to that which can cause mortality after exercise (Wood *et al.*, 1983).

A second potential reason for a lack of effect of acute $CO_2$ exposure on $CT_{max}$ is that regulation of intracellular pH may have occurred within the time frame of our $CT_{max}$ trials, before fish reached thermal tolerance limits. In order to ensure that internal body temperature of trout matched water temperatures during ramping protocols we used a heating rate of 0.05 $^{o}C\ min^{-1}$ (based on equations and parameters from Stevens and Sutterlin, 1976; Pépino *et al.*, 2015). This meant that trial lengths were ~4.75 hours for all treatments. Combined with the ~30 minute treatment exposure pre-$CT_{max}$ trial, trout were exposed to increased $CO_2$ levels for > 5 hours. Previous research with rainbow trout has shown that intracellular pH regulation can take ~12 hours (Milligan and Wood, 1986), and results from our experiment show slower acid-base regulation than observed by Milligan and Wood (1986), so we think it is unlikely

that intracellular pH would be fully regulated within the time frame of our experiments.

Although our results do not identify potential mechanisms underlying critical thermal tolerance limits they do provide further evidence that increased $CO_2$ does not reduce $CT_{max}$ of fish species. This indicates that the natural fluctuation of $CO_2$ in the environment would be unlikely to impact on acute thermal tolerances of fish species. We also did not see impacts of chronic $CO_2$ treatments on $CT_{max,}$ and combined with previous results (Clark *et al.*, 2017; Ern *et al.*, 2017), we believe this indicates that blood chemistry changes which occur when fish regulate acid-base disturbances caused by increased $CO_2$ (e.g. increased $HCO_3^-$, decreased $Na^+$ & $Cl^-$) also do not reduce critical thermal tolerance of fishes.

## 5. Conclusion

Overall, our results demonstrate that acute thermal tolerance limits of rainbow trout are not affected by the respiratory acidosis caused by acute exposure to increased $CO_2$. This suggests that exposure to acute fluctuations in $CO_2$ which may occur in the environment are unlikely to reduce thermal tolerance of fishes. We also confirm results of previous research that changes in aerobic scope do not impact upon thermal tolerance of trout, in contrast to predictions of the OCLTT hypothesis. Despite this, as predictions of the OCLTT hypothesis have been corroborated in some species (e.g. Gomez Isaza *et al.*, 2020), continued research is still needed to identify why responses of some species conform to the hypothesis when others do not. Our results also suggest that mechanisms underlying acute thermal tolerance of fish are not pH sensitive, perhaps lending support to processes such as breakdown of cellular membranes as the mechanism determining acute thermal tolerance of trout. However, it is still unclear if acute or chronic exposure to increased $CO_2$ may reduce tolerance of fish to long term warming or cause sub-lethal impacts at temperatures below critical thermal limits. Given the reliance on $CT_{max}$ as a quick and repeatable tool for determining thermal tolerance limits, understanding whether mechanisms which determine tolerance to acute vs. chronic warming is critical to accurately predicting effects of environmental changes on thermal limits of fish.

# 6. Supplementary Material

**Table S1:** General linear mixed-effects model outputs for analysis of standard metabolic rate (SMR). The best supported model was fitted using a Gaussian distribution and included treatment as the explanatory variable and group ID as a random intercept term. Treatment effects are compared against the control treatment as the reference level. Marginal R2 = 0.10, Condition R2 = 0.15. Confidence intervals for each parameter were determined from function confint in package lme4. Marginal and conditional R2 of the model were determined using function r.squaredGLMM from package MuMIn.

| Parameter | Estimate | Standard Error | Confidence Interval (95 %) | t-value | P value | Variance | Standard Deviation |
|---|---|---|---|---|---|---|---|
| lmer(SMR ~ Treatment + (1\|Batch)) | | | | | | | |
| Intercept | 90.39 | 4.49 | 81.60 – 99.18 | 20.15 | <<0.001 | | |
| Treatment (Acute $CO_2$) | -4.10 | 5.54 | -14.95 – 6.76 | -0.74 | 0.464 | | |
| Treatment (Acute $CO_2$ & Hyperoxia) | -2.37 | 5.68 | -13.50 – 8.76 | -0.42 | 0.678 | | |
| Treatment (Chronic $CO_2$) | -12.13 | 5.54 | -22.98 – -1.28 | -2.19 | 0.034 | | |
| Batch | | | | | | 10.38 | 3.22 |

**Table S2:** General linear mixed-effects model outputs for analysis of maximum metabolic rate (MMR). The best supported model was fitted using a Gaussian distribution and included treatment as the explanatory variable and group ID as a random intercept term. Treatment effects are compared against the control treatment as the reference level. Marginal R2 = 0.52, Condition R2 = 0.52. Confidence intervals for each parameter were determined from function confint in package lme4. Marginal and conditional R2 of the model were determined using function r.squaredGLMM from package MuMIn.

| Parameter | Estimate | Standard Error | Confidence Interval (95 %) | t-value | P value | Variance | Standard Deviation |
|---|---|---|---|---|---|---|---|
| lmer(MMR ~ Treatment + (1\|Batch)) | | | | | | | |
| Intercept | 690.42 | 25.65 | 640.15 – 740.69 | 26.92 | <<0.001 | | |
| Treatment (Acute $CO_2$) | -85.33 | 37.09 | -158.03 – -12.64 | -2.30 | 0.027 | | |
| Treatment (Acute $CO_2$ & Hyperoxia) | 143.62 | 38.04 | 69.06 – 218.19 | 3.78 | 0.001 | | |
| Treatment (Chronic $CO_2$) | -85.15 | 37.09 | -157.84 – -12.46 | -2.30 | 0.027 | | |
| Batch | | | | | | 0 | 0.00 |

**Table S3:** General linear mixed-effects model outputs for analysis of aerobic scope. The best supported model was fitted using a Gaussian distribution and included treatment as the explanatory variable and group ID as a random intercept term. Treatment effects are compared against the control treatment as the reference level. Marginal R2 = 0.51, Condition R2 = 0.51. Confidence intervals for each parameter were determined from function confint in package lme4. Marginal and conditional R2 of the model were determined using function r.squaredGLMM from package MuMIn.

| Parameter | Estimate | Standard Error | Confidence Interval (95 %) | t-value | P value | Variance | Standard Deviation |
|---|---|---|---|---|---|---|---|
| lmer(Aerobic scope ~ Treatment + (1\|Batch)) | | | | | | | |
| Intercept | 600.60 | 25.21 | 551.18 – 650.09 | 23.82 | <<0.001 | | |
| Treatment (Acute $CO_2$) | -81.04 | 36.46 | -152.50 – -9.58 | -2.22 | 0.032 | | |
| Treatment (Acute $CO_2$ & Hyperoxia) | 146.09 | 37.40 | 72.79 – 219.39 | 3.91 | <0.001 | | |
| Treatment (Chronic $CO_2$) | -73.14 | 36.46 | -144.60 – -1.68 | -2.01 | 0.052 | | |
| Batch | | | | | | 0 | 0.00 |

# Chapter VI

## Temperature and $O_2$, but not $CO_2$, interact to affect aerobic performance of European sea bass (*Dicentrarchus labrax*)

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**Author contributions:** D.W.M. was primarily responsible for designing the research, conducting experimental work, data analysis and writing the chapter. R.W.W. assisted with designing the research, conducting experimental work, and commenting on drafts of the paper. G.H.E. assisted with designing the research, data analysis, and commented on drafts of the paper. S.D.S.and S.N.R.B. assisted with designing the research and commented on drafts of the paper.

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## Abstract

Climate change causes warming, decreased $O_2$, and increased $CO_2$ in marine systems. Responses of marine fish will depend on the interactive impacts between combinations of these factors. We provide the first experimental assessment of their combined interactive effects on aerobic performance of a marine fish. European sea bass (*Dicentrarchus labrax*, Linnaeus) were exposed to combinations of temperature (14, 18, or 22 °C) and $CO_2$ (~400 or ~1000 µatm) for at least two weeks. We then measured blood chemistry, Hb-$O_2$ binding affinity ($P_{50}$), and three indicators of aerobic performance: standard metabolic rate (SMR), hypoxia tolerance ($O_{2crit}$), and maximum metabolic rate (MMR) – the latter measured at 'normal' $O_2$ levels (normoxia) and during 2-h exposures to hypoxia (at either ~12 kPa or ~6 kPa $O_2$). Warming increased SMR and $O_{2crit}$ (i.e. reduced hypoxia tolerance) but we observed no interactive effects between $CO_2$ and temperature. Rising temperatures resulted in higher MMR in normoxia. Hypoxia caused a decline in MMR along a limiting $O_2$ curve. Combined, temperature and $O_2$ interacted synergistically so that hypoxia caused larger reductions in aerobic scope at higher temperatures. Increasing $CO_2$ had minimal effects on MMR and there were no interactive effects between $CO_2$ and temperature or $O_2$ on MMR or aerobic scope. Aerobic performance was not linked to changes in blood acid-base chemistry or Hb-$O_2$ binding affinity. However, increased $CO_2$ induced 30 % mortality of fish exercised in low $O_2$ at 22 $^oC$ indicating important threshold effects independent of aerobic performance. Overall, our results show temperature and $O_2$, but not $CO_2$, interact to affect aerobic performance of sea bass, disagreeing with predictions of the oxygen- and capacity-limited thermal tolerance hypothesis. This work suggests that sea bass populations in the North-East Atlantic may benefit from warming but will be more vulnerable to hypoxia in the warmer and higher $CO_2$ conditions typical of future oceans.


## 1. Introduction

Anthropogenic activity is leading to an increase in atmospheric greenhouse gas concentrations, with $CO_2$ levels increasing from ~280 µatm in the pre-industrial era to > 410 µatm today, potentially reaching ~1000 µatm by the end of the century (IPCC, 2014). This results in the warming of surface waters

(Bopp *et al.*, 2013), which in turn is reducing the average $O_2$ content of our oceans and increasing the prevalence and severity of hypoxic (low $O_2$) events (Diaz and Rosenberg, 2008; Breitburg *et al.*, 2018). In addition, marine $CO_2$ levels are rising in parallel with those in the atmosphere (Caldeira and Wickett, 2003). As climate change is resulting in simultaneous warming, reduced $O_2$, and increased $CO_2$ in our oceans, the responses of marine organisms will be a result of simultaneous changes in multiple environmental factors.

Changes in temperature, $O_2$, and $CO_2$ individually can directly impact physiological performance of fish. Concern has been raised that interactions between these three may occur in a non-linear manner, so that their combined impact cannot be predicted from responses to an individual variable (Crain *et al.*, 2008; McBryan *et al.*, 2013; Todgham and Stillman, 2013; Côté *et al.*, 2016). As such, we need to understand how temperature, $O_2$, and $CO_2$ interact to affect the physiology of fish to enable accurate predictions of how climate change will influence fish species (Pörtner and Peck, 2010; Wernberg *et al.*, 2012; Hollowed *et al.*, 2013). One approach is to examine how these environmental factors affect the fish's range of aerobic metabolism (aerobic scope) which is defined as the difference between an animal's maximum rate of $O_2$ consumption (maximum metabolic rate, MMR) and the minimum rate needed to meet basal energy demands (standard metabolic rate, SMR) (Fry, 1971).

It has been proposed that aerobic scope provides a single metric which indicates whole-animal performance in a particular environment, and can be directly linked to processes such as growth and reproduction, and therefore overall organism fitness, through the concept of oxygen- and capacity- limited thermal tolerance, OCLTT (Pörtner, 2012; Pörtner *et al.*, 2017). The OCLTT hypothesis assumes that aerobic scope is highest at an optimal temperature at which an organism's overall fitness is maximised, and therefore anything which reduces aerobic scope will also diminish fitness, which then translates to shifts in distribution of populations (Pörtner and Farrell, 2008). The impact of hypoxia on aerobic scope is intuitive, with reduced environmental availability of $O_2$ limiting the maximum $O_2$ uptake rate possible. Increased $CO_2$ has also been proposed to affect aerobic scope both by increasing the basal energy demand (SMR) of fish (e.g. through increased cost of acid-base regulation) and by

decreasing MMR (potentially because subsequent changes in internal acid-base chemistry can reduce $O_2$ transport capacity of the blood or impair tissue functioning). As a result, the OCLTT hypothesis predicts that increased $CO_2$ and reduced $O_2$ will interact synergistically to reduce aerobic scope across the thermal performance curve. This would result in a lower optimal temperature (where peak aerobic scope occurs) and reduced thermal tolerance. While this concept has been successfully used to explain changes in habitat suitability and population distributions of some fish species (Cucco *et al.*, 2012; Del Raye and Weng, 2015), the assertion that it represents a universal framework to predict effects of climate change on all fish populations (Farrell, 2016) has been widely questioned (Lefevre, 2016; Jutfelt *et al.*, 2018).

The proposal of the OCLTT hypothesis has led to several studies which examine how $O_2$ or $CO_2$ interact with temperature to affect aerobic performance (for examples see Chabot and Claireaux, 2008; Rummer *et al.*, 2013b; Grans *et al.*, 2014). However, to date no experimental work has sought to investigate how combined changes in all three factors (temperature, $O_2$, and $CO_2$) interact to affect aerobic performance of a fish species. Combining all three environmental variables is vital to accurately assess potential interactive effects for three reasons. Firstly, meta-analysis of multi-factor studies indicates that the prevalence of non-linear effects doubles when moving from studies that combine 2 factors to 3 factors (Crain *et al.*, 2008). Secondly, the role of $CO_2$ as a limiting factor of aerobic scope was originally suggested to occur primarily in combination with hypoxia (Fry, 1971). Thirdly, low $O_2$ conditions in the environment always co-occur with increased $CO_2$ (Melzner *et al.*, 2013). As such, experiments investigating effects of $O_2$ or $CO_2$ and temperature on aerobic performance may not accurately reflect interactive effects caused by all three environmental factors.

In the present study we sought to investigate how combined changes in temperature, $O_2$, and $CO_2$ affect aerobic performance of European sea bass (*Dicentrarchus labrax*, Linnaeus), which are distributed in the North-East Atlantic and throughout the Mediterranean and have shown recent northward range expansions thought to be related to warming (Pawson *et al.*, 2007). Two separate populations exist (Souche *et al.*, 2015) and, although the physiological responses of this species to environmental change have been regularly

examined, to date no studies have used fish from the Atlantic population. As such, our experiment had three aims:

i. to assess how aerobic performance of sea bass from the Atlantic population will respond to environmental changes that are in line with those expected to occur over the next 80 years;
ii. to determine whether combinations of hypoxia and increased $CO_2$ interact with temperature to affect aerobic scope, as predicted by the OCLTT hypothesis;
iii. to determine whether changes in aerobic performance are linked to acid-base chemistry and $O_2$ transport capacity of the blood.

## 2. Materials and Methods

### 2.1. Animal Collection and Husbandry

We collected juvenile sea bass from estuaries and coastal lagoons on the south Dorset coast and Isle of Wight in June 2017 and held them in a marine recirculating aquaculture system (RAS) at the University of Exeter for 332 days before experimental work began (see water chemistry data in Table 1). Sea bass were fed a diet of commercial pellet (Horizon 80, Skretting) at a ration of ~1-2 % body mass 3 times per week and supplemented with ~1 % body mass of chopped mussel (*Mytilus edulis*) once per week. All experimental procedures were carried out under a UK Home Office licence P88687E07 and approved by the University of Exeter's Animal Welfare and Ethical Review Board.

**Table 1:** Water chemistry parameters of the recirculating aquaculture system in which sea bass were held prior to experimental work beginning (means ± S.D. shown). Fish were initially held at 15 $^{o}$C before stock systems were raised to 18 $^{o}$C approximately 6 months prior to experimental work beginning. The temperature shown in the table represents the mean temperature for the entire time fish were held in the RAS prior to experimental trials beginning.

| **Time in system (days)** | **Temperature ($^{o}$C)** | **pH (NBS scale)** | **Salinity** | **Total Alkalinity (µM)** | ***p*$CO_2$ (µatm)** |
|---|---|---|---|---|---|
| 332 | 17.1 ± 1.4 | 8.02 ± 0.05 | 32.98 ± 0.65 | 2072.0 ± 130.2 | 534.0 ± 60.2 |

## 2.2. Treatment conditions

Groups of 10 sea bass were transferred to an experimental RAS, in a temperature-controlled room, in which they were acclimated for a minimum of 14 days to treatment conditions. Six treatment conditions were used combining three temperatures and two $CO_2$ levels in a 3 x 2 factorial design (Table 2). Temperature treatments (14, 18 and 22 $^oC$) were chosen to reflect temperature ranges in coastal UK waters from spring to autumn as well as potential future summer temperatures at the end of the century (IPCC, 2014; Tinker *et al.*, 2020). $CO_2$ treatments (~400 & ~1000 µatm) were chosen to reflect annual average ambient atmospheric $CO_2$ levels currently and possible end-of-century ambient atmospheric levels according to an RCP 8.5 scenario (IPCC, 2014). Details on manipulation of temperature and $CO_2$ during the initial 14 day treatment are provided in the supplementary materials (Section 6.1.1.).

Measurements of treatment tank pH (NBS scale), salinity, temperature, and a 12 mL water sample to measure Dissolved Inorganic Carbon (DIC) were taken every 2-3 days. Seawater DIC analysis was conducted using a custom built system described in detail by Lewis *et al.* (2013). Data for pH, salinity, temperature and DIC were then input into the seawater carbon calculator programme, CO2SYS (Pierrot *et al.*, 2006) to calculate $pCO_2$ based on the equilibration constants refitted by Dickson and Millero (1987), and $KSO_4$ dissociation constants from Dickson (1990) (Table 2).

**Table 2:** Mean ± S.D. of water chemistry parameters in treatment tanks during treatments at 3 temperature (14, 18, and 22 $^oC$) and 2 $CO_2$ levels (~400 and ~1000 µatm). Treatment order represents time course of treatments (i.e treatment 1 was conducted first and treatment 6 last). Total Alkalinity (TA) over time as a result of biological activity and so was adjusted periodically by addition of 1.0 M $NaHCO_3$ to restore TA levels back to >2000 µM.

| | **Treatment** | | | | | |
|---|---|---|---|---|---|---|
| **Parameter** | 1 | 2 | 3 | 4 | 5 | 6 |
| Temperature ($^oC$) | 17.9 ± 0.0 | 22.0 ± 0.0 | 13.9 ± 0.1 | 21.7 ± 0.5 | 13.9 ± 0.1 | 18.0 ± 0.1 |
| $pCO_2$ (µatm) | 460 ± 34 | 375 ± 28 | 333 ± 38 | 1065 ± 172 | 1057 ± 65 | 973 ± 114 |
| pH (NBS) | 8.06 ± 0.03 | 8.12 ± 0.02 | 8.14 ± 0.04 | 7.79 ± 0.03 | 7.81 ± 0.04 | 7.83 ± 0.05 |
| Salinity | 32.9 ± 0.6 | 32.5 ± 2.9 | 33.3 ± 0.3 | 32.6 ± 0. 3 | 34.6 ± 0.5 | 33.8 ± 0.6 |
| TA (µM) | 1935 ± 93 | 1745 ± 26 | 1861 ± 88 | 2147 ± 292 | 2393 ± 188 | 2170 |

## 2.3. Respirometry measurements (SMR, MMR, and $O_{2crit}$)

Measurements of oxygen consumption rates ($\dot{M}O_2$) were made as a proxy for metabolic rate using an intermittent-flow respirometer system, details of which can be found in Montgomery *et al.* (2019), set-up following recommendations by Svendsen *et al.* (2016). Sea bass were starved for 72 hours prior to the start of measurements to ensure that metabolism was not affected by the specific dynamic action of digestion (Chabot *et al.*, 2016). Individual sea bass were then transferred to respirometer chambers and left to acclimate for a minimum of 12 hours overnight before measurements of $\dot{M}O_2$ began. For each treatment all respirometry measurements were conducted in two groups (hereafter referred to as respirometry group), with five fish being measured simultaneously for each group. Following the 12 hour acclimation period we measured $\dot{M}O_2$ of each sea bass for ~3-4 hours (from ~6 am to ~10 am) before hypoxia tolerance was assessed using a critical $O_2$ tension ($O_{2crit}$) trial, following protocols set out in Montgomery *et al.* (2019). Carbon dioxide levels in the water were simultaneously increased as $O_2$ declined during $O_{2crit}$ trials to reflect the natural rise in $CO_2$ during hypoxic events in aquatic systems (Melzner *et al.*, 2013; Montgomery *et al.*, 2019). During $O_{2crit}$ trials water pH, temperature, salinity, and DIC were measured every hour to calculate water carbonate chemistry. Changes in system $pCO_2$ and pH during $O_{2crit}$ trials for each treatment are given in supplementary materials (Table S1).

Critical $O_2$ tension trials were stopped once a minimum of 3 consecutive $\dot{M}O_2$ measurements showed a transition from an oxy-regulating to oxy-conforming state for each fish. Following completion of $O_{2crit}$ trials the respirometer system was aerated with ambient air ($CO_2$ ~400 µatm) or a 0.1 % $CO_2$ in air gas mix ($CO_2$ ~1000 µatm) to rapidly restore $O_2$ levels to normoxia and $CO_2$ to the appropriate treatment level. Sea bass were left to recover in respirometers, for a minimum of 1 hour post-trial, until $O_2$ levels reached ~ 21 kPa $O_2$ (~100 % air saturation) before removing the fish and measuring background respiration for a minimum of 1 hour (6 measurement cycles) for all respirometers immediately post trial. Each sea bass was then placed in an individual ~10 L isolation tank which was subsequently fed by the respirometry system sump (at a rate of ~4 L $min^{-1}$) to maintain treatment conditions (with overflowing water from the isolation tanks recirculating back to the sump).

After fish had rested overnight in isolation tanks, MMR was measured for each fish (using an exhaustive chase protocol; Norin and Clark, 2016) on 3 consecutive days (with overnight recovery in between) at three different levels of $O_2$ (100, 60 and 30 % air saturation) with increasing $CO_2$ levels for each $O_2$ level as detailed for $O_{2crit}$ trials. Measurements of water pH, temperature, salinity, and DIC were taken for each isolation tank, the chase tank (after all fish were chased) and the respirometer system (during $\dot{M}O_2$ measurements) to calculate water carbonate chemistry. Changes in system $pCO_2$ and pH for all MMR trials are given in supplementary materials (Table S2). For further details of respirometry system set up for all measurements, water chemistry of respirometer systems, $\dot{M}O_2$ analysis methods, and calculations of SMR, $O_{2crit}$, and MMR see supplementary materials (Section 6.1.2 to 6.1.5).

## 2.4. Blood chemistry and Hb-$O_2$ affinity measurements

Following MMR measurements, sea bass were left overnight in the isolation boxes before blood samples were taken, following methods outlined in Montgomery *et al.* (2019), from each fish in normoxic conditions and at the relevant treatment temperature and $CO_2$ level (see supplementary materials Section 7.1.6. for full details of blood sampling method and Table S3 for water chemistry parameters).

Immediately after sampling, extracellular pH ($pH_e$), haematocrit (Hct) and $TCO_2$ were measured following details set out in Montgomery *et al.* (2019). Plasma $pCO_2$ and $HCO_3^-$ were then calculated from $TCO_2$, temperature and blood pH using the Henderson-Hasselbalch equation with values for solubility and $pK^1_{app}$ based on Boutilier *et al.* (1984, 1985). Measurements of haemoglobin content (Hb), plasma glucose and lactate were also completed following methods from Montgomery *et al.* (2019). We centrifuged whole blood (10,000 g) for 2 minutes at 4 $^oC$, before removing the supernatant and blotted the surface of the leftover red blood cell (RBC) pellet to remove the white blood cell layer. We then followed the freeze-and-thaw method (by snap freezing the RBC pellet in liquid nitrogen for 10 seconds and thawing the pellet for 1 minute in a water bath set to ~ 37 $^oC$) to measure intracellular pH of RBCs ($pH_i$) as described by Zeidler and Kim (1977), and validated by Baker *et al.* (2009). All measurements or storage of blood occurred within 10 minutes of blood sampling. Finally, we measured Hb-$O_2$ affinity using a Blood Oxygen Binding

System (BOBS, Loligo systems), detailed in general in Oellermann *et al.* (2014) and specifically for fish blood in Montgomery et al. (2019) (for further details on Hb-$O_2$ affinity measurements see supplementary materials Section 6.1.6.).

## 2.5. Statistical Analysis

### 2.5.1. Respirometry data analysis

All results are reported as mean ± S.E unless otherwise stated. We conducted all statistical analysis in R v3.6.3 (R Core Team, 2020). The effects of temperature, $O_2$, and $CO_2$ on individual physiological performance metrics (SMR, MMR, and $O_{2crit}$) were analysed using separate general linear mixed-effects models (GLMM) in package 'lme4' (Bates *et al.*, 2015; Pinheiro *et al.*, 2018). All models included respirometry group as a random intercept term to account for potential tank effects introduced during respirometry measurements. For each physiological metric the best supported model was determined as the model with the lowest corrected Akaike's information criterion, AICc (Hurvich and Tsai, 1989; Burnham and Anderson, 1998). Residual diagnostic plots of each GLMM were then assessed using package 'DHARMa' to confirm validity of model fit (Hartig, 2020). For full details of comparisons of model structures see supplementary materials (Section 6.1.7 and Table S4).

The best supported model used to analyse SMR included temperature and $CO_2$ as explanatory variables (supplementary materials Table S5), with temperature being the respirometer temperature at the time of measurement and $CO_2$ being the mean level experienced over the course of the treatment period for each respirometry group. The random variable of respirometer group had no impact on SMR. The best supported model of $O_{2crit}$ included temperature, $CO_2$ and SMR as explanatory variables (supplementary materials Table S6). The model used the respirometer system temperature during the $O_{2crit}$ trial and the $CO_2$ level at the start of $O_{2crit}$ trial for each respirometry group. Standard metabolic rate was included as an explanatory variable because there was a statistically significant correlation between SMR and $O_{2crit}$ across all temperature and $CO_2$ treatments (Kendall's tau correlation, $z = 3.12$, $\tau = 0.29$, $p = 0.0018$). The random variable of respirometer group had minimal impacts on $O_{2crit}$.

Temperature, $O_2$, and $CO_2$ were included as explanatory variables in the best supported model used to analyse MMR (supplementary materials Table S7), utilising the temperature of the respirometer system at time of MMR measurement, the mean $CO_2$ level each fish experienced immediately prior to exhaustive exercise, and the mean $O_2$ level each fish experience inside the respirometer during the measurement period. To account for observed non-linear effects of $O_2$ on MMR, $O_2$ was included as a 2$^{nd}$ order polynomial term in the GLMM. All fixed effects were centred and scaled to improve model fit. In addition, the $O_2$ level at which $O_{2crit}$ was determined for each individual fish was used as the point at which MMR equalled the estimated SMR for each fish. This gave MMR measurements for each individual fish at four separate $O_2$ levels. As such, in addition to including respirometry group as a random variable, fish ID was included as a random intercept in the GLMM to account for possible effects of repeated measurements and inter-individual variation on MMR. We also observed that the slope of the response of MMR to $O_2$ level differed between individuals so the effect of $O_2$ was included as a random slope for each fish. The random variable of respirometer group had minimal impacts on MMR whereas fish ID significantly impacted model predictions.

Once the best supported model for each physiological parameter was identified predictions were made across a range of temperatures, $O_2$ levels, and $CO_2$ levels to visualise combined effects of these variables on the physiology of seabass. We then used function bootMer from lme4 (Pinheiro *et al.*, 2018) to calculate 95 % confidence intervals of model predictions.

### 2.5.2. Blood chemistry and Hb-$O_2$ affinity data analysis

Measurements of blood chemistry parameters ($pH_e$, $pH_i$, $pCO_2$, $HCO_3^-$, $P_{50}$, Hills' number, Hct, Hb, lactate, and glucose) were analysed using the ambient water temperature of the treatment and using a categorical $CO_2$ level of low (i.e. ~400 µatm treatment) or high (i.e. ~1000 µatm treatment). Measurements were analysed using a type III sum of squares two-way ANOVA (to account for unequal sample sizes) before assumptions of normality and equal variances of model residuals were checked. Post hoc-tests were conducted on least-square means generated by package 'emmeans' (Lenth, 2020), with Tukey adjusted p-values for multiple comparisons.

Some measurements did not meet statistical assumptions for two-way ANOVA. In these cases data transformations were attempted using box-cox transformations from package 'MASS' (Venables and Ripley, 2002). Transformed data were then analysed using two-way ANOVA before model residuals were checked. This method allowed measurements of $pO_2$ to be analysed using two-way ANOVA after data had been box-cox transformed ($\lambda = -0.6$). Box-cox transformation did not prevent violations of model assumptions for measurements of lactate and glucose. These measurements also did not meet assumptions of one-way ANOVA and so were analysed using a non-parametric Kruskal-Wallis test.

Blood-oxygen binding parameters ($P_{50}$ and Hills' number) could not be obtained for fish in the 14 $^{o}$C and high $CO_2$ treatment as a result of an equipment failure. As such these data were analysed using a one-way ANOVA. If statistical assumptions of one-way ANOVA were not met then data were analysed using the non-parametric Kruskal-Wallis test.

# 3. Results

## 3.1. Standard metabolic rate and hypoxia tolerance

The best supported model for SMR included both temperature and $CO_2$ as fixed effects (Linear Mixed-Effects Model, marginal $R^2 = 0.70$, conditional $R^2 = 0.70$, see supplementary table S5 for full model summary). Standard metabolic rate approximately doubled between 14 and 22$^{o}$C, exhibiting a $Q_{10}$ temperature coefficient of 2.09 (Figure 1). The best supported model indicated that $CO_2$ had a small effect on SMR with predicted SMR reducing by 7.4 $mgO_2\,kg^{-1}\,h^{-1}$, ~10 % of the lowest SMR recorded, (95 % CI = -1.65 to 16.43 $mgO_2\,kg^{-1}\,h^{-1}$) across all temperatures at ~1000 µatm $CO_2$ (Figure 1). However, the model including temperature but not $CO_2$ had a ΔAICc <2 indicating that including the effect of $CO_2$ in the model did not lead to a large improvement in model fit (supplementary material Table S4). There was no evidence of an interactive effect between increasing temperatures and increasing $CO_2$.

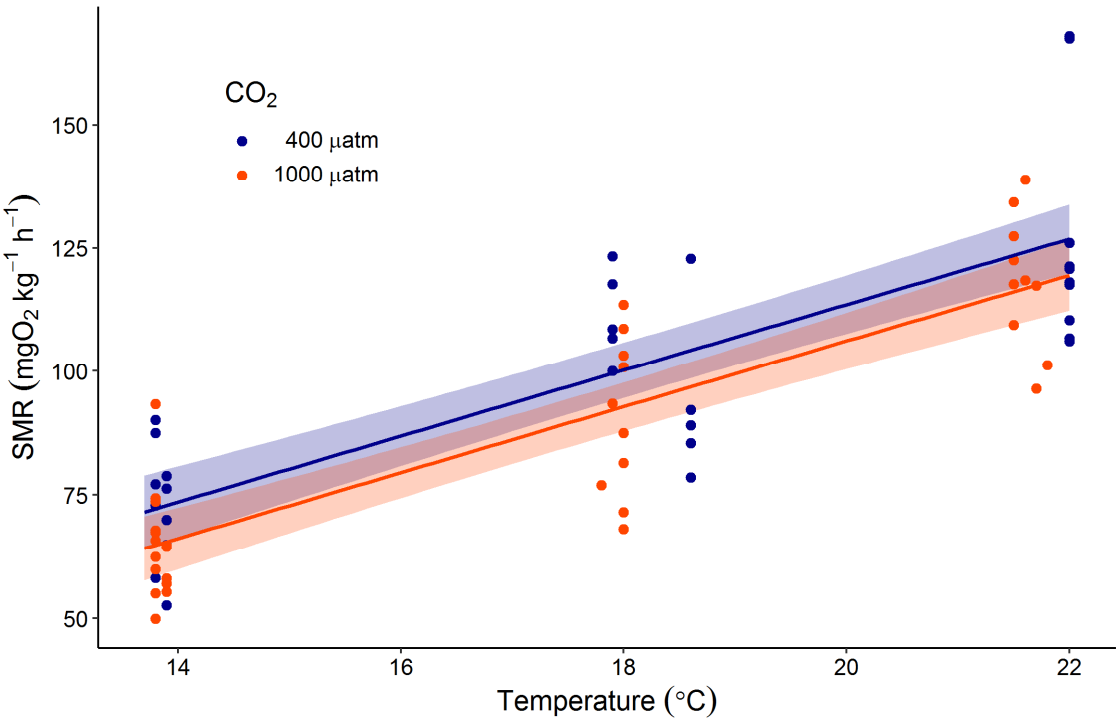


**Figure 1**: Impact of temperature and $CO_2$ on standard metabolic rates (SMR) of juvenile sea bass. The best supported model (marginal $R^2$ = 0.70, conditional $R^2$ = 0.70) to explain variation in SMR included temperature and $CO_2$ as explanatory variables but not their interaction. Points represent calculated SMR for individual fish, lines represented predicted SMR at two $CO_2$ levels (blue = ambient ~400 µatm $CO_2$, orange = end of century ~ 1000 µatm $CO_2$) from the best supported model, and shaded areas represent bootstrapped 95 % CI of predictions (n = 1000).

The best supported model of $O_{2crit}$ included the effects of temperature, $CO_2$ and SMR but no interactions (Linear Mixed-Effects Model, marginal $R^2$ =0.72, conditional $R^2$ = 0.77, see supplementary table S6 for full model summary). Hypoxia tolerance ($O_{2crit}$) of sea bass was affected by the SMR of individual sea bass. A doubling in SMR from 60 to 120 $mgO_2\ kg^{-1}\ h^{-1}$ is predicted to increase $O_{2crit}$ by 2.16 kPa $O_2$ (95 % CI = 1.66 to 2.66 kPa $O_2$). Independent of their effects on SMR, both temperature and $CO_2$ were included in the best model of $O_{2crit}$. The effect of temperature meant that for a given value of SMR $O_{2crit}$ would reduce as temperature increased. For instance, a fish at 14 $^oC$ is predicted to have an $O_{2crit}$ 0.50 kPa $O_2$ higher (95 % CI = 0.13 to 0.87 kPa $O_2$) than a fish with the same SMR at 18 $^oC$ (Figure 2A). Combined the effects of SMR and temperature suggest rising from 14 $^oC$ to 22 $^oC$ is predicted to increase $O_{2crit}$ by

0.91 kPa $O_2$ (95 % CI = 0.53 to 1.29 kPa $O_2$, Figure 2B). The additional effect of $CO_2$ is predicted to increase $O_{2crit}$. Despite this, when taking into account the prediction for $CO_2$ to reduce SMR (Figure 1), and the effect of SMR and temperature on $O_{2crit}$, the resulting effect of $CO_2$ causes an increase in $O_{2crit}$ of only 0.04 kPa $O_2$ (95 % CI = -0.32 to 0.40 kPa $O_2$, Figure 2B).

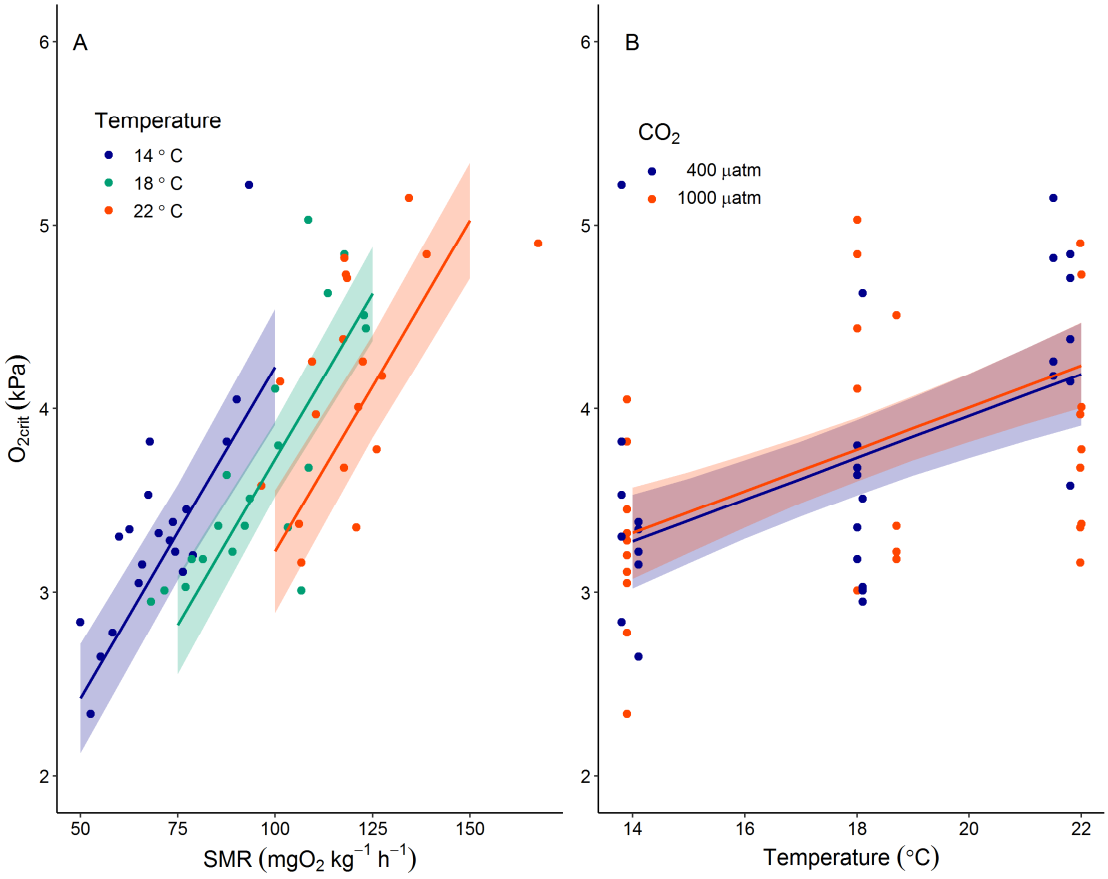


**Figure 2**: Combined impacts of SMR, temperature and $CO_2$ on hypoxia tolerance ($O_{2crit}$) of juvenile European sea bass. **A.** The best supported model (marginal $R^2$ = 0.72, conditional $R^2$ = 0.77) predicted a positive effect of increasing SMR on $O_{2crit}$ with the effect of increased temperature resulting in lower $O_{2crit}$ for a given value of SMR. **B.** Combined effects of SMR and temperature result in an increase in $O_{2crit}$ between 14 ºC and 22 ºC. The predicted positive effect of increased $CO_2$ on $O_{2crit}$ is small compared to changes in SMR and temperature. Points represent calculated $O_{2crit}$ for individual fish, lines represented predicted $O_{2crit}$ from the best supported model, and shaded areas represent bootstrapped 95 % CI of predictions (n = 1000).

## 3.2. Maximum metabolic rate and aerobic scope

Maximum metabolic rate of sea bass was affected by temperature, $O_2$, and $CO_2$ level. Warming from 14 to 22 $^oC$ caused a 50 % increase in MMR in normoxia combined with normocapnia from 293.5 ± 10.4 to 435.9 ± 18.9 $mgO_2\ kg^{-1}\ h^{-1}$, with a $Q_{10}$ of 1.64. In high $CO_2$ the temperature effect on MMR was very similar ($Q_{10}$ = 1.60). The best supported model predicted that $O_2$ had a non-linear quadratic effect on MMR, so that the same reduction in $O_2$ caused a progressively larger reduction in MMR as $O_2$ levels declined, as well as interactive effects between $O_2$ and temperature (Figure 3; Linear Mixed Model, marginal $R^2$ = 0.91, conditional $R^2$ = 0.96, see supplementary Table S7 for full model summary). For example a 5 kPa reduction in $O_2$ at 18 $^oC$ (in normal $CO_2$) from air saturated levels (~ 20 to ~15 kPa $O_2$) results in a predicted decrease in MMR of 8.0 $mgO_2\ kg^{-1}\ h^{-1}$ (95 % CI = -7.2 to 23.2 $mgO_2\ kg^{-1}\ h^{-1}$) whereas the same reduction in $O_2$ from 10 to 5 kPa results in a 15-fold larger predicted decrease in MMR of 123.8 $mgO_2\ kg^{-1}\ h^{-1}$ (95 % CI = 115.1 to 132.5 $mgO_2\ kg^{-1}\ h^{-1}$). In addition, the non-linear effect of $O_2$ interacted with the effect of temperature so that the same reduction in $O_2$ causes a larger reduction in MMR as temperature increased (Figure 3A & B). This is particularly noticeable between 15 and 10 kPa $O_2$ where fish at 22 $^oC$ (in normal $CO_2$) exhibit a predicted decline in MMR that was 60 % larger than fish at 14 °C, 80.9 $mgO_2\ kg^{-1}\ h^{-1}$ (95 % CI = 63.2 to 98.6 $mgO_2\ kg^{-1}\ h^{-1}$) versus 50.8 $mgO_2kg^{-1}h^{-1}$ (95 % CI = 35.9 to 65.7 $mgO_2\ kg^{-1}\ h^{-1}$). Finally, environmental $CO_2$ level had a small, negative additive effect independent of interactions between temperature and $O_2$. As a result of this an increase in $CO_2$ of 1000 µatm is predicted to reduce MMR by 8.5 $mgO_2\ kg^{-1}\ h^{-1}$ (95 % CI = -18.2 to 35.3 $mgO_2\ kg^{-1}\ h^{-1}$) irrespective of temperature and $O_2$ (Figure 3C, D, & E).

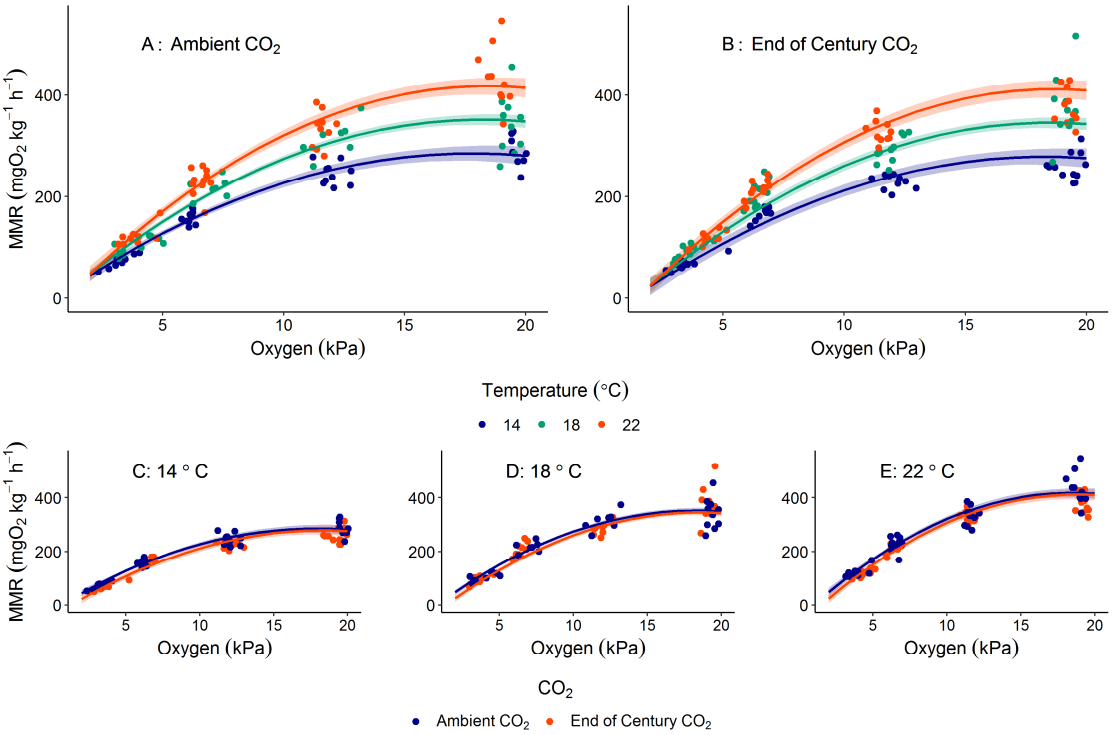


**Figure 3:** Effects of combinations of temperature, $O_2$, and $CO_2$ on the maximum metabolic rate (MMR) of European sea bass. There was a synergistic interactive effect of temperature and $O_2$ on MMR for sea bass exposed to both **A.** ambient $CO_2$ conditions (ambient $CO_2$ = 400 µatm at ~20 kPa $O_2$) and **B.** end of century $CO_2$ conditions (end of century $CO_2$ = 1000 µatm at ~ 20 kPa $O_2$). The additive effects of increased $CO_2$ levels and reduced $O_2$ are displayed for sea bass at **A.** 14 $^oC$ **B.** 18 $^oC$ and **C.** 22 $^oC$. Points represent calculated MMR for individual fish, lines represent predicted MMR from the best supported model, and shaded areas represent bootstrapped 95 % confidence intervals (n = 1000).

We predicted the impacts of temperature, $O_2$, and $CO_2$ on aerobic scope from the best supported models fitted to measurements of SMR and MMR (Figure 4). At normoxia (~20 kPa $O_2$) aerobic scope is predicted to increase by 80.2 $mgO_2\ kg^{-1}\ h^{-1}$ (95 % CI = 24.8 to 135.7 $mgO_2\ kg^{-1}\ h^{-1}$) as fish move from 14 to 22 $^oC$ independent of changes in $CO_2$ level (Figure 4A & B). Interactive effects between temperature and $O_2$ on MMR are reflected in predictions of aerobic scope (Figure 4A &B). As increasing $CO_2$ has the same direction of effect on both SMR and MMR the impact of $CO_2$ on aerobic scope is minimal (Figure 4C, D, & E).

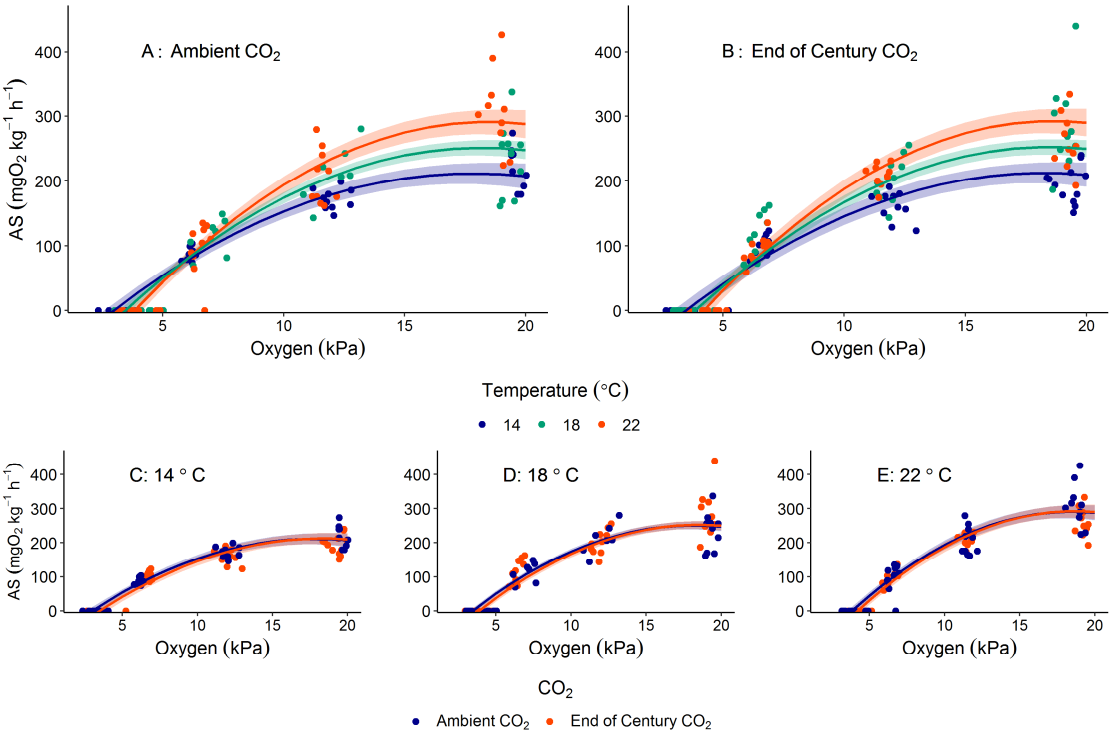


**Figure 4:** Effects of combinations of temperature, $O_2$, and $CO_2$ on the aerobic scope (AS) of European sea bass. There was a non-linear interactive effect of temperature and $O_2$ on aerobic scope for sea bass exposed to both **A.** ambient $CO_2$ conditions (ambient $CO_2$ = 400 µatm at ~20 kPa $O_2$) and **B.** end of century $CO_2$ conditions (end of century $CO_2$ = 1000 µatm at ~ 20 kPa $O_2$). The additive effects of increased $CO_2$ levels and reduced $O_2$ are displayed for sea bass at **A.** 14 $^oC$ **B.** 18 $^oC$ and **C.** 22 $^oC$. Points represent aerobic scope of individual fish derived from calculated RMR and MMR of that individual, lines represent predicted aerobic scope calculated by subtracting model predictions of RMR and MMR and shaded areas represent 95 % confidence intervals calculated from bootstrapped standard errors of predicted RMR and MMR (n = 1000).

### 3.3. Blood chemistry & Hb-$O_2$ affinity

As expected increasing $CO_2$ levels from ~400 µatm to ~1000 µatm significantly increased plasma $pCO_2$ levels (Two-way ANOVA, F = 15.84, df = 1, $p < 0.001$), whereas increasing temperature from 14 $^oC$ to 22 $^oC$ did not (F = 0.772, df = 2, p = 0.468) and there was no interactive effect noted (F = 2.067, df = 2, p = 0.138). Despite the significant overall effect of increased $CO_2$ on plasma $pCO_2$ pairwise comparisons (Figure 5) only revealed a significant increase in plasma $pCO_2$ in bass at 18 $^oC$ exposed to ~1000 µatm (0.53 ± 0.05

kPa $pCO_2$) compared to ambient conditions (0.29 ± 0.05 kPa $pCO_2$) (Pairwise comparisons of least square means, t = 3.688, df = 1, p = 0.008).

To compensate for increased plasma $pCO_2$ in ~1000 µatm $CO_2$ treatments sea bass accumulated ~3 mM extra $HCO_3^-$ (Two-way ANOVA, F = 44.34, df = 1, p << 0.001) (Figure 5), compared to fish in ambient $CO_2$ conditions at 14 and 18 °C. Fish exposed to ~1000 µatm at 22 ºC showed a non-significant increase in $HCO_3^-$ of just under 2 mM (95 % CI = 0.60 – 2.84 mM) when compared to fish at ~400 µatm $CO_2$ (t = 2.66, df = 1, p = 0.103). There was no temperature effect on plasma $HCO_3^-$ (F = 2.538, df = 2, p = 0.0903) and no interactive effect between temperature and $CO_2$ (F = 0.969, df = 2, p = 0.387).

As a result of compensatory accumulation of $HCO_3^-$, blood $pH_e$ was well regulated in response to high $CO_2$ (F = 3.56, df = 1, p = 0.066) and sea bass did not show significant effects of temperature on $pH_e$ (Two way ANOVA, F = 1.425, df = 2, p = 0.251). However, there was a significant interactive effect of temperature and $CO_2$ (F = 3.952, df = 2, p = 0.026). This interactive effect was caused by a significant reduction in $pH_e$ in sea bass exposed to ~1000 µatm $CO_2$ at 18 ºC (7.80 ± 0.03) when compared to fish at ambient $CO_2$ levels (7.97 ± 0.04) (pairwise comparisons of least square means, t = 3.242, df = 1, p = 0.026). We did not find significant differences between $pH_e$ across all other treatment groups. Additionally, intracellular pH ($pH_i$) showed no significant differences between all treatments (Kruskal-Wallis test, $\chi^2$ = 6.79, df = 5, p = 0.237) (Figure 5).

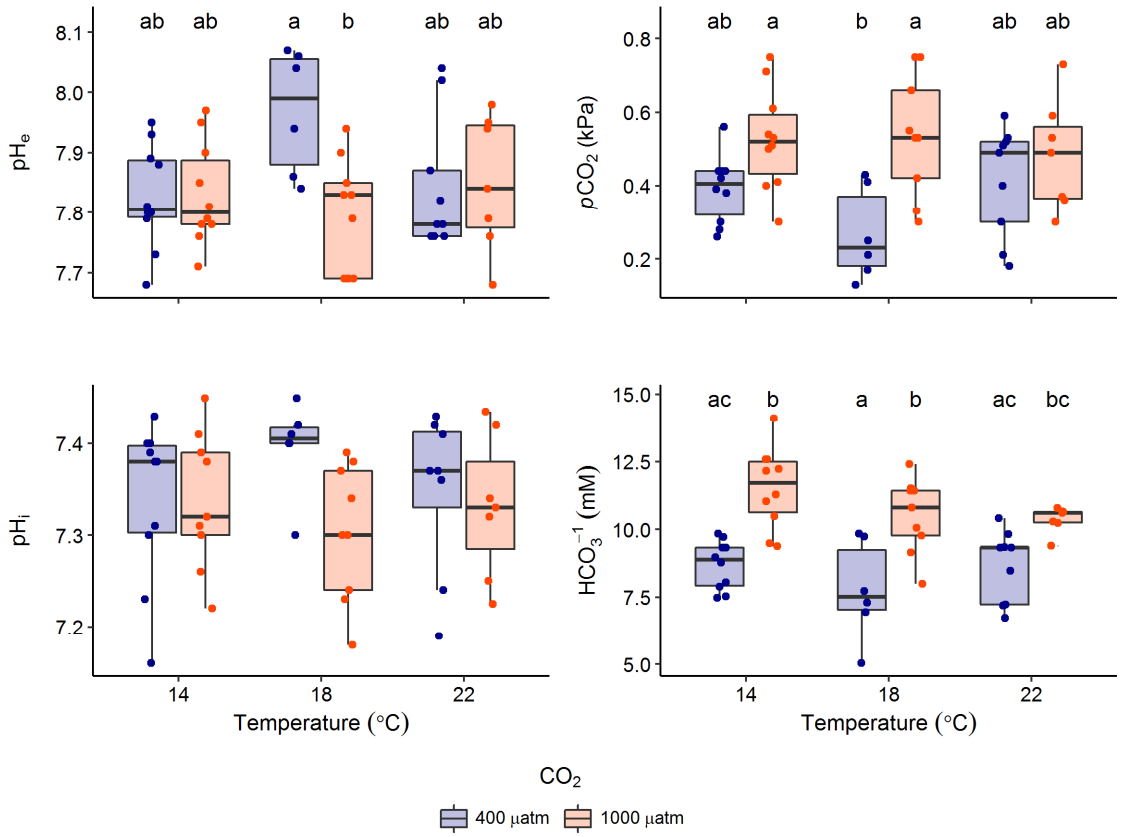


**Figure 5:** Impact of temperature and $CO_2$ on blood acid-base characteristics of European sea bass. Significant differences in pairwise comparisons of least square means between treatments are indicated by different letters (a,b,c). No significant differences between any treatments were noted for measurements of $pH_i$.

There were no consistent significant differences in any of our measurements of Hb-$O_2$ affinity, Hct, Hb, lactate or glucose concentrations as a result of temperature or $CO_2$ treatments and no evidence for any interactive effects between temperature and $CO_2$ (for full summaries of results see supplementary materials Section 6.2).

### 3.4. Mortality

Three out of ten fish (i.e. 30 %) died ~1 hour post-chase after exposure to ~30 % air saturation at 22 $^{o}$C in the high $CO_2$ treatment. No fish died post-chase in any other treatment combinations.

## 4. Discussion

Our results demonstrate, for the first time, the interactive effects of temperature, $O_2$, and $CO_2$ on the aerobic performance of an active predatory marine fish. We show that temperature and $O_2$ have a non-linear interactive effect on aerobic performance of European sea bass but that the effect of $CO_2$ is minor and independent. Both SMR and MMR increased as temperature rose from 14 $^oC$ to 22 $^oC$, but changes in MMR were greater which led to positive effects on absolute aerobic scope. These results suggest that European sea bass populations in the North-East Atlantic (where temperatures are typically < 22 $^oC$) could physiologically benefit from expected global warming. However, hypoxia tolerance reduced at higher temperatures, and hypoxia reduced MMR and aerobic scope by a greater amount at high temperatures, indicating that in warmer waters European sea bass will be more susceptible to simultaneous declines in $O_2$.

We confirm similar findings from previous research investigating effects of temperature and $O_2$ on European sea bass. For example, the increase in SMR we observed closely follows patterns of SMR observed in European sea bass from aquaculture sources (Claireaux and Lagardère, 1999). That is the $Q_{10}$ temperature coefficient dropped from 2.33 between 14 and 18 $^oC$ to 1.82 between 18 and 22 $^oC$. This drop in $Q_{10}$ as temperature increases provides further evidence that thermal acclimation results in a change in the thermal performance curve (TPC) of SMR from an exponential relationship with acute exposure to a linear relationship with chronic exposures (Schulte *et al.*, 2011; Sandblom *et al.*, 2014). Responses of MMR to temperature showed similar temperature quotients (i.e. $Q_{10}$) indicating a linear increase in MMR between 14 and 22 $^oC$. This also closely corresponds with observations from Claireaux and Lagardère (1999) from which MMR was predicted to increase approximately linearly over temperatures between 14 $^oC$ and 22 $^oC$, before peaking between 22 and 24 $^oC$ and declining at higher temperatures. Despite this the SMR of fish in our study was higher at a given temperature than for fish produced in aquaculture from Mediterranean stocks (Claireaux and Lagardère, 1999). This may fit with the theory of metabolic cold adaptation, that basal energy demand in fish from warmer environments will be lower than in fish from cold environments when measured at the same temperature (Krogh, 1916). This has

recently been supported by evidence from wild populations of three-spine stickleback (*Gasterosteus aculeatus*, Linnaeus) (Pilakouta *et al.*, 2020). Further work with sea bass from Mediterranean stock has shown that specific growth rate and feed conversion efficiency peak at ~25 $^{o}$C and decline at higher temperatures (Person-Le Ruyet *et al.*, 2004), roughly in line with the changes in metabolic scope shown by Claireaux and Lagardère (1999). Finally, the most recent research with aquaculture-produced sea bass found that acute temperature rise resulted in an Arrhenius break point temperature (the point where maximum heart rate can no longer exponential increase with temperature, potentially signalling optimal cardiac performance) of heart rate at ~21.5 $^{o}$C with development of arrhythmia beginning at ~26 $^{o}$C (Crespel *et al.*, 2019). This apparent consistency in temperature of peak performance in fish from genetically distinct sub-populations and with vastly different previous environmental experience offers support for the idea that fish species face concrete ceilings to physiological performance in the face of environmental change (Sandblom *et al.*, 2016).

Declining $O_2$ caused a decrease in MMR along a limiting oxygen curve, similar to that seen in previous research with sea bass (Lagardère *et al.*, 1998; Claireaux and Lagardère, 1999) and other fish species (Claireaux *et al.*, 2000; Mallekh and Lagardère, 2002; Lefrancois and Claireaux, 2003; Chabot and Claireaux, 2008). This is notable as it does not fit with the recent assumptions made by Seibel and Deutsch (2020) that MMR of fish should decrease linearly from maximum levels near 21 kPa $O_2$ down to $O_{2crit}$ values. Presumably this curved, rather than linear, response of MMR occurs because as $O_2$ declines fish utilise several compensatory mechanisms (e.g. increased ventilation, cardiac output, gill lamellar perfusion and surface area, and haematocrit) to maintain $O_2$ supply to tissues (Farrell and Richards, 2009). While these adjustments may limit reductions in MMR during mild to moderate hypoxia they may reach their performance limits as $O_2$ approaches critical levels and so result in a steeper decline in MMR. For example, in moderate hypoxia rainbow trout (*Oncorhynchus mykiss*, Walbaum) can increase cardiac output via increased stroke volume but in severe hypoxia cardiac output cannot be increased further as fish suffer from bradycardia (Sandblom and Axelsson, 2005).

Impacts of temperature and $O_2$ on metabolism of sea bass interacted synergistically so that impacts of hypoxia on MMR were greater at higher temperatures (Figure 3). Again this result concurs well with previous research (Claireaux and Lagardère, 1999). However, sea bass in our study displayed higher MMR at similar temperature and $O_2$ levels when compared to bass from Claireaux and Lagardère (1999). In addition, the $O_{2crit}$ (which can otherwise be defined as the point at which SMR = MMR) of our sea bass increased (i.e. hypoxia tolerance decreased) between 14 $^oC$ and 22 $^oC$ (Figure 2) whereas results from previous work suggested that hypoxia tolerance increases (i.e. $O_{2crit}$ declines) or remains constant across this temperature range (Claireaux and Lagardère, 1999). The reduced hypoxia tolerance of sea bass with temperature was primarily a result of its strong positive correlation with increased SMR (Figure 1). This relationship between SMR and $O_{2crit}$ has been shown for numerous fish species, and most recently in work with black sea bass (*Centropristus striata*, Linnaeus) (Slesinger *et al.*, 2019). However, our results also indicate that temperature had a secondary effect on $O_{2crit}$ which resulted in lower $O_{2crit}$ at higher temperatures for a given SMR. This suggests that temperature affects $O_{2crit}$ of sea bass via another mechanism (or mechanisms) independent of SMR. This is unlikely to be related to $O_2$ transport capacity of the blood as there were few consistent effects of temperature on Hct, Hb or $P_{50}$ of sea bass (Figure 5 and supplementary materials Figure S1 and S2). It has previously been observed that improved hypoxia tolerance after warm acclimation is correlated to increased gill lamellar surface area (McBryan *et al.*, 2016) or changes in the structure of the heart (Anttila *et al.*, 2015). Recent research has shown that these cardiorespiratory responses can improve $O_2$ supply capacity (Gomez Isaza *et al.*, 2021). Combined, these results suggest that acclimation to higher temperatures can cause structural changes to the gills and the heart that improve performance in low $O_2$ conditions and, therefore, mitigate the negative effect of temperature induced increases in SMR on hypoxia tolerance.

The independent effect of rising $CO_2$ reduced MMR. Previous research has not shown consistent effects of $CO_2$ on MMR or SMR of fish species, with the majority showing no effects of $CO_2$ (Lefevre, 2016, 2019). Interestingly the most recent research with European sea bass found that long term exposure to

elevated $CO_2$ causes increased MMR. This may indicate that negative effects of short exposures can be overcome by acclimation over longer time periods (Crespel *et al.*, 2019). It is important to note that the effect of $CO_2$ on metabolic rate in our study was only identifiable by including the expected rise in $CO_2$ during decline in $O_2$. Without the additional data from these increased $CO_2$ exposures at lower $O_2$ levels the effect of $CO_2$ on MMR at normoxia was not identified as significant. Although the best model of SMR also included a negative effect of $CO_2$ this was predicted to be small with confidence intervals overlapping zero (Figure 1). Additionally, removal of $CO_2$ as an explanatory variable from the model of SMR did not greatly impair model fit ($\Delta$AICc < 2) which indicates that the effect of $CO_2$ on SMR was not critically important for overall model performance. As $CO_2$ increases in the environment when $O_2$ declines the negative effect of $CO_2$ has a greater impact on MMR at lower $O_2$ levels (Figure 3 C, D, and E). Decreased MMR when fish are exposed to acutely increased $CO_2$ is usually thought to be a result of an internal acidosis causing a decrease in Hb-$O_2$ binding affinity, and therefore reducing the capacity of $O_2$ transport in the blood (Heuer and Grosell, 2016a). However, blood sampling showed that sea bass in normoxia had fully compensated for the effects of increased environmental $CO_2$ on blood pH by compensatory accumulation of extra $HCO_3^-$, and that this pH regulation resulted in no changes in Hb-$O_2$ binding affinity (Figure 5 and supplementary materials Figure S1). Additionally, we have recently shown that sea bass regulate blood pH when exposed to concurrent progressive hypercapnia during progressive hypoxia over the course of several hours and have higher Hb-$O_2$ binding affinity in these conditions when compared to fish exposed to progressive hypoxia with no concurrent hypercapnia (Montgomery *et al.*, 2019). This increase in Hb-$O_2$ binding affinity after exposure to hypercapnia is thought to be a result of catecholamine release which stimulates RBCs to remove $H^+$ and increase intracellular pH (Hannan and Rummer, 2018). In addition, we did not see significant changes in Hb or Hct (Figure 7). As such, we conclude that the negative effect of increased $CO_2$ on MMR is unlikely to be related to changes in $O_2$ transport in the blood. Whilst beyond the scope of the present study we can speculate that instead $CO_2$ affects MMR via changes in mitochondrial metabolism (Strobel *et al.*, 2012, 2013b; Leo *et al.*, 2017), cardiac performance

(Perry and Abdallah, 2012; Crespel *et al.*, 2019) or shifts from aerobic to anaerobic metabolic pathways (Michaelidis *et al.*, 2007).

While the effect of $CO_2$ on MMR was part of the best supported model it is uncertain whether it would cause biologically relevant impacts. Previous research has linked declines in MMR caused by increased $CO_2$ with decreased swimming performance (Lefevre, 2019) but it is unknown if the relatively small changes in MMR shown in the present study would translate to changes in other aspects of whole animal performance. This is especially the case as predictions of aerobic scope from the combined best supported models of MMR and SMR essentially show no effect of $CO_2$ on aerobic scope at $O_2$ levels >10 kPa (Figure 4 C, D, and E). This occurs because predicted effects of $CO_2$ act in the same direction for both SMR and MMR. As changes in aerobic scope are normally said to predict environmental impacts on processes such as growth and reproduction (Clark *et al.*, 2013; Pörtner *et al.*, 2017) we would predict that climate change relevant $CO_2$ increases have negligible effects on these endpoints. However, the effect of $CO_2$ may have important consequences which are not reflected in changes in aerobic scope. In our most extreme treatment (22 $^{o}$C, ~30 % air saturation, high $CO_2$) we observed 30 % mortality of sea bass after exhaustive exercise. Fish exercised at the same temperature and $O_2$ levels in ambient $CO_2$ conditions showed no mortality (and no mortality was observed in any other treatment combinations) – consequently it appears the additional $CO_2$ increase during hypoxia in a future ocean scenario (which was approximately 1100 µatm higher than in the present ocean $CO_2$ scenario) may impair recovery from exercise when $O_2$ is limiting. Mortality in fish post-exercise has been theorised to result from intracellular acidosis caused by end products generated by anaerobic respiration (i.e. lactate and succinate) (Wood *et al.*, 1983). Therefore, the greater increase in $CO_2$ during hypoxia in the future ocean $CO_2$ scenario may either exacerbate the intracellular acidosis caused by anaerobic activity or impair the ability of fish to process anaerobic end products.

### 4.1. Evidence to support OCLTT?

The OCLTT hypothesis suggests that climate change will affect fish because combined effects of increasing $CO_2$ and reduced $O_2$ will synergistically interact to lower aerobic scope across its thermal performance curve (Pörtner and Peck, 2010), and that changes in aerobic scope can be used as a single metric for

predicting whole animal performance (Pörtner, 2012). There have been active discussions about whether this framework is accurate for all fish and how best to explore it (Pörtner, 2014; Pörtner *et al.*, 2017; Jutfelt *et al.*, 2018), but to date few studies have attempted to investigate whether combined effects of hypoxia and $CO_2$ on the thermal performance curve of aerobic scope in fish match predictions from the OCLTT. Our data provides some support for the OCLTT hypothesis as the observed synergistic relationship between increased temperature and reduced $O_2$ on aerobic scope would be expected to result in changes to the thermal performance curve as predicted by Pörtner and Farrell (2008). However, our highest temperature treatment did not result in decreased MMR or aerobic scope and so we cannot confirm whether interactive effects between temperature and hypoxia would continue to follow predictions of the OCLTT above optimum temperatures. In contrast to hypoxia, the effects of $CO_2$ did not follow predictions from the OCLTT as $CO_2$ did not interact with either temperature or hypoxia and had minimal impacts on aerobic scope.

The interactive effects of $O_2$ and temperature on MMR of sea bass closely resemble the metabolic niche framework which Ern (2019) proposed as an update to the OCLTT hypothesis. In particular the concept of aerobic scope isopleths (where aerobic scope remains constant across a range of either temperatures or $O_2$ levels as a result of changes in the second factor) is supported by our data, which show that aerobic scope of sea bass would be expected to remain constant across an 8 $^oC$ temperature range at an $O_2$ level of ~6 kPa (Figure 3 A and B). As such, we would support Ern's suggestion to experimentally assess how aerobic scope affects important processes, such as growth, independently of changes in temperature and $O_2$ by utilising these isopleths.

### 4.2. How might effects on physiological performance affect future sea bass populations?

Initially our results suggest that rising temperatures in the North-East Atlantic associated with climate change will physiologically benefit European sea bass as aerobic scope increased from 14 $^oC$ to 22 $^oC$. This result indicates that wild populations in this region do not show reductions in thermal optimum of aerobic scope when compared to populations in the West Mediterranean or fish produced from aquaculture. However, the synergistic interaction between

warming and reduced $O_2$ which decreased MMR, and therefore aerobic scope, indicates that sea bass will be more susceptible to hypoxic events in a warmer future environment. Although increased aerobic scope has long been linked to improved individual fitness there is a still a lack of evidence to suggest that responses of aerobic scope to environmental variables are reflected in changes in ecologically important processes. As such, assuming that the thermal optimum of aerobic scope indicates improved peak physiological performance may be erroneous.

Recently Deutsch *et al.* (2015) have suggested that changes in the environment will affect physiological suitability of a habitat via a metabolic index relating the ratio of $O_2$ supply to resting metabolic demand. The southern distribution limits of several species correspond to a metabolic index of ~2-5 (Deutsch *et al.*, 2015) and recent experimental work with black sea bass has revealed that the northward shift of the population along the east coast of USA can be linked to changes in the metabolic index of habitats (Slesinger *et al.*, 2019). Applying the principle of the metabolic index to our data suggests that temperatures of 22 $^{o}$C approach levels which are associated with upper temperature limits of population distributions (metabolic index at 22 $^{o}$C and 20 kPa $O_2$ was ~5). This indicates that further environmental disturbance such as relatively moderate hypoxia or periodic marine heatwaves could cause reductions in the metabolic index to values of ~2. As a result, sea bass populations in the Atlantic may temporarily disperse from regions where seasonal temperatures are >22 °C.

## 5. Conclusions

In summary, our research shows that aerobic scope of European sea bass will increase with expected warming in the North-East Atlantic, and that even extreme summer temperatures (~22 $^{o}$C) at the end of the century will positively impact on the aerobic performance of sea bass. However, synergistic interactions between warming and reduced $O_2$ indicate that hypoxic conditions will have greater impacts on sea bass in future oceans. Increased $CO_2$ levels showed no interactions with either temperature or $O_2$ changes but were predicted to cause a small decline in MMR – although this had little impact on aerobic scope because increased $CO_2$ caused a trend for decreased SMR. Sea

bass fully compensated blood pH for increased $CO_2$ levels and increases in SMR and reductions in MMR with temperature were not linked to changes in $O_2$ transport in the blood. Despite end of century $CO_2$ levels having minimal effects on aerobic scope, they did cause increased mortality of fish recovering from exercise in the more extreme hypoxic scenario (~30 % air saturation) at 22 $^oC$. Thus, environmentally relevant changes in $CO_2$ during hypoxia may lead to important threshold effects that would not be observed if experiments only consider changes in $CO_2$ related to atmospheric concentrations. Mortality was not observed in any other treatments. Interactive effects of temperature and $O_2$ support predictions from the oxygen-and temperature-limited metabolic niche framework proposed as an update to the OCLTT hypothesis by Ern (2019) however the effect of $CO_2$ did not support predictions of the OCLTT (Pörtner and Farrell, 2008). Changes in the metabolic index proposed as a physiological constraint by Deutsch *et al.* (2015) suggest that despite increases in MMR and aerobic scope future climate change may result in conditions which will begin to constrain growth and reproduction of sea bass in areas where temperatures increase above 22 $^oC$. However, there is a vital need for increased research to link changes in aerobic performance measures such as aerobic scope to population relevant metrics such as growth and reproduction to better assess what environmental impacts on aerobic performance may mean for wider populations.

# 6. Supplementary materials

## 6.1. Methods

### 6.1.1. Treatment conditions

Sea bass were always transferred into the experimental RAS at a temperature of 18 $^{o}$C (to match the temperature in the stock system). For temperature treatments of 14 and 22 $^{o}$C the experimental RAS was then cooled or warmed at a rate of ~2 $^{o}$C per day to reach the desired treatment. A header tank (~500 L) was used to adjust $CO_2$ to the desired level prior to entering the fish holding tank (~500 L). For ambient $CO_2$ treatments (~400 µatm) air was passed through a soda lime ($CaHNaO_2$) scrubber to reduce $CO_2$ levels before aerating the header tank and off-gassing increased $CO_2$ (produced by the biological respiration in the RAS) to near ambient levels. For end of century $CO_2$ treatments (~1000 µatm) an Aqua Medic pH computer was used to adjust RAS water to an appropriate pH (7.8). Each pH computer controlled an electronic solenoid valve, which was opened to deliver $CO_2$ to a diffuser in the header tank when pH levels rose above 7.82 and closed when pH levels dropped below 7.78. Additionally, the treatment tank was aerated with a gas mix of 99.94 % air and 0.06 % $CO_2$ (i.e. total mix = 0.1% $CO_2$) using mass flow controllers (GFC mass flow controller, Aalborg, USA) at a rate of 10 L $min^{-1}$ to reduce fluctuations in $CO_2$ levels that sea bass experienced.

### 6.1.2. Respirometer system set-up

Respirometry was conducted in a semi-closed system consisting of three 100 L experimental tanks fed by a 100 L sump, with overflowing water from the experimental tanks recirculating back to the sump. The sump was temperature controlled using a heater/chiller unit (Grant TX150 R2, Grant Instruments, Cambridge, UK) attached to a temperature exchange coil to maintain respirometer temperatures equal to treatment temperatures. For end of century $CO_2$ treatments an Aqua Medic pH computer was used to adjust sump pH to match the pH of the treatment (~pH 7.8). In addition, the experimental tanks were aerated either using ambient air (~400 µatm $CO_2$) or a gas mix of 99.4 % air and 0.06 % $CO_2$ (i.e. total mix 0.1 % $CO_2$) to maintain normoxic $CO_2$ levels in the respirometer system equivalent to treatment levels. Together these tanks

formed a 400 L system with the same temperature, $O_2$ and water chemistry parameters as treatment conditions for all respirometers.

### 6.1.3. $O_{2crit}$ trials

During $O_{2crit}$ trials $O_2$ was regulated by gassing the sump and experimental tanks with a mix of $N_2$, $O_2$ and $CO_2$ (G400 Gas mixing system, Qubit Biology Inc.) at a rate of 10 L $min^{-1}$ following a pre-set automated protocol (Flowvision, Alicat software). Predicted increases in $CO_2$ during hypoxia were calculated for each treatment temperature as well as the starting $CO_2$ level at normoxia (i.e. ~400 µatm and ~1000 µatm $CO_2$). Carbon dioxide levels were then increased in the respirometer system by increasing the level of $CO_2$ included in the gas mix feeding the system (Montgomery *et al.*, 2019).

**Table S1:** Measurements of $pCO_2$ (µatm, top row) and pH (NBS scale, bottom row) as $O_2$ declined during $O_{2crit}$ trials for each treatment. All values are mean ± S.D. apart from for Treatment 3 at ~30 % dissolved $O_2$ where only one measurement of $pCO_2$ and pH was made and calculations of mean and S.D. were not possible.

| | **Treatment** | | | | | |
|---|---|---|---|---|---|---|
| **~Dissolved $O_2$ (% air saturation)** | 14 ºC Ambient $CO_2$ | 18 ºC Ambient $CO_2$ | 22 ºC Ambient $CO_2$ | 14 ºC Future $CO_2$ | 18 ºC Future $CO_2$ | 22 ºC Future $CO_2$ |
| 100 | 377 ± 23 | 483 ± 20 | 455 ± 35 | 1019 ± 50 | 1070 ± 10 | 1172 ± 89 |
| | 8.14 ± 0.02 | 8.09 ± 0.02 | 8.10 ± 0.04 | 7.79 ± 0.01 | 7.78 ± 0.00 | 7.80 ± 0.03 |
| 80 | 418 ± 9 | 508 ± 10 | 486 ± 47 | 1149 ± 2 | 1156 ± 86 | 1209 ± 18 |
| | 8.10 ± 0.01 | 8.07 ± 0.01 | 8.07 ± 0.04 | 7.74 ± 0.01 | 7.75 ± 0.02 | 7.79 ± 0.02 |
| 60 | 477 ± 25 | 613 ± 90 | 497 ± 23 | 1383 ± 125 | 1315 ± 73 | 1335 ± 92 |
| | 8.05 ± 0.02 | 7.99 ± 0.01 | 8.07 ± 0.02 | 7.67 ± 0.02 | 7.69 ± 0.02 | 7.75 ± 0.03 |
| 40 | 625 ± 44 | 746 ± 40 | 577 ± 46 | 1637 ± 83 | 1488 ± 24 | 1658 ± 237 |
| | 7.94 ± 0.03 | 7.92 ± 0.02 | 8.01 ± 0.04 | 7.59 ± 0.01 | 7.64 ± 0.00 | 7.66 ± 0.05 |
| 30 | 822 | 1104 ± 155 | 647 ± 18 | 1973 ± 159 | 1964 ± 134 | 2179 ± 63 |
| | 7.83 | 7.76 ± 0.06 | 7.96 ± 0.01 | 7.51 ± 0.04 | 7.53 ± 0.02 | 7.55 ± 0.01 |
| 20 | 1162 ± 2 | 2103 ± 79 | 808 ± 35 | 2491 ± 238 | 2416 ± 265 | 2547 ± 266 |
| | 7.69 ± 0.00 | 7.50 ± 0.02 | 7.88 ± 0.02 | 7.42 ± 0.05 | 7.45 ± 0.03 | 7.48 ± 0.06 |
| 15 | 1734 ± 92 | 2431 ± 663 | 1318 ± 108 | 3099 ± 448 | 2579 ± 4 | 3380 ± 354 |
| | 7.53 ± 0.02 | 7.45 ± 0.11 | 7.68 ± 0.04 | 7.32 ± 0.07 | 7.41 ± 0.00 | 7.36 ± 0.06 |

### 6.1.4. MMR measurements

After fish had rested overnight in isolation tanks, maximum metabolic rate (MMR) was of fish was measured on 3 consecutive days (with overnight recovery in between) at three different levels of $O_2$ (100, 60 and 30 % air

saturation) with increasing $CO_2$ levels for each $O_2$ level as detailed for $O_{2crit}$ trials.The appropriate $O_2$ and $CO_2$ level was achieved by gassing isolation boxes with a mix of $N_2$, $O_2$ and $CO_2$ (G400 Gas mixing system, Qubit Biology Inc.) at a rate of 5 L $min^{-1}$. New $O_2$ and $CO_2$ levels took ~ 1 hour to stabilise and fish were exposed to these levels for a further hour before being transferred to a circular chase tank (60 cm diameter with ~15 cm water depth) with the appropriate temperature, $O_2$, and $CO_2$ levels for the treatment. We then chased sea bass using an exhaustive chase protocol based on guidelines set out by Norin and Clark (2016), followed immediately by transfer to a respirometer chamber (with temperature, $O_2$, and $CO_2$ levels appropriate to the treatment) and measurement of $\dot{M}O_2$ (average time from end of chase to beginning of $\dot{M}O_2$ measurement was ~ 1 minute). Measurements of $\dot{M}O_2$ were conducted for a minimum of 2 hours before fish were returned to normoxic $O_2$ and $CO_2$ treatment levels (if not already at normoxia) for 1 hour in the respirometers before then being transferred back to the flow-through isolation chambers. Following completion of $\dot{M}O_2$ measurements, background respiration in each respirometer chamber was measured for a minimum of one hour. Measurements of water pH, temperature, and salinity, as well as samples of water for DIC measurement, were taken for each isolation tank, the chase tank (after all fish were chased) and the respirometer system (during $\dot{M}O_2$ measurements). These parameters were then used to calculate water carbonate chemistry conditions. Temperature, $O_2$, and $pCO_2$ levels fish were exposed to in isolation boxes pre-chase, during the chase protocol, and during recovery from exercise in the respirometer chamber are given in Table S2.

**Table S2:** Temperature (ºC), $pCO_2$ (µatm), and $O_2$ (kPa) conditions sea bass were exposed to pre-chase, during chase, and when recovering from exercise during measurements of MMR. All values are presented as mean ± S.E. - indicates measurements for which data was not collected. For treatment 1 missing pre-chase values would be expected to be extremely similar to values recorded during chase as shown by values for all other treatments.

| | | Treatment | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 14 ºC Ambient $CO_2$ | | | 18 ºC Ambient $CO_2$ | | | 22 ºC Ambient $CO_2$ | | | 14 ºC Future $CO_2$ | | | 18 ºC Future $CO_2$ | | | 22 ºC Future $CO_2$ | | |
| | | Pre-chase | During chase | Recovery | Pre-chase | During chase | Recovery | Pre-chase | During chase | Recovery | Pre-chase | During chase | Recovery | Pre-chase | During chase | Recovery | Pre-chase | During chase | Recovery |
| ~100 % air saturation | Temp (ºC) | 13.9 ± 0.1 | 13.9 ± 0.1 | 13.9 ± 0.1 | - | 18.3 ± 0.5 | 18.3 ± 0.5 | 22.0 ± 0.0 | 21.8 ± 0.1 | 22.0 ± 0.0 | 13.9 ± 0.0 | 14.2 ± 0.5 | 13.9 ± 0.1 | 18.0 ± 0.1 | 18.5 ± 0.0 | 18.0 ± 0.2 | 21.6 ± 0.1 | 22.3 ± 1.1 | 21.9 ± 0.1 |
| | $pCO_2$ (µatm) | - | - | 364 ± 12 | - | 522 ± 105 | 522 ± 105 | - | - | 404 ± 0 | 1056 ± 27 | 1148 ± 171 | 1040 ± 4 | 1085 ± 57 | 1129 ± 44 | 1195 ± 44 | 1092 ± 27 | 1168 ± 177 | 1100 ± 52 |
| | $O_2$ (kPa) | - | - | 19.6 ± 0.1 | 20.8 | 20.7 | 19.3 ± 0.2 | - | - | 18.8 ± 0.2 | 20.0 ± 0.3 | 20.7 ± 0.2 | 19.2 ± 0.6 | 20.8 ± 0.4 | 20.4 ± 0.6 | 19.1 ± 0.3 | 20.5 ± 0.2 | 20.3 ± 0.1 | 19.2 ± 0.9 |
| ~60 % air saturation | Temp (ºC) | 13.3 ± 0.1 | 13.6 ± 0.1 | 13.9 ± 0.1 | - | 17.7 ± 0.1 | 18.7 ± 0.1 | 20.5 ± 0.1 | 21.4 ± 0.2 | 22.0 ± 0.0 | 13.7 ± 0.2 | 13.8 ± 0.4 | 14.0 ± 0.2 | 17.5 ± 0.2 | 17.7 ± 0.2 | 18.0 ± 0.1 | 21.2 ± 0.4 | 21.2 ± 0.3 | 21.9 ± 0.1 |
| | $pCO_2$ (µatm) | 542 ± 67 | - | 501 ± 69 | - | 619 ± 98 | 674 ± 37 | 554 ± 25 | - | 579 ± 93 | 1533 ± 62 | 1577 ± 67 | 1528 ± 44 | 1457 ± 137 | 1486 ± 168 | 1540 ± 38 | 1468 ± 67 | 1436 ± 58 | 1468 ± 60 |
| | $O_2$ (kPa) | 13.9 ± 0.3 | 14.5 ± 0.2 | 12.0 ± 0.1 | 14.3 ± 0.3 | 14.3 ± 0.6 | 11.8 ± 1.0 | 14.3 ± 0.2 | 14.8 ± 0.0 | 11.6 ± 0.2 | 14.5 ± 0.2 | 14.4 ± 0.0 | 12.0 ± 0.1 | 14.4 ± 0.3 | 14.4 ± 0.2 | 11.9 ± 0.2 | 14.5 ± 0.2 | 14.5 ± 0.1 | 11.5 ± 0.1 |
| ~30 % air saturation | Temp (ºC) | 13.1 ± 0.2 | 13.4 ± 0.0 | 13.8 ± 0.1 | - | 17.6 ± 0.5 | 18.7 ± 0.1 | 20.5 ± 0.1 | 21.4 ± 0.1 | 22.0 ± 0.0 | 12.9 ± 0.2 | 13.1 ± 0.6 | 13.9 ± 0.1 | 17.6 ± 0.0 | 17.6 ± 0.1 | 18.3 ± 0.2 | 20.9 ± 0.1 | 20.9 ± 0.1 | 21.9 ± 0.1 |
| | $pCO_2$ (µatm) | 1173 ± 95 | - | 839 ± 53 | - | 854 ± 40 | 793 ± 118 | 929 ± 85 | - | 898 ± 101 | 2575 ± 243 | 2467 ± 110 | 2219 ± 216 | 2213 ± 135 | 2278 ± 179 | 2123 ± 272 | 2400 ± 126 | 2399 ± 34 | 2027 ± 100 |
| | $O_2$ (kPa) | 8.3 ± 0.3 | 8.8 ± 0.0 | 6.1 ± 0.1 | 8.4 ± 0.5 | 8.6 ± 0.3 | 6 .8 ± 0.8 | 8.5 ± 0.3 | 8.8 ± 0.2 | 6.6 ± 0.2 | 9.1 ± 0.3 | 8.9 ± 0.0 | 6.7 ± 0.1 | 8 .8 ± 0.4 | 9.1 ± 0.1 | 6.4 ± 0.3 | 8.7 ± 0.5 | 8.7 ± 0.3 | 6. 4 ± 0.5 |

### 6.1.5. $\dot{M}O_2$ analysis and SMR, $O_{2crit}$, and MMR calculation

Following each measurement period $\dot{M}O_2$ was automatically calculated by the AquaResp3 software. A linear regression was fitted to the $O_2$ versus time data for each measurement period. The slope (s) of this regression (kPa $O_2$ $h^{-1}$) was then used to calculate $\dot{M}O_2$ (mg $O_2$ $kg^{-1}$ $h^{-1}$) using the equation outlined by Svendsen *et al.*(2016):

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

where $V_{resp}$ is the respirometer volume minus the volume of the fish (L), α is the solubility of $O_2$ in water ($mgO_2$ $L^{-1}$ $kPa^{-1}$) for the relevant salinity and temperature, and m is the mass of the fish (kg). Calculations of $\dot{M}O_2$ where s had a $R^2$ of < 0.98 were removed from subsequent analysis. For the purpose of establishing the impacts of reduced $O_2$ on MMR and determining $O_{2crit}$ values, the $O_2$ level of each measurement period was defined as the mean dissolved $O_2$ measurement over the measurement period. The mean background respiration for each respirometer over the 1 hour post-trial measuring period (average was < 2 % of fish $\dot{M}O_2$) was subtracted from $\dot{M}O_2$ measurements. We decided to control for effects of body mass on metabolic rate by scaling background corrected $\dot{M}O_2$ measurements to an average individual mass of 120 g (this corresponded closely with the mean mass, 120.1 g, of all fish used for $\dot{M}O_2$ measurements) using the following equation:

$$\dot{M}O_2\,(120) = \dot{M}O_2 \times \left(\frac{M}{120}\right)^{(1-A)}$$

where $\dot{M}O_2$ (120) is the predicted oxygen consumption ($mgO_2$ $kg^{-1}$ $h^{-1}$) of an individual scaled to a body mass of 120 g, $\dot{M}O_2$ is the measured oxygen consumption of a sea bass with mass of M, and A is the mass exponent describing the relationship between metabolic rate and body mass. We chose to use a mass exponent of 0.89 because it best describes the most recent analysis of the relationship between standard metabolic rate (SMR) and mass in fish (Jerde *et al.*, 2019).

We then calculated the SMR of each fish in R v.3.6.3 (R Core Team, 2020) using function 'calcSMR' in package 'fishMO2' (Chabot *et al.*, 2016). All $\dot{M}O_2$ values from the SMR measurement period and the beginning of the $O_{2crit}$

trial in which mean dissolved $O_2$ saturation was >80 % air saturation were included for SMR calculations. We estimated SMR for all fish as the mean of the lowest 10 $\dot{M}O_2$ measurements included in SMR calculations. We chose to use this method to estimate SMR as it more accurately matched the consistent low $\dot{M}O_2$ of oxy-regulating fish at values of $pO_2$ above the $O_{2crit}$ point than estimates using the methods which are recommended by Chabot *et al.* (2016). A minimum of 3 hours of measurements were used to determine SMR for all fish. Despite the short time period over which $\dot{M}O_2$ measurements were made to determine SMR we believe the values we calculated are representative for each fish. We believe this because estimates of SMR closely matched the consistent low $\dot{M}O_2$ measurements of oxy-regulating fish at $O_2$ levels between 80 % saturation and the $O_{2crit}$ point. Also, using a similar 3 hour time period of $\dot{M}O_2$ measurements did not significantly change determinations of SMR when compared to measurements taken over a 14-16 hour period in previous work with this respirometer system and the same stock of sea bass (SMR from 3 hours of $\dot{M}O_2$ measurements = 114.3 ± 6.2 $mgO_2\ kg^{-1}\ h^{-1}$, SMR from 14-16 hours of measurements = 109.6 ± 5.7 $mgO_2\ kg^{-1}\ h^{-1}$, n = 16).

The critical $O_2$ tension ($O_{2crit}$) of each individual fish was calculated using $\dot{M}O_2$ measurements from $O_{2crit}$ trials with function ‘calcO2crit’ from package ‘fishMO2’, using the estimated SMR of each individual, as detailed in the supplementary material of Claireaux & Chabot (2016). This function identifies the portion of the $O_{2crit}$ test where metabolic rate data follows an $O_2$ conforming relationship and fits a linear regression line through this data, $O_{2crit}$ was then calculated as the $O_2$ level at which this regression line crosses the calculated SMR of the individual fish. Calculations were conducted using a gap limit of 0.83 kPa $O_2$ (~4 % air saturation) and a maximum number of 7 $\dot{M}O_2$ points to fit the regression line through the $O_2$-conforming component of the data used to estimate $O_{2crit}$.

Finally, we defined MMR as the single highest measurement of $\dot{M}O_2$ in the one hour period immediately following exercise to exhaustion (Norin and Clark, 2016). This point usually occurred during the first measurement period immediately after each fish was moved to the respirometer chamber i.e. ~ 2-5 minutes after the cessation of the chase protocol. However, for some fish in normoxia spontaneous activity inside the respirometer chamber during SMR or $O_{2crit}$ trials resulted in instantaneous measurements of $\dot{M}O_2$ higher than those

noted following chase protocols. In these occasions this higher value of $\dot{M}O_2$ was used as the estimated MMR for that fish (n = 2 out of 65 fish).

### 6.1.6. Blood chemistry and Hb-$O_2$ affinity measurement

We anaesthetized each fish in-situ using a dose of 100 $mgL^{-1}$ of benzocaine (temperature and $pCO_2$ conditions given in Table S3). Once fish were sufficiently anaesthetized (no longer responded to a pinch of the anal fin) they were transferred within 5 seconds to a gill irrigation table (at the same temperature and $pCO_2$ level of the respective treatment, and containing 37.5 mg $L^{-1}$ of benzocaine). Gill ventilation was artificially maintained by a micro-pump, so that the operculum of the fish were just open and exhalant water flow could be visualised. Once a stable gill water flow was established blood was sampled by caudal vessel puncture using a 1 mL heparinised syringe and 23G needle. This method has been demonstrated to obtain accurate measurements of blood gas and acid-base variables comparable to those achieved using cannulation (Davison & Wilson, University of Exeter, personal communication).

**Table S3:** Temperature and $pCO_2$ levels fish were exposed to when they were anaesthetised immediately prior to blood sampling. All measures are given as mean ± S.D.

| **Parameter** | **Treatment** | | | | | |
|---|---|---|---|---|---|---|
| | 14 °C Ambient $CO_2$ | 18 °C Ambient $CO_2$ | 22 °C Ambient $CO_2$ | 14 °C Future $CO_2$ | 18 °C Future $CO_2$ | 22 °C Future $CO_2$ |
| Temperature (°C) | 13.1 ± 0.1 | 17.4 ± 0.1 | 21.2 ± 0.3 | 13.3 ± 0.3 | 17. 8 ± 0.1 | 21.3 ± 0.2 |
| $pCO_2$ (µatm) | 350 ± 13 | 445 ± 52 | 398 ± 28 | 1130 ± 33 | 1103 ± 47 | 1221 ± 35 |

For each individual fish the gas mix that blood was exposed to by the BOBS matched the *in vivo* temperature and calculated $pCO_2$ of the blood sample. The change in absorption of light at a wavelength of 435 nm was used to assess changes in oxygenation of Hb, as previously used by Verhille & Farrell (2012). Background changes in absorption of the blood sample were corrected using the isosbestic wavelength of 390 nm (Verhille and Farrell, 2012). Following measurements the BOBS calculated the $O_2$ equilibrium curve of the sample using Hill's formula. This was then used to estimate affinity of Hb for $O_2$ as the partial pressure to achieve 50 % saturation, or $P_{50}$ (Oellermann *et al.*, 2014), as well as the Hills' number (which indicates the cooperativity of ligand binding).

### 6.1.7. Statistical analysis of SMR, $O_{2crit}$, and MMR

Comparisons of AICc between models were conducted using the MuMin package (Table S4; Barton, 2020). Where AICc is reduced by >2 it indicates that the model with the lower AICc is much more likely to be supported given the data available (Burnham, 2002; Burnham and Anderson, 2002), by comparing models with different combinations of explanatory variables we can infer which variables are important for explaining variation in physiological metrics.

**Table S4:** Structure of models and model comparison results explaining variation in SMR, $O_{2crit}$, and MMR of sea bass exposed to differing combinations of temperature, $O_2$, and $CO_2$. The best supported model for each response variable is highlighted in bold.

| Response Variable | Explanatory Variables Structure | AICc | ΔAICc | d.f. |
|---|---|---|---|---|
| SMR | **Temperature + $CO_2$** | **546.3** | **0** | **5** |
| | Temperature | 548.2 | 1.9 | 4 |
| | Temperature*$CO_2$ | 548.7 | 2.4 | 6 |
| | $CO_2$ | 578.4 | 32.1 | 4 |
| $O_{2crit}$ | **Temperature + $CO_2$ + SMR** | **72.7** | **0** | **6** |
| | Temperature * SMR + $CO_2$ | 75.2 | 2.5 | 7 |
| | Temperature * $CO_2$ + SMR | 75.2 | 2.5 | 7 |
| | Temperature + $CO_2$ * SMR | 75.3 | 2.6 | 7 |
| | Temperature + SMR | 76.2 | 3.5 | 5 |
| | Temperature*SMR | 78.6 | 5.9 | 6 |
| | Temperature * $CO_2$ * SMR | 83.0 | 10.3 | 10 |
| | SMR | 84.0 | 11.3 | 4 |
| | SMR + $CO_2$ | 84.8 | 12.1 | 5 |
| | SMR*$CO_2$ | 87.2 | 14.5 | 6 |
| | Temperature | 134.2 | 61.5 | 4 |
| | Temperature + $CO_2$ | 136.4 | 63.7 | 5 |
| | Temperature*$CO_2$ | 138.8 | 66.1 | 6 |
| | $CO_2$ | 144.0 | 71.3 | 4 |
| MMR | **$CO_2$ + Temperature*$O_2$ + Temperature*$(O_2)^2$** | **2240.8** | **0** | **12** |
| | $CO_2$*Temperature*$O_2$ + Temperature*$(O_2)^2$ | 2243.0 | 2.2 | 15 |
| | $CO_2$ + Temperature*$O_2$ + $(O_2)^2$ | 2247.5 | 6.7 | 11 |
| | $CO_2$*Temperature*$O_2$ + $(O_2)^2$ | 2248.8 | 8.0 | 14 |
| | Temperature + $CO_2$*$O_2$ + $CO_2$*$(O_2)^2$ | 2280.1 | 39.3 | 12 |
| | $CO_2$*Temperature*$O_2$ | 2349.8 | 109.0 | 13 |
| | Temperature + $CO_2$*$O_2$ | 2360.7 | 119.9 | 10 |
| | $CO_2$ + Temperature*$O_2$ | 2362.8 | 122.0 | 10 |
| | $CO_2$*$O_2$ | 2365.1 | 124.3 | 9 |
| | Temperature*$CO_2$ + $O_2$ | 2371.4 | 130.6 | 10 |
| | Temperature + $CO_2$ + $O_2$ | 2379.7 | 138.9 | 9 |
| | Temperature + $CO_2$ | 2464.3 | 223.5 | 8 |
| | Temperature + $O_2$ | 2464.7 | 223.9 | 8 |
| | Temperature *$CO_2$ | 2465.4 | 224.6 | 9 |
| | Temperature*$O_2$ | 2467.0 | 226.2 | 9 |
| | $CO_2$ | 2478.3 | 237.5 | 7 |
| | $O_2$ | 2488.8 | 248.0 | 7 |
| | Temperature | 2638.1 | 397.3 | 7 |

Model summaries of each of the best supported models used to analyse SMR, $O_{2crit}$ and MMR are given in Table S5, S6, and S7 respectively.

**Table S5**: General linear mixed-effects model outputs for analysis of standard metabolic rate. The best supported model was fitted using a Gaussian distribution and included the parameters temperature and $CO_2$ as explanatory variables and group ID as a random intercept term. Parameter effects are compared against a reference level where temperature and $CO_2$ are 0. Marginal $R^2$ = 0.70, Condition $R^2$ = 0.70.Confidence intervals for each parameter were determined from function confint in package lme4. Marginal and conditional $R^2$ of the model were determined using function r.squaredGLMM from package MuMIn.

| Parameter | Estimate | Standard Error | Confidence Interval (95%) | t-value | Variance | Standard Deviation |
|---|---|---|---|---|---|---|
| Best supported model <- lmer(SMR ~Temperature + $CO_2$ + (1\|Group) | | | | | | |
| Intercept | -14.77 | 11.62 | -37.35 – 7.81 | -1.27 | | |
| Temperature | 6.66 | 0.57 | 5.55 – 7.78 | 11.61 | | |
| $CO_2$ | -0.012 | 0.006 | -0.024 – -0.001 | -2.04 | | |
| Group | | | | | 0.0 | 0.0 |

**Table S6:** General linear mixed-effects model outputs for analysis of $O_{2crit}$. The best supported model was fitted using a Gaussian distribution and included the parameters temperature,$CO_2$, and SMR as explanatory variables and group ID as a random intercept term. Parameter effects are compared against a reference level where temperature, $CO_2$, and SMR are 0. Marginal $R^2$ = 0.72, Condition $R^2$ = 0.77. Confidence intervals for each parameter were determined from function confint in package lme4. Marginal and conditional $R^2$ of the model were determined using function r.squaredGLMM from package MuMIn.

| Parameter | Estimate | Standard Error | Confidence Interval (95%) | t-value | Variance | Standard Deviation |
|---|---|---|---|---|---|---|
| Best supported model < - lmer(O2crit~ Temperature+$CO_2$+SMR) + (1\|Group) | | | | | | |
| Intercept | 2.174 | 0.421 | 1.401 – 2.945 | 5.16 | | |
| Temperature | -0.126 | 0.031 | -0.185 – -0.068 | -4.00 | | |
| $CO_2$ | 0.0005 | 0.0002 | 0.0001 – 0.0009 | 10.75 | | |
| SMR | 0.036 | 0.003 | 0.030 – 0.043 | 2.37 | | |
| Group | | | | | 0.03 | 0.18 |

**Table S7**: General linear mixed model outputs for analysis of maximum metabolic rate. The best supported model was fitted using a Gaussian distribution and included the parameters temperature and $CO_2$ as explanatory variables and group ID as a random intercept term. Parameter effects are compared against a reference level where temperature and $CO_2$ are 0. Marginal $R^2$ = 0.91, Condition $R^2$ = 0.96.Confidence intervals for each parameter were determined from function confint in package lme4. Marginal and conditional $R^2$ of the model were determined using function r.squaredGLMM from package MuMIn.

| Parameter | Estimate | Standard Error | Confidence Interval (95%) | t-value | Variance | Standard Deviation |
|---|---|---|---|---|---|---|
| Best supported model < - lmer(MMR ~ scale(Temperature)*scale($O_2$) + scale(Temperature)*scale($O_2^2$) + scale($CO_2$) + ($O_2$\|Fish ID) + (1\|Group) | | | | | | |
| Intercept | 231.99 | 2.58 | 227.01 – 236.98 | 89.96 | | |
| scale(Temperature) | 32.53 | 2.55 | 27.60 – 37.46 | 12.76 | | |
| scale($O_2$) | 240.35 | 9.50 | 221.92 – 258.74 | 25.31 | | |
| scale(Temperature*$O_2$) | 41.95 | 8.60 | 25.26 – 58.62 | 4.88 | | |
| scale($O_2^2$) | -157.69 | 8.68 | -174.50 – -140.85 | -18.17 | | |
| scale(Temperature*$O_2^2$) | -25.10 | 8.36 | -41.31 – -8.86 | -3.00 | | |
| scale($CO_2$) | -7.52 | 2.58 | -12.08 – -2.97 | -3.20 | | |
| Fish ID (Intercept) | | | | | 90.02 | 9.49 |
| Fish ID ($O_2$) | | | | | 6.53 | 2.56 |
| Group | | | | | $4.78 \times 10^{-6}$ | 0.00 |

## 6.2. Results

### 6.2.1. $O_2$ transport capacity

Our measurements of haemoglobin-$O_2$ binding affinity ($P_{50}$) (Kruskal-Wallis, $\chi^2$ = 6.503, df = 4, p = 0.165) and the Hills' number (One-way ANOVA, F = 0.878, df = 4, p = 0.487) were not significantly different between any treatments (Figure S1). Due to an equipment failure, measurements of $P_{50}$ were not completed for sea bass exposed to ~1000 µatm $CO_2$ at 14 $^oC$ during the original experimental period. However, this was measured at a later date and no significant differences between $P_{50}$ or Hills' number were noted for sea bass exposed to ~1000 µatm or ambient levels of $CO_2$ at 14 $^oC$.

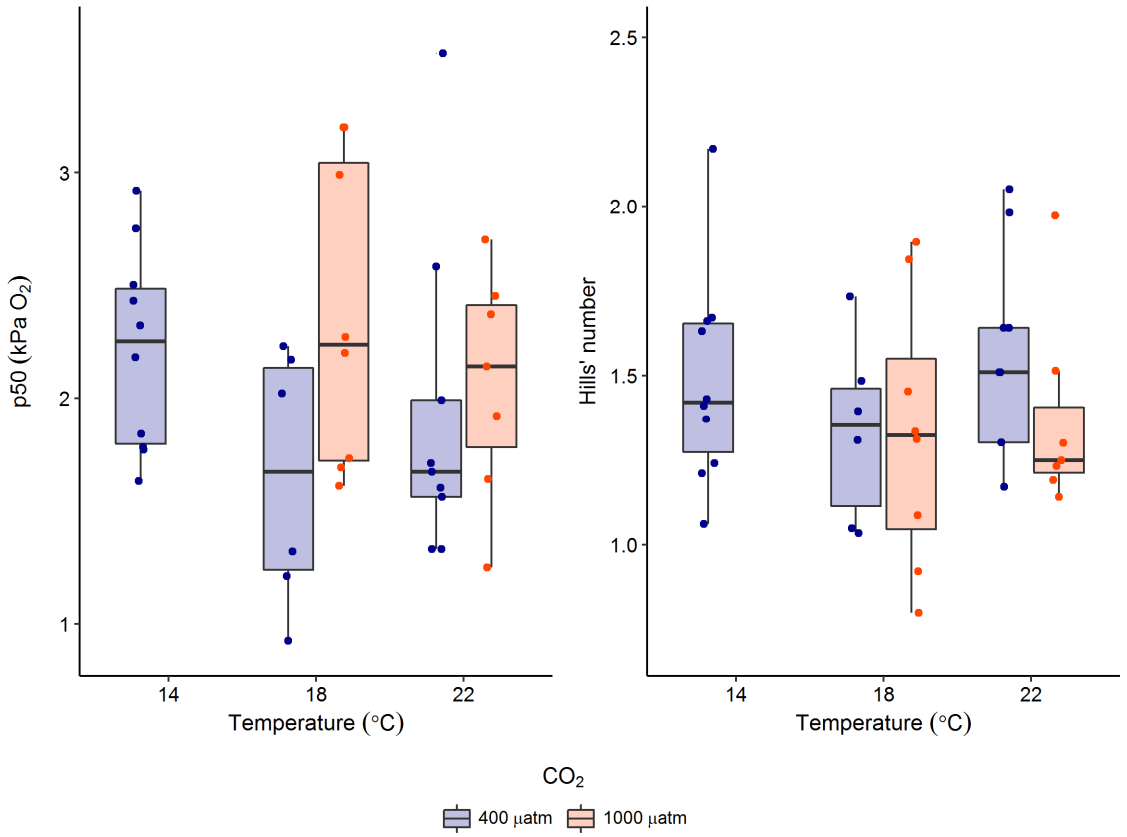


**Figure S1:** No impacts of temperature and $CO_2$ were observed for measurements of haemoglobin-$O_2$ binding affinity (measured using $P_{50}$) and Hills' number. Due to an equipment failure no measurements were possible during the original experimental period for fish at 14 ºC exposed to ~1000 µatm $CO_2$.

Haematocrit was not affected by either temperature (Two-way ANOVA, F = 0.832, df = 2, p = 0.442) or $CO_2$ (F = 2.945, df = 1, p = 0.093) and no interactive effect between these factors was evident (F = 0.069, df = 2, p = 0.934). Haemoglobin levels were affected by temperature (Two-way ANOVA, F = 7.094, df = 2, p = 0.002) with fish samples at 22 ºC having Hb levels 0.20 and 0.24 mM higher than fish sampled at 18 and 14 ºC respectively. Haemoglobin levels were not affected by $CO_2$ (F = 0.690, df = 1, p = 0.411) and there was no interactive effect (F = 3.067, df = 2, p = 0.057) (Figure S2).

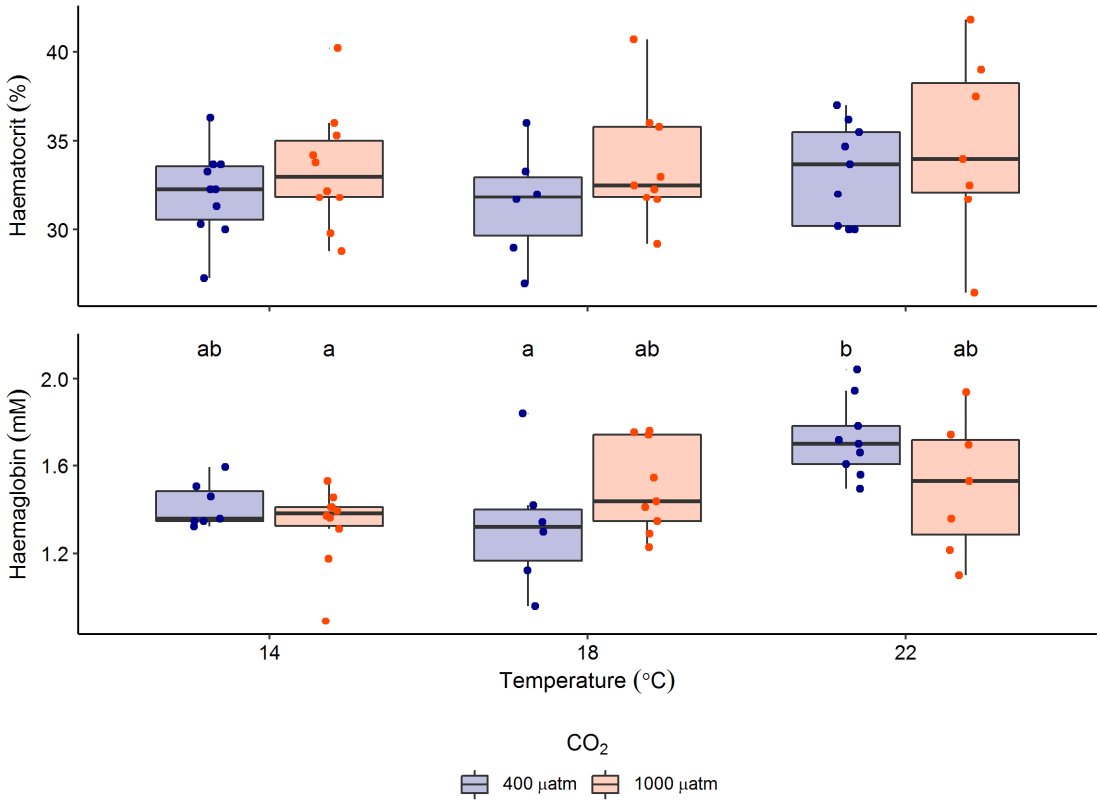


**Figure S2**: Impact of temperature and $CO_2$ on haematological parameters of sea bass. No significant difference in haematocrit were observed between any treatments. Significant difference in haemoglobin content were noted between fish sampled at different temperature and $CO_2$ treatments and are represented by different lower case letters.

### 6.2.2. Blood lactate and glucose

There were no significant differences in lactate levels across all treatments (Kruskall-Wallis, $\chi^2$ = 6.40, df = 5, p = 0.269), with mean lactate for all fish of 0.42 ± 0.06 mM (± S.E.). Glucose levels (mean for all fish of 4.30 ± 0.14 mM, ± S.E.) were not significantly affected by temperature (Two-way ANOVA, F = 0.864, df = 2, p = 0.429), $CO_2$ (F = 0.0138, df = 1, p = 0.907) or the interaction between temperature and $CO_2$ (F = 1.052, df = 2, p = 0.358).

# Chapter VII

## General Discussion and Conclusions

The results of the experimental work presented in this thesis contributes to the rapidly growing evidence for interactive effects between warming, hypoxia, and hypercapnia on marine fish (**chapter II**). Based on existing hypotheses I decided to focus my investigations on the $O_2$ transport system, aerobic metabolism, and physiological tolerance limits of fish as any interactive effects that act on these end points may have broad implications for organism fitness and hence population level responses to environmental change. The majority of the thesis (**chapter III, IV, and VI**) focusses on potential interactive effects of acute changes in temperature, $O_2$, and $CO_2$. In these sections we highlight the potential for interactive effects on aerobic performance during extreme events and relate these to shifts in whole organism physiological tolerance limits. In contrast, **chapter V** investigates interactive effects that occur as a result of longer term environmental changes. The combination of these approaches gives insight into mechanisms and consequences of interactive effects which occur across different timescales.

### 1. Overview of Major Thesis findings

In the first study of the thesis (**chapter II**) I conducted a systematic search (conducted in 2019) of the literature to identify experimental studies which had quantified combined effects of interactions between changes in at least two of temperature, $O_2$, and $CO_2$ on the biology of marine fish. This resulted in the identification of 68 peer-reviewed papers involving 53 species and covering 412 individual end measurements. Of these, the majority (38 studies, 31 species, and 311 end measurements) of research focussed on interactions between temperature and $CO_2$, ~1/3 (22 studies, 20 species, and 52 end measurements) investigated interactions between temperature and $O_2$, with relatively little focus on interactions between $O_2$ and $CO_2$ (7 studies, 8 species, 31 end measurements). Additionally, despite leading hypotheses predicting synergistic interactions between combined changes in temperature, $O_2$, and $CO_2$ the literature search found only one study has included manipulations of all three simultaneously. The focus of experimental work on interactions between

temperature and $CO_2$ is not surprising given the global rise in $CO_2$ and associated increase in temperatures. However, the lack of studies investigating interactions between $O_2$ and $CO_2$ is a surprise given the known relationship between these environmental variables. Indeed, we highlight the increase in $CO_2$ which co-occurs during hypoxia in the chapter and predict that global increases in $CO_2$ will exacerbate increased $pCO_2$ levels during hypoxia in future oceans.

In contrast to previous research which suggested that non-linear interactions between multiple stressors may occur in ~2/3 to 3/4 of measurements (Crain *et al.*, 2008; Darling and Côté, 2008), only ~1/3 of total end measurements from identified studies (in which an interaction was quantifiable) showed evidence for non-linear impacts. This trend is driven primarily by studies including interactions between temperature and $CO_2$ with non-linear interactions more common when studies investigated interactions involving $O_2$. However, assessing trends in proportion of non-linear interactions are difficult to interpret given the variability in methods, treatments, and species used between studies. The difficulty in identifying clear patterns of where and when non-linear interactions occur is clearly a major barrier to enhancing the accuracy of predictions of impacts of future environmental changes. In general one of the major challenges for fish physiology research is the low statistical power of most experiments, which is a result of logistical difficulties related to animal husbandry, maintenance of treatment conditions, the complex and time consuming nature of physiological measurements, and the costs of larger, more robust studies (Boyd *et al.*, 2018). As a result low-power studies are often unsuitable for detecting non-linear interactions when treatment magnitudes are relatively small, durations are short, and effects are subtle (for an example of this see Bouyoucos *et al.*, 2020). To combat this problem one option may be to increase the number and magnitude of treatment conditions. For example, instead of classic 2 x 2 factorial designs having three levels of each treatment would allow for ambient conditions, moderate magnitude treatments, and a further high magnitude treatment. In the context of $CO_2$ this would allow for a treatment at ambient $CO_2$ conditions (~400 µatm), a moderate treatment based on global change predictions (~1000 µatm), and a more extreme treatment (~5000-10,000 µatm) which may induce larger effect sizes. For temperature and

$O_2$ treatments this would involve including treatments that cover the majority of the species physiological niche. Using this approach for temperature and $O_2$ has already been shown to offer strong predictive ability of species distributions (Slesinger *et al.*, 2019; Duncan *et al.*, 2020) when combined with the metabolic index (the ratio of $O_2$ supply to resting $O_2$ demand; Deutsch *et al.*, 2015). Overall I hope that the collation of these studies into one database will aid future researchers to identify key knowledge gaps and facilitate future studies to produce more accurate quantification of the potential for non-linear interactions between temperature, $O_2$, and $CO_2$.

**Chapter III** focussed on the time course of acid-base regulatory responses of European sea bass, *Dicentrarchus labrax*, after acute exposure to elevated $CO_2$. To date the majority of research investigating the time course of acid-base regulation in fish has been conducted with freshwater species. This is probably because researchers investigating impacts of $CO_2$ on marine fish have primarily focussed on ocean acidification where increasing $CO_2$ will occur over many decades. As such, the responses of marine fishes to acute $CO_2$ changes has been relatively understudied. Studies that have been conducted with marine fish show higher rates of acid-base regulation than are typically seen in freshwater species (see examples in Brauner *et al.*, 2019), which has been linked to differences in ionic composition of marine vs freshwater. These ionic differences are thought to affect ion gradients between the fish and the water and ultimately affect the rate of $H^+$ excretion from the gills (Larsen and Jensen, 1997; Weakley *et al.*, 2012; Tovey and Brauner, 2018). However, some work has suggested that marine fish may utilise direct $HCO_3^-$ uptake from the environment as well as $H^+$ excretion during acid-base regulation (Esbaugh *et al.*, 2012). Therefore, our objectives in this study were to investigate the time-course of acid-base regulation in sea bass, impacts of acid-base disturbance on $O_2$ transport in the blood, and effects of reduced environmental $HCO_3^-$ on rate of acid-base regulation.

The results of this study indicated that sea bass regulated blood pH within ~135 minutes of exposure to acute $CO_2$ – this is the second fastest rate of acid-base regulation recorded in a fish. Intracellular pH of red blood cells was restored faster than extracellular blood pH (within ~40 minutes) which resulted in Hb-$O_2$ affinity returning to control levels after an initial ~3-fold decline 10

minutes after acute $CO_2$ exposure. This increased speed of intracellular pH regulation is likely a result of β-adrenergic stimulation of NHE (Harter *et al.*, 2018). Overall, the extremely rapid acid-base regulation seen in sea bass enables protection of blood $O_2$ transport capacity. The ability to rapidly protect $O_2$ transport capacity may enable sea bass to maintain aerobic energy expenditure (e.g. for activity or digestion) if they encounter large variations in $CO_2$ over acute time periods (diel/tidal) while foraging in shallow nearshore habitats such as saltmarshes and estuaries (Doyle *et al.*, 2017). We also observed that decreased environmental $HCO_3^-$ completely prevented acid-base regulation by sea bass. While I initially thought this might signal a role for environmental $HCO_3^-$ uptake in the acid-base regulatory response, after further consideration this is probably a result of decreased environmental pH (which occurs in association with decreased $HCO_3^-$) creating an unfavourable gradient for $H^+$ excretion by gill $Na^+/H^+$ exchangers (NHE). Indeed, initial analysis of gill tissue samples taken from sea bass shows apical localisation of NHE3b in mitochondria-rich (MR) cells, likely indicating that NHE3 is involved in acid-base regulation as proposed for other marine fish (Esbaugh, 2017). Although our results provide further insight into acid-base regulatory responses of sea bass, including highlighting protection of $O_2$ transport capacity as a key benefit of rapid regulatory responses, they do beg the question as to what causes variation in the rate of acid-base regulation between species when environmental ion concentrations are not different. An obvious explanation is that differences in trial temperatures will partly explain differences in regulation rates but surprisingly to my knowledge the effect of temperature on rate of acid-base regulation has not yet been investigated. Acid-base regulation rates could also be linked to the ecology of fish – species that show faster rates of acid-base regulation tend to be active, predatory species which may experience regular metabolic acid-base disturbances (from anaerobic exercise during prey capture or digestion of large protein rich food sources) and therefore have adaptations to increase rates of acid-base regulation (for example they could potentially have a greater proportion of MR cells in gills). Reduced acid-base regulation rates in species which do not experience large or frequent metabolic acid-base disturbances may indicate that increased regulation rates come at a cost (for example increased SMR). In my opinion investigations into these questions would provide an intriguing avenue for further study.

**Chapter IV** investigated impacts of increased $CO_2$ on the hypoxia tolerance of sea bass. As highlighted in **chapter II,** hypoxic events in aquatic systems are almost ubiquitously associated with increased $CO_2$ levels – this is because $CO_2$ is produced as a by-product of aerobic respiration (Robinson, 2019). Previous research has suggested that impacts of increased $CO_2$ during low $O_2$ result in decreased hypoxia tolerance of fish (Miller *et* al., 2016; DePasquale *et al.*, 2015) potentially as a result of impacts of increased $CO_2$ on $O_2$ transport capacity. These findings have been corroborated by previous research at the University of Exeter conducted with European flounder (*Platichthyes flesus*) – although changes in blood $O_2$ transport were not quantified (Rogers, 2015). Thus, our initial hypothesis at the start of this study was that including environmentally realistic increased $CO_2$ during hypoxia tolerance trials would increase $O_{2crit}$ (i.e. reduce hypoxia tolerance) as a result of changes in blood acid-base chemistry and Hb-$O_2$ affinity. In contrast to our hypothesis we observed a ~20 % decrease in $O_{2crit}$ when sea bass where exposed to a concurrent increase in $CO_2$ during $O_{2crit}$ trials compared to trials where $CO_2$ was maintained at ambient atmospheric levels. There was no difference in blood pH between fish exposed to increasing $CO_2$ and fish exposed to ambient $CO_2$ during $O_2$ declines as sea bass compensated for the doubling of blood $pCO_2$ by the ~2-fold accumulation of $HCO_3^-$. Despite no change in blood pH we did see a 32 % fall in $P_{50}$ in fish exposed to increasing $CO_2$ in combination with hypoxia. This result likely indicates allosteric modulation of Hb-$O_2$ affinity in these fish. For example, erythrocyte pH is likely protected by β-adrenergic stimulated NHE when blood $pCO_2$ is initially increased. This can lead to an 'overshoot' and intracellular alkalosis which could explain increased Hb-$O_2$ affinity (i.e. reduced $P_{50}$). Additionally, changes in intracellular $Cl^-$ which occur as a result of increased plasma $HCO_3^-$ in bass exposed to increasing $CO_2$ may also contribute to reduced $P_{50}$ (Wells, 2009). Although results from this chapter contrast with previous studies (e.g.DePasquale *et al.* 2015), the observed interactive effects of reduced $O_2$ and increased $CO_2$ add to the evidence base from **chapter II** that suggests that interactions between $O_2$ and $CO_2$ may be more prevalent than interactions between $CO_2$ and temperature. Overall, the findings from this chapter indicate that interactive effects of rising $CO_2$ and decreased $O_2$ are important to consider when assessing impacts of hypoxia on fish species and, combined with evidence from other research, highlight that

more research of interactions between $CO_2$ and $O_2$ is needed in comparison to interactions between temperature and $CO_2$. This is especially important because increased $CO_2$ will always occur when $O_2$ reduces in aquatic environments (as shown in **chapter II**).

In **Chapter V** I sought to determine whether respiratory acidosis as a result of acute exposure to increased $CO_2$ can affect the critical thermal maximum ($CT_{max}$) of fish. One of the major predictions of the OCLTT hypothesis is that reductions in $O_2$ supply capacity caused by hypoxia or increased $CO_2$ will reduce upper thermal tolerance limits of ectotherms. For fish there has been contrasting evidence of effects of hypoxia on upper thermal tolerance limits (Ern *et al.*, 2016) and studies investigating effects of increased $CO_2$ have typically shown no impacts (Clark *et al.*, 2017; Ern *et al.*, 2017), although these studies only investigated impacts of long term exposure to $CO_2$ which may not actually reduce $O_2$ supply capacity. Combined, these results suggest that limitation of $O_2$ supply capacity may not be the mechanism underlying acute thermal tolerance limits in fish and alternative mechanisms have been suggested (for examples see Somero and Dahlhoff, 2008; Sidhu *et al.*, 2014; Ern *et al.*, 2015; Jørgensen *et al.*, 2017; O'Sullivan *et al.*, 2017; Bowler, 2018; Jutfelt *et al.*, 2019). Physiologists have primarily focussed on impacts of chronic $CO_2$ exposures on fish because increases in ambient $CO_2$ associated with climate change will occur over many decades. However, acute $CO_2$ changes do occur in nature (Helmuth *et al.*, 2014, 2016; Baumann *et al.*, 2015) and so understanding if acute changes in $CO_2$ can affect thermal tolerances of fish is also important. In fact, acute $CO_2$ exposure may be more likely to impact thermal tolerance limits than chronic $CO_2$ exposure – either via changes in $O_2$ supply capacity (e.g. Hb-$O_2$ affinity) known to occur as a result of respiratory acidosis or, potentially, via direct effects of reduced pH.

I hypothesised that effects of acute $CO_2$ may impact $CT_{max}$ of trout as a result of respiratory acidosis but not as a result of changes in $O_2$ supply capacity. To test this I exposed trout to increased $CO_2$ (acutely and chronically) with normoxia (~100 % air saturation) and acutely increased $CO_2$ with hyperoxia (~200 % air saturation) before measuring critical thermal maximum ($CT_{max}$). Exposure to an acute increase in $CO_2$ (~1 %) resulted in a ~0.43 mean decrease in blood pH and a corresponding ~0.18 decrease in erythrocyte $pH_i$.

Respiratory acidosis in fish exposed to acutely increased $CO_2$ caused an ~2-fold reduction in Hb-$O_2$ affinity. However, reduced Hb-$O_2$ affinity only caused a marginal reduction in aerobic scope when compared to control trout. In contrast, trout exposed to an acute increase in $CO_2$ in combination with hyperoxia showed an ~20 % increase in aerobic scope compared to control trout and ~30 % increase compared to fish acutely exposed to increased $CO_2$ in normoxia. Despite the large changes in blood pH, acid-base chemistry, and aerobic scope between treatments we saw no changes in $CT_{max}$. While this result does not support the OCLTT hypothesis, it should be considered whether $CT_{max}$ is an appropriate measure of thermal tolerance when determining impacts of long term climate change. Regardless, the lack of changes in $CT_{max}$ indicates that the mechanism underlying acute thermal tolerance of trout is not linked to $O_2$ supply and is not sensitive to changes in pH. This suggests that cardiac failure (Sidhu *et al.*, 2014; Jørgensen *et al.*, 2017), ion imbalances (O'Sullivan *et al.*, 2017), and impairment of neural functions (Ern *et al.*, 2015; Jutfelt *et al.*, 2019) may not be the principle reason for physiological failure at high temperatures in rainbow trout. Instead effects of temperature on membrane fluidity (both cellular and mitochondrial) may be the underlying cause of acute tolerance limits (Bowler, 2018; Chung and Schulte, 2020).

In the final study (**chapter VI**) I aimed to characterise the interactions between temperature, $O_2$, and $CO_2$ on aerobic scope of European sea bass. The OCLTT hypothesis predicts that impacts of decrease $O_2$ and increased $CO_2$ will interact synergistically to reduce aerobic scope of an organism across its thermal performance curve (see Figure 3 in **chapter I**). While interactive effects of $O_2$ and temperature on aerobic scope are well documented (e.g. see Claireaux and Lagardère, 1999; Claireaux *et al.*, 2000; Lefrancois and Claireaux, 2003) evidence for interactive effects between temperature and $CO_2$ is limited (Lefevre, 2016). However, to date no research has sought to investigate combined interactions between all three factors (temperature, $O_2$, and $CO_2$) on aerobic scope. Including all three environmental factors is important to determine whether synergistic interactions occur when fish are exposed to all three factors simultaneously because non-linear interactions are predicted to become more common when assessing combined impacts of three

as opposed to two factors (Crain *et al.*, 2008) and effects of $CO_2$ on aerobic scope where originally suggested in connection with hypoxia (Fry, 1971).

Increasing temperature resulted in a linear increase in standard metabolic rate and $O_{2crit}$ of sea bass. Increased $O_{2crit}$ at higher temperatures was driven by increased SMR – in fact accounting for changes in SMR the effect of temperature was predicted to decrease $O_{2crit}$. This may be because of temperature induced remodelling of the heart (to increase thickness of the compact myocardium and increase cardiac output; Nyboer and Chapman, 2018) and gills (to reduce interlamellar cell mass and increase gill surface area; Sollid and Nilsson, 2006). Similar effects of temperature on gill and cardiac morphology have been proposed to offset impacts of reduced Hb-$O_2$ affinity and increased methaemoglobin concentration in silver perch exposed to high nitrate levels (Gomez Isaza *et al.*, 2021). Temperature also resulted in a linear increase in MMR and there was negative synergistic interaction between increased temperature and reduced $O_2$ – this resulted in greater impacts of $O_2$ declines on MMR at higher temperatures. In contrast to temperature, increased $CO_2$ resulted in a small decline in SMR but there were no interactive effects between temperature and $CO_2$. Increased $CO_2$ also resulted in small decreases in MMR but there were no interactive effects with either temperature or $O_2$.

One of the initial reasons proposed by Pörtner *et al.* (2005) that increased $CO_2$ would reduce MMR (and hence aerobic scope) of water breathing ectotherms is a result of reduced $O_2$ binding affinity of respiratory pigments. Despite this, there is no experimental evidence to suggest that long term exposure to increased environmental $CO_2$ reduces Hb-$O_2$ binding affinity of fish (Hannan and Rummer, 2018). In **chapter III** we demonstrated that after exposure to increased $CO_2$ for less than an hour sea bass restore Hb-$O_2$ affinity to 'normal' levels. Building on that result, in this chapter we saw no impact of longer term exposures to increased $CO_2$ on Hb-$O_2$ affinity. The lack of effect of increased $CO_2$ on Hb-$O_2$ affinity seen in sea bass supports the lack of effects of increased $CO_2$ on MMR in most fish (Lefevre, 2019). Together our results from this chapter and **chapter III** provide some of the first evidence that increased environmental $CO_2$ is unlikely to significantly affect blood $O_2$ transport as a result of effects on Hb-$O_2$ affinity, in contrast to assertions of the OCLTT hypothesis. This may also partly explain why there is more evidence of effects

of $CO_2$ on aerobic scope of water breathing invertebrates which generally have less ability to maintain blood pH, and therefore $O_2$ transport of respiratory pigments, when compared to fish. Instead I would suggest that the negative effects of $CO_2$ on MMR of fish seen in some studies (including in this chapter) may be a result of impacts on cardiac or mitochondrial performance (e.g see Strobel *et al.*, 2013; Crespel *et al.*, 2019).

Overall impacts of temperature and $O_2$ on SMR and MMR resulted in a negative synergistic relationship between increased temperatures and decreased $O_2$ on aerobic scope, whereas increased $CO_2$ had little impact and did not show interactive effects with either temperature or $CO_2$. Our results indicate that $O_2$ does interact synergistically with temperature to affect aerobic scope as suggested by the OCLTT hypothesis. However, increased $CO_2$ does not have the impacts on aerobic scope that the OCLTT hypothesis predicts. The results of this chapter suggest that climate change will primarily impact fish species via warming temperatures and reduced $O_2$ as opposed to increased $CO_2$. One promising approach is the metabolic index – defined as the ratio of $O_2$ supply capacity to an organisms $O_2$ demand (Deutsch *et al.*, 2015) – which can be calculated from measurements of MMR and $O_{2crit}$ across an organisms temperature range and has been shown to accurately predict distributions of black sea bass (Slesinger *et al.*, 2019) and Roman seabream (Duncan *et al.*, 2020). Consequently, the metabolic index may represent a mechanism for predicting distribution shifts of species as a result of warming and decreased $O_2$ in future oceans.

Combined the results of this thesis build our knowledge of where and when interactive effects between temperature, $O_2$, and $CO_2$ occur however there are still significant challenges in predicting responses of fish to climate change. Overall, my findings show little support for the OCLTT hypothesis as a unifying framework to predict interactive effects of temperature, $O_2$, and $CO_2$ on fish. This is for primarily two reasons – the first is that combined changes in temperature, $O_2$, and $CO_2$ do not impact upon aerobic performance as predicted by the OCLTT hypothesis. For example, no interactive effects of $CO_2$ and temperature were noted upon aerobic performance of sea bass (**chapter V)** and elevated $CO_2$ does not reduce $O_2$ transport capacity other than initially after sudden exposure (**chapter II, III, IV**, and **VI**). Secondly, changes in $O_2$ supply

capacity (i.e. aerobic scope) did not change thermal tolerance as predicted by the OCLTT hypothesis (shown in **chapter VI**). Together these findings support previous literature (i.e. Clark *et al.* 2013; Lefevre, 2016, 2019) which highlight relatively little support for the OCLTT hypothesis in data from fish. Instead interactive effects between temperature and $O_2$ observed in this thesis (**chapter VI**) may better support the use of frameworks such as the oxygen- and temperature-limited metabolic niche framework (Ern, 2019) or metabolic index (Deutsch *et al.* 2015, 2020) to investigate impacts of climate change on fish. Both frameworks require greater experimental testing to validate the physiological mechanisms underlying their predictions.

## 2. General thesis limitations

Although the results of this thesis provide increased knowledge of the impacts of combined environmental changes on the physiology of fish there are a number of general limitations with the experimental approaches employed that should be considered when placing these results in a wider context. Key among these relate to the time scale of experimental work, constraints of laboratory experiments, and generality of observed responses.

Perhaps the key limitation of all the experimental work presented in this thesis is the time scale over which fish were exposed to treatments. All of the experiments reported in this thesis involved treatment durations ranging from minutes to days (maximum treatment duration = 19 days). While these time scales are representative of acute environmental changes that occur in aquatic habitats (e.g. over tidal and diel cycles, or during extreme events such as hypoxia or marine heatwaves) they do mean that care should be taken when translating results from experiments in this thesis to responses to long term climate change. This is for two reasons: firstly if impacts of environmental change are subtle then short time scales may not be long enough for them to be observed (e.g. see Murray and Baumann, 2020), secondly short time scales of experiments cannot account for effects of long-term acclimation, phenotypic plasticity, or transgenerational acclimation on physiological responses of fish. For instance, in **chapter VI** metabolic rates were measured after treatment exposures of 14-19 days but in some species metabolic thermal compensation after exposure to increased temperatures has been observed to occur over

periods as long as 8 weeks (Sandblom *et al.* 2014). In addition, exposure to climate change over multiple generations will also change physiological responses when compared to short term responses of individuals. Fish exposed to increased temperatures (Donelson *et al.* 2012), low $O_2$ (Ragsdale *et al.* 2020), or high $CO_2$ (Allan *et al.* 2014; Snyder *et al.* 2018) show improved performance if their parents also experienced these conditions when compared to fish with naïve parents. As such, physiological measurements reported in this thesis after exposure to treatments for shorter time periods may not reflect long term responses and this will influence upon observed interactive effects between combined environmental changes on the physiology of fish.

The detailed physiological measurements presented in this thesis are also limited by issues related to the laboratory-based nature of such experiments. A necessity of the research presented in this thesis was the capture of wild fish, transfer of fish to an aquarium facility where they were maintained in captivity, the handling of fish periodically for husbandry purposes, and the isolation and confinement of individual fish during measurements. These actions will all have caused either chronic or acute stress to individual fish, triggering the physiological stress response and release of cortisol into the blood (Pottinger, 2008). Elevated cortisol levels lead to a variety of secondary effects including upon metabolism, osmoregulation, growth, molecular responses and immune function (Pickering and Pottinger, 1989; Basu *et al.* 2001; Barton, 2002) and as such could have impacted upon measurements reported in this thesis. Conversely, the high water quality, lack of predators, treatment of parasites and diseases, and provisioning of food of fish in captivity may all reduce stress and increase fitness of captive compared to wild fishes (Plante *et al.* 2002). As a result physiological performance of fish in laboratory settings may differ from fish in natural environments.

A variety of other limitations also affect the generality of results presented in this thesis. Studies of single species should not be used to draw generalised conclusions for all fish. This is because species that inhabit different habitats, occupy different ecological roles, and have differing life history characteristics will have evolved physiological mechanisms adapted to the challenges they individually face. As a result changes in temperature, $O_2$, and $CO_2$ are unlikely to show the same interactive effects in all species. Indeed, even within a

species differences in prior environmental experiences between populations can have large influences upon physiological responses when faced with similar environmental changes (for example see a recent example from Gervais *et al.* 2020). Furthermore, even within the same population short term variations in environmental conditions can alter physiological responses over annual and seasonal time periods (Murray *et al.* 2014). These limitations of single-species studies mean that general conclusions or theories need to be developed and tested using results from numerous studies to build a strong evidence base and ensure findings hold across the majority of species. Recent advances in meta-analyses provide the optimal way to do this (for example see Lefevre, 2016).

Finally, limitations related to laboratory logistics should also be considered. Laboratory based experimental work is often restricted to work with juvenile fish because of sourcing, husbandry, and experimental approaches when working with other life stages. As each life stage (e.g. eggs, embryo, larvae, juveniles, and adults) has different physiological requirements responses observed in juvenile fish may not reflect responses of other life stages (e.g. Dahlke *et al.* 2020). Laboratory based physiological experiments also often suffer from low statistical power as a result of small sample sizes, particularly when investigating interactive effects, as a result of the complexity of experimental measurements, number of treatments required, and logistical constraints. As a result if effect sizes are small or there is high intra-individual variability in responses then interactive effects may not be identified in statistical analysis (e.g. Bouyoucos *et al.* 2020).Overall, the general limitations mentioned above should be considered to avoid over-interpretation of the results presented in this thesis. By acknowledging these limitations we can also seek to increase the robustness of future experiments and formulate research to reduce the influence of limitations and uncertainties in existing studies.

## 3. Future research directions

The findings in this thesis increase our understanding of how combinations of temperature, $O_2$, and $CO_2$ may interact to affect physiological performance of fish. The original aim of the thesis was to characterise interactive effects between these three factors in order to improve understanding of how fish will respond to global environmental change. The findings here highlight the

difficulties in generalising interactive effects between temperature, $O_2$, and $CO_2$ to all fish species. This is because interactive effects appear to be context and species dependent. I believe this indicates two crucial areas in which further research is required – determining what causes variations in responses to combined factors and translating physiological impacts of combined effects to population levels responses.

### 3.1. Variation in responses

A key barrier for predicting interactive effects of changes in temperature, $O_2$, and $CO_2$ on fish is this the lack of consistency in results from laboratory based experiments. Understanding why different responses are observed between studies is vital to enhance accuracy of predictions. General aspects commonly thought to increase variation between studies include species used, source of experimental organisms, laboratory conditions, experimental methods, and treatment magnitudes and duration. These factors also restrict the ability to make direct comparisons between studies in order to identify if variations in results are truly a result of differing biological responses. While adopting standardised best practices for methods and treatment conditions in experimental work may allow greater comparability between studies (Riebesell *et al.*, 2011) this will not reduce inconsistency of results if the source of variation is a result of biological as opposed to methodological factors.

#### 3.1.1. Combined effects of hypoxia and $CO_2$

There is growing evidence that combined effects of decreased $O_2$ and increased $CO_2$ can impact upon aerobic performance and physiological tolerance limits of fish (Gobler and Baumann, 2016). However, to date there has been little research focussed on effects of these combined factors (**chapter II**). Results from **chapter IV** confirm that effects of increased $CO_2$ can impact acute hypoxia tolerance of sea bass although, surprisingly, the direction of impact was opposite to that seen in previous research with European flounder (Rogers, 2015). Why did sea bass respond differently to European flounder? One possibility is that differences in acid-base regulatory capacities may modulate responses of fish to combined exposure to increased $CO_2$ and hypoxia. For example, sea bass have high acid-base regulatory capacity (**chapter III**) and so were able to fully compensate for impacts of increased $CO_2$ on $O_2$ transport

capacity essentially in real time during concurrent exposure to increased $CO_2$ and decreased $O_2$. Shared mechanisms underlying physiological adaptations to increased $CO_2$ and low $O_2$ may then promote cross-tolerance to increased $CO_2$ – for example adrenergic stimulated responses which increase blood $O_2$ carrying capacity when fish experience low $O_2$ or increased $CO_2$ may be stronger when fish acutely experience combined changes in $O_2$ and $CO_2$. Research in which acid-base regulatory capacity is manipulated in a single species (e.g. via changes in water ion composition) or comparing responses of species with known differences in acid-base regulatory capacity (e.g. between species which show fast vs. slow rates of acid-base regulation or species that don't vs. do possess adrenergic stimulation of erythrocyte $pH_i$ regulation) may confirm if variation in combined impacts between species can be linked to differences in acid-base regulatory responses.

As well as differences in acute responses to combined changes in $O_2$ and $CO_2$ the results from **chapter IV** differ from effects of longer term (i.e. days rather than hours) exposures (DePasquale *et al.*, 2015; Schwemmer *et al.*, 2020). This may occur if initial responses to acute changes in $O_2$/$CO_2$ improve physiological performance but are unsustainable over longer time periods. Research charting how responses to combined changes in $O_2$ and $CO_2$ alter over time may help explain differences in responses reported between studies.

### 3.1.2. Impacts of $O_2$ and $CO_2$ on thermal tolerance

**Chapter V** provides further evidence of a lack of effect of aerobic scope on thermal tolerance of rainbow trout and also shows that mechanisms underlying acute thermal tolerance are pH insensitive. However, whether these mechanisms set acute thermal tolerance limits of all fish species is debatable as some species do show impacts of aerobic scope on $CT_{max}$, for example in silver perch, *Bidyanus bidyanus* (Gomez Isaza *et al.*, 2020). Understanding why acute thermal tolerance limits of some species are affected by changes in aerobic scope whilst other species are not is clearly an area where further research should focus. It may be that the short time scale of $CT_{max}$ tests means that some species are able to maintain energy production (either via aerobic or anaerobic respiration) at temperatures higher than the thermal tolerance limits of other systems (e.g. membrane structure). If this were the case then species which show no impacts of aerobic scope on acute thermal tolerances might be

expected to show impacts of aerobic scope on long term thermal tolerance limits.

### 3.2. Translating impacts of combined effects on individuals to population level responses

As well as the need to understand what causes differences in responses to combined effects of temperature, $O_2$, and $CO_2$, another key challenge is to translate experimental data from laboratory measurements to population responses in nature. The use of aerobic scope as a whole organism measure of performance has been widely adopted by experimental biologists (Clark *et al.*, 2013) to predict effects of environmental change on fish populations. Despite this, there is little experimental evidence that changes in aerobic scope drive changes in whole organism fitness and population distributions as suggested by hypothesis such as the OCLTT. In order to have more confidence that responses measured in the laboratory will translate to 'real-world' consequences future experimental work should seek to link changes in aerobic scope to impacts on whole organism performance such as growth, swimming performance, and reproductive output.

Experimental approaches could also attempt to improve realism of treatment conditions to increase accuracy of predictions of species responses in nature. For example, laboratory experiments usually feed organisms to satiation or at a sufficient ration that study animals are in a high level of physical condition before conducting experiments. In contrast, organisms in the wild may be challenged by changes in food abundance or quality. Sea bass exposed to combined warming and increased $CO_2$ showed differing interactive effects between these environmental stressors depending on the food ration they were given (Cominassi *et al.*, 2020) highlighting the need for future research to account for changes in both biotic and abiotic factors. A second area where experimental research could be made more realistic is in the treatment conditions used. Treatment conditions are often static and based on changes in mean conditions whereas we know temperature, $O_2$, and $CO_2$ are highly variable in nature (Helmuth *et al.*, 2014; Bates *et al.*, 2018). Incorporating realistic natural variation in treatment conditions can result in different biological responses to multi-factor interactions (Laubenstein *et al.*, 2020) which has been further highlighted by the results of **chapter IV** and **chapter VI**. However, it may

be impractical to incorporate abiotic factors and natural variability into experiments seeking to quantify interactive effects between temperature, $O_2$ and $CO_2$ on physiological performance because of the logistical challenges associated with the increased complexity of experimental designs and problems of low statistical power. Therefore it may be necessary to use alternative methods to determine if combined effects measured in laboratory settings translate to fish in the wild – including use of biomarkers or telemetry to estimate physiological performance of fish in the field (Brijs *et al.*, 2019; Chung *et al.*, 2019).

## 4. Conclusion

Fish species are being exposed to rapid and concurrent increases in temperature, decreases in $O_2$, and increases in $CO_2$ as a result of global climate change. Key to making accurate predictions of the impacts of climate change on fish is understanding interactive effects between combined changes in environmental conditions on physiological performance. While theoretical frameworks have suggested that impacts of combined effects of warming, reduced $O_2$, and increased $CO_2$ will act via common mechanisms for all species experimental data is needed to validate the accuracy of these predictions. Results from this thesis highlight the importance of quantifying interactive effects by demonstrating context specific responses to combined environmental changes. The lack of consistent combined effects suggest that theoretical frameworks currently lack the mechanistic support to provide universal predictions of the impacts of climate change on fish species. The contributions of this thesis to the rapidly growing evidence base of interactive effects will hopefully provide greater understanding of the biological mechanisms through which multiple environmental changes will act on fish species. Ultimately, identifying the physiological mechanisms behind observed interactive effects will enable accurate and specific predictions of population responses needed to manage and conserve fish species in the changing world of the future.

# Chapter VIII

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