TRACKING TRENDS IN HYPOXIA:

A FRESHWATER PERSPECTIVE

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Cover Image: Collecting and measuring field caught golden perch (*Macquaria ambigua*) at Chowilla floodplain, South Australia, Australia. Photo credit: Kayla Gilmore

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Kayla L. Gilmore

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THESIS ABSTRACT

Hypoxia or low dissolved oxygen occurs in both marine and freshwater systems and is a threat to aquatic life. Historically hypoxia arose naturally and intermittently however, anthropogenic influences and climate change have exacerbated its severity and frequency. I define a hypoxic event as one where levels of dissolved oxygen in the water are low enough to cause a reduction in overall fish health based on the most sensitive species in the system. The primary aims of my thesis were to determine the long-term physiological impacts of hypoxia to freshwater fish and develop the science to trace its occurrence through time.

I examined the physiological effects and tolerance of fish to long-term hypoxia exposure at different temperatures. Higher water temperatures limit the amount of available dissolved oxygen occurring in water therefore, it was likely that high temperatures in combination with hypoxic conditions would also limit performance. I investigated the effects of hypoxia on three key species from the Murray Darling Basin, Australia, golden perch (*Macquaria ambigua*), silver perch (*Bidyanus bidyanus*) and Murray cod (*Maccullochella peelii*). In my first data chapter I found golden perch had a reduced metabolic scope for activity after long-term exposure to hypoxia, which was also influenced by temperature. Additionally, golden perch exhibited an acclimation response, whereby prolonged hypoxic exposure improved tolerance to low oxygen conditions. However, silver perch, a sympatric species, had a poor tolerance to hypoxia and all individuals died after one month's exposure. In my next chapter, I investigated if acclimation ability was affected by exposure time (7, 14 and 30 days) for Murray cod. Similarly long-term exposure to hypoxia improved the tolerance of Murray cod suggesting fish had acclimated. However, acclimation was inversely related to exposure time.

After documenting the physiological impact hypoxia exposure had on fish, I investigated a means to track its occurrence through time. Otoliths or the ear bones of fish accrete daily layers of material on a calcium carbonate matrix that reflect the environmental conditions experienced by fish. I investigated elemental signatures that represented hypoxic occurrence under controlled conditions. I reared juvenile golden

perch under combined differing temperature and oxygen conditions for a month and analysed trace element concentrations in the otoliths. Trace elements measured in the otoliths, however, did not differ among hypoxic and normoxic treatments. By running transects along the otoliths of golden perch and Murray cod from modern and historic collections of fish that either died or experienced a hypoxic event I could reconstruct the long-term occurrence of hypoxic events. Records of hypoxia frequency in most waterways only go back a few decades so this technique could determine hypoxia histories of water bodies that would be unattainable using traditional methods.

I highlight that any prolonged exposure to hypoxic conditions benefits individuals' ability to remain in low oxygen environments longer, and that coexisting fish have species-specific responses. Furthermore, I highlight otoliths acting as natural tracers of hypoxia, such that given the right conditions elements routinely physiologically regulated act as natural tracers for low-oxygen events. Thus, our ability to reconstruct hypoxia through time using otoliths is reliant on the physiological disruption it creates.

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Students and demonstrators sampling freshwater fish from the Chowilla floodplain, South Australia, Australia. Photo credit: Kayla Gilmore

CHAPTER ONE

GENERAL INTRODUCTION

Aquatic systems are highly productive zones that support a large diversity of plants and animals. Stressors associated with anthropogenic change are impacting these systems on a global scale. Increasing water temperatures, changes in dissolved oxygen levels, pH fluctuations, and pollution (eutrophication, nutrient loading and waste waters) are among the most prevalent changes (Richardson *et al.* 2001, Wong *et al.* 2018). These disruptions to the natural environment can affect the distribution and abundances of marine and freshwater organisms (Poloczanska 2018). Yet, in nature they rarely act in isolation. Understanding the cumulative effects of such stressors is key to assessing their impacts on aquatic organisms (Richardson *et al.* 2001).

Hypoxia in aquatic systems is an increasing threat worldwide, and occurs when dissolved oxygen is depleted. Throughout this manuscript, hypoxia is defined as an incidence where concentrations of dissolved oxygen in water are low enough to reduce the overall health of fish based upon a system's most sensitive species. While hypoxia is often caused by natural events, the influence of climatic shifts, anthropogenic disruptions, eutrophication and organic pollution, are considered to be critical threats that exacerbate hypoxic conditions (Pollock *et al.* 2007, Saari *et al.* 2018, Wong *et al.* 2018). The impacts of low dissolved oxygen range from sub-lethal effects on reproduction, behaviour, dispersal and declines in fisheries productivity, to severe effects which cause mass mortalities and widespread dead zones (Pollock *et al.* 2007, King *et al.* 2012, Whitworth *et al.* 2012, Jeppesen *et al.* 2018). The negative impacts of hypoxia have increased over recent years and are expected to further intensify without future intervention (Pollock *et al.* 2007).

FACTORS THAT INFLUENCE HYPOXIA

Current rates of biodiversity loss rival those previously observed in global mass extinction events (Birnie-Gauvin *et al.* 2017). Freshwater fish are no exception with populations declining worldwide in response to habitat degradation (Hamilton *et al.* 2017, Harris *et al.* 2017). Globally freshwater vertebrates have suffered a decline in

abundance of 76% over the past 40 years (Harris *et al.* 2017), and in the Murray-Darling Basin of Australia, it is estimated that the abundance of native fish have declined by ~90% in the past 200 years (Hamilton *et al.* 2017). Hypoxia is a recurring environmental stressor in many marine, estuarine and freshwater systems worldwide (Roberts *et al.* 2012). The challenge then for researchers is to predict the likely responses of species and communities to hypoxic events under both natural and anthropogenically-influenced conditions. Herein, I focus on the hypoxic response of freshwater fish and the factors that influence hypoxia tolerance and occurrence.

Water Temperature

Under hypoxic conditions, water temperature is one of the critical factors that can determine whether or not an event will result in mortalities (Baldwin and Whitworth 2009, Whitworth *et al.* 2012). Water temperature influences the physiological performance, development, growth rate and reproductive output of aquatic organisms (Gehrke and Fielder 1988, Pollock *et al.* 2007, McBryan *et al.* 2016), and as temperature increases beyond the optimum level for a species, negative effects manifest. High water temperatures diminish the capacity for oxygen to be dissolved, causing declines in production associated with increased food requirements, spread of disease and mortalities (Ruby *et al.* 2017). Additionally, continued anthropogenic interference in waterways exacerbates already diminished conditions by restricting natural flow regimes and limiting the adaptive capacity of biota to respond to changing temperatures (Balcombe *et al.* 2011, Saari *et al.* 2018). Therefore, determining how fish respond to the combination of elevated temperature and low dissolved oxygen is fundamental to understanding their future resilience (Stehfest *et al.* 2017, McBryan *et al.* 2016).

Anthropogenic Factors

Anthropogenic factors have dramatically impacted hypoxic events through changes to flow regimes, environmental water allocation, agricultural run-off, encroaching populations, flood timing and damming of rivers (Pollock *et al.* 2007, Baldwin and Whitworth 2009, King *et al.* 2012, Whitworth *et al.* 2012). Whilst these anthropogenic factors are not the sole driver of hypoxia, they have modified the frequency and severity of these events in natural systems (Jenny *et al.* 2016). In many instances, an improved understanding of the environmental requirements of aquatic

organisms would allow anthropogenic impacts to be better managed. For example, in late 2010, in south-eastern Australia, drought-breaking rains caused the inundation of extensive floodplains across the lower Murray River region, resulting in a prolonged large scale hypoxic blackwater event (so named due to the dark colour of the water, often caused by large quantities of dissolved organic carbon from organic leaf litter; King *et al.* 2012, Whitworth *et al.* 2012). The event lasted for six months and affected over 2000km of river channels and forested floodplains (Whitworth *et al.* 2012). Modelling of systems similar to this, suggested that frequent smaller inundations of forested floodplains, which do not receive natural water flows due to upstream damming and water regulation, would significantly reduce the severity of such an event (Baldwin and Whitworth 2009, Whitworth *et al.* 2012). However, due to an ever-increasing human population and the associated demand for water resources, priority is often given to shorter-term social and economic objectives, rather than aquatic health. Left unmanaged, anthropogenic influences often negatively impact organisms sometimes resulting in mass mortalities of fauna. Such outcomes can be mostly attributed to poor management of waterways and a lack of applicable knowledge regarding the physiological and ecological requirements of aquatic organisms (Baumgartner *et al.* 2017).

Extended Hypoxic Exposure

Organisms have developed numerous physiological, biological and behavioural adaptions to cope with hypoxic exposure (Chapman *et al.* 2002, Sollid *et al.* 2005, Crocker *et al.* 2013, Small *et al.* 2014). Lethal endpoints have often been used to assess safe levels of hypoxic conditions for fish (Feng *et al.* 2016, Speers-Rosech *et al.* 2013). However, adaptive responses to sub-lethal hypoxic exposure are more valuable to determine sensitivity relative to other important functions (e.g. reproduction, growth and mortality) (Feng *et al.* 2016, Dwyer *et al.* 2014, McBryan *et al.* 2016). When faced with hypoxic exposure, adaptive ability (sometimes referred to as phenotypic plasticity) is advantageous as it can provide immediate alleviation to stressors, however it may not always be lasting (Cote *et al.* 2012, Collins *et al.* 2016). Heritable adaptations also exist and improve long-term survival particularly to cyclic hypoxic exposure, but require multiple generations before becoming beneficial (Cote *et al.* 2012, Collins *et al.* 2016).

If hypoxic conditions cannot be avoided (i.e. migration), the length and timing of a hypoxic event is a key factor in the success of an organism's response, and thus capacity to survive (Crocker *et al.* 2013). A common response by fish to short-term (hours) hypoxic exposure is to maintain oxygen delivery by reducing activity (Crocker *et al.* 2013, Small *et al.* 2014). Long-term, this method would be insufficient in maintaining the homeostatic balance of the organism, and some species may not have the capacity to change their mechanism for oxygen delivery (Crocker *et al.* 2013). Along with changes in activity some other mechanisms to cope with short-term hypoxia are through increased air breathing, increased use of aquatic surface respiration (species specific), and through vertical or horizontal habitat changes or avoidance (Dwyer *et al.* 2014, Stehfest *et al.* 2017). Long-term survival is predominantly driven through performance modifications in cells and tissues and is generally inherited (Dwyer *et al.* 2014, Cook *et al.* 2013). Some species may acclimate to hypoxic conditions, but limited research has been conducted in this area (Crocker *et al.* 2013, Small *et al.* 2014).

The severity of a hypoxic event is mediated by the current environmental conditions. For example, a long dry period in freshwater rivers results in a build-up of leaf litter in wetlands and floodplains (King *et al.* 2012, Dean and Richardson 1999). A subsequent flood during this period may rapidly induce a hypoxic event due to the high carbon load for micro-organisms and low oxygen transfer in slow flowing water bodies. The timing of hypoxic events may also be important on a finer temporal scale (i.e. daily) (Crocker *et al.* 2013, Dan *et al.* 2014). Thus, environmental water releases that result in a sudden increase in the frequency and severity of hypoxia in the system could lead to fish mortalities (Thiem *et al.* 2017). Understanding the extent of acute hypoxic exposure organisms can tolerate, as well as their long-term responses and/or adaptations, is necessary to improve restoration and conservation efforts in freshwater systems.

MEASURING HYPOXIC INFLUENCES ON FISH

While the causes of a hypoxic event are generally understood in freshwater and floodplain systems (McMaster and Bond 2008, King *et al.* 2012, Whitworth *et al.* 2012), comparatively little is known of the effects on aquatic life. Fish mortalities are among the most readily observable and immediate effects of hypoxia. Yet, the

vulnerability of different species remains poorly understood; this gap in knowledge affects our ability to predict and prevent fish kills occurring. Dissolved oxygen levels of ~8mg L^{-1} are normal for most systems (Vaquer-Sunyer and Duarte 2008). As dissolved oxygen levels decline below 4-5mg L^{-1} many species show signs of stress and sometimes death (King *et al.* 2012). During blackwater events, such as the one in 2010, dissolved oxygen concentrations can quickly reach levels lower than 2mg L^{-1}, with survival under these conditions typically less than 48 hours (Gehrke and Fielder 1988). Lower levels of hypoxic stress influence growth, survival and presumably also reproductive output in some fish species; and more sensitive species are likely to be further influenced long-term, however, these affects have not been studied (Gehrke *et al.* 1993, Landman *et al.* 2005). At present, research into the hypoxia tolerance of fish can be separated into two broad categories; field-based presence/absence surveys which are often conducted following hypoxic events and short-term physiological/behavioural studies, with the latter being the most dominant. In both cases few studies have considered the long-term effects of exposure to hypoxic conditions. Fewer still have attempted to track hypoxic events through time to determine long-term tolerance of fish species.

Presence-absence data

At present, studies investigating short and long-term tolerance are commonly presence-absence studies that link the presence of an organism to the environmental conditions at the time of sampling. Such studies commonly compare affected and non-affected sites in terms of hypoxia, and generally have a limited spatial coverage. For example, a study concerning the aforementioned hypoxic blackwater event of 2010 found that affected sites had a significantly lower number of native fish species in comparison to non-affected sites, but both had similar abundances of invasive species (King *et al.* 2012). The abundance of small-bodied fish was also significantly lower at the affected sites after the major flooding event and during the seasonal recruitment period (King *et al.* 2012). Sites affected by hypoxic blackwater also lacked the large-bodied natives often associated with these areas such as golden perch, Murray cod and silver perch, though these species were found at the non-affected sites.

Quantitative studies that document the physiological impact and extent of hypoxic events on native fish populations in these systems are limited. A general absence of data on natural fish assemblages prior to hypoxic events is often a key limiting factor, although some attempts have been made to reconstruct these from past catch records and fishing logs (Disspain *et al.* 2011). Sampling native populations during and following hypoxia events can be confounded by other factors including mass mortality of species, movement of aquatic fauna away from areas into un-sampled sites, and potentially large scale relocation to non-affected sites for refuge and feeding (when sampling during hypoxic events, King *et al.* 2012). An ability to track the effects of hypoxic events on native fauna through time would enhance our understanding of its impacts on both fish migrations and species assemblages.

Physiology and Behaviour

Physiological and behavioural studies focus on the ability of organisms to cope and adapt to environmental stressors (Zhang *et al.* 2010, Roche *et al.* 2013). Respirometry is commonly used in physiological studies and is typically based on one of two methods: swimming or resting respirometry (Zhang *et al.* 2010, Roche *et al.* 2013). These methods facilitate the measurement of oxygen consumption rates ($\dot{M}o_2$) at both the lower and upper bounds of a fish's capacity to uptake oxygen. The short-term effects of different stressors on metabolic performance and behavioural responses on individual fish species are well researched; however, few studies have considered long-term (chronic) exposures to one or more stressors (Richardson *et al.* 2001, Small *et al.* 2014). Physiological and behavioural studies suggest fish tend to reduce their energetic requirements under hypoxic conditions by restricting movement and reducing their feeding rates. These behavioural changes can have a significant impact on growth, reproduction and development, all of which are found to be negatively affected under extended hypoxic exposure (Gehrke and Fielder 1988, Gehrke *et al.* 1993, McMaster and Bond 2008, Landman and Ling 2011).

Otoliths – Reconstructing Environmental Change

Otoliths or the ear bones of fish are paired calcified structures found within the inner ear canal typically used for hearing and balance. These structures occur mainly as an aragonitic form of calcium carbonate (Radtke 1989, Campana 1999, Campana and Thorrold 2001, Elsdon *et al.* 2008). Otoliths are unique compared to other calcified structures found in marine organisms as they accrete layers of crystalline and protein material on their surface daily (Radtke 1989, Campana 1999). Minor and trace elements are incorporated within these layers providing a chronological record of the biological, physical and chemical environments, which the fish has encountered over its lifetime. An extensive number of studies have used otoliths to track fish migrations, climatic shifts as well as other environmental conditions by analysing the concentrations of specific elements and isotopes in the otolith (Campana 1999, Campana and Thorrold 2001, Elsdon and Gillanders 2003, Elsdon *et al.* 2008, Gillanders and Munro 2012, Limburg *et al.* 2018). The uptake of strontium and barium has been documented to change with temperature and salinity (Campana and Thorrold 2001, Elsdon and Gillanders 2002, Limburg *et al.* 2011). Manganese has

been linked to hypoxic and anoxic conditions, however, to our knowledge few studies have attempted to reconstruct the hypoxia histories of fish over variable timescales (but see Limburg *et al.* 2011). Dissolved manganese may become available for accumulation into the otolith in two ways; as a product of redox reactions in sediment during low oxygen events and by becoming biologically available through disruptions in physiological regulation during exposure to hypoxic stress (Limburg *et al.* 2015, Walther *et al.* 2017, Sturrock *et al.* 2015). Researchers validated the use of manganese as an indicator to reconstruct hypoxia exposure in Baltic cod (*Gadus morhua*), as its presence could be matched to those years with hypoxic conditions, and was generally absent in years lacking hypoxia events (Limburg *et al.* 2011). Studies such as this highlight the potential for tracking and reconstructing long term trends in hypoxia exposure for freshwater fish, information almost unattainable without utilising otolith chemistry.

HYPOXIA AND THE MURRAY RIVER REGION

The Murray River region is the largest freshwater catchment in Australia and covers ~14% of the continents total surface area (Balcombe *et al.* 2011). The Murray River region is the life blood of Australia and supports many social, cultural and economic interests as well as a large biodiversity of organisms (Balcombe *et al.* 2011, Koehn 2015). Hypoxia is widespread in the Murray River region. The frequency and severity of hypoxic events is dictated by climate and the management of environmental flows (e.g. timing, seasonality and frequency of inundation). The Murray River region has historically been affected by severe moisture stress due to a combination of climatic drought and the over-allocation of upstream water resources. For example, the average total annual discharge for the Murray River at Lock 9, located downstream of all major tributaries of the southern Murray Darling Basin, between 1980 and 1999 was 5579 GL, and between 2000 and 2009 prior to the blackwater event in 2010, average discharge was only 1360 GL, a ~75% reduction of natural flow (Whitworth *et al.* 2012). The use of artificial flow regulation in the Murray region has significantly altered the natural flooding and drying cycles of the forests and floodplains, changing the natural dynamics of the river region. There is a limited amount of available environmental water for allocation and much of this is recycled right through the system. When water is transported from one location to

another the quality of that water decreases. Before river regulation across the Murray River region, floodplains were naturally inundated during winter and early spring. However, following regulation much of this water is captured upstream limiting flooding to smaller events in spring and early summer (King *et al.* 2012). A lack of adequate inundation increases chances of hypoxic flows, due to increased litter fall, summer temperatures and microbial activity (King *et al.* 2012). Current management is focused on environmental water flows, with an emphasis on increasing the extent, duration and frequency of flooding in the Murray River region (King *et al.* 2012). The results and subsequent benefits of these plans could be greatly enhanced by an informed understanding of native species physiological requirements and a baseline for historic hypoxic occurrence.

The Murray River region is home to many endemic species listed from Vulnerable to Critically Endangered by the International Union for the Conservation of Nature (IUCN 2011). Reasons for this are broad, ranging from poor connectivity of waterways to natal habitats, introduced predators, climate shifts, fishing pressure and pollution. Fish kills due to hypoxic blackwater have been recorded in a number of different aquatic habitats in the Murray River region and across Australia (Gehrke *et al.* 1993, Townsend and Edwards 2003, Baldwin and Whitworth 2009, King *et al.* 2012, Whitworth *et al.* 2012). Increased understanding of tolerances and effects of long-term exposure of native fish to hypoxia is necessary in order to begin the development of better preventative strategies for mass mortalities.

THE HYPOXIA DILEMMA

Sufficient oxygen availability is key to the survival of all fish species, but the influence of dissolved oxygen levels on fish health is often overlooked and poorly understood due to difficulties associated with testing. How then do species survive hypoxia, is it through a natural tolerance, acclimation, avoidance activity or is mortality the most common outcome? Analysis of the metabolic performance of fish has shown varied results among different families and species, with some of those tested showing large tolerance to low dissolved oxygen, while others begin surface respiration at levels just below normal (McNeil and Closs 2007, Nilsson *et al.* 2009). Obtaining representative data requires sampling of a range of species. Observational data is dependent on sampling a portion of the population at one moment in time.

These studies are easily confounded by species occupying the area at time of sampling (e.g. presence of a schooling species might suggest a greater abundance than would normally occur). Therefore, it is increasingly important that we conduct similar physiologically and behaviourally driven studies on a range of native species to determine their response to these environmental stressors. Freshwater species that have been assessed for hypoxia tolerance in Australia are typically limited to smaller bodied native species and other non-natives (Gehrke and Fielder 1988, Gehrke *et al.* 1993, McNeil and Closs 2007, McMaster and Bond 2008). Research to date has also focused on those species most frequently exposed to extreme and sustained hypoxic conditions (Gehrke and Fielder 1988, Gehrke *et al.* 1993, McNeil and Closs 2007, McMaster and Bond 2008). Nevertheless, many species experience hypoxic conditions infrequently and may have adapted to these natural events through behavioural avoidance. In this instance, species are unlikely to have built up long-term tolerances to severe hypoxic conditions. Informative data sources are required to understand the long-term effects of hypoxic exposure on typically mobile species. Our current understanding of responses to hypoxia in the Murray River region is predominantly based on presence/absence species assemblage data, with a paucity of pre-disturbance data and limited understanding regarding the true physiological impacts on native species.

Furthermore, very little work has been done to track changes in dissolved oxygen in the water over variable time periods. Otoliths have the potential to trace trends in fish exposure to hypoxia over broad temporal scales. Changes in elemental tracers or a combination of elements in the otoliths of fish may be useful as a proxy for reconstructing a timeline of hypoxia. A short-term history could be achieved using modern record keeping of dissolved oxygen levels. Reconstructing long-term trends of hypoxia in Australian freshwater systems could utilise archival collections of otoliths from native fish.

SCOPE AND OUTLINE OF THE THESIS

This thesis summarises my doctoral research focused on tracking hypoxic effects at two levels; the tolerance of individuals through physiological measures and the environmental occurrence of hypoxia using otoliths as tracers of hypoxic events. The thesis focuses predominantly on three native Australian fish species (Murray cod,

Maccullochella peelii, golden perch, *Macquaria ambigua* and silver perch, *Bidyanus bidyanus*) endemic to the Murray Darling Basin. All three species have experienced dramatic population declines and are classed from vulnerable to critically endangered on the IUCN Red list. First, I identify the sub-lethal impacts of reduced dissolved oxygen at different temperatures, with a focus on metabolic performance and acclimation ability, and second, I reconstruct past hypoxic events in the Murray River region over decadal and centennial timescales using chemical tracers in fish ear bones (otoliths).

My aim is to:

1. Better understand the sub-lethal impacts of combined low dissolved oxygen (hypoxia) and temperature on fish health in several native species from the Murray River region
2. Determine if exposure time exacerbates the impacts of hypoxia and elicits an acclimation response
3. Validate a key elemental tracer, or combination of elemental tracers to track changes in hypoxia over time using otolith chemistry: and,
4. Investigate the use of elemental tracers in otoliths to track long-term trends in hypoxic exposure in the Murray River region.

Although these research aims are addressed in different thesis chapters and form an independent body of work, collectively they build upon our understanding of hypoxic exposure on freshwater fish and its historical occurrence in the environment. This thesis addresses the gap in our understanding of long-term hypoxic exposure in freshwater species and the prevalence of hypoxic events in an inland system that has been heavily impacted by anthropogenic stressors and can serve as an example for other systems worldwide.

CHAPTER OUTLINE

Chapter 2

Chapter 2 investigates the effects of long-term exposure to combined temperature and hypoxic conditions on fish physiological tolerance. I examine the responses of golden perch and silver perch to sub-lethal combinations of temperature and hypoxia sustained over 10 months. Resting respirometry was used to observe a physiological response and loss of equilibrium was used to measure a behavioural response. I found one species had a higher tolerance to hypoxic exposure even showing an acclimation response while the other species failed to survive beyond one-month exposure to conditions that were anticipated to be sub-lethal. This chapter illustrates the disparity in response between sympatric species and highlights the need for reconsideration of generic management strategies for all species.

Chapter 3

Chapter 3 expands on the findings regarding acclimation responses after prolonged sub-lethal exposure to combined temperature and hypoxic conditions from my first chapter. Herein, I assess the responses of Murray cod and golden perch to sub-lethal combinations of temperature and hypoxia from 7 to 30 days. I use the same measures of behavioural and physiological tolerance as before, but to address a different question. Is acclimation achievable and if so, how long does it take? I found that longer exposure to hypoxia improved hypoxia tolerance, however, metabolic measures alone were not sufficient in explaining this response. This chapter illustrates that moving forward, researchers would benefit from considering the long-term effects of hypoxia on species instead of only short-term studies that typically address the immediate impacts of hypoxic events.

Chapter 4

Chapter 4 involves analysis of golden perch otoliths (ear bones) from juvenile fish that had long-term exposure to different combinations of temperature and hypoxia. As otoliths are metabolically inert and accrete material that reflects the environment on a daily basis, they are ideal for long-term environmental tracking where no other data exists. However, the environmental impact of interest, in this case hypoxia, first needs to be assigned a validated chemical tracer. The intent of this chapter was to

experimentally examine whether manganese or another element could be used as a tracer of hypoxia. I found that manganese in otoliths was not associated with hypoxia, nor were they affected by physiological regulation. I concluded that a lack of sediment in experimental tanks did not provide the reducing conditions required to release manganese into solution for incorporation onto the otoliths of fish.

Chapter 5

My final chapter builds upon the use of otoliths to trace hypoxic events through time. I do this using a combination of modern and historic otoliths collected from golden perch and Murray cod throughout the Murray Darling Basin. The species I chose are long-lived natives endemic to the Murray River with individuals aged from 2 to 42 years. I sampled transects running from the core of the otolith to the edge using laser ablation inductively coupled plasma mass-spectrometry (LA ICP-MS). I found elevated concentrations of manganese across different years of growth and combined this with information from digitised newspapers with recorded instances of hypoxic events, droughts and floods to illustrate the use of otoliths in tracing hypoxic occurrence in the Murray River over time. This research validates the use of this method for tracing environmental events over decadal and centennial scales.

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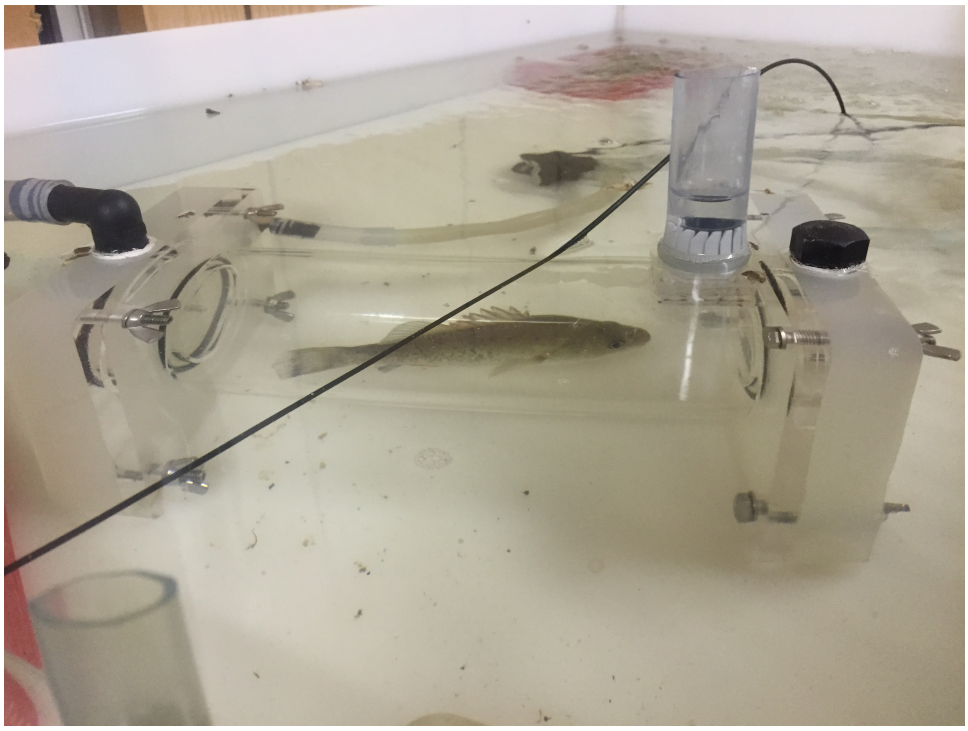
CHAPTER TWO

TESTING HYPOXIA: PHYSIOLOGICAL EFFECTS OF LONG-TERM EXPOSURE IN TWO FRESHWATER FISHES

Kayla L. Gilmore, Zoe A. Doubleday and Bronwyn M. Gillanders



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Above: Experimental setup used on native fish being trialled on Golden perch (*Macquaria ambigua*). Photo credit: Kayla Gilmore

STATEMENT OF AUTHORSHIP

This paper *Testing hypoxia: physiological effects of long-term exposure in two freshwater fishes*, has been published in *Oecologia*, and all authors made a contribution. Bronwyn M. Gillanders, Zoe A. Doubleday and myself conceived the experiment. I was responsible for the design of the experiment, performing the experiment and analysing data. I wrote the manuscript; Bronwyn M. Gillanders and Zoe A. Doubleday were crucial in reviewing work and provided editorial advice.

This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis.

Signatures of authors:

Kayla . Overall contribution 80%

Overall contribution 10%

Professor Bronwyn M. Gillanders Overall contribution 10%

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PHYSIOLOGICAL ECOLOGY - ORIGINAL RESEARCH

Testing hypoxia: physiological effects of long-term exposure in two freshwater fishes

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Abstract Hypoxic or oxygen-free zones are linked to large-scale mortalities of fauna in aquatic environments. Studies investigating the hypoxia tolerance of fish are limited and focused on marine species and short-term exposure. However, there has been minimal effort to understand the implications of long-term exposure on fish and their ability to acclimate. To test the effects of long-term exposure (months) of fish to hypoxia we devised a novel method to control the level of available oxygen. Juvenile golden perch (*Macquaria ambigua ambigua*), and silver perch (*Bidyanus bidyanus*), two key native species found within the Murray Darling Basin, Australia, were exposed to different temperatures (20, 24 and 28 °C) combined with normoxic (6–8 $mgO_2\ L^{-1}$ or 12–14 kPa) and hypoxic (3–4 $mgO_2\ L^{-1}$ or 7–9 kPa) conditions. After 10 months, fish were placed in individual respirometry chambers to measure standard and maximum metabolic rate (SMR and MMR), absolute aerobic scope (AAS) and hypoxia tolerance. Golden perch had a much higher tolerance to hypoxia exposure than silver perch, as most silver perch died after only 1 month exposure. Golden perch acclimated to hypoxia had reduced MMR at 20 and 28 °C, but there was no change to SMR. Long-term exposure to hypoxia improved the tolerance of golden perch to hypoxia, compared to individuals held under normoxic conditions suggesting that golden perch can acclimate to levels around 3 $mgO_2\ L^{-1}$ (kPa ~ 7) and lower. The contrasting tolerance of two sympatric fish species to hypoxia highlights our lack of understanding of how hypoxia effects fish after long-term exposure.

Keywords Metabolic scope · Sub-lethal · Threshold limit · Acclimation · Water management

Introduction

Hypoxia occurs when dissolved oxygen in the water goes below a level that can sustain the life of an organism and its natural capacity to function physiologically. Under such conditions organisms are unable to carry out vital processes such as feeding, reproduction, growth, migration and predator avoidance (Dean and Richardson 1999; Pollock et al. 2007). Hypoxia is not a new issue; in fact, it commonly occurs as a natural process, with anthropogenic activities contributing greatly to the severity and duration of hypoxic events in more recent times (King et al. 2012; Whitworth et al. 2012). Over 500 hypoxic areas or dead zones worldwide have been documented, predominantly associated with anthropogenic pressures and are expected to increase exponentially (by 5.54% per year Díaz and Rosenberg 2011; Vaquer-Sunyer and Duarte 2008).

The response of fish to hypoxia and temperature change is dependent on species and context, with conspecifics responding differently both physiologically and behaviourally (Collins et al. 2013; McNeil and Closs 2007; Nilsson et al. 2009). Fish exposed to hypoxia encounter a number of problems: short term (0.5–96 h) they face problems with maintaining oxygen uptake to meet basic metabolic

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maintenance requirements, reductions in aerobic scope, bactericidal activity, and antibody levels, the production of disease-fighting reactive oxygen species and a build-up of anaerobic by products (Díaz and Rosenberg 2011; Pörtner and Lannig 2009); long term (96 + h) they suffer reduced growth and fecundity, as well as altered behaviour and mortality (Breitburg et al. 2009; Pörtner and Lannig 2009). Indirectly, hypoxia can result in habitat loss (from forced migrations), increased predation pressure, and overall trophic changes, with some effects being irreversible or requiring extensive time for recovery (Collins et al. 2013; McCarthy et al. 2014; Vaquer-Sunyer and Duarte 2008). Fish can counter hypoxia by breathing air at the water's surface (aquatic surface respiration, ASR), escaping hypoxic areas if possible and reducing oxygen demand by decreasing activity; as well as increasing the oxygen carrying capacity of haemoglobin (Hb), depressing their metabolism and changing cardiac function (Cook et al. 2013; Timmerman and Chapman 2004; Rogers et al. 2016). Cellular and tissue modifications can also improve performance under hypoxic conditions, evident through lowering of the critical oxygen tension (P_{crit}), defined as the partial pressure of oxygen (Po_2) below which a stable rate of oxygen uptake can be maintained and is not reliant upon on ambient oxygen availability (Cook et al. 2013; Timmerman and Chapman 2004; Sollid et al. 2005). Furthermore, increased temperatures reduce the solubility of oxygen in water, thus reducing a fish's capacity to supply oxygen to tissues, compounding the problem of a low-oxygen environment (Dean and Richardson 1999; Farrell 2016; McCarthy et al. 2014). Thus, the combined effects of temperature and hypoxia can be devastating and may affect ecological communities in complex ways and elicit highly species-specific responses.

The response of organisms to hypoxia can be difficult to measure, particularly in combination with temperature. Much of our understanding is based upon abundance presence–absence studies and short-term physiological and behavioural studies (King et al. 2012; Zhang et al. 2010). Presence–absence studies are limited by populations being present at time of sampling, typically small numbers and sizes of sample sites, and limited prior knowledge of fish assemblages of the sample area (King et al. 2012). While physiological and behavioural studies have focussed on short-term effects of different stressors (i.e. < 100 h exposure to hypoxic conditions) to metabolic performance and behavioural responses of individual fish species, few studies have considered the longer term (chronic) exposure to multiple stressors (Richardson et al. 2001).

Respirometry, a physiological approach used to measure oxygen consumption rates ($\dot{M}o_2$) of aquatic organisms presents a unique opportunity to predict an organism's response to long-term exposure to environmental stressors (Roche et al. 2013). The metabolic scope of an individual is of particular interest as it shows the total capacity for energy use by aerobic pathways and can be estimated indirectly through measurements of oxygen consumption (Norin and Clark 2016). Furthermore, it can be influenced by a number of intrinsic and extrinsic factors such as activity level, body mass, temperature, food consumption and environmental conditions like hypoxia (Norin and Clark 2016; Chabot et al. 2016). Metabolic scope is calculated using the standard (resting) metabolic rate (SMR or $\dot{M}_{O_2,\min}$) and the maximum metabolic rate (MMR or $\dot{M}_{O_2,\max}$). The SMR represents the minimum rate of oxygen consumption (minimal cost of living) of a resting fish in a post-absorptive state at a given temperature (McBryan et al. 2013; Roche et al. 2013), while the MMR represents the maximum rate at which oxygen from the environment can be transported to the organism for consumption (McBryan et al. 2013; Roche et al. 2013). A fish's total aerobic scope for activity, the range of metabolic energy available for aerobic activity, can then be determined from SMR and MMR; this is referred to as the absolute aerobic scope (AAS)(Roche et al. 2013). Fish exposed to hypoxic conditions will suffer a reduction to their total metabolic scope. As such, it is necessary to understand the severity of this reduction and if prolonged hypoxia exposure would allow organisms to initiate an acclimation response.

Understanding the thresholds of fish to hypoxic conditions is crucial for establishing management targets to avoid high mortalities (Vaquer-Sunyer and Duarte 2008). There have been few attempts to determine thresholds of hypoxia for fish species and identify a limit for management purposes (Dean and Richardson 1999). Of the possible limits proposed the majority of the literature refers to a value of ~ $2 mg O_2 L^{-1}$ for all aquatic environments (Breitburg et al. 2009; Helz and Adelson 2013; Vaquer-Sunyer and Duarte 2008). Species-specific responses to hypoxic conditions suggest that this value may be inadequate in supporting whole system survival, and suggests that ecosystems may require independent reviews before instituting a limit for management (Dean and Richardson 1999; Vaquer-Sunyer and Duarte 2008).

To date, there are no known studies that have considered freshwater species and their tolerance to the combined effects of elevated temperatures and hypoxia. We investigated the independent and interactive effects of long-term exposure to low dissolved oxygen and temperature on the metabolic scope and tolerance of two freshwater fish species. We used juvenile golden perch (Percichthyidae: *Macquaria ambigua ambigua*) and silver perch (Terapontidae: *Bidyanus bidyanus*) these are key species found throughout Australia's largest inland freshwater river system, the Murray Darling Basin (MDB) and are classified as vulnerable, threatened or endangered dependent on region (for more species information see Supp. SSI 1, Lintermans 2007) were used. The MDB is a highly regulated system subject to extreme variations

in environmental conditions. Prolonged periods of severe drought are punctuated by periods of high rainfall and flooding, conditions that can result in hypoxic events particularly during summer. Golden perch and silver perch were exposed to prolonged hypoxic conditions at different temperatures; however, due to mortality of silver perch physiological tests were only performed on golden perch. We expect long-term exposure to hypoxic conditions will allow fish to acclimate to hypoxia. Furthermore, higher temperature treatments are likely to limit the metabolic scope and acclimation ability of fish even after prolonged hypoxic exposure.

Methods

Experimental design

Silver perch (*Bidyanus bidyanus,* average length 45 mm, average body mass 2.7 ± 0.5 g) and golden perch (*Macquaria ambigua ambigua*, average length 35 mm, average body mass 2.1 ± 0.5 g) were sourced from aquaculture stock from the NSW Hatchery Quality Assurance Scheme (HQAS) accredited Silverwater Native Fish Hatchery, Grong Grong, NSW, in March 2014. Upon arrival at the University of Adelaide, fish were held in 250-L holding tanks at 20 °C. Aged (dechlorinated) tap water was used in tanks throughout the pre-experimental and experimental periods. Silver perch were fed hatchery pellet food until satiation, with any excess siphoned out an hour after feeding. Golden perch were fed live black worm (*Lumbriculus variegatus*), with waste siphoned out 24 h after feeding. Diets of both fish were matched to those at the hatchery: silver perch were fed pellets (sourced from Silverwater Native Fish Hatchery) and golden perch were fed live blackworm (sourced from Seaview Aquarium Centre, Plympton, SA). Fish were exposed to a 12:12-h light:dark cycle and room temperature was maintained at 20 °C. Water quality was monitored every second day for pH, ammonia and nitrite, with 25% water changes made daily for silver perch and every other day for golden perch. For both pre-experimental and experimental periods all tanks were aerated, and water in each tank was filtered using independent submersible aquarium filters for the duration of the experiment. Evaporation was minimised by covering tanks with clear plexiglass lids.

Fish were randomly assigned to 20-L treatment tanks 10 days after arrival to give sufficient time to adjust to the new conditions, with about 11 fish per tank. The experimental design consisted of two oxygen treatments, normoxic (6–8 $mgO_2\ L^{-1}$ or 12–14 kPa) and hypoxic (3–4 $mgO_2\ L^{-1}$ or 7–9 kPa), combined with up to three temperature treatments (20, 24 and 28 °C). Treatments included all possible combinations of temperature and oxygen for golden perch, with duplicate tanks for each treatment ($n = 12$ tanks), while silver perch included all possible combinations of two temperatures (20 °C and 28 °C) and oxygen with duplicate tanks for each treatment ($n = 8$ tanks). The experimental design differed for silver perch as there were less individuals available. The temperatures chosen reflected a portion of the natural thermal range experienced by both species (from 4 to 34 °C across their entire natural range) and are most likely to be affected by hypoxic conditions (Lintermans 2007). All tanks with temperatures ≥ 24 °C were heated independently using submersible aquarium heaters; temperature was monitored regularly. The desired temperatures were reached by adjusting heaters by 2 °C per day. Oxygen levels were based on the globally accepted tolerance limit of 2 $mgO_2\ L^{-1}$ which is expected to cause high levels of stress and mortality for most species (Vaquer-Sunyer and Duarte 2008). The experiment was designed to provide long-term exposure yet still subject species to low levels of oxygen; therefore, the hypoxic treatments were higher than this limit, enough to cause slight discomfort but not mortalities. At the completion of all experiments fish were measured and weighed to calculate a simple condition index, Fulton's K, which assumes that the weight of a fish is proportional to the cube of its length:

$$K = 100\left(W/L^3\right),$$

where W is body wet weight (g) and L the total standard length (cm), 100 is used to bring the factor close to a value of one. Fulton's K condition factor is widely used in fish biology studies to describe the condition of the individual (Nash et al. 2006).

Control of hypoxia

For the hypoxic treatments a simple and novel degassing system was developed, which used nitrogen gas to remove oxygen from the water (see Fig. 1). A combination of 9 L/min of nitrogen, split across 3 G-Class nitrogen cylinders (food grade, 3 L/min per cylinder) was mixed with 9 L/min of air in a loosely sealed 35-L mixing tank. Cylinders were changed on average every 3 days. The mixing tank contained two electric air pumps, with a combined flow rate of less than 18 L/min, which pumped the mixed gas to individual aquarium tanks, using air hosing (of equal distance) connected to a single air stone (of the same size) in each tank. Air was pumped into the mixing tank from an air compressor. Tanks were covered with plexiglass lids to minimise turbulence and limit diffusion of surrounding atmospheric air. This method allowed oxygen levels to be simultaneously controlled in all tanks over an extended period, and could be adjusted to suit both larger and smaller experiments.

Maximum exposure periods for silver perch and golden perch were 87 and 247 days, respectively. Exposure times varied as physiology experiments were carried out over

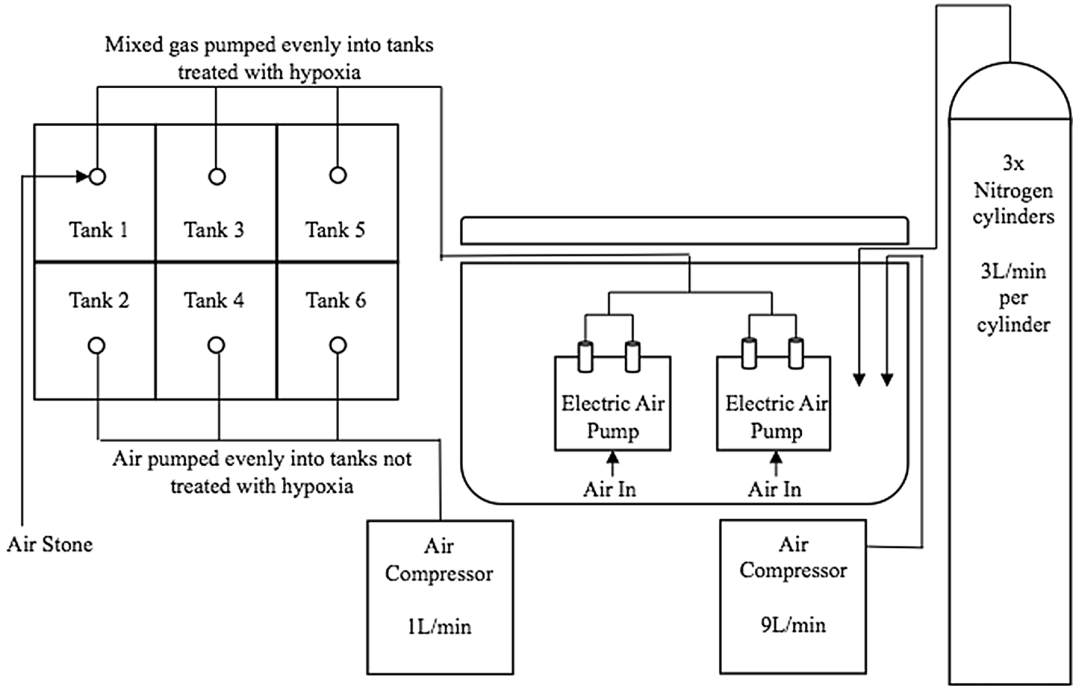

Fig. 1 Schematic of degassing system used for the control of hypoxia showing setup for six tanks

20 weeks, which included the initial experimental trials. Physiological trials were randomised such that there was little variation of exposure to experimental conditions among fish relative to the total exposure time.

Resting respirometry

Resting respirometry was only conducted on golden perch due to high mortalities of silver perch in the initial experiment. All fish were fasted for 24 h prior to experimental trials to evacuate the digestive tract so that only oxygen consumption rates ($\dot{M}o_2$) were recorded. Approximately 12 fish, per treatment, were randomly selected and subjected to respirometry experiments.

A four-chamber system was designed so that multiple fish could be tested simultaneously. Each resting chamber was custom made to fit the size of the fish based on a 1:10 ratio (1 kg of animal for every 10 L of water), and was 300 mL in volume. All four chambers were submerged in a larger water bath (139 × 52 × 20 cm), which was used to control temperature and oxygen levels. Individual chambers used a closed recirculation loop to pump low flowing water over the fish. A fibre optic oxygen probe (Pyroscience, OXROB3, Aachen, Germany) was fitted to the recirculation loop in each chamber; this recorded oxygen consumed during each $\dot{M}o_2$ determination. Each chamber was connected to a flushing pump that circulated water intermittently after each $\dot{M}o_2$ determination to completely replenish the chamber with oxygenated water from the water bath. A 5-min flushing period and a 20-min determination period was used for the 20 °C normoxic and hypoxic treatments; however, this was reduced to a 2-min flush and 20-min determination period for the remaining treatments as the 5-min flush caused stress for fish at higher temperatures. Stress was indicated by fish not maintaining a neutral or central position in the middle of the chamber during flushing. During the 20-min determination period oxygen was not reduced to less than 1 $mgO_2\ L^{-1}$ and was above the background respiration rates. Dissolved oxygen concentration was recorded using four fibre optic oxygen probes (OXROB3) in a four-channel FireSting O_2 Optical Oxygen Meter (Pyroscience, Aachen, Germany), with one probe fitted to the recirculation loop of each chamber.

To determine maximum metabolic rate (MMR), we used a method described by Roche et al. (2013), where fish were subjected to a 3-min exhaustive chase followed by a 1-min air exposure prior to being placed in respirometry chambers. This method forces fish to reach their MMR in a short time and can be determined using the highest $\dot{M}o_2$ determination value. However, the 3- and 1-min combination was too stressful for the golden perch at higher temperatures, such that they were unable to recover from

3 min of chase and 1 min of air exposure; these times were, therefore, reduced to a 2-min exhaustive chase with 40-s air exposure for the 24 and 28 °C treatments. During the exhaustive chase, individual fish were placed in a 25-L bucket and were encouraged to continue swimming by gently touching the tip of the tail; fish were only encouraged if they slowed down or stopped swimming. Once the exhaustive chase was complete fish were suspended in a mesh aquarium net out of the water for 40–60 s and then immediately placed into a chamber. The first determination period was started 1 min after each fish was placed in a chamber, whereby maximum metabolic rate (MMR) was measured. Following this determination period, fish were left in the chamber for 8–12 h to allow fish to reach a resting state. Once the resting state was reached, the standard metabolic rate (SMR) was calculated using the equation below.

$\dot{M}o_2$ ($mgO_2\ kg^{-1}\ h^{-1}$) was calculated for each determination cycle using the equation:

$$\dot{M}O_2 = \left([O_2]_{t0} - [O_2]_{t1}\right) \cdot \frac{V}{t} \cdot \frac{1}{BW},$$

where (t_0) is the oxygen content of the water measured at the end of a flushing cycle, and (t_1) is the oxygen content measured at the end of a determination period, just prior to the flush, both measured in mg O_2/L. V is the volume of the chamber minus the volume of the experimental animal in L, t is t_0–t_1 and BW is body weight of the experimental animal in kg. The average of the lowest 10% of total measurements was used to calculate SMR. Three determinations were run before and after the testing period to record background values of bacterial respiration. Background rates were subtracted from $\dot{M}o_2$ values. To reduce background respiration water was pumped through a heater/chiller unit fitted with a UV lamp that sterilized the water. The absolute aerobic scope (AAS) for activity of fish was calculated by subtracting SMR from MMR (MMR − SMR). The whole system was rinsed every third day to ensure background consumption of oxygen remained below 15% of the resting metabolic rate of fish.

Determining tolerances to hypoxia

To record tolerance limits among the different treatments, fish were left in chambers for an additional period of time with the intermittent flushing cycle turned off, which normally replenishes the system with oxygen. We defined the tolerance limit as the point at which an individual fish showed signs of stress at low oxygen levels. A sign of stress was indicated by a significant burst reaction in the chamber or loss of equilibrium. Fish were observed constantly during this period. At the first signs of stress, the oxygen level and time were recorded and the fish was immediately removed from the chamber. A tolerance limit was recorded for each fish.

Critical oxygen tension or P_{crit}

The critical oxygen tension or P_{crit} was measured using data from the closed respirometry phase of the experiment. To minimise the effects of CO_2 accumulation, metabolic products and reductions in pH the chamber was flushed after acclimation. The P_{crit} was defined as the point at which $\dot{M}o_2$ was reduced below SMR and fish shifted to an oxy-conforming state. The P_{crit} was determined for each fish by fitting a segmented regression using RStudio Version 1.0.143 (RStudio Team 2016). The critical tension was recorded as the point of intersection of the two lines as this indicated the breakpoint at which oxy-regulating changed to oxy-conforming. This measure differed from the hypoxia tolerance measure as it occurred prior to fish losing equilibrium.

Statistical analyses

Statistical analyses were conducted using PRIMER 6 and PERMANOVA + software (www.primer-e.com). Temperature, DO and oxygen saturation of the water, and Fulton's K were analysed for both species at all possible temperature and treatment levels and for tank effects using a three-way permutational univariate analysis of variance (ANOVA) with unrestricted permutations. Temperature and hypoxia treatment levels (hypoxic or normoxic) were treated as fixed factors with replicate tanks treated as a random factor nested in temperature and hypoxia treatment. The same ANOVA design was used to analyse data regarding MMR, SMR, AAS, P_{crit} and tolerance limits. Where tank effects were not detected, data were pooled and re-analysed using a two-way permutational univariate ANOVA with unrestricted permutations. Post hoc pairwise tests were conducted where significant differences were found. All analyses included Monte Carlo tests to ensure that there were sufficient permutations to detect significant differences.

Results

Rearing conditions

Treatment conditions remained consistent throughout the experimental period for both species and fish length and weight were similar among treatments for each species (< 0.5 g/cm, Table S1). Significant tank effects were detected; however, these were generally less than the variation among treatments (Table S2). All significant effects of temperature and hypoxia on water conditions were in line with the experimental treatment designs and changes

had minimal variation (temperature < 0.7 °C, dissolved oxygen < 1 mgO_2 L^{-1} and saturation < 8%) over the course of the experiment (Table S1).

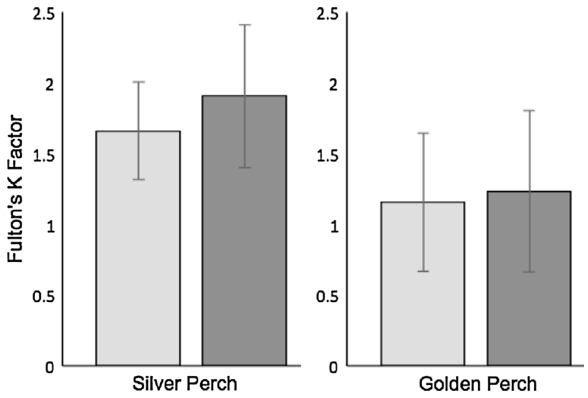

Fig. 2 Mean (± SE) Fulton's K for silver perch (*Bidyanus bidyanus, n* = 93), and golden perch (*Macquaria ambigua ambigua, n* = 127), by hypoxia treatment (light grey bars represent hypoxic conditions, dark grey bars represent normoxia). Data for tank and temperature have been pooled

Fish condition

Body condition, Fulton's K, did not vary for golden perch despite long-term exposure to varied environmental conditions (Table S3, $P \leq 0.05$). Silver perch in hypoxic conditions had a significantly lower Fulton's K than those in normoxic conditions (Table S3, Fig. 2). A between-species comparison also shows that golden perch overall had a poorer body condition than silver perch; this may be because of the length of time species were exposed to treatment conditions (Fig. 2).

Survivorship

Mortality recorded during the rearing period for both species showed variation between the two species (golden perch and silver perch) (Fig. 3). Silver perch suffered significant high levels of mortality during the rearing period with no fish surviving beyond week 14 ($P < 0.0001$, Kaplan–Meier, IBM SPSS Statistics 24.0.0.1). Silver perch treated under hypoxic conditions suffered more than 50% mortality by week 2 at 28 °C, and by week 3 at 20 °C. In contrast, silver perch in normoxic conditions suffered more than 50% mortalities in week 5 at 28 °C and week 12 at 20 °C. Comparatively,

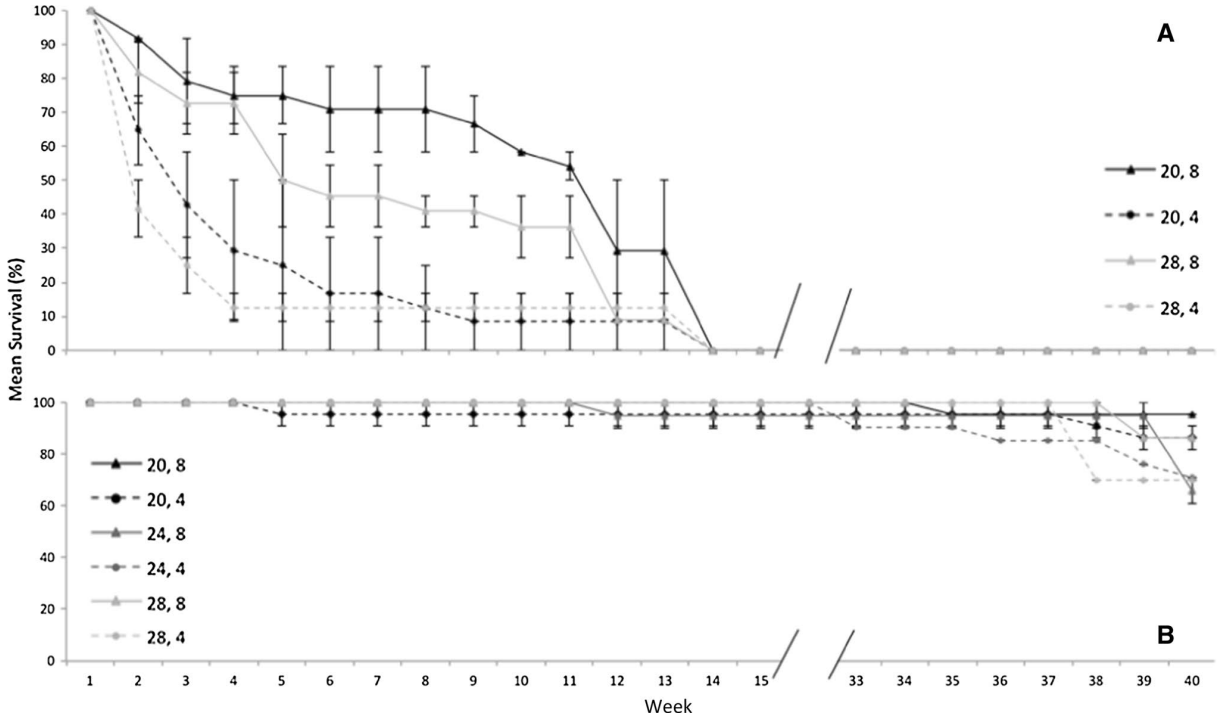

Fig. 3 Weekly mean survival (± SE) for replicate tanks of each treatment for **a** silver perch (*n* = 93) and **b** golden perch (*n* = 127). Survival is calculated as a percentage of fish surviving in each tank and then the mean of the two tanks is shown. Silver perch were only exposed to two of the three temperature treatments

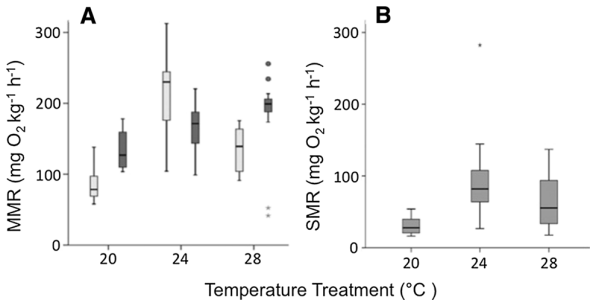

Fig. 4 Boxplots (95% quantile) indicating maximum metabolic rate (MMR) and standard metabolic rate (SMR) recorded for golden perch at all temperatures and hypoxia treatments; replicate tanks are pooled. A) MMR where light grey bars represent hypoxic conditions (4 mg L^{-1}), and dark grey bars represent normoxia (8 mg L^{-1}, n = 69). **b** SMR where data were pooled for each temperature because significant differences were not found for hypoxia at each temperature (n = 69). Circles (○) represent outliers that fell between 1.5 and 3 interquartile ranges from the nearest edge of the box and stars (*) represent outliers beyond 3 interquartile ranges from the nearest edge of the box, and lines in the centre of box represent the median point

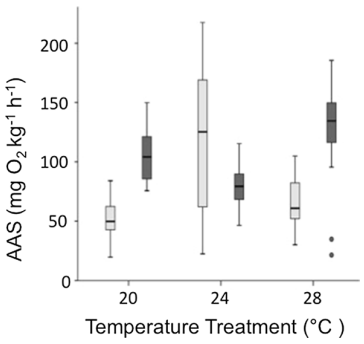

Fig. 5 Boxplot (95% quantile) showing absolute aerobic scope (AAS) for golden perch at all temperature and hypoxia treatments; replicate tanks are pooled (n = 69). Light grey bars represent hypoxic conditions (4 mg L^{-1}), and dark grey bars represent normoxia (8 mg L^{-1}). Circles (○) represent outliers, and lines in the centre of box represent the median point

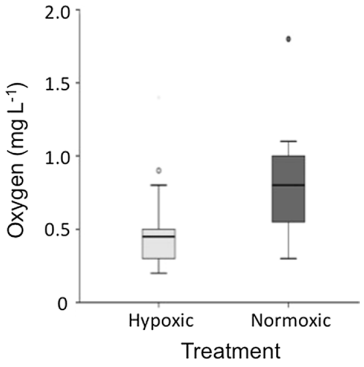

Fig. 6 Boxplot (95% quantile) showing the hypoxia tolerance limits for individual golden perch at normoxic (dark grey, 8 mg L^{-1}) and hypoxic (light grey, 4 mg L^{-1}) conditions; replicate tanks and temperatures are pooled, as there were no significant temperature or tank effects detected (n = 69). Circles (○) represent outliers, and lines in the centre of box represent the median point

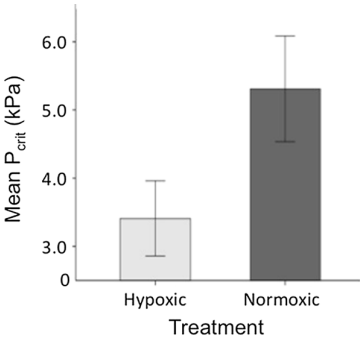

Fig. 7 Mean (± SE) P_{crit} for golden perch by hypoxia treatment (light grey bar represents hypoxic conditions and the dark grey bar represents normoxic). Data for tank and temperature have been pooled (n = 60)

golden perch treated under the same conditions (Table S2) suffered few mortalities over the full 40-week period.

Metabolic scope

As silver perch suffered high mortalities during the rearing period, metabolic variables were only measured on golden perch. Long-term exposure to hypoxia at 20 and 28 °C resulted in lower SMR and MMRs compared to 24 °C for golden perch (Fig. 4, Table S4). Maximum metabolic rate and SMR showed no significant effects of hypoxia although there was a significant interaction between temperature and hypoxia for MMR (Table S4). Significant differences were detected among temperature treatments for both SMR and MMR (Table S4, Fig. 4). Lower AAS measures occurred at 20 and 28 °C in fish exposed to hypoxia long term compared to those treated under normoxia, while fish exposed to hypoxia at 24 °C had higher AAS than those treated under normoxia (Fig. 5).

Hypoxia tolerance limits and P_{crit}

Hypoxia tolerance limits and critical oxygen tension (P_{crit}) of fish were significantly higher at normoxic conditions but did not vary among temperatures (Table S5, Figs. 6 and 7).

Discussion

Our study showed that sympatric species have different responses to thermal and hypoxic stress. Despite exposure

to the same conditions as golden perch in our initial experimental rearing period, silver perch were unable to cope and suffered high levels of mortality (50% mortality by week 3 in hypoxic treatments). Golden perch, however, suffered few mortalities over the whole experimental period. Additionally, golden perch were able to tolerate hypoxic conditions after prolonged exposure. The behaviour and tolerance of fish exposed to hypoxia often differs among and within species and also with size (Eliason and Farrell 2016; McCarthy et al. 2014; Metcalfe et al. 2016). Multiple studies have demonstrated highly species-specific responses to thermal and hypoxic stress, in some cases influenced by fish making physiological trade-offs associated with specific natal conditions (Eliason and Farrell 2016; McNeil and Closs 2007). We observed that thermal stress accelerated the decline of silver perch, particularly those treated under hypoxic conditions. Due to mortality during the initial experiment, physiological performance of silver perch was not tested; however, the effect of hypoxia on body condition of this species may indicate that exposure to hypoxic conditions quickly degenerates their overall health. In comparison, there was minimal impact on the body condition of golden perch held under the same conditions, but their overall condition was lower than silver perch. As all measures on body condition were conducted after the 40-week exposure period differences in condition between species may be attributed to golden perch withstanding the full 40 weeks of exposure. Differences in the diet between the two species may have also driven differences in body condition. Silver perch were fed a pellet food specifically designed to enhance fitness in this species (Rowland 2008, 2009; Rowland and Tully 2004), while golden perch were fed unenriched live food. Golden perch and silver perch inhabit similar zones in the MDB, including lowland, turbid and slow-flowing rivers with snags and rocky outcrops (Lintermans 2007). Historically, the distribution of silver and golden perch was similar; however, changes to river regulation, reproductive behaviour, migration patterns through the addition of weirs and dams, threat of alien species, thermal pollution, hypoxic episodes and flow regime have resulted in a severe decline in silver perch distribution, while golden perch are still widespread, albeit at reduced numbers (for more species information see Supp. SSI 1, Koehn and Nicol 2016; Lintermans 2007). Without knowing the complete genetic history of silver perch used in our study it is possible they do not fully reflect wild populations, although the hatchery fish were sourced from are one of many involved in restocking wild populations throughout the MDB. However, given the dramatic decline seen in this species over the last 50-year exposure to hypoxia and high temperatures in our laboratory experiment may explain why their natural range has diminished (Lintermans 2007; Rowland 2008, 2009; Rowland and Tully 2004).

Long-term exposure to hypoxia directly affected the tolerance thresholds of golden perch. Fish exposed to hypoxia were able to tolerate lower levels of dissolved oxygen, while a lack of exposure (long-term normoxia) resulted in fish reaching their tolerance thresholds at higher levels of dissolved oxygen. Critical oxygen tensions (P_{crit}) of golden perch also followed the same pattern, suggesting that long-term exposure to hypoxia induced an acclimation response (for other examples see: Cook et al. 2013, Timmerman and Chapman 2004; Rogers et al. 2016). Additionally, higher temperatures had no effect on the hypoxia tolerance of golden perch, which may be due to a thermal acclimation response from prolonged exposure (McMaster and Bond 2008). Although golden perch may persist in low levels of dissolved oxygen and partial pressure (Po_2) it is necessary to consider at what cost this occurs and if they use other options in the wild such as aquatic surface respiration (ASR), increasing gill ventilation or simply move away. For example, a study on the tolerance of zebrafish (*Danio rerio*) found that individuals could persist to levels of 1 $mgO_2\ L^{-1}$ and lower; however, it was at the cost of poor-performing antioxidant enzymes, which resulted in oxidative damage (Feng et al. 2016). Environmental factors such as temperature, food intake and diet composition may also act to change this value in wild fish; therefore, oxygen thresholds of fish should always be used carefully for management. For example, our results suggest golden perch would be protected under the current universal threshold limit (2 $mgO_2\ L^{-1}$), but silver perch would be unlikely to survive (Vaquer-Sunyer and Duarte 2008). The threshold limit for silver perch may be higher than 4 $mgO_2\ L^{-1}$ given the high mortalities during the experimental rearing period even under normoxia; however, they are known to continue feeding at dissolved oxygen concentrations below 3$mgO_2\ L^{-1}$ (Rowland 2009). Even though the global limit provides a standard for managers to work from it is difficult to predict what this value means for wild populations. Adapting this information to existing models for the MDB would allow managers to predict larger scale impacts of hypoxic events and appropriate dissolved oxygen levels and Po_2 for highly oxygenated water releases to combat hypoxic events spreading. Models could further incorporate physiological details such as thermal thresholds, oxygen carrying capacity, aerobic scope, and the growth, digestion and reproductive requirements of fish to provide a comprehensive way of predicting impacts on local populations. There are some examples of models being used effectively in this way for predicting how future environmental conditions will impact Pacific salmonids (Eliason and Farrell 2016; Hague et al. 2011; Rand et al. 2006). However, there may be some limits to this method due to the clear species-specific reactions to hypoxia, thus

models considering the most sensitive species among local populations would be most appropriate. Other researchers have also observed fish under hypoxia reaching tolerance points much faster than those treated under normoxia (Dean and Richardson 1999; Fu et al. 2011).

Temperature is widely considered the principal controlling factor for aerobic and metabolic capacity, while hypoxia is considered the primary limiting factor (Claireaux and Chabot 2016; Pörtner and Lannig 2009). Long-term exposure to hypoxia typically limited the aerobic and metabolic capacity (AAS and MMR) of golden perch at 20 and 28 °C, while overall fish had a lower basal oxygen demand (standard metabolic rate, SMR) at the same temperatures. Therefore, aerobic and metabolic capacity may be limited beyond these points, such that it deleteriously impacts normal functioning (Farrell 2016; Neuheimer et al. 2011). However, at 24 °C prolonged hypoxia exposure typically improved the aerobic and metabolic capacity of golden perch, compared to those fish treated under normoxia. However, possible thermal acclimation of golden perch could confound some of our results, particularly the higher MMRs observed under normoxia at 28 °C (Farrell 2016; McBryan et al. 2013; McMaster and Bond 2008). Thermal acclimation can be achieved by reducing general metabolism via reduced feeding and movement, the downregulation of protein synthesis or the decrease and/or modification of certain regulatory enzymes in aerobic and anaerobic pathways (McMaster and Bond 2008; Wu 2002). For example, Chilko, *Oncorhynchus nerka*, have an enhanced cardiac capacity due to a higher density of adrenaline-binding ventricular β-adrenoreceptors, giving them a broader thermal range compared to co-migrators Nechako, *O. nerka* (Eliason et al. 2011). When fish are also exposed to hypoxia the capacity for oxygen transfer through haemoglobin and the circulatory system is reduced, this would seem to be the case for golden perch AAS and MMR, suggesting their thermal range may be limited by prolonged hypoxia exposure. The lower SMRs observed in golden perch overall at 28 °C could also be a result of thermal acclimation as fish at higher temperatures would be expected to grow and metabolise faster than those at lower temperatures. In a marine system, elevated levels of ambient CO_2 would have a similar limiting effect as hypoxia, highlighting the necessity for future physiological studies to consider the synergistic effects of environmental factors (Pörtner and Lannig 2009).

Fish respond to hypoxia in many different ways and while the lethal endpoint has been used in the past to assess safe levels of dissolved oxygen for fish, the sub-lethal tolerance limits are likely to be the most useful (Feng et al. 2016). Fish tolerance to a sub-lethal point will indicate the sensitivity of other vital functions such as growth and reproduction (Feng et al. 2016). Organisms recovery from hypoxic events can be partially attributed to the availability of nearby refuges and species capacity to adapt, exploit oxygen-rich zones and recolonize an area successfully after an event (Conley et al. 2009; McMaster and Bond 2008). However, if those organisms are unable to relocate or survive a hypoxic event there is very little chance of system recovery. Conley et al. (2009) suggested that systems which have been previously exposed to hypoxia are more prone to experience it in the future and suffer a slower recovery with each incidence. For example, hypoxic events resulting in large-scale losses of benthic communities lead to a change in overall trophic structure, with smaller, fast-growing species recolonizing an area first, impacting not only community structure but complete system functioning with deleterious effects to the storage capacity of sediments (Conley et al. 2009; Diaz and Rosenberg 2008). Only ~ 4% of the 500-plus systems affected by hypoxia worldwide have shown signs of recovery (Diaz and Rosenberg 2008).

Supply of oxygen to aquatic organisms worldwide is going to be affected by climate change, with models predicting substantial warming and deoxygenation throughout much of the world's oceans and terrestrial water bodies (Deutsch et al. 2015). Due to the disparity observed among our two case study species, it will be necessary to consider management targets carefully to ensure the survival of all species within any one system (Vaquer-Sunyer and Duarte 2008). We show some species may be able to develop natural resistance to poor oxygen conditions over time; however, this may be limited to only those with a naturally higher tolerance to hypoxia. Furthermore, future research should be targeted towards understanding the individual tolerance of known sensitive species within a system. We recommend that it is valuable to consider each system individually in terms of species and the effects of large-scale water allocation on dissolved oxygen content. Poor management of reallocated waters will influence local fauna where managers do not consider the complete spectrum of organism tolerance to hypoxia. Sympatric populations of fish under hypoxic stress in our system exhibited distinctly different responses to prolonged hypoxia exposure, and while it appears acclimation is achievable it remains species specific.

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Author contribution statement KLG, BMG and ZAD conceived the experiment, KLG was responsible for the design of the experiment, performing the experiment and analysing data. KLG wrote the

manuscript; BMG and ZAD were crucial in reviewing work and provided editorial advice.

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SUPPLEMENTARY INFORMATION

Study Species Information 1:

Percichthyidae: *Macquaria ambigua* (Golden perch, Yellowbelly, Callop, Murray perch)

Terapontidae: *Bidyanus bidyanus* (Silver perch, Black bream, Silver bream, Bidyan)

Golden perch and silver perch are two sympatric endemic species found throughout the Murray Darling Basin, Australia. Golden perch grow as large as 76cm in length with weights recorded up to 23kg. Silver perch grow as large as 50cm in length with weights recorded up to 8kg (Lintermans 2007). Golden perch males reach maturity at two years and females at four years, and silver perch reach maturity between 3-5 years for both sexes (Lintermans 2007). The Murray-Darling system encompasses five separate states and is the largest catchment in Australia covering approx. one million km^2 (Koehn and Nicol 2016). Golden perch are opportunistic carnivores while silver perch are omnivores (Lintermans 2007). Both species are found predominantly in lowland, turbid and slow flowing rivers, it has also been suggested that golden perch prefer deeper pools found within these habitats (Lintermans 2007). Golden perch and silver perch are both bred artificially by government and commercial hatcheries and are widely stocked in farm dams, lakes, streams and reservoirs (Lintermans 2007). Abundance of these species has been drastically reduced across their natural range due to both natural (e.g. high temperature, hypoxia) and anthropogenic factors (e.g. extensive barriers in place which limit fish passage and environmental watering, Koehn and Nicol 2016).

Table S1. Summary of the rearing conditions and length and weight data of the fish within treatment tanks for the experiments. Data are displayed as means ± standard error (SE) with *n* represents the number of fish assigned to each treatment. For temperature, dissolved oxygen and saturation, *n* denotes the number of recordings taken throughout experimental period; this differed as the experiment with golden perch ran for a longer time.

Water Condition	Hypoxia, mg L^{-1}	Tank	Temperature °C	Dissolved Oxygen, mg L^{-1}	Saturation, %	Fish Length, cm	Fish Weight, g	*n*
Silver Perch			(*n*=50)	(*n*=50)	(*n*=50)			
20	Hypoxic	1	19.4±0.20	3.92±0.09	57.2±0.96	4.8±0.18	1.9±0.28	11
		2	19.2±0.15	3.59±0.09	49.6±1.38	5.2±0.21	2.5±0.48	12
	Normoxic	1	18.3±0.13	6.63±0.05	89.2±1.56	5.4±0.39	2.9±0.85	12
		2	18.4±0.25	6.73±0.05	91.0±0.57	5.5±0.35	3.1±0.44	12
28	Hypoxic	1	28.3±0.03	3.18±0.07	51.6±1.11	5.3±0.44	2.3±0.74	12
		2	27.1±0.04	3.02±0.07	48.0±1.05	5.0±0.35	2.2±0.55	12
	Normoxic	1	27.9±0.08	5.60±0.04	89.9±0.58	5.5±0.46	3.4±0.71	11
		2	27.7±0.02	5.54±0.02	86.1±1.04	5.5±0.37	3.4±0.63	11
Golden Perch			(*n*=100)	(*n*=100)	(*n*=100)			
20	Hypoxic	1	19.3±0.09	3.82±0.07	53.0±0.94	5.4±0.28	1.8±0.39	11
		2	18.8±0.08	4.17±0.10	55.5±1.30	5.6±0.22	1.8±0.30	11
	Normoxic	1	19.7±0.07	6.70±0.05	92.4±0.92	5.4±0.03	2.3±0.50	10
		2	19.1±0.16	6.74±0.04	92.3±0.56	5.7±0.26	2.3±0.75	11
24	Hypoxic	1	24.3±0.03	3.36±0.05	49.6±0.69	6.0±0.39	2.4±0.65	10
		2	24.7±0.07	3.32±0.06	50.0±0.94	5.6±0.16	1.9±0.28	11
	Normoxic	1	23.4±0.03	5.96±0.03	88.2±0.36	5.9±0.36	1.9±0.56	10
		2	24.1±0.05	5.65±0.03	86.2±0.42	5.7±0.25	2.1±0.50	11
28	Hypoxic	1	27.3±0.05	3.30±0.05	51.1±0.72	5.7±0.43	2.1±0.90	10
		2	27.4±0.03	3.03±0.06	47.3±0.91	5.6±0.52	2.6±0.50	10
	Normoxic	1	26.9±0.05	5.63±0.03	88.1±0.36	5.2±0.15	1.8±0.62	11
		2	26.4±0.12	5.59±0.03	88.5±0.41	5.6±0.31	2.3±0.47	11

Table S2. Analysis of variance for the effects of temperature, dissolved oxygen (DO), oxygen saturation (SAT) and hypoxia in the rearing water.

Water	Source of Variation	df	MS	F	P
Silver Perch					
Temp	Temp	1	7612.9	809.12	≤0.001*
	Hypoxia	1	17.75	1.89	>0.050
	Temp X Hypoxia	1	24.85	2.64	>0.050
	Tank (Temp X Hypoxia)	4	9.43	11.56	≤0.001*
	Residuals	377	0.82		
DO	Temp	1	74.12	90.11	≤0.001*
	Hypoxia	1	697.81	847.3	≤0.001*
	Temp X Hypoxia	1	5.09	6.19	>0.050
	Tank (Temp X Hypoxia)	4	0.83	4.15	<0.050*
	Residuals	377	0.21		
SAT	Temp	1	781.29	1.55	>0.050
	Hypoxia	1	1.34	266.65	≤0.001*
	Temp X Hypoxia	1	52.07	0.10	>0.050
	Tank (Temp X Hypoxia)	4	504.94	8.75	≤0.001*
	Residuals	377	57.72		
Golden Perch					
Temp	Temp	2	5886.9	506.02	≤0.001*
	Hypoxia	1	39.31	3.38	>0.050
	Temp X Hypoxia	2	32.84	2.82	>0.050
	Tank (Temp X Hypoxia)	6	11.64	20.02	≤0.001*
	Residuals	1148	0.58		
DO	Temp	2	99.88	41.30	≤0.001*
	Hypoxia	1	1881.5	778.35	≤0.001*
	Temp X Hypoxia	2	2.14	0.89	>0.050
	Tank (Temp X Hypoxia)	6	2.42	8.68	≤0.001*
	Residuals	1148	0.28		
SAT	Temp	2	2787.1	13.96	<0.050*
	Hypoxia	1	4.23	2118.9	≤0.001*
	Temp X Hypoxia	2	73.60	0.37	>0.050
	Tank (Temp X Hypoxia)	6	199.81	3.57	≤0.001*
	Residuals	1148	55.91		

** Denotes significant effect*

Table S3. Analysis of variance for the effects of tank, temperature and hypoxia on body condition, calculated using Fulton's K, of silver perch and golden perch.

Fulton's K Condition Index	Sources of Variation	df	MS	F	P
Silver Perch	Temp	2	0.39	2.48	>0.050
	Hypoxia	1	1.36	8.66	<0.050*
	Temp X Hypoxia	2	0.39	2.51	>0.050
	Tank(Temp X Hypoxia)	6	0.16	0.63	>0.050
	Residuals	85	0.24		
Golden Perch	Temp	2	0.81	3.81	>0.050
	Hypoxia	1	2.49	0.12	>0.050
	Temp X Hypoxia	2	5.34	0.25	>0.050
	Tank(Temp X Hypoxia)	6	0.21	0.70	>0.050
	Residuals	63	0.30		

**Denotes significant effect*

Table S4. Analysis of variance for the effects of temperature and hypoxia on the metabolic rates of golden perch. All possible temperature (20, 24 and 28°C) and hypoxic treatments (normoxic and hypoxic) are considered. No tank effects were detected so data were pooled.

Metabolic Rate	Sources of Variation	df	MS	F	P
MMR	Temp	2	35709	18.12	≤0.001*
	Hypoxia	1	3347.6	1.71	>0.050
	Temp X Hypoxia	2	41421	10.51	≤0.001*
	Residuals	63	1970.4		
SMR	Temp	2	19515	13.76	≤0.001*
	Hypoxia	1	1638.8	1.16	>0.050
	Temp X Hypoxia	2	216.69	0.15	>0.050
	Residuals	63	1418.1		
AAS	Temp	2	2460	1.69	>0.050
	Hypoxia	1	9671	6.64	≤0.001*
	Temp X Hypoxia	2	17408	11.96	≤0.001*
	Residuals	63	1456.2		

** Denotes significant effect.*

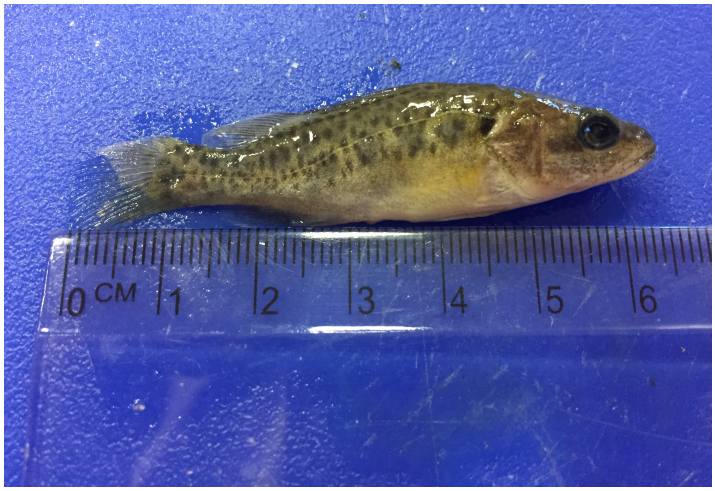
Table S5. Analysis of variance for the effects of temperature and hypoxia on the ability of golden perch to tolerate hypoxic conditions (recorded once fish exhibited bursting or equilibrium loss) and their critical oxygen tension (P_{crit}). All possible temperature (20, 24 and 28°C) and hypoxic combinations (normoxic and hypoxic) were considered. No tank effects were detected so data were pooled.

Hypoxia Tolerance	Sources of Variation	df	MS	F	P
Tolerance Limits	Temp	2	0.12	1.73	>0.050
	Hypoxia	1	1.48	22.95	≤0.001*
	Temp X Hypoxia	2	0.13	2.0068	>0.050
	Residuals	64	0.0065		
P_{crit}	Temp	2	4.93	1.53	>0.050
	Hypoxia	1	52.48	16.24	≤0.001*
	Temp X Hypoxia	2	2.52	0.78	>0.050
	Residuals	50	3.23		

** Denotes significant effect*

CHAPTER THREE

PROLONGED EXPOSURE TO LOW OXYGEN IMPROVES HYPOXIA TOLERANCE IN A FRESHWATER FISH



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Above: Murray cod (*Maccullochella peelii*) juvenile being measured before dissection. Photo credits: Kayla Gilmore

STATEMENT OF AUTHORSHIP

This paper *Prolonged exposure to hypoxia improves hypoxia tolerance in a freshwater fish*, has been re-submitted for publication to *Conservation Physiology* following revision, and all authors made a contribution. Bronwyn M. Gillanders, Zoe A. Doubleday and myself conceived the ideas for the manuscript. I designed the methodology and collected and analysed the data. I led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis.

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Overall contribution 10%

Professor Bronwyn M. Gillanders Overall contribution 10%

ABSTRACT

Persistent hypoxic conditions in aquatic systems are becoming more frequent worldwide, causing large-scale mortalities to aquatic fauna. It is poorly understood, however, whether species can acclimate to long-term hypoxic conditions. In two experiments we exposed juvenile freshwater fish (Murray cod, *Maccullochella peelii*), to low-oxygen conditions and investigated acclimation effects. Experiment 1 determined how responses could be modified by exposure to different temperatures (20, 24 and 28°C) and oxygen conditions (normoxia/control 6-8mgO_2 L^{-1} and hypoxia/low-oxygen 3-4mgO_2 L^{-1}), over 30 days. Experiment 2 determined the acclimation ability of fish exposed to two temperatures (20 and 28°C) and low-oxygen conditions (hypoxia/low-oxygen 3-4mgO_2 L^{-1}) for three different acclimation periods (7, 14 and 30 days). Responses were measured by determining critical oxygen tension (P_{crit}), loss of equilibrium and aerobic capacity using resting respirometry. In experiment 1 resting oxygen requirements were negatively affected by long-term low-oxygen exposure except at the highest temperature (28°C). However, long-term acclimation in low-oxygen improved tolerance as measured by loss of equilibrium but not P_{crit}. In experiment 2 fish could tolerate lower oxygen levels before reaching loss of equilibrium after 7 days acclimation, but this declined overtime. Murray cod were most tolerant to low-oxygen at the lowest temperature (20°C) and shortest exposure time (7 days). Extended low-oxygen exposure resulted in reduced aerobic capacity of fish particularly at the lowest temperature. While prior exposure to low-oxygen may allow fish to cope with hypoxic conditions better in the long-term, acclimation time was inversely related to tolerance, suggesting that resistance to hypoxia might decrease as a function of exposure time. Our study fills a much-needed gap in our understanding of how freshwater species acclimate to hypoxia; in particular how exposure to prolonged periods of low-oxygen and elevated temperatures affect organisms physiologically.

INTRODUCTION

Hypoxia can be fatal to many organisms including mammals, birds, fish, reptiles and invertebrates (Hermes-Lima and Zenteno-Savín 2002, Bickler and Buck 2007, Ramirez *et al.* 2007). However, countless species have become adapted to periods of hypoxia ranging from hours to months (Hermes-Lima and Zenteno-Savín 2002, Bickler and Buck 2007). Extensive research has shown many of the biochemical and physiological mechanisms that allow animals to endure oxidative stress (for reviews see: Hochachka and Lutz 2001, Hermes-Lima and Zenteno-Savín 2002, Bickler and Buck 2007, Ramirez *et al.* 2007). However, the focus has mostly been on the cellular response pathways, protein synthesis, gene expression and metabolic constraints of organisms under short-term (hours) exposure to hypoxia with few studies considering long-term exposure to hypoxia and the potential for a species to acclimate to low-oxygen conditions.

Fishes have adapted to almost all aquatic habitats on Earth and can be found living in some of the most extreme environments, yet they are also considered to be some of the most sensitive taxa to hypoxia (Vaquer-Sunyer and Duarte 2008, Gräns *et al.* 2014). However, the ability of fish to acclimate and adapt to hypoxia has received little attention. Furthermore, fish species have not evolved to tolerate all conditions simultaneously and often exhibit species-specific responses, for example, certain species tolerate temperatures from -2°C in the polar regions to +44°C in African lakes and from $1 mgO_2 L^{-1}$ of oxygen to $8 mgO_2 L^{-1}$ (Gräns *et al.* 2014). Phylogenetic comparisons of fish species show that hypoxia tolerance has arisen independently many times among different lineages and geographical locations (Hochachka and Lutz 2001, Mandic *et al.* 2009, Killen *et al.* 2016). Research on the effects of hypoxia is mainly focussed on marine and estuarine species, with considerably less attention given to freshwater species (Diaz and Rosenberg 2011, Rogers *et al.* 2016). The paucity of research examining acclimation of fish, particularly for freshwater species, highlights the need for additional research.

Resting respirometry is one method used to determine the thermal tolerance of fish, and more recently, hypoxia tolerance (Roche *et al.* 2013, Nelson and Lipkey 2015). Thermal tolerance, in addition to hypoxia tolerance, is an important consideration for physiological and behavioural studies as it affects both oxygen demand and the amount of dissolved oxygen available in the water (McBryan *et al.* 2013, Claireaux and Chabot 2016). For example, every 10°C increase in temperature results in a 10 to 20% decrease in dissolved oxygen (Farrell and Richards 2009). Therefore, fish that experience a broad thermal range, such as freshwater and estuarine species, will be strongly influenced by changes in oxygen levels and temperature. Resting respiromentry represents an ideal experimental solution to predict and test organism responses to multiple levels of environmental conditions like hypoxia and temperature.

Rapid changes in water partial oxygen pressure (PO_2), can have dire consequences on aquatic fauna as their capacity to respond to hypoxia is dictated by functioning physiological and biochemical systems in place at the time of exposure (Farrell and Richards 2009). If fish are unable to extract oxygen efficiently from the environment during progressive hypoxia exposure they become less tolerant (Farrell and Richards 2009). Some fish may be able to acclimate by initiating physiological and biochemical changes to enhance body function and extend survival, however, the temporal scope of this resistance is poorly understood. Furthermore, as increased temperatures result in a reduction in available dissolved oxygen in the water, temperature may also diminish the resistance of fish to hypoxia, as it increases metabolism in ectotherms. The temporal resistance of fish to hypoxia and their acclimation ability has been largely overlooked in the literature.

We investigated whether prior exposure to hypoxia or low-oxygen could improve the tolerance of freshwater fish to hypoxic conditions. We used Murray cod (*Maccullochella peelii*), a susceptible freshwater fish native to the Murray Darling Basin (MDB), an extensive river system that is frequently influenced by natural and anthropogenic hypoxic events (for further species information see SI). First, we tested how Murray cod responded to long-term low-oxygen exposure at different temperatures, and then we tested how low oxygen exposure for different lengths of time and different temperatures modified fish responses. We measured aerobic capacity, the critical oxygen limit of fish (P_{crit}) and loss of equilibrium to determine if

there was an acclimation response to low-oxygen. We predicted that a) the combination of high temperatures and prolonged exposure to low-oxygen would exacerbate the effects of hypoxia and limit a fish's ability to acclimate and b) we predicted that duration of exposure to low-oxygen would alter the acclimation capacity of fish such that, longer duration of exposure would decrease acclimation capacity.

METHODS

Experimental Design

Juvenile Murray cod (*Maccullochella peelii*), approximately 55mm in length and 1.5g average body mass, were obtained from aquaculture stock from the NSW Hatchery Quality Assurance Scheme (HQAS) accredited Silverwater Native Fish Hatchery, Grong Grong, NSW, in March 2015. Fish were kept in 250L holding tanks at 20°C at the University of Adelaide before being assigned to 20L tanks for the experimental treatments. All tanks (250L holding tanks and 20L experimental tanks) were filled using aged (dechlorinated) tap water and aerated and filtered for the duration of the experiments; evaporation was minimised using plexiglass lids. Fish were fed hatchery pellet food until satiation with excess food siphoned out an hour after feeding. Room temperature was maintained at 20°C and fish were exposed to a 12:12hr light:dark cycle. Water changes of 25% were made daily and water quality was monitored every second day for temperature, oxygen levels and saturation, pH, ammonia and nitrite.

For Experiment 1 we exposed fish for 30 days to two oxygen treatments (normoxic 6-8mgO_2 L^{-1} or 12-14 kPa and low-oxygen 3-4mgO_2 L^{-1} or 7-9 kPa), and three temperature treatments reflective of the species' natural thermal range; (20, 24 and 28°C; Lintermans 2007) in an orthogonal design (n=6 treatments). Each treatment was duplicated resulting in 12 tanks. Murray cod experience temperatures ranging from 4-34°C across their natural range; temperatures chosen reflect those most likely to be experienced at the higher end of the thermal range as this range would be most affected by low-oxygen exposure (Lintermans 2007). Experiment 2 consisted of three acclimation treatments (7, 14 and 30 days exposure to low-oxygen conditions 3-4mgO_2 L^{-1} or 7-9 kPa) and two temperature treatments (20 and 28°C) again with duplicate tanks in an orthogonal design (n=6 treatments, 3 acclimation durations x 2 temperatures). However, only 8 new tanks (including duplicates) were used for Experiment 2 as fish from low-oxygen treatments in Experiment 1 at 20 and 28°C were used to measure 30 days of acclimation. Experiment 2 was run following the completion of Experiment 1. Fish were randomly assigned to tanks (20L), with about 11 fish per tank. Fish in treatments ≥24°C were acclimated 2°C per day by adjusting the submersible aquarium heaters. To ensure survival of fish during experimentation, low-oxygen levels (3-4mgO_2 L^{-1} or 7-9 kPa) were higher than the globally accepted

tolerance limit of $2mgO_2\ L^{-1}$, which is believed to frequently result in mortality of aquatic species (Vaquer-Sunyer and Duarte 2008).

The experiment was designed to provide long-term exposure yet still subject fish to low-oxygen conditions and minimise mortality of individuals (i.e. sub-lethal treatments) while allowing physiological responses to be tested. For the low-oxygen treatments we developed a simple method to deoxygenate the water (Gilmore *et al.* 2018). Nitrogen gas (9L/min split across 3 food grade G-Class nitrogen cylinders, at 3L/min/cylinder) was mixed with 9L/min of air (from an air compressor) in a loosely sealed 35L mixing chamber. Two electric air pumps running within the mixing chamber pumped the mixed gas into relevant individual tanks using air hosing (of equal distance) connected to single air stones (of the same size) with a combined flow rate of ~18L/min. Plexiglass lids covered all tanks to minimise turbulence and limit diffusion of surrounding atmospheric air. Oxygen levels in all 14 'low-oxygen' tanks could be simultaneously controlled for extended periods (Experiment 1 low-oxygen tanks = 6, control = 6, Experiment 2 low-oxygen tanks = 8, the additional 4 tanks required in this analysis were from Experiment 1).

Length and weight of each fish was measured at the completion of the experiments and used to calculate a simple condition index, Fulton's K, which assumes the weight of a fish is proportional to the cube of its length:

$$K = 100\ (W/L^3)$$

Where W is body wet weight (g) and L the total standard length (McMaster and Bond), 100 is used to bring the factor close to a value of one. Fulton's K condition index is widely used in fish biology studies to describe condition of the individual and has been used in our experiment to show how condition may have changed in the different treatments (Nash *et al.* 2006).

Experimental treatment conditions were maintained consistently for all experimental periods (SUPP. Table 1, 2). Fish lengths and weights showed little variation among treatments (SUPP. Table 1).

Intermittent Respirometry

Following 7, 14 or 30 days exposure, fish were fasted for 24hrs prior to experimental trials to evacuate the digestive tract so that only oxygen consumption rates ($\dot{M}o_2$) were recorded. Fasting fish were held in an isolated container in the larger water bath where respirometry experiments were conducted, to ensure there was no shock experienced prior to being placed in resting chambers. Twelve fish per treatment were randomly selected and subjected to respirometry experiments.

Three fish were tested simultaneously using a 4-chamber system (each 300mL volume), custom made to fit the fish (1kg animal: 10L water). All chambers were submerged in a larger water bath (139x52x20cm), where temperature and oxygen levels were controlled and set to match experimental treatments. A closed recirculation loop pumped low flowing water over the fish in individual chambers. To reduce background respiration water was pumped through a heater/chiller unit fitted with a UV lamp to sterilise the water. Further, the whole system was rinsed every third day to ensure background consumption of oxygen remained below 15% of the resting metabolic rate of fish. The remaining chamber was used to record background respiration each day; and was randomised each day of recording.

Each chamber was fitted with a fibre optic oxygen probe (FireSting, Pyroscience, OXROB3), which recorded oxygen consumed during each $\dot{M}o_2$ determination ($mgO_2kg^{-1}h^{-1}$). A $\dot{M}o_2$ determination period uses the slope of the line of oxygen consumption by fish for each 20 minute determination period before water is replenished to the chamber for 2 minutes (flushing period). Water was circulated intermittently after each $\dot{M}o_2$ determination using a flushing pump connected to all chambers to completely replenish the chamber with oxygenated water from the water bath. During the 20min determination period oxygen was not reduced to less than $1mgO_2\ L^{-1}$ and was above the background respiration rates. Maximum and standard metabolic rates (MMR and SMR) were determined using a modified version of the method described by Roche *et al.* (2013), where fish were chased to exhaustion for 2min or until fish stopped responding and exposed to the air for 40sec before being placed inside chambers. For the exhaustive chase individual fish were placed in a 25L bucket and encouraged to swim continuously by gently touching the tip of the tail. At the completion of the exhaustive chase fish were suspended in air in a mesh net for

40sec and then placed immediately inside a chamber. MMR was measured during the first determination period. Fish were then left in the chamber for ~24hrs to allow them to reach a resting state. The SMR and $\dot{M}o_2$ was calculated for each determination cycle using the equation:

$$\dot{\mathrm{M}}O_2 = ([O_2]_{t0} - [O_2]_{t1}).\frac{V}{t}.\frac{1}{BW}$$

where (t0), is the oxygen content (mgO_2/L) of the water at the conclusion of a flushing cycle, and (t1), is the oxygen content measured at the end of a determination period, prior to the next flushing cycle. V is the volume of the chamber minus the volume of the experimental animal in L, t, is t0-t1, and BW, is body weight of the experimental animal in kg. The lowest 10% of measurements were averaged to calculate SMR. Background rates were subtracted from $\dot{M}o_2$ values upon calculation. The absolute aerobic scope of fish was calculated by subtracting SMR from MMR (MMR-SMR).

Determining Tolerance to Low-Oxygen and Critical Oxygen Tension (P_{crit})

In order to record tolerance limits of fish among the different treatments we left fish in chambers with the intermittent flushing cycle turned off with only access to the oxygen available from water in the chamber (closed respirometry). Fish were observed constantly during this period. Fish reached a low-oxygen tolerance limit when they lost equilibrium, at which point oxygen level in $mgO_2\ L^{-1}$ was recorded and fish were immediately removed from the chamber. The critical oxygen tension or P_{crit} of fish was measured using data from this closed respirometry phase. P_{crit} was defined as the point at which $\dot{M}_{O2}$ was reduced below SMR and fish shifted to an oxy-conforming state. P_{crit} was determined for each fish by fitting a segmented regression using RStudio Version 1.1.419 (segmented package, https://cran.r-project.org/web/packages/segmented/segmented.pdf), a method adapted from Yeager and Ultsch (1989) and Cook *et al.* (2013). The critical tension was recorded as the point of intersection of the two lines as this indicated the breakpoint at which oxy-regulating individuals changed to oxy-conforming individuals. This measure differed from the low-oxygen tolerance point as it occurred prior to fish losing equilibrium.

Statistical Analyses

A linear mixed effects model (lmm) was fit to the experimental data for MMR, SMR, AAS, P_{crit} and the low-oxygen tolerance at loss of equilibrium using the R-package lmerTest (Kuznetsova *et al.* 2017). Factors included temperature and oxygen for Experiment 1 and temperature and acclimation duration for Experiment 2. Prior to model fitting the distribution of the response variable was inspected using quantile comparison plots. Each of the response variables closely followed a normal distribution, and hence this was considered the most appropriate distribution to model the data and warranted the use of the lmm. All treatment levels of temperature, oxygen (normoxic or low-oxygen) and acclimation duration (7, 14 or 30 days) were treated as fixed factors in both experiments. *Post hoc* pairwise tests were conducted using the least squares means of the fixed effects where significant effects of the fixed factors were evident in the linear mixed effects model. Tank was treated as a random effect. To assess the variance component of the models the residuals were plotted against the fitted values. The random scatter observed around zero indicated constant variance across the fitted values for each model. All model analyses were undertaken using R-Studio Version 1.1.419 (R Core Team 2018).

Rearing water temperature, dissolved oxygen (DO) and oxygen saturation in the experimental tanks, as well as Fulton's K factor, were analysed at all possible treatment levels (temperature, oxygen and acclimation duration) using a 2-factor permutational univariate analysis of variance (ANOVA) with unrestricted permutations using PRIMER 6 & PERMANOVA+ software (www.primer-e.com). All PERMANOVA+ analyses included Monte Carlo permutation tests to derive the probability value and ensure there were sufficient permutations to detect significant differences in all tests. All PERMANOVA+ statistical analyses were initially conducted as 3-factor permutational univariate ANOVAs with tank as the third factor treated as a random factor nested in temperature, oxygen or acclimation duration dependent on the experiment. No effects of tank were detected for rearing water or during any of the experimental responses in either experiment therefore data were pooled and 2-factor permutational univariate ANOVAs were conducted removing tank as a factor.

RESULTS

Experiment 1: Effects of temperature and low-oxygen on fish physiology after 30 days exposure

Metabolic Scope

There was an interactive effect between temperature and oxygen treatments on SMR, with fish having higher SMR within the low-oxygen treatments except at the highest temperature (28°C, p=<0.050 Table S3, Figure 1A). SMR was higher in low-oxygen treatments than in normoxic treatments at 20°C (p=0.020, Figure 1A, Table S3). MMR and AAS were not affected by low-oxygen exposure or temperature treatments (Table S3).

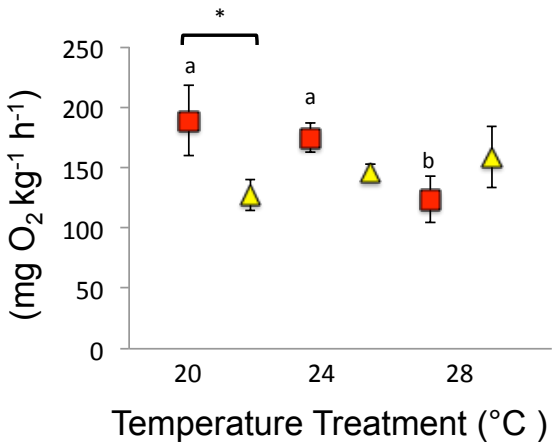

Figure 1. Mean (±SE) standard metabolic rate (SMR) for individual Murray cod at all temperatures (20, 24, and 28°C) and treatments (low-oxygen, 3-4mgO_2 L^{-1} or 7-9 kPa, and normoxia, 6-8mgO_2 L^{-1} or 12-14 kPa), replicate tanks were pooled. Multifactorial pairwise comparisons are indicated by letters and asterisks where significant differences occur ($p<0.05$). Letters indicate significant differences occurring among temperatures for each oxygen level (normoxic or low-oxygen). Brackets and asterisks indicate significant differences occurring between oxygen levels for each temperature. Red squares represent low-oxygen, 20°C n=8, 24°C n=11 and 28°C n=9, and yellow triangles represent normoxia 20°C n=11, 24°C n=11 and 28°C n=10.

Low oxygen Tolerance Limits & P_{crit}

Fish exposed to low-oxygen maintained equilibrium for longer than those exposed to normoxic conditions (p=0.022, Figure 2A, Table S4). The P_{crit} of fish was unaffected by hypoxia or temperature (hypoxia p=0.121 and temperature p=0.332, Table S6).

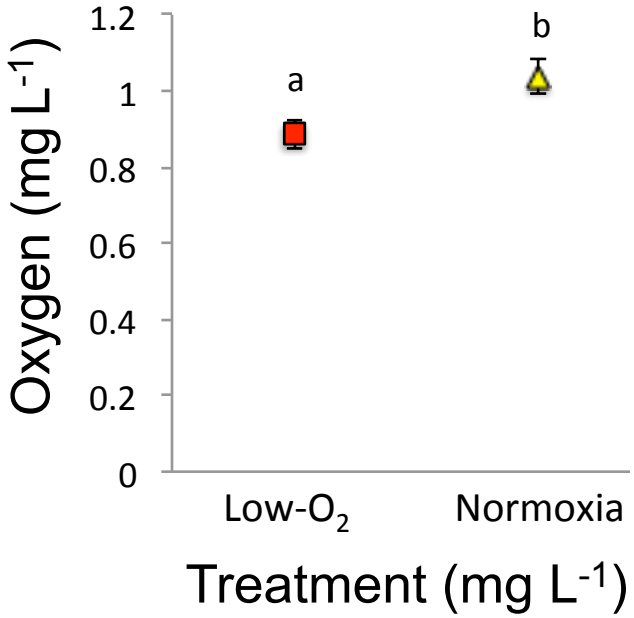

Figure 2. Mean (±SE) low-oxygen tolerance at loss of equilibrium from exposure to low-oxygen or normoxia for individual Murray cod (low-oxygen 3-4mgO_2 L^{-1} or 7-9 kPa, n=28 and normoxia 6-8mgO_2 L^{-1} or 12-14 kPa, n=34). Multifactorial pairwise comparisons are indicated by letters where significant differences occur ($p<0.05$). Red squares represent low-oxygen and yellow triangles represent normoxia.

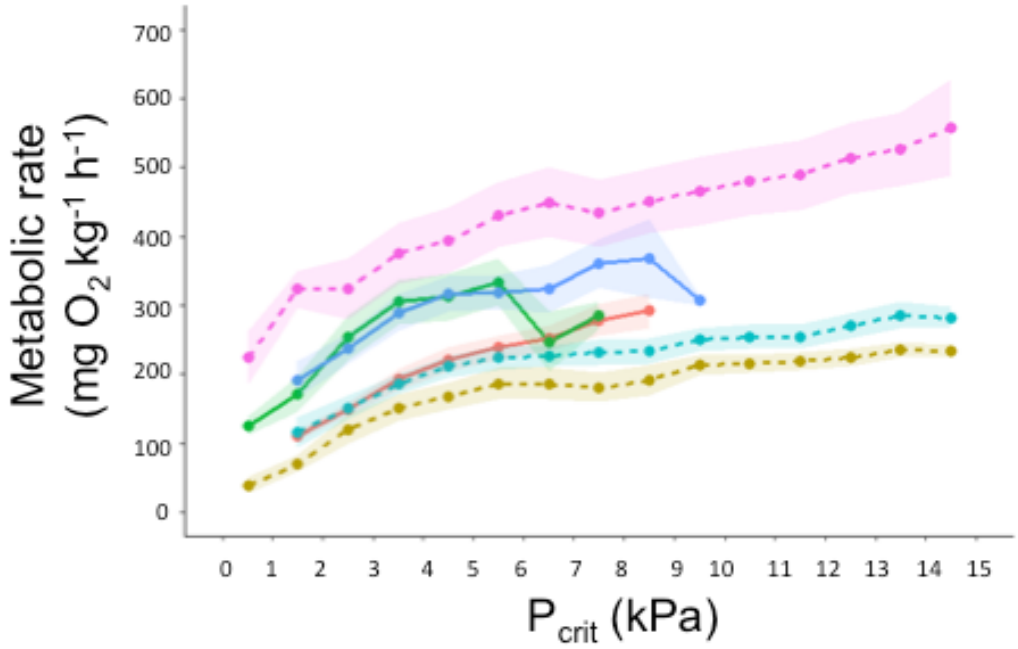

Figure 3. P_{crit} and average metabolic rate of Murray cod at different kPa's, temperature's (20, 24 and 28°C) and oxygen exposures (low-oxygen, 3-4mgO_2 L^{-1} or 7-9 kPa, solid lines or normoxia, 6-8mgO_2 L^{-1} or 12-14 kPa, dashed lines). Treatments are distinguished by colour with yellow representing 20°C under normoxia (n=10), red representing 20°C under low-oxygen (n=8), teal representing 24°C under normoxia (n=11), green representing 24°C under low-oxygen (n=11), pink representing 28°C under normoxia (n=9) and blue representing 28°C under low-oxygen (n=9). Shading around the lines indicates standard error.

Experiment 2: Acclimation of fish at two temperatures under low-oxygen conditions

Metabolic Scope

Murray cod had the highest aerobic capacity (AAS) after 14 days exposure to low-oxygen and the lowest after 30 days exposure (p=<0.001, Figure 4A, Table S5). No effect of temperature was detected for AAS (p=0.06, Table S5). There was an interaction between temperature and exposure time for both measures of metabolic rate (SMR and MMR) with fish held at 28°C, and 14 days exposure to low-oxygen having the highest metabolic rates (p=<0.001, Figure 4B & C, Table S5). Fish held at 20°C and 7 days exposure had the lowest metabolic rates (p=<0.001, Figure 4B & C, Table S5). Additionally, fish had higher metabolic rates after 7 and 14 days exposure at 28°C compared to 20°C in both SMR and MMR (p=<0.001, Figure 4B & C, Table S5).

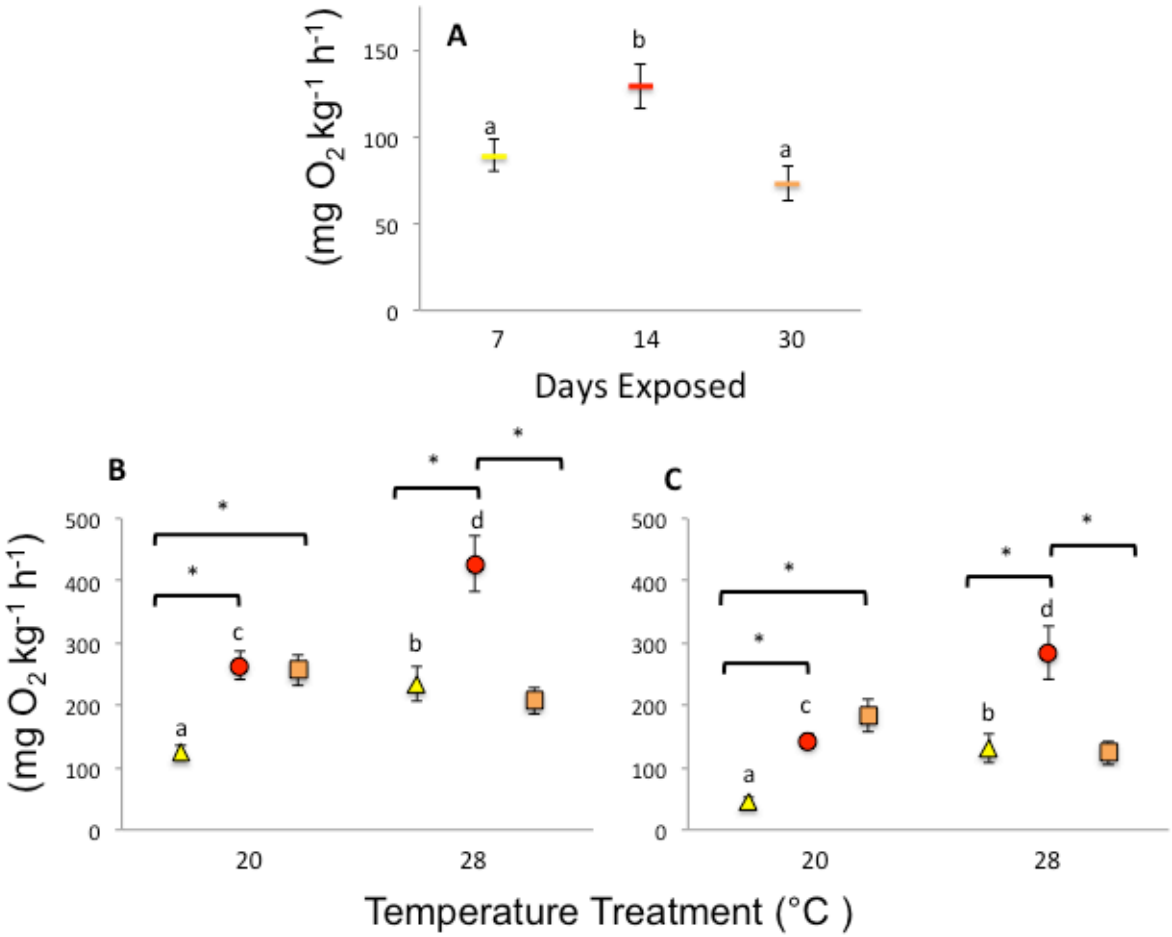

Figure 4. Mean (±SE) A) absolute aerobic scope (AAS) for Murray cod exposed to low-oxygen for different acclimation times (7, n=20, 14, n=16, and 30 days, n=17); temperature treatments and replicate tanks have been pooled as no significant effects were detected. B) Maximum metabolic rates (MMR) and C) standard metabolic rates (SMR) for Murray cod exposed to low-oxygen at 20 and 28°C, for three different acclimation times (7, 14 and 30 days). Shapes and colours have been used to differentiate acclimation times (yellow triangle = 7 days, red circle = 14 days and orange square = 30 days), except in A) where rectangles were used as temperatures were pooled. Multifactorial pairwise comparisons are indicated by letters and asterisks where significant differences occur ($p<0.05$). Letters indicate significant differences occurring between temperatures for each acclimation time (7, 14 or 30 days). Brackets and asterisks indicate significant differences occurring among acclimation times for each temperature. For both B) and C) yellow triangles represent seven days exposure at 20°C n=11 and 28°C n=9, red circles represent fourteen days exposure at 20°C n=10 and 28°C n=6, and orange squares represent thirty days exposure at 20°C n=9 and 28°C n=9.

Loss of Equilibrium & P_{crit}

Fish exhibited the greatest tolerance to low-oxygen at 20°C after only 7 days exposure, however, fish held at 28°C had the greatest tolerances after 30 days exposure to low-oxygen (Post-hoc; between 20 & 28°C after 7 days, p=0.005, and between different acclimation times at 20°C, 7 & 14 days, p=0.006 and 7 & 30 days, p=0.013 and at 28°C between 14 & 30 days, p=0.019; Figure 5, Table S6). The P_{crit} of fish was unaffected by acclimation time or temperature (acclimation time p=0.160 and temperature p=0.376, Table S6).

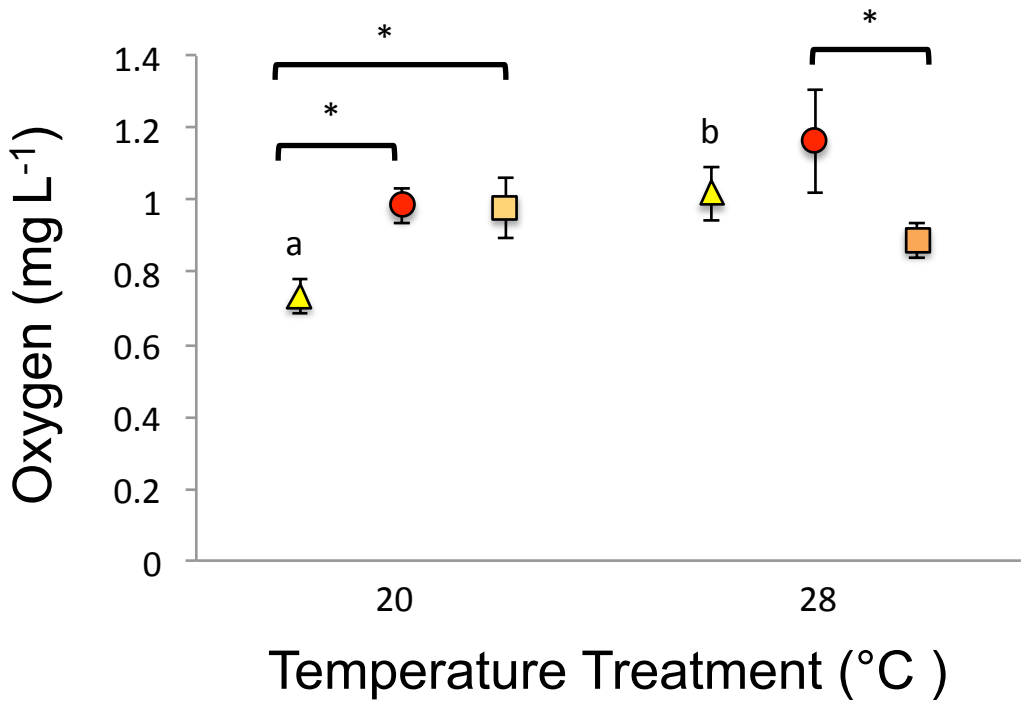

Figure 5. Mean (±SE) low-oxygen tolerance at loss of equilibrium for individual Murray cod exposed to low-oxygen at 20 and 28°C, for three different acclimation times (7, 14 and 30 days). Multifactorial pairwise comparisons are indicated by letters and asterisks where significant differences occur ($p<0.05$). Letters indicate significant differences between temperatures for each acclimation time (7, 14 or 30 days). Brackets and asterisks indicate significant differences occurring among the three acclimation times for each temperature. Yellow triangles represent seven days exposure at 20°C n=11 and 28°C n=9, red circles represent fourteen days exposure at 20°C n=13 and 28°C n=6, and orange squares represent thirty days exposure at 20°C n=9 and 28°C n=9.

Fish Condition

Fish condition (Fulton's K) was lowest at 28°C after exposure to low-oxygen for one month (Post-hoc; after exposure to low-oxygen between 20 & 28°C p=0.001 and 24 & 28°C p=0.001; Table S7, Figure S1A). During the acclimation trials, fish exposed to low-oxygen for 7 days were healthier (had a higher Fulton's K) than fish exposed for longer (Post-hoc; between 7 & 14 days p=0.001 and 7 & 30 days p=0.001), and fish reared at 20°C fared much better during all acclimation periods than those reared at 28°C (p=0.001; Table S7, Figure S1B).

DISCUSSION

Our results show that prior exposure to low-oxygen could improve the tolerance of fish to hypoxic conditions. Additionally we showed that time spent under low-oxygen conditions impacted physiological performance. However, counter to expectations, physiological performance (aerobic ability) did not improve with exposure to low-oxygen compared with fish exposed to normoxia.

Prolonged exposure to low-oxygen improved the tolerance of fish relative to those exposed to normoxia, allowing them to tolerate lower levels of dissolved oxygen before losing equilibrium. Additionally, acclimation time influenced low-oxygen tolerance such that fish exposed for greater periods of time had poorer tolerance, though critical oxygen tension (P_{crit}) remained unaffected. However, the difference between average loss of equilibrium after low-oxygen exposure in our treatments was small compared to normoxic treatments (<1mg L^{-1}), suggesting that it may not equate to a significant response in the wild. Acclimation of fish to hypoxia may be a naturally selected trait for species living in areas prone to low oxygen. For example, acclimation to seasonal hypoxia and diel hypoxia exposure has improved the tolerance of a number of fish species (Collins *et al.* 2016, McBryan *et al.* 2016, Rogers *et al.* 2016). However, chronic and daily exposure over long time periods does not always result in improved tolerance particularly in sensitive fish species (Cook *et al.* 2013, Remen *et al.* 2013). Our results suggest that prior exposure to low-oxygen conditions may improve tolerance of fish to hypoxia but that prolonged acclimation time to those conditions may significantly reduce survival. Multiple studies have found unique adaptations to hypoxia based on life history and habitat use (McBryan *et*

al. 2016). Species which experience a greater fluctuation of environmental conditions such as those inhabiting temperate, estuarine and freshwater systems, are likely to have a higher level of plasticity than species which remain in stable/slow-changing environments. Freshwater species may well have improved hypoxia tolerance due to more frequent exposure than saltwater species; this has been supported by differences in P_{crit} between the two groups (Rogers *et al.* 2016). Our study species, Murray cod, has a broad geographical distribution and likely adapted to a wide range of thermal and hypoxic conditions, and this study provides new hypoxia data for a species of conservation priority (for further species information see SI; Koehn and Nicol 2016).

As ectotherms, stress created through temperature changes directly effects growth and metabolic rate (Neuheimer *et al.* 2011). Combined low oxygen and high temperatures can be physiologically challenging, making mild hypoxic conditions potentially lethal at higher temperatures (McBryan *et al.* 2016, Sinclair *et al.* 2016). Prolonged low-oxygen exposure improved resting oxygen requirements (SMR) at 28°C compared to the normoxic control in our study, suggesting an acclimation response however; other metabolic rate measures at the same temperature were unaffected. Fish responses to changes in temperature and hypoxia vary significantly among and within species (Pörtner and Farrell 2008, Healy and Schulte 2012, Sandblom *et al.* 2014, Rogers *et al.* 2016). Gill remodelling to increase gill surface area has improved oxygen uptake in response to temperature and hypoxia for some species (Sollid *et al.* 2005, McBryan *et al.* 2016). Other influences which may impact hypoxia tolerance include: variation in oxygen consumption influenced by ATP production, oxygen carrying capacity of blood, and environmental influences such as changes to food intake, diet composition and ambient conditions (Salin *et al.* 2015, Collins *et al.* 2016, Rogers *et al.* 2016). Prolonged exposure to low-oxygen at the lower end of the species thermal range reduced aerobic capacity. Additionally, varied effects of temperature were observed after exposure to hypoxia for different durations under measures of metabolic capacity of Murray Cod. In contrast, a study that investigated thermal acclimation in Murray cod found they were temperature-independent, such that they had a greater capacity to transport oxygen to tissues regardless of higher temperatures (Clark *et al.* 2005). Aerobic scope values in our study appeared to be similar to aerobic scope values observed by Clark *et al.* (2005), however, our results did not indicate that Murray cod was temperature independent. The difference in our findings regarding

temperature may be explained by a difference in method as Clark, *et al.* (2005), investigated swimming respirometry rates under a higher flow, while we investigated resting rates of this species, as well as exposing fish for a prolonged period to low-oxygen conditions. Declines in hypoxia tolerance have been attributed to elevated temperatures raising metabolic demands in some species (McBryan *et al.* 2016), however; multiple species have displayed improved tolerance to hypoxia following temperature stress (Todgham *et al.* 2005, Burleson and Silva 2011, McBryan *et al.* 2013, Fu *et al.* 2014). Fitness of other species has also been shown to decline due to repeated exposure to high temperatures; this is particularly prominent in lizards and insects (Bickler and Buck 2007).

Activity profiles of organisms (i.e. active versus sedentary lifestyles) are associated with contrasting levels of aerobic capacity, such that there is a trade-off for locomotive performance and tolerance to low resource availability, in particular oxygen (Killen *et al.* 2016). Species better adapted to low levels of oxygen have lower aerobic capacity and are able to initiate changes to increase oxygen extraction and transport by adjusting gill surface area, oxygen affinity of haemoglobin and muscle mitochondrial density (Nilsson and Ostlund-Nilsson 2008, Killen *et al.* 2016). Prolonged exposure to low-oxygen improved the tolerance of Murray cod, although in nature this may have had minimal impact on hypoxic tolerance, and was not reflected in our metabolic tests. Aerobic ability of fish was linked to long-term low-oxygen exposure, such that, fish exposed for a longer period did not show any marked improvement in low-oxygen tolerance and only showed improvement in aerobic ability after 14 days of acclimation. Therefore, resistance to hypoxia may be likely to decrease as a function of exposure time. Furthermore, our results suggest that the duration of low-oxygen exposure may play an important role in hypoxia tolerance and post-hypoxic exposure metabolism. The lack of distinct effects of temperature and low-oxygen on the metabolic rate and aerobic scope could still indicate acclimation ability. Another study showed that temperature dramatically reduced tolerance to hypoxia, even when aerobic scope was minimally effected (McBryan *et al.* 2013). Acclimation responses of fish remain largely unknown. Fish may move and encounter areas with low oxygen, but can actively avoid them (except during widespread events), thereby, not acclimating to those conditions, leaving them less likely to survive future hypoxic events. Transgenerational transfers of hypoxia tolerance

among individuals is also evident in some species (Rogers *et al.* 2016). Acclimation to hypoxic conditions may not be viable for all species as some may be unable to make selective trade-offs to cope with a changing climate. Understanding physiological responses of fish to environmental stressors is crucial for predicting future ecological impacts between environmental change and population level effects which will aid in setting conservation targets (Rogers *et al.* 2016).

Acclimation to hypoxia is likely to be accompanied by changes to the oxygen transport capacity of blood (Collins *et al.* 2016, Rogers *et al.* 2016). Some species exhibit increased haemoglobin and haematocrit after chronic hypoxia exposure resulting in improved tolerance to hypoxia (Collins *et al.* 2016, Rogers *et al.* 2016) however; tolerance to hypoxia does not always change (Collins *et al.* 2016, Rogers *et al.* 2016). In our study, acclimation of fish at higher temperatures could have been improved by increases in haemoglobin leading to increased oxygen carrying capacity of the blood improving oxygen uptake during chronic exposure. Increases in temperature reduce haemoglobin oxygen binding affinity, as the haemoglobin molecule is thermally sensitive, therefore acclimation of fish to higher temperatures could counteract reduced transport efficiency by increasing the amount of oxygen picked up at the gills (McBryan *et al.* 2016). Increased transport of haemoglobin may explain the possible acclimation of our species at the highest temperature after the longest acclimation time. Manipulation of the oxygen carrying capacity of blood in relation to hypoxia and temperature is likely to be species-specific, and may be affected by life-history traits and the nature of the hypoxic event (Collins *et al.* 2016). Therefore, future research would benefit from testing the oxygen carrying capacity of haemoglobin when investigating hypoxia tolerance.

Progressive warming and an increased propensity for hypoxic events in aquatic environments is of critical conservation concern for fish as these stressors are associated with shifts in phenology, distribution, abundance and reproduction, as well as large scale mortalities (Breitburg *et al.* 2009, Norin *et al.* 2014, Rogers *et al.* 2016). Riverine ecosystems have been largely degraded throughout the world, due to flow mismanagement and the construction of barriers that limit fish movements (Dwyer *et al.* 2014, Small *et al.* 2014, Koehn and Nicol 2016). For example, the Murray-Darling Basin is home to 46 native species, including Murray cod, but only represents

10% total abundance of their pre-European settlement populations (Koehn and Nicol 2016). To manage the effects of combined temperature and hypoxia we need to understand the relationship between instantaneous physiological performance (the focus of most physiologically targeted studies) and long-term fitness as well as post-hypoxic exposure responses (Sinclair *et al.* 2016). Physiological information on species hypoxia tolerance and overall aerobic capacity can be incorporated into models to predict the long-term outcomes of deliberate water releases and natural flooding events on aquatic life. Models have successfully predicted changes in populations due to different stressors, and there is a growing trend to incorporate multi-species data into models to maximise the benefits of future conservation management plans (Sherman *et al.* 2007, Koehn and Nicol 2014). Data, which could be incorporated to aid model efficiency, include longitudinal studies in nature or molecular and physiological markers of performance (Sinclair *et al.* 2016). Presently, a number of management actions exist for the recovery of Murray cod such as stock enhancements, translocation efforts, habitat rehabilitation, legislative protection, remediation of barriers to fish passage, improved water quality and flow management and control of alien species (Lintermans 2013). However, if hypoxic events cannot be controlled or managed these efforts will provide little relief for this iconic species. Our study showed that Murray cod could persist in low-oxygen conditions, particularly after prior exposure, with temperature having minimal effect on physiological response. However, prolonged exposure to low-oxygen conditions may reduce long-term survival after greater duration periods. By informing water managers we can aid in meeting conservation conditions for species like Murray cod. For example, environmental water flows could be controlled for release to alleviate low-oxygen conditions that persist for longer than 14 days, which may impact Murray cod.

Aerobic scope measures alone were not sufficient in explaining hypoxia acclimation in our study. Conclusions based on oxygen consumption rates alone (metabolic scope) could thus lead to erroneous interpretations about the acclimation abilities of fish when faced with environmental stressors such as hypoxia and elevated temperatures. Behavioural tests on the tolerance of fish to low-oxygen illustrated the possible acclimation ability of Murray cod, in particular how prolonged exposure to low-oxygen conditions may physiologically reduce tolerance long-term. Other species

may be similarly affected by prolonged periods of low-oxygen conditions, and our results provide much needed hypoxia data on a species of conservation concern. Future research should target numerous species and their ability to acclimate to hypoxia in combination with other stressors. In particular, species recovery from hypoxia has been largely overlooked and will aid in understanding the capabilities of fish to not only withstand but also endure hypoxic events. Furthermore, development of a universal method to measure acclimation response to hypoxia exposure would allow direct comparisons among different species as research in this field continues.

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SUPPLEMENARY DATA

Supplementary Information 1:

Study Species

Percichthyidae: *Maccullochella peelii* (Murray cod)

Murray cod is Australia's largest solely freshwater fish reaching sizes of 1800mm in length with weights recorded up to 113.6kg. It reaches maturity at around 4-5 years for both sexes (Couch *et al.* 2016). This species occurs throughout the Murray-Darling system, a system that encompasses 5 separate states and is the largest catchment in Australia covering approx. 1 million km^2 (Koehn and Nicol 2016). At present there are active national recovery programs in place for the conservation of this species, which is listed, as vulnerable under the *Environmental Protection and Biodiversity Conservation* Act 1999 (EPBC) and as critically endangered on the IUCN Red List (www.iucnredlist.org, Couch *et al.* 2016). This species has a predominantly sedentary lifestyle, and commonly uses a sit-and-wait predation technique, but is also well known for making large migrations (>1km, Clark *et al.* 2005). Murray cod inhabit a wide variety of habitats including clear, rocky streams to slow flowing, turbid rivers and billabongs, with a strong preference for structural woody habitats (Koehn and Nicol 2016). Their abundance has been drastically reduced across their natural range due to both natural (e.g. increased temperature, hypoxia) and anthropogenic factors (e.g. extensive barriers in place which limit fish passage and environmental watering, Koehn and Nicol 2016).

SUPP Table 1. Summary of the rearing conditions and length and weight data of the fish within treatment tanks for physiological experiments. Data are displayed as means ± standard error (SE) with *n* representing the sample size of fish. For temperature, dissolved oxygen and saturation, *n* denotes the number of recordings taken throughout the experimental period; this differed with exposure length.

Desired Treatments	Oxygen, mg L^{-1}	Tank	Temperature, °C	Dissolved Oxygen, mg L^{-1}	Saturation, %	Fish Length, cm	Fish Weight, g	*n*
7 Days Exposure			(*n*=5)	(*n*=5)	(*n*=5)			
20	4	1	20.0±0.09	3.92±0.02	56.4±0.26	5.7±0.19	2.3±0.18	6
		2	20.2±0.07	3.96±0.01	57.2±0.09	5.9±0.08	2.4±0.23	5
28	4	1	28.0±0.14	3.96±0.02	53.6±0.15	6.3±0.23	2.2±0.23	6
		2	28.3±0.05	3.88±0.01	55.4±0.13	5.8±0.23	2.2±0.18	3
14 Days Exposure			(*n*=9)	(*n*=9)	(*n*=9)			
20	4	1	19.9±0.10	3.80±0.01	51.0±0.31	5.8±0.11	2.0±0.16	7
		2	19.8±0.03	3.87±0.01	53.5±0.12	5.7±0.15	1.8±0.19	6
28	4	1	28.2±0.04	3.94±0.01	54.5±0.11	5.1±0.01	1.4±0.01	2
		2	27.8±0.04	3.90±0.01	56.4±0.12	5.6±0.16	1.1±0.21	5
30 Days Exposure			(*n*=17)	(*n*=17)	(*n*=17)			
20	4	1	20.4±0.08	4.07±0.01	50.0±0.15	6.1±0.02	2.2±0.16	5
		2	20.1±0.04	3.95±0.01	52.5±0.09	5.8±0.06	1.9±0.16	3
	8	1	20.2±0.06	6.81±0.01	90.6±0.08	6.0±0.10	1.6±0.06	6
		2	20.9±0.04	6.75±0.01	91.3±0.07	5.8±0.18	1.9±0.17	5
24	4	1	24.3±0.04	3.79±0.01	53.6±0.07	5.6±0.05	1.6±0.10	5
		2	24.4±0.03	3.94±0.01	56.8±0.06	6.1±0.16	2.4±0.18	6
	8	1	24.2±0.04	6.78±0.01	89.4±0.06	6.2±0.24	2.1±0.18	5
		2	24.1±0.03	6.85±0.01	92.1±0.04	5.9±0.12	1.8±0.18	6
28	4	1	28.0±0.03	3.89±0.01	57.4±0.06	6.7±0.17	2.3±0.18	5
		2	28.2±0.04	3.78±0.01	55.5±0.06	6.6±0.19	2.1±0.18	4
	8	1	28.3±0.03	6.90±0.01	92.2±0.06	5.5±0.06	1.3±0.05	4
		2	27.9±0.03	6.58±0.01	91.5±0.05	5.5±0.13	1.4±0.13	8

SUPP Table 2. Analysis of variance examining the effects of measured temperature (temp), dissolved oxygen (DO), and oxygen saturation (SAT) on temperature and low oxygen treatments in the rearing water for the month long exposure and acclimation to low oxygen. * refers to P values <0.05

Water	Source of Variation	df	MS	F	P
Month long exposure					
Temp	Temp	2	1013.3	15427	≤0.001*
	Oxygen	1	0.14	2.18	>0.050
	Temp X Oxygen	2	1.29	19.69	≤0.001*
	Residuals	198	$6.5x10^2$		
DO	Temp	2	0.21	26.08	≤0.001*
	Oxygen	1	420.8	53053	≤0.001*
	Temp X Oxygen	2	0.15	18.35	≤0.001*
	Residuals	198	$7.9x10^3$		
SAT	Temp	2	158.9	120.49	≤0.001*
	Oxygen	1	69446	52656	≤0.001*
	Temp X Oxygen	2	97.34	73.81	≤0.001*
	Residuals	198	1.32		
Acclimation to low oxygen					
Temp	Temp	1	1560.4	28298	≤0.001*
	Days Exposed	2	0.55	9.91	≤0.001*
	Temp X Days Exposed	2	0.13	2.35	>0.050
	Residuals	118	$5.5x10^2$		
DO	Temp	1	$4.1x10^2$	11.99	≤0.001*
	Days Exposed	2	$2.6x10^2$	7.69	≤0.001*
	Temp X Days Exposed	2	0.20	58.58	≤0.001*
	Residuals	118	$3.5x10^3$		
SAT	Temp	1	99.59	72.76	≤0.001*
	Days Exposed	2	26.33	19.23	≤0.001*
	Temp X Days Exposed	2	106.86	78.07	≤0.001*
	Residuals	118	1.37		

SUPP Table 3. Linear mixed effects model examining the effects of temperature and oxygen on the metabolic rates of Murray cod. All possible temperatures (20, 24 and 28°C) and oxygen treatments (normoxic 6-8 and low oxygen 3-4mg L^{-1}) are considered for fish exposed to treatments for 30 days.

Metabolic Rate	Sources of Variation	df	MS	F	P
MMR	Temp	2	3451.9	0.71	>0.050
	Oxygen	1	1996.1	0.41	>0.050
	Temp X Oxygen	2	4580.4	0.95	>0.050
SMR	Temp	2	2168.5	0.70	>0.050
	Oxygen	1	5039.2	1.63	>0.050
	Temp X Oxygen	2	11468	3.71	<0.050*
AAS	Temp	2	159.1	0.08	>0.050
	Oxygen	1	5107.0	2.61	>0.050
	Temp X Oxygen	2	3017.8	1.54	>0.050

* *Denotes significant difference.*

SUPP Table 4. Linear mixed effects model examining the effects of temperature and oxygen on the ability of Murray cod to tolerate low-oxygen conditions. All possible temperatures (20, 24 and 28°C) and oxygen treatments (normoxic 6-8 and low oxygen 3-4, mg L^{-1}) are considered.

Low-oxygen Tolerance	Sources of Variation	df	MS	F	P
Pcrit	Temp	2	2.57	1.39	>0.050
	Oxygen	1	6.44	3.49	>0.050
	Temp X Oxygen	2	5.24	2.84	>0.050
Loss of equilibrium	Temp	2	0.01	0.27	>0.050
	Oxygen	1	0.33	5.57	<0.050*
	Temp X Oxygen	2	0.05	0.88	>0.050

* *Denotes statistical significance*

SUPP Table 5. Linear mixed effects model for the effects of temperature and length of exposure (7,14 or 30 days) to low oxygen on the metabolic rates of Murray cod. All possible temperatures (20 and 28°C) are considered.

Metabolic Rate	Sources of Variation	df	MS	F	P
MMR	Temp	1	73412	13.94	≤0.001*
	Days Exposed	2	120506	22.88	≤0.001*
	Temp X Days	2	52306	9.93	≤0.001*
SMR	Temp	1	40165	10.19	≤0.001*
	Days Exposed	2	67884	17.22	≤0.001*
	Temp X Days	2	46046	11.68	≤0.001*
AAS	Temp	1	7157.3	3.73	>0.050
	Days Exposed	2	14621.8	7.62	≤0.001*
	Temp X Days	2	10.6	0.005	>0.050

** Denotes significant difference.*

SUPP Table 6. Linear mixed effects model for the effects of temperature and days exposure (7, 14 or 30 days) on the ability of Murray cod to tolerate low-oxygen conditions. All possible temperatures (20, 24 and 28°C) are considered.

Low-oxygen Tolerance	Sources of Variation	df	MS	F	P
Pcrit	Temp	1	1.49	0.62	>0.050
	Days Exposed	2	1.75	0.73	>0.050
	Temp X Days	2	0.65	0.27	>0.050
Loss of equilibrium	Temp	1	0.21	4.5	<0.050*
	Days Exposed	2	0.18	3.95	<0.050*
	Temp X Days	2	0.17	3.78	<0.050*

** Denotes statistical significance*

SUPP Table 7. Analysis of variance for the effects of oxygen exposure over a month and acclimation to low oxygen on Fulton's K condition factor. Month long exposure (Experiment 1) concerns fish exposed to either low oxygen or normoxia for 30 days under three different temperatures (20, 24 and 28°C). Acclimation to low oxygen (Experiment 2) concerns fish exposed only to low oxygen conditions for differing numbers of days (7, 14 and 30 days) and under two different temperatures (20 and 28°C).

Fulton's K Condition Index	Sources of Variation	df	MS	F	P
Exp 1. Temperature	Temp	2	0.26	9.16	≤0.001*
	Oxygen	1	$3.26x10^2$	2.32	>0.050
	Temp X Oxygen	2	$6.88x10^2$	4.89	≤0.001*
	Residuals	56	$1.41x10^2$		
Exp 2. Acclimation	Temp	1	0.97	37.57	≤0.001*
	Days Exposed	2	0.32	12.33	≤0.001*
	Temp X Days Exposed	2	$7.62x10^3$	0.29	>0.050
	Residuals	50	$2.59x10^2$		

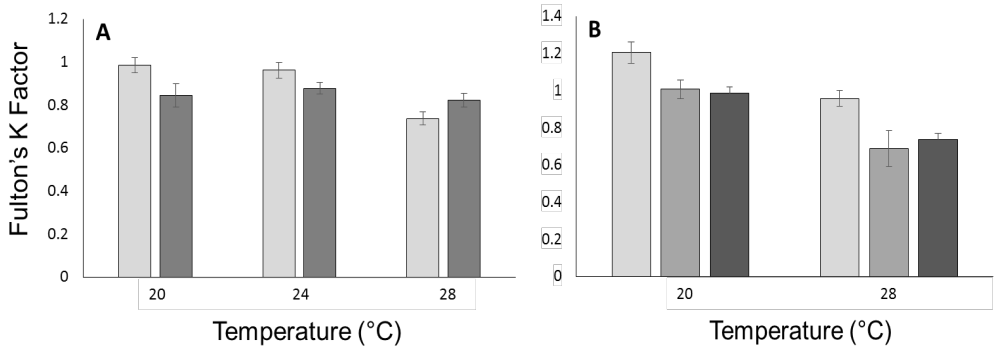

Figure SUPP 1. Mean (±SE) Fulton's K factor A) temperature experiment with exposure to low oxygen (light grey) or normoxia (grey) separated by temperature treatments, and B) after acclimation to low oxygen at two temperatures after 7 (light grey), 14 (grey) or 30 days (dark grey) (n=54).

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CHAPTER FOUR

TRACKING FRESHWATER HYPOXIA USING FISH OTOLITHS: AN EXPERIMENTAL APPROACH

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Above: Golden perch (*Macquaria ambigua*) juvenile being measured before dissection. Photo credits: Kayla Gilmore

STATEMENT OF AUTHORSHIP

This paper *Tracking freshwater hypoxia using fish otoliths: an experimental approach*, has been submitted for publication to *Freshwater Science*, and all authors made a contribution. Bronwyn M. Gillanders, Zoe A. Doubleday and myself conceived the ideas for the manuscript. I designed the methodology and collected and analysed the data. I led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis.

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Kayla . Overall contribution 80%

Overall contribution 10%

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ABSTRACT

Determining the effects of hypoxia or low oxygen levels in water can be problematic due to difficulties tracking the location, timing and duration of hypoxic events. Otoliths (ear stones) of fish may provide a means to track hypoxia due to daily accretion of material that reflects the environmental conditions experienced by fish. But first, it is necessary to identify elemental tracers that indicate hypoxic conditions. Using a controlled laboratory experiment, juvenile golden perch (*Macquaria ambigua*) were reared under different temperature treatments (20, 24 and 28°C) crossed with normoxic and hypoxic conditions for one month. Additionally, physiological experiments were conducted to determine effects of hypoxic conditions on otolith chemistry. Water samples and otoliths were analysed for a suite of elements to determine specific tracers associated with hypoxic conditions using laser ablation inductively coupled plasma-mass spectrometry (LA ICP-MS). Trace elements in otoliths of golden perch were not linked to hypoxic conditions and there was no relationship between trace elements and several physiological variables. We highlight the potential for Mn in otoliths as an environmental indicator of hypoxia as it was not physiologically regulated. If this elemental tracer is validated as a hypoxia tracer it will be key to reconstructing long term trends in hypoxia using freshwater fish otoliths, information that would be almost unattainable using traditional methods.

INTRODUCTION

Low oxygen or hypoxic conditions in freshwater systems are increasing in frequency and severity on a global scale (Diaz and Rosenberg 2008, Collins *et al.* 2013, Limburg *et al.* 2015). Although hypoxic conditions can be caused by both natural processes and human activities, it is believed that worsening conditions are due to the increasing influence of human activities in freshwater systems (i.e. damming, pollution and eutrophication, Diaz and Rosenberg 2008). Instrumental records of oxygen only span a few decades (Diaz and Rosenberg 2008, McCarthy *et al.* 2014), therefore, to determine if human activities have increased the frequency and severity of hypoxia it is necessary to find a way to measure its historic occurrence over longer time scales.

Otoliths, the ear stones of fish, can provide a record of environmental changes. Otoliths are metabolically inert aragonitic structures, which accrete layers of calcareous material on a daily basis (Campana 1999, Elsdon *et al.* 2008, Limburg *et al.* 2015). They form part of the hearing and balance system in teleost fishes and are surrounded by the endolymph fluid. Trace elements incorporated into otoliths can reflect environmental conditions. For example, salinity and temperature vary with strontium and barium concentrations in ambient water, which modifies relationships between otoliths and water in predictable ways (Elsdon *et al.* 2008, Collingsworth *et al.* 2010, Aschenbrenner *et al.* 2016). Analysis of specific trace elements incorporated into the otoliths of fish provides a broader temporal record of the life history of individual fish when matched to growth increments (e.g. Izzo *et al.* 2016). Otolith studies are increasing our understanding of fish life histories and may even be used to retrospectively track how environmental conditions have changed (Limburg *et al.* 2011, Limburg *et al.* 2015, Izzo *et al.* 2016). Furthermore, sclerochronological and biochronological techniques can be applied to otoliths found in scientific collections, sedimentary deposits, fossil and archaeological sites to extend the temporal record as otoliths suffer minimal diagenetic changes (Disspain *et al.* 2012, Izzo *et al.* 2016). Therefore, determining a chemical tracer for hypoxic conditions in otoliths could place current changes in hypoxic frequency and severity into context.

Elements that show promise as markers of hypoxia, include those that are abundant in low oxygen environments. Manganese (Mn) has been associated with hypoxia in marine environments (Campana 1999, Miller 2009, Limburg *et al.* 2015, Aschenbrenner *et al.* 2016). Dissolved Mn is available as a redox product and could be indicative of low-oxygen concentrations (Limburg *et al.* 2015). Actual concentration of bottom water Mn depends on the duration of hypoxia and build-up of Mn oxides in sediment and availability of particulates that remain dissolved in solution for days at a time (Miller 2009, Limburg *et al.* 2011, Mohan *et al.* 2014, Limburg *et al.* 2015). Dissolved Mn fluxes out of hypoxic or anoxic sediments and is then available for uptake by fish that move into these hypoxic regions (Limburg *et al.* 2011, Limburg *et al.* 2015). Conceivably then if fish are present during redox conditions they may be able to incorporate these elements into otoliths or other biomineralized structures (Lu *et al.* 2010, Limburg *et al.* 2015). However, the underlying mechanisms for Mn incorporation into otoliths are still being researched, and flux out of sediment may not be the only available source of Mn. Currently there has been no known validation of Mn or other elements as a tracer of hypoxic conditions for freshwater fish from sediments or through other means.

Markers of hypoxia may also be elements that reflect reduced physiological regulation of a fish's internal environment. Due to regulatory differences between freshwater and marine fish, physiological barriers may impact incorporation of Mn or other elements into the otolith (Woodcock *et al.* 2012). For example, saltwater fish gain ions from food and by drinking seawater, and excrete excess ions in concentrated urine and excess salts actively across the gill epithelium. In contrast, freshwater fish gain ions actively through the gills and from food with excess ions lost through urine and some from diffusion at the gills (Webb *et al.* 2012). Once fish are exposed to hypoxia they become physiologically stressed, and barriers usually in place that regulate elemental uptake in the organism could be overcome or ignored in favour of more important physiological functions. Therefore, elements abundant within a fish's internal environment, such as magnesium obtained readily from food (Woodcock *et al.* 2012), may flood in when physiological regulation (homeostasis) breaks down. Water temperature could also influence physiological regulation as hypoxic conditions are exacerbated at higher temperatures, increasing metabolic stress and

potentially allowing greater concentrations of elements to be incorporated onto the otolith.

Our aim was to determine if trace elements in otoliths could be used as a natural tracer of hypoxic events in freshwater systems. A suite of elements (Ba, Mg, Mn, Sr, Na and Zn) that are a by-product of chemical and redox reactions were investigated. In a controlled laboratory setup, fish were exposed to sub-lethal levels of hypoxia, as well as normoxia, combined with different temperatures. To investigate physiological regulation of key elements, we also linked elemental concentration to physiological performance of the fish. Our specific hypotheses were that A) manganese would act as an indicator of hypoxic conditions in otoliths of fish exposed for a prolonged period; B) the ability of fish to regulate ions would breakdown under stressful low oxygen conditions; and C) manganese incorporation was not reliant on sediment or driven through physiological regulation.

MATERIALS & METHODS

Animal Husbandry

Juvenile golden perch (*Macquaria ambigua*) approximately 40mm SL, were obtained from the Silverwater Native Fish Hatchery, Grong Grong, NSW. The species chosen is a key native long-lived freshwater fish endemic to the Murray Darling Basin, Australia that is listed as threatened under different bodies including the IUCN Red List (Lintermans 2007, Couch *et al.* 2016). Golden perch is known for large-scale migrations and is usually found actively swimming in the main channel of the river where is adopts a demersal lifestyle (Clark *et al.* 2005, Koehn and Nicol 2016). The Murray Darling Basin is increasingly exposed to fluctuating hypoxic conditions and high temperatures and golden perch have generally low tolerances to hypoxia, experiencing mortalities from 3 mg O_2 L^{-1} and lower putting them at risk (Small *et al.* 2014, Gilmore *et al.* 2018).

Fish were bred from a common brood stock to reduce the influence of genetic variability on otolith chemistry. Upon arrival at the University of Adelaide, fish were held in 250L holding tanks equipped with filtration and aeration, at 20°C. Aged (dechlorinated) tap water was used in the tanks. Golden perch were fed live blackworm (*Lumbriculus variegatus*) to satiation once a day; waste was siphoned out an hour after feeding. Fish were exposed to a 12:12h light:dark cycle and room temperature was maintained at 20°C. Ammonia, nitrite and pH levels were monitored and 25% water changes (approximately 4L from an aged water bath held under experimental hypoxia or normoxia to reduce water chemistry changes and thus otolith elemental composition) were made every second day to maintain fish health. All tanks were aerated, water was filtered for the duration of the experiment and evaporation was minimised by covering tanks with clear plexiglass lids.

Otolith Marking Technique

Eight days after arrival the fish were marked using alizarin complexone, which allows experimental otolith growth to be distinguished from growth prior to the experiment. The marking involved immersing the fish in an aerated tank and adding alizarin complexone (20mg/L); fish were then left overnight (Van der Walt and Faragher 2002). Respirometry experiments were commenced after fish were held in

experimental conditions for a minimum of 30 days. At the completion of the respirometry experiments (for details see Metabolic Rate Measures) fish were euthanized in an ice-slurry, and standard length and weight recorded (SUPP Table 1). The alizarin mark was observed on the dissected otoliths under a microscope to ensure sufficient otolith material had been laid down during the experiment. Additional otolith material had been accreted between the alizarin mark and end of the experiment/otolith edge (mean experimental growth was 208.6μm for hypoxic treatments held at 20°C and 217.2μm at 28°C, and 243.1μm for normoxic treatments held at 20°C and 252.3μm at 28°C) such that spot samples (26μm) were completely within the experimental period for golden perch. There was sufficient otolith growth at all temperatures allowing experimental growth to be distinguished from non-experimental.

Experimental Treatments

Fish were randomly assigned to 20L treatment tanks ten days after arrival to give fish sufficient time to acclimate, with approximately 11 fish per tank (SUPP Table 1). The experimental design consisted of two oxygen treatments (normoxic 6-8mg O_2 L^{-1} or 12-14 kPa and hypoxic 3-4mg O_2 L^{-1} or 7-9 kPa), combined with three temperature treatments (20, 24 and 28°C). Golden perch can tolerate a range of water temperatures however hypoxic conditions are exacerbated by warmer water temperatures. Our chosen temperatures are reflective of the species' natural upper thermal range (Lintermans 2007). The temperatures chosen reflect common temperatures along the Murray river during summer, the seasonal period where hypoxic events have the most devastating impacts. While water temperature can reach up to 30°C in summer, this is uncommon, therefore our thermal range reflects the most likely maximum temperatures fish would encounter in the wild. All possible combinations of temperature and oxygen were included, with duplicate tanks for each treatment (n=12 tanks). Tanks held at ≥ 24°C were heated independently using submersible aquarium heaters; temperature was measured every second day to ensure temperature treatments were maintained. Temperatures were increased by 2°C per day until the required experimental temperature was reached. Hypoxic oxygen levels were chosen for long-term exposure (≥30 days) without mortality of fish. The globally accepted hypoxia tolerance limit of 2 mg O_2 L^{-1} for fish causes high levels of stress and mortality for most species (Vaquer-Sunyer and Duarte 2008). The experimental hypoxia levels

chosen were slightly higher than this tolerance level to ensure survival of fish. A simple degassing system was used to control oxygen levels in hypoxic treatments, using nitrogen gas to deoxygenate the water (see Gilmore *et al.* 2018, for details). Oxygen levels in all 'hypoxic' tanks could be simultaneously controlled for extended periods using this method. Fish were exposed to experimental conditions for ≥30 days to ensure sufficient otolith growth occurred prior to chemical analysis.

Metabolic Rate Measures

To understand the physiological regulation of elements under stress, physiological performance and elemental concentrations in the same fish were measured. Resting respirometry represents the ideal system for manipulating environmental influences like hypoxia. Resting respirometry allows the user to measure resting rates (standard metabolic rate, SMR), exhaustive rates (maximum metabolic rate, MMR) and the total capacity for activity (absolute aerobic scope, AAS) of oxygen consumption without putting fish under oxidative pressure from exercising (Roche *et al.* 2013). More specifically AAS, is a physiological measure of the total capacity of an organism for activity and also specific dynamic action; low aerobic scope indicates high stress and high aerobic scope indicates low stress. SMR represents the basic resting oxygen requirements of a fish; low SMR means basic oxygen requirements take longer to be reached creating low stress, and high SMR means basic oxygen requirements are reached earlier creating stress and reducing the ability of fish to carry out other activities i.e. feeding and reproduction. MMR measures the oxygen requirements under exhaustive activity; low MMR means exhaustive activity is limited under stress and exhaustion occurs earlier after activity, and high MMR means fish can endure exhaustive activity longer with low stress. Physiological responses to hypoxia were measured using resting respirometry, a method used to determine metabolic oxygen consumption rates ($\dot{M}O_2$) of fish under different environmental conditions. Approximately 12 fish per treatment were randomly selected and subjected to respirometry experiments (SUPP Table 1). Fish were fasted for 24 hours prior to tests to avoid the influence of digestion on $\dot{M}O_2$ estimates.

Multiple fish were tested simultaneously using a custom designed four-chamber system with each chamber made to fit the size of the fish based on a 1 kg animal: 10 L of water ratio. The four chambers were submerged in a larger water bath that was

used to control temperature and oxygen levels. Three chambers were used to measure fish oxygen consumption with the fourth (which was randomly assigned each test) measuring background respiration rates. Individual chambers used a closed recirculation loop to pump low flowing water over the fish. Each chamber was fitted with a fibre optic oxygen probe (Pyroscience, OXROB3, Aachen, Germany) to measure oxygen consumed during each $\dot{M}O_2$ determination.

A $\dot{M}O_2$ determination period uses the slope of the line of oxygen consumption by fish for each 20-minute determination period before water is replenished to the chamber for 2 minutes (flushing period). Water was circulated intermittently after each $\dot{M}O_2$ determination using a flushing pump connected to all chambers to completely replenish the chamber with oxygenated water from the water bath. During the 20min determination period oxygen did not decline below 1 mg O_2 L^{-1} and was held above the background respiration rates of microorganisms (background rates were continuously measured in one of the four resting chambers during each experimental trial with the chamber used alternated throughout trials). Maximum resting routine and standard metabolic rates, measures of the highest and lowest capacity for activity by fish (MMR and SMR), were determined using a modified version of the method described by Roche *et al.* (2013), where fish were chased to exhaustion for 2min in a 25L bucket and exposed to the air in a mesh net for 40sec before being placed immediately inside chambers. MMR was measured during the first determination period. Fish were then left in the chamber for ~24hrs to allow them to reach a resting state. The SMR and $\dot{M}O_2$ was calculated for each determination cycle using the equation:

$$\dot{\mathrm{M}}O_2 = ([O_2]_{t0} - [O_2]_{t1}).\frac{V}{t}.\frac{1}{BW}$$

where (t0), is the oxygen content (mg O_2/L) of the water after a flushing cycle, and (t1), is the oxygen content measured at the end of a determination period, prior to the next flushing cycle. V is the volume of the chamber minus the volume of the experimental animal in L, t is time (t1-t0 in hr), and BW, is body weight of the experimental animal in kg. The lowest 10% of measurements were averaged to calculate SMR. Background rates were subtracted from $\dot{M}o_2$ values upon calculation. The absolute aerobic scope (AAS) of fish was calculated by subtracting SMR from

MMR (MMR-SMR), a high AAS indicates low stress and a low AAS indicates high stress as fish reach the critical oxygen requirements for basic survival.

Physiological measures of absolute aerobic scope and standard metabolic rate (AAS and SMR) were compared to Mn:Ca concentrations in otoliths, as Mn has the potential to be under physiological control and could be used as an indicator of hypoxia (see Limburg *et al.* 2015). Maximum metabolic rate measures were used to calculate AAS, but as total aerobic capacity, AAS, and basal resting oxygen requirements, SMR, are critical to understanding hypoxic responses our study focussed on these two metrics.

Otolith Preparation and Analyses

At the completion of the respirometry experiments fish were euthanized in an ice-slurry, and standard length and weight recorded (SUPP Table 1). Both sagittal otoliths were dissected from fish, cleaned in Milli-Q water and air-dried. An otolith from each fish was embedded in epoxy resin (Epofix, Struers) that had been spiked with 40ppm indium, and then sectioned transversely through the core to a thickness of approximately 300 μm using a low speed diamond saw. Sections were polished using 9 and 3-μm lapping film, cleaned in Milli-Q water, dried overnight in a laminar flow cabinet, and mounted on glass microscope slides using indium-spiked thermo-plastic glue (Crystal Bond^{TM} 509). Slides were stored in clean sealable plastic bags until analysis.

The concentration of elements in the otolith (^{88}Sr, ^{137}Ba, ^{55}Mn, ^{24}Mg, ^{23}Na, ^{43,44}Ca, ^{34}S, ^{57}Fe, ^{66}Zn and ^{115}In) were determined using a ASI M50 laser connected to an Agilent 7700cs inductively coupled plasma-mass spectrometer (ICP-MS) (see SUPP Table 2 for operating parameters). To correct for machine drift, a reference standard (National Institute of Standards and Technology, NIST 612) was analysed after every 10 samples and a carbonate standard (MACS3) was analysed to calculate accuracy and precision of analyses at the beginning and end of the day. Spots were sampled from the edge of the otolith representing experimental growth, based on viewing the position of the alizarin complexone mark (a single spot was taken per sample, see also Otolith Marking Technique). A pre-ablation was done to remove any surface contamination. Prior to each ablation, background levels of elements in the ablation chamber were measured for 30s. The element:Ca ratio was calculated by converting

the elemental concentration in mols, and then dividing the element in mols by Ca (mols). Ca was treated as an internal standard. The element:Ca ratio was used as elements (^{88}Sr, ^{137}Ba, ^{55}Mn, ^{24}Mg, ^{23}Na and ^{66}Zn) either substitute for Ca or are found in the interstitial spaces or the organic component in otoliths (Campana 1999, Doubleday *et al.* 2015). ^{115}In was analysed so otolith material could be distinguished from epoxy resin and to confirm otolith material was constantly ablated. Precision, calculated as the mean coefficients of variation of repeated measures, for all elements based on the NIST 612 standard were <8%. The coefficients of variation of the elements ^{34}S and ^{57}Fe were above this level and were not considered further due to poor precision. The ablation chamber was purged for 20s after each ablation to remove any background gas or sample particles that could contaminate future samples.

Water Analyses

Element concentrations in the rearing water were monitored by taking two replicate 25mL water samples from each tank, one at the beginning and one at the conclusion of the experimental period. All samples were filtered through a 0.45 μm filter into acid washed vials, acidified using 500 μL of ultrapure nitric acid, and refrigerated until analysis. Vials were acid washed by soaking in 10% nitric acid prior to use and rinsed several times in Milli-Q water.

The water samples were analysed by the National Measurement Institute using an ICP-AES (inductively coupled plasma - atomic emission spectrometer; Varian-Vista Pro ICP-AES) or a Quadrupole ICP-MS (inductively coupled plasma-mass spectrometer; Elan DRC-2, Perkin-Elmer). The concentration of each element (the specific isotopes used were ^{88}Sr, ^{137}Ba, ^{55}Mn, ^{24}Mg, ^{23}Na, and ^{66}Zn) was expressed as an element:Ca ratio to estimate actual elemental concentrations.

Statistical Analyses

Statistical analyses of otolith and water sample data were conducted using PRIMER 6 & PERMANOVA+ software (www.primer-e.com). Element:Ca ratios in the water and otolith material were analysed individually in a 3-way permutational univariate analysis of variance (ANOVA) with unrestricted permutations of the data. Temperature and hypoxia were treated as fixed factors with replicate tanks treated as

a random factor nested in the interaction between temperature and hypoxia. Post hoc pairwise tests were conducted where significant differences were detected in the main tests to determine when the differences occurred among treatments or tanks. Monte Carlo tests were included in all analyses to ensure that there were sufficient permutations to detect significant differences in all tests. If significant tank effects were not detected in either otolith or water data for individual elements a 2-way permutational ANOVA was performed without tank as a nested factor. Similar results were found for the main effects and interaction for both 2-way and 3-way tests therefore only the findings from the 3-way tests are reported (see Supplementary Tables).

RESULTS

Rearing Conditions

Treatment conditions were maintained for golden perch throughout the experimental period (SUPP. Table 3). Fish lengths and weights showed little variation among treatments (SUPP. Table 3). Significant differences in temperature and dissolved oxygen were detected among relevant treatments, in line with the experimental design (SUPP. Table 4). In general, element:Ca ratios in the rearing water did not differ among treatments.

Trace elements and hypoxia

Concentrations of the elements did not vary due to hypoxic exposure (Mn, Mg, Na, Zn, Sr and Ba). Otolith Mn:Ca was not significantly influenced by hypoxia in this study (Figure 1). No differences in otolith Mn:Ca, Mg:Ca or Zn:Ca were found among treatments for golden perch ($p>0.05$, Table 1). Concentrations of Na:Ca ($p<0.05$) in the otoliths of golden perch were negatively affected by increasing temperatures, but not by hypoxia (Figure 2, Table 1). Significant effects on Ba:Ca, Sr:Ca and Na:Ca were detected among replicate tanks for golden perch (Ba & Sr:Ca $p<0.001$ and Na:Ca $p<0.05$, Figure 2, Table 1). Otolith experimental growth was negatively affected at both temperatures while under hypoxic conditions (mean experimental growth was 208.6µm at 20°C and 217.2µm 28°C under hypoxia and 243.1µm at 20°C and 252.3µm at 28°C under normoxia).

Table 1. Analysis of variance for the effects of temperature and hypoxia exposure on trace elements found in the otoliths of golden perch. All possible temperatures (20, 24 and 28°C) and hypoxic treatments (4 mg L^{-1} and 8 mg L^{-1}) are considered.

Trace Element	Sources of Variation	df	MS	F	P
Na:Ca	Temp	2	25.94	8.18	<0.050*
	Hypoxia	1	3.16	1	>0.050
	Temp X Hypoxia	2	0.82	0.26	>0.050
	Tank (Temp X Hypoxia)	6	3.19	2.29	<0.050*
	Residuals	95	1.39		
Mg:Ca	Temp	2	5.31x10^-2	3.08	>0.050
	Hypoxia	1	7.66x10^-3	0.44	>0.050
	Temp X Hypoxia	2	3.35x10^-2	1.94	>0.050
	Tank (Temp X Hypoxia)	6	1.72x10^-2	0.99	>0.050
	Residuals	95	1.74x10^-2		
Mn:Ca	Temp	2	5.35x10^-8	1.21	>0.050
	Hypoxia	1	2.08x10^-9	4.72x10^2	>0.050
	Temp X Hypoxia	2	2.73x10^-8	0.61	>0.050
	Tank (Temp X Hypoxia)	6	4.39x10^-8	0.87	>0.050
	Residuals	95	5.03x10^-8		
Zn:Ca	Temp	2	1.18x10^-2	3.32	>0.050
	Hypoxia	1	1.19x10^-3	0.33	>0.050
	Temp X Hypoxia	2	2.92x10^-3	0.82	>0.050
	Tank (Temp X Hypoxia)	6	3.58x10^-3	1.82	>0.050
	Residuals	95	1.97x10^-3		
Sr:Ca	Temp	2	7.74x10^-2	0.41	>0.050
	Hypoxia	1	0.46	2.51	>0.050
	Temp X Hypoxia	2	0.25	1.38	>0.050
	Tank (Temp X Hypoxia)	6	0.18	16.71	≤0.001*
	Residuals	95	1.13x10^-2		
Ba:Ca	Temp	2	6.78x10^-5	2.88	>0.050
	Hypoxia	1	3.18x10^-5	1.35	>0.050
	Temp X Hypoxia	2	5.29x10^-5	2.24	>0.050
	Tank (Temp X Hypoxia)	6	2.38x10^-5	5.28	≤0.001*
	Residuals	95	4.51x10^-6		

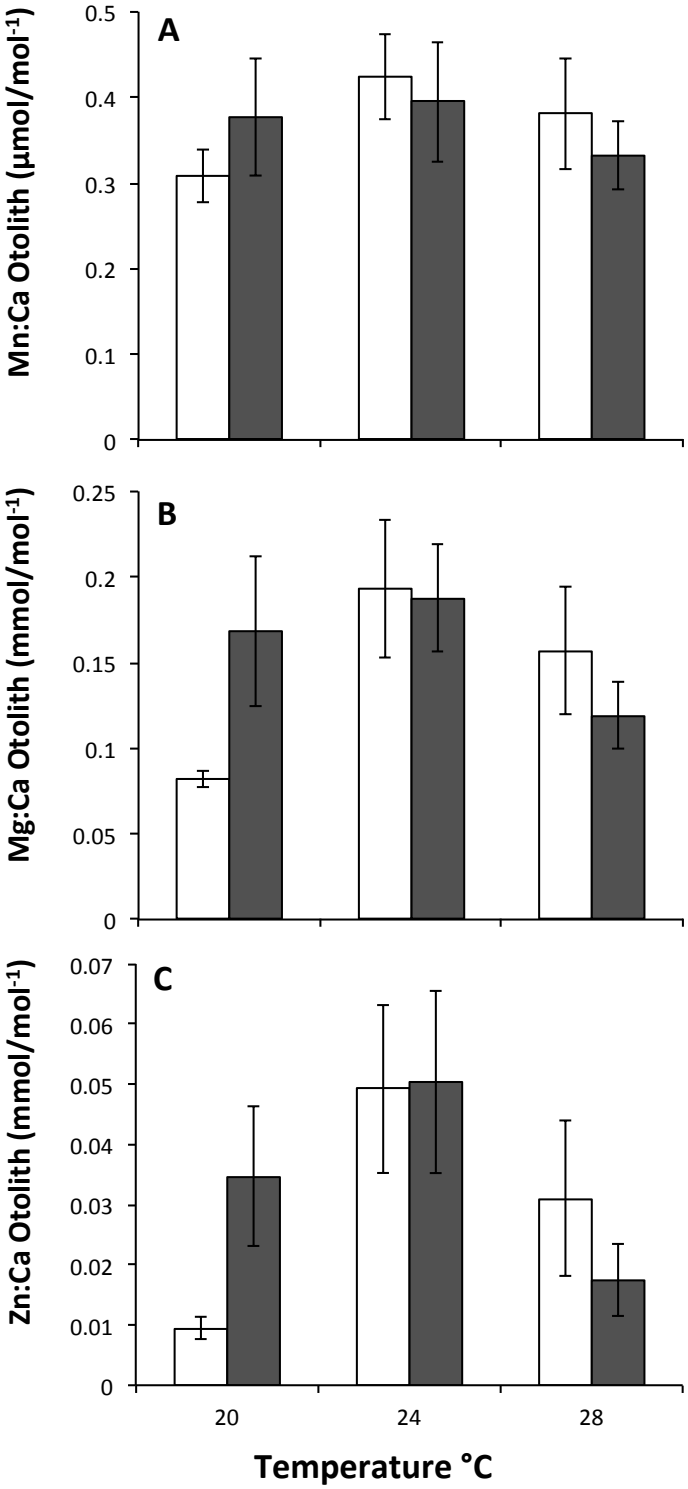

Figure 1: Mean (±SE) amounts of A) Mn:Ca B) Mg:Ca and C) Zn:Ca within otoliths of golden perch reared at different temperatures and oxygen levels. White bars represent fish reared under hypoxic conditions (4 mg O_2 L^{-1}) and grey bars denote those reared under normoxia (8 mg O_2 L^{-1}). Replicate tanks have been pooled, as there were no significant tank effects. There were no significant effects of temperature, hypoxia or an interaction between the two.

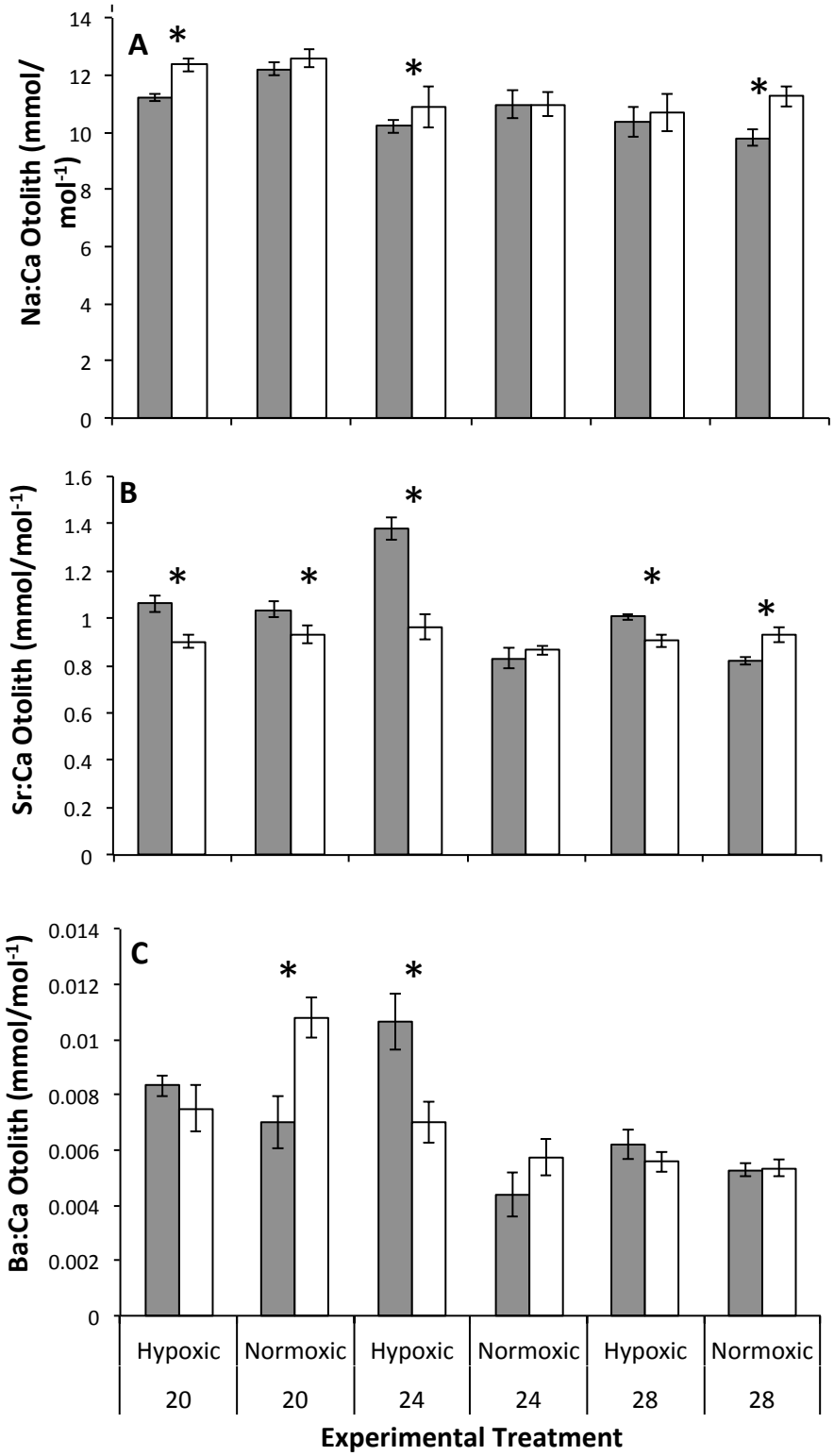

Figure 2: Mean (±SE) concentrations of Na:Ca (A), Sr:Ca (B) and Ba:Ca (C) within otoliths of golden perch reared at different oxygen levels (hypoxic, 4 mg L^{-1} and normoxic, 8 mg L^{-1}) and temperatures (20, 24 and 28°C). Coloured bars denote replicate tanks, grey (tank 1) and white (tank 2), with significant tank effects denoted by an asterisk (*).

Trace elements and metabolic stress

Metabolic stress, linked to hypoxia, was not associated with an increase in Mn:Ca concentrations for golden perch (Figure 3). No discernible patterns among metabolic rate and otolith chemistry were detected for golden perch (Figure 3).

Trace elements in Otoliths vs. Water

No linear relationships between the concentration of elements in the water and those found in the otolith were observed (SUPP Figure 5). Water condition data showed that there was variability in water conditions among treatments and tanks, but all were within the expected variability intentionally created to meet experimental parameters (SUPP Table 3, 4, 5 and 6). As such, chemical differences in otoliths are likely driven by physiological change rather than changes in the water chemistry.

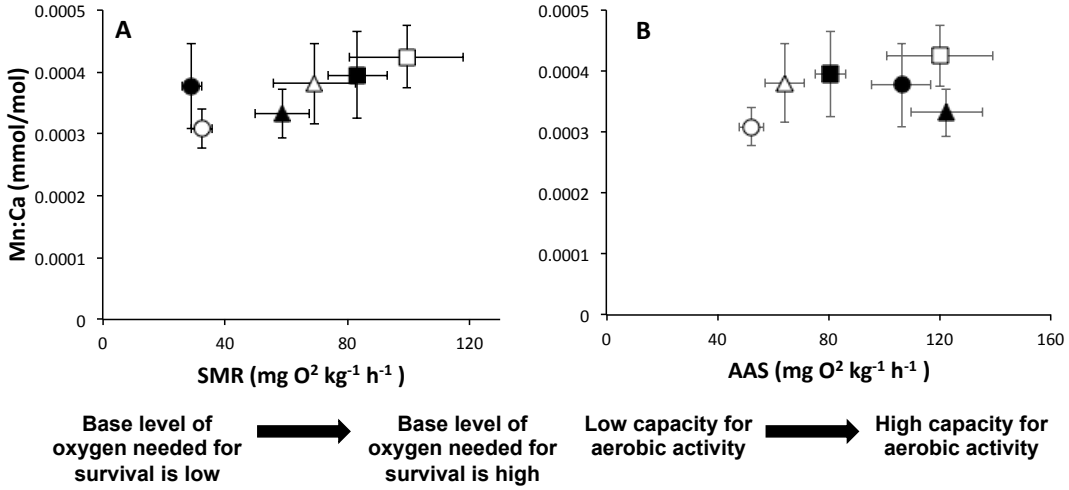

Figure 3: Mean (±SE) concentrations of Mn:Ca within otoliths and A) standard metabolic rate (SMR, energy consumption at rest) and B) absolute aerobic scope (AAS, total capacity for energy) of golden perch reared at different temperatures and oxygen levels. White symbols represent fish reared under hypoxic conditions (4 mg O_2 L^{-1}) and black symbols denote those reared under normoxia (8 mg O_2 L^{-1}). Symbol shape represents temperature treatment (● 20°C, ■ 24°C, ▲ 28°C) Replicate tanks have been pooled, as there were no significant tank effects.

DISCUSSION

Environmental reconstructions based on elements found within the hard parts of organisms provide an unparalleled ability for scientists to elucidate modern and historical environmental changes. Herein, we were unable to link trace elements in otoliths of golden perch with hypoxic conditions. Comparisons of physiological performance to concentrations of trace elements in otoliths under hypoxic conditions also found no discernable patterns. Of the elements sampled within the otoliths Mn was expected to show the most promise as a marker of hypoxia. However, as aquaria did not contain any added sources of Mn (i.e. from sediment or elemental spiking) we were unable to validate Mn as a useful environmental indicator as physiological processes did not affect it, an interaction that to our knowledge has rarely been studied.

Contrary to previous studies based in marine environments, Mn was not associated with hypoxic conditions (Limburg *et al.* 2011, Limburg *et al.* 2015, Aschenbrenner *et al.* 2016). The mechanism of Mn uptake in otoliths remains uncertain, as Mn is found in high concentrations in the primordia (nucleation points in the core) of otoliths along with some other trace elements (Miller 2009, Limburg *et al.* 2011, Limburg *et al.* 2015). Maternal transfer of Mn is one possible explanation for a high concentration in the primordia but does not explain high concentrations of Mn in otoliths in later years (Limburg *et al.* 2015). Elevated dissolved Mn in the water is the most likely cause of elevated Mn in otoliths of fish later in life; however, to date there is little empirical evidence of this relationship (Limburg *et al.* 2011, Mohan *et al.* 2014, Limburg *et al.* 2015). Enriching the water with Mn typically has no impact on otolith Mn concentrations, with a lack of sediment in controlled aquaria suspected as the cause (Elsdon and Gillanders 2003, Miller 2009, Collingsworth *et al.* 2010). The chemical characteristics of Mn (monoisotopic) may be the cause of contrary findings between lab and field based studies (Limburg *et al.* 2015). The speciation and production of Mn can be inhibited by parameters that affect oxygenation and pH (Limburg *et al.* 2015). Furthermore, sediment redox reactions may not take place except under very low dissolved oxygen levels (DO <1mg L^{-1}, Collingsworth *et al.* 2010, Limburg *et al.* 2015). Therefore, a lack of sediment in aquaria tanks, or dissolved oxygen intentionally kept at 3-4mg L^{-1} to ensure long term survival, may have affected the incorporation of Mn into the otoliths in this study.

No source of Mn was provided in this study (e.g. no sediment, nor enrichment of Mn in water) allowing us to test if there was a physiological effect of hypoxic stress on Mn incorporation. While incorporation of Mn was generally elevated in otoliths of fish exposed to hypoxia (at 24 and 28°C), this was not significant, suggesting a Mn signature is not influenced by physiological stress (i.e. endogenous factors do not outweigh exogenous factors). Incorporation of Mn then is likely due to environmental factors, for example enhanced Mn flux in hypoxic systems. Mn incorporation could be physiologically regulated and our experiment designed to maintain sub-lethal concentrations may have limited a physiological response. However, if the impact of internal stress dynamics on Mn incorporation is low, as indicated in our study, it will be a useful indicator for environmental reconstructions of hypoxia. Our results lend further support to lab based studies where Mn incorporation was not detected and also lacked sediment and Mn enrichment (Mohan *et al.* 2014), and to field based caging experiments detecting environmentally-derived Mn (Forrester 2005, Dorval *et al.* 2007, Mohan *et al.* 2012). Future studies should focus on the mechanism of uptake of this element to determine if it is physiologically regulated under hypoxic extremes or requires more specific conditions, as well as sampling from different size and age classes of wild caught fish known to have survived hypoxic events.

Mn is not an exclusive indicator of hypoxic encounters in fish; other elements may also be used as indicators but often have limited validation. Magnesium levels in otoliths are often related to somatic growth and diet (Limburg *et al.* 2011, Woodcock *et al.* 2012, Aschenbrenner *et al.* 2016). Few studies have validated it as a tracer of environmental conditions as it is considered to be under physiological control (Woodcock *et al.* 2012, Barnes and Gillanders 2013). Our study found no correlation between elevated levels of Mg in the otoliths of golden perch and hypoxia. Uptake of Mg and otolith growth continues even when fish are starved suggesting the process that drives otolith accretion is closely linked to metabolic rate, and that the rate of otolith accretion is directly proportional to the metabolic rate of the fish (Limburg *et al.* 2018). If the physiological regulation of Mg is influenced by hypoxic conditions it could provide a valuable record of the physiological health of the fish, as well as an indicator of hypoxic events (Woodcock *et al.* 2012). Recently, it was proposed that the mechanism allowing uptake of Mg onto otoliths was a two-step process driven by active metabolic transport (Limburg *et al.* 2018). Higher levels of metabolic activity

are thought to drive more movement of Mg through the endolymph fluid to the otolith (Limburg *et al.* 2018). Inherent to this theory is the idea that Mg uptake is scaled with metabolism, such that limiting conditions such as high temperatures and hypoxia mirror incorporation rates, or reflect bioenergetic controls (e.g. consumption, metabolism, excretion, growth) (Limburg *et al.* 2018). Mg incorporation associated with physiological performance in this study was not related to high levels of metabolic activity. Golden perch are more tolerant of hypoxic conditions than other species being able to acclimate to levels from 3 mgO_2 L^{-1} and lower (Gilmore *et al.* 2018), which suggests low oxygen conditions during the experiment may not have been stressful enough to induce a physiological response to allow elevated incorporation of Mg onto the otoliths of golden perch. Further support for this theory comes from a study investigating elemental spiking, where concentrations of Mg did not increase in otoliths despite water spiking, suggesting some physiological regulation (Gilmore unpub, 2013). Additionally, dietary influences were kept to a minimum as all fish were fed the same during experimentation, however similar experiments in the future could measure dietary influences. Mg uptake in otoliths may be less heavily regulated under hypoxic stress allowing elevated concentrations to be accreted onto the otolith potentially indicative of an environmental event. Alternatively, Mg uptake is under complete physiological control, such that, hypoxia changes physiology and the level of Mg uptake in the otolith. However, there has been no experimental validation of this direct relationship to date (Limburg *et al.* 2018).

Other elements may also bind to the surface of the otolith under periods of high stress (Sturrock *et al.* 2015, Gronkjaer 2016, Limburg *et al.* 2018). Fe in the otoliths of fish has received minimal attention, presumably because it is difficult to measure using ICP-MS due to argon interferences, as was the case in our study (Limburg *et al.* 2015). The rarity of dissolved forms of reduced Fe^{2+} in the water column and its affinity with oxygen has often led researchers to suspect it is unlikely to be useful as an indicator of hypoxia (Limburg *et al.* 2015). Fe was enriched in the blood of fish compared to that of the endolymph fluid similarly to Mg in one study, which may suggest it is also physiologically regulated by the organisms through partitioning (Melancon *et al.* 2009). Inorganic iodine present as iodide (I^-, reduced form) and iodate (IO^{3-}, oxidised form) has also been used as an indicator of deoxygenated waters

in marine systems using biogenic carbonates such as corals and foraminifera shells (Lu *et al.* 2010, Limburg *et al.* 2015). It is likely to also be present in otoliths, but has rarely been studied (Lu *et al.* 2010, Limburg *et al.* 2015). Phosphorus as a by-product of dissolved organic carbon which is released during the breakdown of organic matter, may also be useful as an indicator of hypoxic events particularly in freshwater systems where flushing of organic matter is less regular (McBryan *et al.* 2013). Incorporation of any elements indicating sub-lethal hypoxic exposure requires survival of the species through a hypoxic event and roughly a month to become apparent on the otolith (Campana 1999, Elsdon and Gillanders 2003, Elsdon and Gillanders 2006). Elements naturally occurring in low quantities in fish habitats then may not be useful indicators of hypoxic conditions. Further research will be necessary to determine the cause of elemental discrimination on otolith incorporation in fish and determine the physiological mechanisms that drives them (Woodcock *et al.* 2012).

Environmental interactions are going to effect physiological reactions in all fish. Most challenging is using otoliths and the trace elements incorporated on their matrix as indicators of environmental cues where trace elements are physiologically controlled. Herein, we were unable to find a link between otolith chemistry and poor physiological performance when fish were exposed to hypoxia. Future research should continue to tease apart the interactions of physiological controls on trace element concentrations in otoliths. We also suggest that future experimental studies including Mn as a measure of hypoxic exposure should include sediment in tanks to allow realistic redox reactions to occur. Otoliths can be preserved over long time periods and have untapped potential as tracers of environmental events like hypoxia. Elements that are not subject to physiological regulation will provide reliable records of environmental change over long timescales, information that may be unattainable by other means.

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AUTHOR CONTRIBUTIONS

K.L.G., Z.A.D. and B.M.G. conceived the ideas for the manuscript. K.L.G. designed the methodology and collected and analysed the data. K.L.G. led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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SUPPLEMENTARY INFORMATION

SUPP Table 1: Summary of number of golden perch, showing experimental treatments and sample sizes (*n)* for each stage of experimentation. Fish length and weight refers to fish in tanks length and weight at the completion of the experiment.

	Hypoxia	Tank	Fish in Tanks, *n*	Otoliths analysed, *n*	Physiological measures, *n*	Fish Length, cm	Fish Weight, g
20	4	1	11	11	10	5.4±0.28	1.8±0.39
		2	11	10	5	5.6±0.22	1.8±0.30
	8	1	10	9	4	5.4±0.03	2.3±0.50
		2	11	9	3	5.7±0.26	2.3±0.75
24	4	1	10	11	5	6.0±0.39	2.4±0.65
		2	11	9	7	5.6±0.16	1.9±0.28
	8	1	10	6	4	5.9±0.36	1.9±0.56
		2	11	11	8	5.7±0.25	2.1±0.50
28	4	1	10	6	6	5.7±0.43	2.1±0.90
		2	10	8	4	5.6±0.52	2.6±0.50
	8	1	11	9	9	5.2±0.15	1.8±0.62
		2	11	8	4	5.6±0.31	2.3±0.47

SUPP Table 2. Operating parameters for the Resonetic ASI M50 laser connected to the Agilent 7700cs inductively coupled plasma-mass spectrometer (ICP-MS).

Laser	
Wavelength	213 nm
Mode	Q-switch
Frequency	5 Hz
Spot size	26 µm
Spot scan rate	3 µm s^{-1}
Laser energy	100mJ
Carrier	Ar (0.92 L·min^{-1})
Attenuator value	25%T
ICP-MS	
Optional gas	He (58%)
Cone	Pt
Dwell times (ms)	^{23}Na (100), ^{137}Ba (500), ^{88}Sr (200), ^{43}Ca (30), ^{44}Ca (30), ^{115}In (100), ^{55}Mn(1000), ^{24}Mg(500), ^{66}Zn (500)

SUPP Table 3. Summary of the rearing conditions of golden perch within treatment tanks. Data are displayed as means ± standard error (SE) with *n* representing the sample size of fish. For temperature, dissolved oxygen and oxygen saturation, *n* denotes the number of recordings taken throughout experimental period.

Water Conditions	Hypoxia	Tank	Temperature, °C	Dissolved Oxygen, mg L^{-1}	Saturation, %	*n*
			(*n*=100)	(*n*=100)	(*n*=100)	
20	4	1	19.3±0.09	3.82±0.07	53.0±0.94	11
		2	18.8±0.08	4.17±0.10	55.5±1.30	11
	8	1	19.7±0.07	6.70±0.05	92.4±0.92	10
		2	19.1±0.16	6.74±0.04	92.3±0.56	11
24	4	1	24.3±0.03	3.36±0.05	49.6±0.69	10
		2	24.7±0.07	3.32±0.06	50.0±0.94	11
	8	1	23.4±0.03	5.96±0.03	88.2±0.36	10
		2	24.1±0.05	5.65±0.03	86.2±0.42	11
28	4	1	27.3±0.05	3.30±0.05	51.1±0.72	10
		2	27.4±0.03	3.03±0.06	47.3±0.91	10
	8	1	26.9±0.05	5.63±0.03	88.1±0.36	11
		2	26.4±0.12	5.59±0.03	88.5±0.41	11

SUPP Table 4. Analysis of variance for the effects of temperature and hypoxia on temperature, dissolved oxygen (DO), oxygen saturation (SAT) and hypoxia in the rearing water of golden perch.

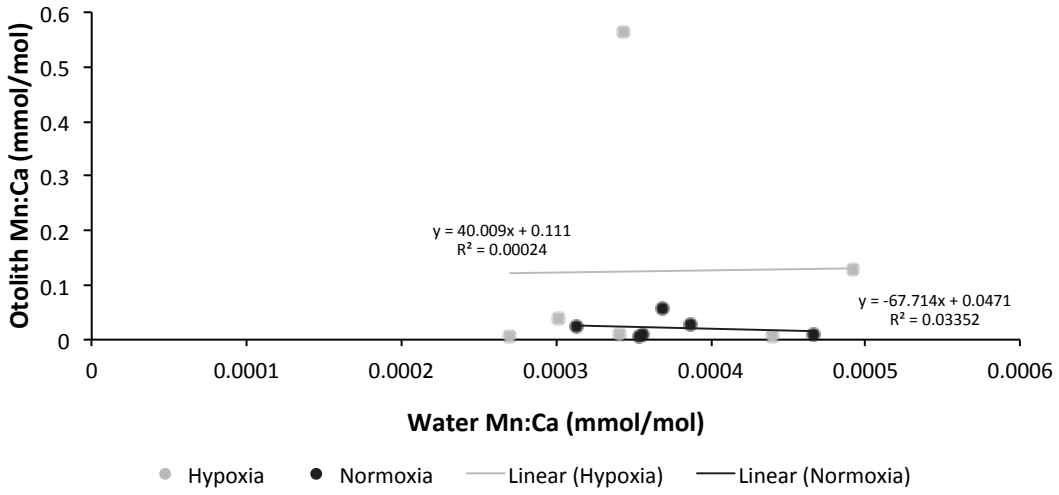
Water	Source of Variation	df	MS	F	P
Temp	Temp	2	5886.9	506.02	≤0.001*
	Hypoxia	1	39.31	3.38	>0.050
	Temp X Hypoxia	2	32.84	2.82	>0.050
	Tank (Temp X Hypoxia)	6	11.64	20.02	≤0.001*
	Residuals	1148	0.58		
DO	Temp	2	99.88	41.30	≤0.001*
	Hypoxia	1	1881.5	778.35	≤0.001*
	Temp X Hypoxia	2	2.14	0.89	>0.050
	Tank (Temp X Hypoxia)	6	2.42	8.68	≤0.001*
	Residuals	1148	0.28		
SAT	Temp	2	2787.1	13.96	<0.050*
	Hypoxia	1	4.23	2118.9	≤0.001*
	Temp X Hypoxia	2	73.60	0.37	>0.050
	Tank (Temp X Hypoxia)	6	199.81	3.57	≤0.001*
	Residuals	1148	55.91		

SUPP Table 5. Analysis of variance for the effects of temperature and hypoxia on levels of trace elements in the experimental rearing water of golden perch. All temperature and hypoxia treatments are considered here.

Trace Element	Sources of Variation	df	MS	F	P
Ba:Ca	Hypoxia	1	0.008	2.61	>0.050
	Temp	2	0.002	0.79	>0.050
	Hypoxia x Temp	2	0.003	1.02	>0.050
	Tank(Hypoxia x Temp)	6	0.003	0.52	>0.050
	Res	12	0.006		
Mg:Ca	Hypoxia	1	3.1	0.00	>0.050
	Temp	2	1521.3	0.91	>0.050
	Hypoxia x Temp	2	1885.0	1.13	>0.050
	Tank(Hypoxia x Temp)	6	1671.7	0.73	>0.050
	Res	12	2277.2		
Mn:Ca	Hypoxia	1	0.06	1.20	>0.050
	Temp	2	0.05	0.91	>0.050
	Hypoxia x Temp	2	0.03	0.57	>0.050
	Tank(Hypoxia x Temp)	6	0.05	1.01	>0.050
	Res	12	0.05		
Na:Ca	Hypoxia	1	121330	1.75	>0.050
	Temp	2	946700	13.65	>0.050
	Hypoxia x Temp	2	63675	0.92	>0.050
	Tank(Hypoxia x Temp)	6	69369	0.23	>0.050
	Res	12	307750		
Sr:Ca	Hypoxia	1	0.22	2.97	>0.050
	Temp	2	0.04	0.55	>0.050
	Hypoxia x Temp	2	0.06	0.75	>0.050
	Tank(Hypoxia x Temp)	6	0.07	3.84	>0.050
	Res	12	0.02		

SUPP Table 6. Summary of the elemental water conditions golden perch were exposed to within treatment tanks. Data are displayed as means ± standard error (SE) with *n* representing the sample size of water measures (beginning of experiment, middle of experiment, conclusion of experiment).

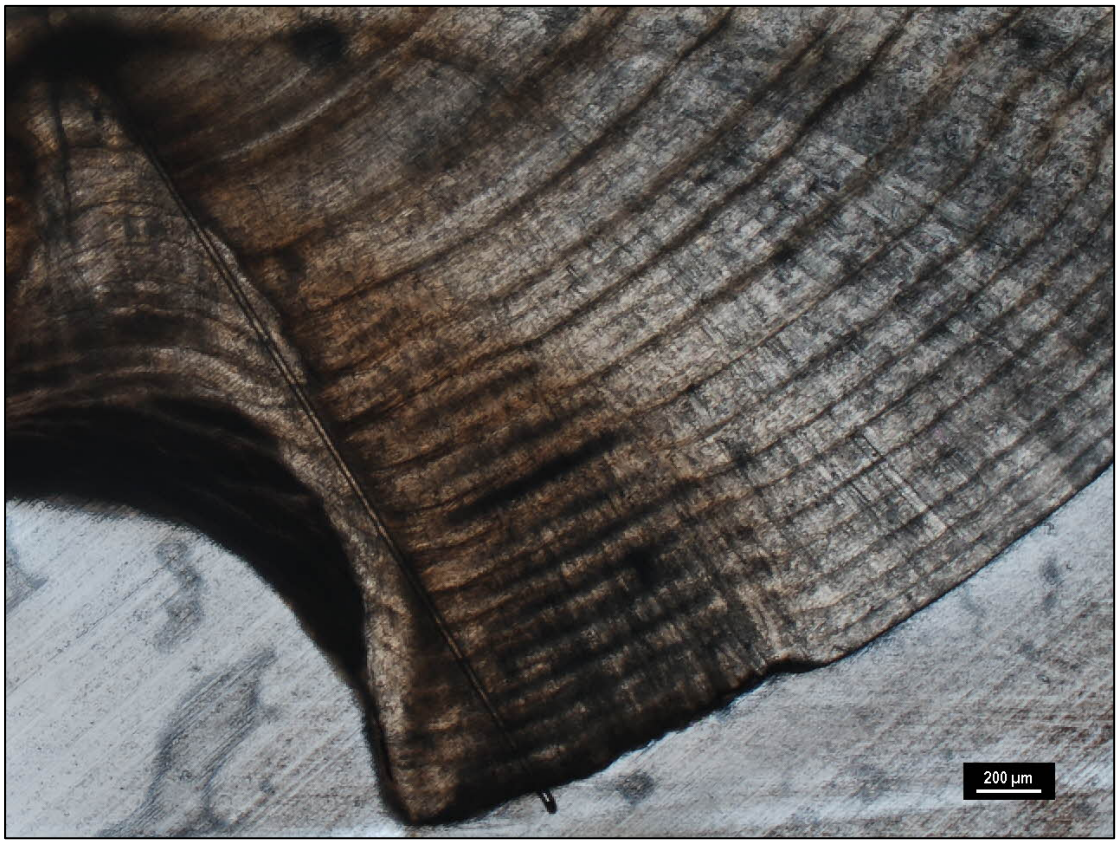
Water Conditions	Hypoxia	Tank	Ba:Ca (mmol/mol)	Mg:Ca (mol/mol)	Sr:Ca (mmol/mol)	Mn;Ca (mmol/mol)	Na:Ca (mol/mol)
Golden Perch			(*n*=3)	(*n*=3)	(*n*=3)	(*n*=3)	(*n*=3)
20	4	1	0.24±0.03	0.42±0.03	1.68±0.02	0.56±0.55	2.98±0.31
		2	0.2±0.04	0.43±0.02	1.47±0.07	0.01±0	2.93±0.22
	8	1	0.15±0.08	0.38±0	1.72±0.16	0.03±0.02	2.97±0.1
		2	0.16±0.03	0.4±0	1.26±0.05	0.06±0.04	2.9±0.16
24	4	1	0.28±0.07	0.39±0.02	1.89±0.17	0.13±0.12	3.08±0.03
		2	0.17±0.04	0.42±0.01	1.49±0.08	0.01±0	3.16±0.05
	8	1	0.17±0.06	0.46±0.04	1.34±0.04	0.01±0	3.71±0.51
		2	0.18±0.03	0.39±0.04	1.27±0.07	0.01±0	3.2±0.15
28	4	1	0.19±0.05	0.43±0.04	1.42±0.08	0.04±0	3.62±0.57
		2	0.13±0.05	0.41±0.06	1.48±0.13	0.01±0	3.54±0.58
	8	1	0.18±0.08	0.47±0.02	1.42±0.11	0.02±0.01	3.87±0.68
		2	0.16±0.06	0.41±0.06	1.26±0.08	0.01±0	3.5±0.52



SUPP Figure 1: Mean otolith element:Ca ratio versus water element:Ca ratio separated by oxygen conditions (hypoxic 3-4mg L^{-1}, or normoxic 6-8 mg L^{-1}) among tank and temperatures during the experimental period. Graph shows golden perch otolith/water Mn:Ca (mmol/mol).

CHAPTER FIVE

USING FISH EAR BONES TO TRACK HYPOXIA IN FRESHWATER



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Above: Image of Murray cod (*Maccullochella peelii*) modern otolith collected at Lock 2 on the Murray river, South Australia, Australia. Photo credit: Kayla Gilmore

STATEMENT OF AUTHORSHIP

This paper *Using fish ear bones to track hypoxia in freshwater*, is unpublished and unsubmitted, all authors made a contribution to the manuscript. Bronwyn M. Gillanders, Zoe A. Doubleday and myself conceived the ideas for the manuscript. I designed the methodology and collected and analysed the data. I led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis.

Signatures of authors:

Kayla . Overall contribution 80%

Overall contribution 10%

Professor Bronwyn M. Gillanders Overall contribution 10%

ABSTRACT

Tracing the oxygen history of fish throughout their life is difficult, but the otoliths (ear bones) of fish may provide a novel option. Otoliths grow incrementally and accrete $CaCO_3$ along with trace elements over the whole life of the fish. Our aim was to determine if trace elements in the otoliths of fish could be used as a natural tracer of hypoxic events in freshwater systems. We examined otoliths from modern and historical collections of field-caught fish that could have experienced or died from hypoxic events. We measured the trace elements in otoliths using laser ablation inductively coupled plasma-mass spectrometry (LA ICP-MS). Transects across field caught fish otoliths allowed us to correlate spikes in manganese, an element previously used to indicate hypoxia, to particular years of growth. We also examined historic newspaper articles describing hypoxia or events associated with hypoxia (major floods and drought) using the National Library of Australia database to determine if there was a link between manganese spikes in otoliths and hypoxic events. Our study is the first to explore the link between hypoxia and otolith chemistry in a freshwater environment, and adds to the use of elemental tracers and archived articles for detecting hypoxic conditions and tracking its occurrence through time.

INTRODUCTION

Ecosystems worldwide have been dramatically changed from their historic baseline conditions through human activities, climate change and other natural events (Izzo *et al.* 2016, Disspain *et al.* 2018). Human activities in particular, have exacerbated the occurrence, extent and severity of natural events, such as drought, floods, and hypoxia (low levels of dissolved oxygen in water, Diaz and Rosenberg 2011, Whitworth *et al.* 2012, Limburg *et al.* 2018). Effective conservation and management requires baseline information on long-term patterns of environmental and ecological community structure to set realistic and sustainable goals for environmental improvement (Carder and Crock 2012, Haidvogl *et al.* 2015, Jenny *et al.* 2016). However, in many cases these historic baselines do not exist.

Hypoxia is a naturally occurring event that has received global attention as its prevalence increases in aquatic systems (Diaz and Rosenberg 2008, Diaz and Rosenberg 2011, Limburg *et al.* 2011). Establishing a historical baseline for comparison is difficult, as the technology to record its presence is relatively new (Diaz and Rosenberg 2011). Increased temperatures, eutrophication, environmental water releases, accumulation of dissolved organic matter and restrictions to water flow all contribute to hypoxic occurrence (Baldwin and Whitworth 2009, Whitworth *et al.* 2012, Limburg *et al.* 2015). At present we understand what hypoxia or low levels of dissolved oxygen are, how they occur and what mechanisms aid in altering its impact (Limburg *et al.* 2011). However, our understanding of the long-term effects of hypoxia to fish populations, fisheries and ecosystems is limited.

Rivers and lakes were modified through human activities and settlement many years before modern ecological monitoring began (Haidvogl *et al.* 2015, Jenny *et al.* 2016). In fact, instrumental records of oxygen only span a few decades (Diaz and Rosenberg 2008, McCarthy *et al.* 2014, Haidvogl *et al.* 2015). Archaeological sites, historic and modern scientific collections, provide the opportunity to collect data about past environmental conditions and fishing practices, including resources such as preserved bone and tissue samples, photographs and anecdotal records (Disspain *et al.* 2012, Alleway *et al.* 2016, Izzo *et al.* 2016). Furthermore, when combined with archival written records (e.g. boating and fishing ledgers, menus and newspapers) they enable

the evaluation of long-term environmental changes (Disspain *et al.* 2011, Alleway *et al.* 2016, Izzo *et al.* 2016).

Otolith (ear bone) chemistry is a valuable tool for unravelling critical questions in fish ecology and past environmental conditions (Disspain *et al.* 2011, Walther *et al.* 2017). Otoliths are found in the inner ear of teleost fishes and form prior to hatching, growing continuously throughout their life (Campana 1999, Campana and Thorrold 2001). Although fish use these for hearing and balance, the daily accretion of material on the surface of the otolith in alternating layers of calcium carbonate and protein have further applications for scientific research (Campana 1999, Elsdon *et al.* 2008). Many studies have used otoliths for reconstructions of age and growth of fish, but increasingly their chemical properties and microstructure are also used to reconstruct environmental histories (Limburg *et al.* 2011, Limburg *et al.* 2015, Disspain *et al.* 2016). Exploration of the chemical elements occurring in otoliths has been dominated by a few key elements (strontium (Sr) and barium (Ba)), generally believed to derive from water and be readily substituted for Ca in the calcium carbonate matrix (Elsdon *et al.* 2008, Doubleday *et al.* 2014, Walther *et al.* 2017). Both Sr and Ba have been utilised as effective tracers of past environmental variation in temperature and salinity explaining the proliferation of studies using these chemical markers (Reis-Santos *et al.* 2012, Walther and Limburg 2012, Izzo *et al.* 2017, Walther *et al.* 2017).

Physiological factors may also influence the uptake of elements in addition to environmental factors, opening the doors to other chemical elements to explain environmental variation (Limburg *et al.* 2011, Walther *et al.* 2017, Altenritter *et al.* 2018). Manganese (Mn) has been the focus of recent studies as it can be affected by physiological regulation and may indicate different environmental patterns (Limburg *et al.* 2011, Limburg *et al.* 2015, Walther *et al.* 2017). Hypoxic conditions create physiological stress, and change regular physiological function in fish; therefore, uptake of manganese in otoliths could be linked to physiological stress caused by hypoxia (Limburg *et al.* 2015, Sturrock *et al.* 2015, Gilmore *et al.* 2018). Dissolved manganese is also available as a product of redox reactions and thus, useful as an indicator of low oxygen conditions (Limburg *et al.* 2015). At present there remains a lack of experimental validation of the uptake and incorporation dynamics of both new and previously explored elements in different physicochemical settings such as hypoxia, pH differences, water pollution and upwelling (Mohan *et al.* 2014, Walther

et al. 2017). Element choice, as well as studies that validate environmental and biological mechanisms driving observed chemical patterns are crucial to expanding the use of otolith chemistry (Walther *et al.* 2017).

This paper combines qualitative data collected from digitised newspaper articles and quantitative data collected from otolith archives. The archives represent two time periods, early 1900s and early 2000s, and are collected from the lower Murray Darling Basin, Australia. The specific aims of this paper were to A) validate Mn as a chemical tracer of hypoxia in a freshwater system known to experience natural and anthropogenically induced hypoxia, B) examine the efficacy of Mn as a tracer among different species, C) utilize Mn to record a changing baseline of hypoxic water conditions throughout the lives of the fish, and C) highlight the value of qualitative data in aiding interpretation of trace element analysis of historic and modern otoliths.

Figure 1. Map showing sites of collected samples within the Murray Darling Basin (Australian Capital Territory, ACT). Image (adapted from map on murrayriver.com.au).

BACKGROUND OF THE STUDY AREA

The Murray Darling Basin is the largest freshwater catchment in Australia covering approx. 1 million km^2 or 14% of Australia's land area (see Figure 1, Koehn 2015, Koehn 2016, Koehn and Nicol 2016). It spans six legislative jurisdictions and a myriad of different governmental departments and agencies each with separate interests and disparate responsibilities. This complex political landscape often results in area specific management targets reflecting short-term interests, with little consideration of flow on effects (Koehn 2015). Fed from headwaters in the Snowy Mountains, the Murray incorporates Australia's three longest rivers the Darling (2740 km), Murray (2530 km) and Murrumbidgee (1690 km) rivers (Koehn 2015), and flows southwest through the Mallee Trench and Mallee Gorge to the Lakes and Coorong district and finally, to the Southern Ocean (Disspain *et al.* 2012). The region is highly dynamic experiencing periods of severe drought and floods and significant anthropogenic influences (Figure 2). Human occupation in the region dates back from c.8,500 years ago, and further archaeological records support that this area was among the most densely populated in Australia at the time of European arrival (Disspain

et al. 2012). Specifically, the construction of barrages in the 1940s, as well as further construction of locks and weirs and the use of water for agricultural irrigation in subsequent years, has led to significantly altered water flows and the system is generally in poor health (Disspain *et al.* 2012, Koehn 2015). Traditionally the area was surrounded by forest and scrubland but is now dominated by agricultural lands.

The Murray Darling Basin has been subjected to periods of hypoxia (Whitworth *et al.* 2012, Koehn and Nicol 2016), which are believed to have increased in frequency and severity since European settlement in the 1840s (Disspain *et al.* 2012). Furthermore, increases in summer rainfall, which exacerbate hypoxic events and more frequent climatic extremes, are also expected due to climate change (Whitworth *et al.* 2012, Koehn 2015). A recent example of hypoxia in this system occurred following the 10-year Millennium Drought (Figure 2). The basin experienced large-scale floods and prolonged periods of hypoxic blackwater, affecting 2000kms of the river with widespread fish kills (Whitworth *et al.* 2012).

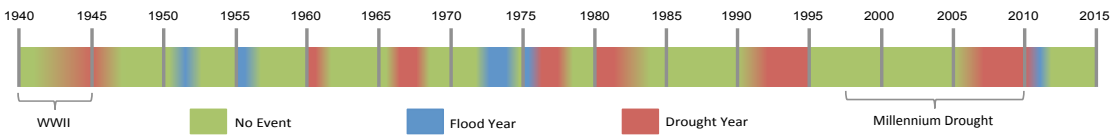


Figure 2. Timeline showing major droughts and floods over the sampling period across the lower Murray river (information for timeline collected from samemory.sa.gov.au, murrayriver.com.au and floodvictoria.vic.gov.au, Koehn 2015).

METHODS

Study species

The fish of the Murray Darling Basin are culturally important and contribute to tourism and recreational fishing (Koehn 2015). However, native fish in the Murray Darling Basin have been estimated to be at 10% of their pre-European abundance, due to natural (e.g. increased temperatures and hypoxia) and anthropogenic factors (e.g. barriers to water flow and environmental watering, Koehn and Nicol 2016). Two native long-lived species endemic to the Murray River region were chosen for this study; Murray cod (Percichthyidae: *Maccullochella peelii*) and golden perch (Percichthyidae: *Macquaria ambigua*). They are the largest species in the MDB

weighing up to 114 and 23 kg, respectively (Lintermans 2007, Koehn and Nicol 2016). Both species are opportunistic carnivores however, they have quite different lifestyles (Lintermans 2007). Murray cod are predominantly sedentary, and have a strong preference for structural woody habitats, often inhabiting the same area for many years, however, there are also records of this species typically making small migrations (>1km, Clark *et al.* 2005, Koehn and Nicol 2016). In comparison, golden perch are typically found in the main channel from fast flowing upper reaches to lowland, turbid and slow flowing reaches and in deep pools within these habitats and regularly make large migrations (>1000km, Lintermans 2007).

Both species have been bred by government and commercial hatcheries as part of ongoing conservation efforts and active national recovery programs currently in place in Australia (Lintermans 2007, Couch *et al.* 2016). Murray cod are listed as critically endangered on the International Union for Conservation of Nature (IUCN) Red List (www.iucnredlist.org, Couch *et al.* 2016), and golden perch is identified as vulnerable in Australia (Lintermans 2007).

Otolith Preparation

Archived otolith collections from Australian government agencies were mostly used in this study (see Table 1). Otoliths from archived collections were classified as either modern (collected <15 years ago) or historic (collected >15 years ago). Ninety-four percent of Murray cod and 73% of golden perch otoliths came from archived samples, with the remaining opportunistically collected from natural fish kills. Collections included samples from the middle to lower portion of the Murray River including from New South Wales, NSW, Victoria, VIC, and South Australia, SA. Otoliths were first rinsed using ultrapure water and then left to air-dry overnight. Otoliths were then embedded in latex molds in indium spiked resin (40ppm) to allow discrimination of otolith and resin, and placed in an oven maintained at 54.5°C overnight to harden. Otoliths were then sectioned transversely through the core using a low-speed diamond saw lubricated with ultrapure water. Sections were approximately 300µm and were polished to 200µm using 30- and 9-µm lapping film. Sections were mounted on slides using crystal bond, cleaned and placed in plastic bags for storage.

For the two species a total of 140 sectioned otoliths (Murray cod n=91 and golden perch n=49), were ablated to investigate the trace element data across 59 years (see Figure 3) for signs of elevated levels of manganese (spikes >5µmol/mol Mn:Ca, see *Chemical analysis* for further information). The age at time of death ranged from 2 to 28 years for golden perch and from 3 to 41 years for Murray cod (see also Figure 3 for distribution of sampled years). The average age of golden perch (n=49) was 7.2 years with the majority of samples aged between 4 and 8 years (75%; n=37). The average age of Murray cod (n=91) was 14.1 years with the majority of samples aged between 5 and 16 years (68%; n=62). Modern golden perch were mostly collected in the colder months (n=11 of 13 fish), while the historic golden perch were mostly collected in the warmer months of the year (n=24 of 36 fish). The opposite was true of Murray cod samples where the modern collection were gathered during the warmer months of the year (n=46 of 47 fish) and the historic samples were collected during the cooler months (n=25 of 44 fish). All of the sectioned otoliths could be aged as annuli were clearly visible and two independent counters checked ages. Ages were then backdated from year of death. Both species have been validated as forming increments on an annual basis (Anderson *et al.* 1992).

Table 1: Details of golden perch (M, Modern = 13 samples; H, Historic = 36 samples) and Murray cod (M = 47 samples; H = 44 samples) otolith samples. Shown are collection locations and state (New South Wales, NSW; Victoria, VIC; and South Australia, SA), year collected; season (southern hemisphere) and years the otolith collection spans based on when they were collected; as well as age information for samples.

State	Location	# fish	Collected	Years that collection spans	Fish age	Season collected
Golden perch						
NSW	Molongolo Reach (M)	2	2012	2012-2000	6-13	Summer
NSW	Lake Ginnindera (M)	3	2005	2005-1995	2-11	Autumn
NSW	Barmah (H)*	12	1952	1952-1925	4-28	Autumn-Winter
VIC	Boundary Bend (H)*	12	1949	1949-1940	4-10	Spring
NSW	Moorna Woolshed, Wentworth (H)*	12	1954	1954-1945	4-10	Summer
SA	Swan Reach (M)	8	2014	2014-1999	4-16	Autumn
Murray cod						
NSW	Yerrabi Pond (M)	5	2009 & 2014	2014-2007	3-5	Winter-Spring
VIC	Bundalong (H)	5	1953	1953-1947	3-7	Winter-Spring
NSW/ VIC	Yarrawonga Weir (H)	5	1953	1953-1943	6-11	Winter
VIC	Murray/Ovens river junction (H)	9	1953	1953-1942	5-12	Winter
VIC/ NSW	Moira Lakes/Barmah (H)*	3	1952	1952-1935	9-18	Winter
VIC	Boundary Bend (H)*	13	1949	1949-1941	4-8	Spring
NSW/ VIC	Moorna Woolshed, Wentworth/Lock 8 (H)*	9	1954	1954-1942	6-13	Summer-Winter
SA	Berri (M)	10	2011	2011-1977	13-35	Summer
SA	Lock 4 / Lock 3-4 (M)	3	2012	2012-1976	35-37	Summer
SA	Loxton (M)	10	2011	2011-1992	15-20	Summer
SA	Lock 3 (M)	3	2009	2009-1976	13-34	Spring
SA	Lock 2 (M)	6	2009 & 2012	2012-1977	15-34	Spring-Summer
SA	Lock 1 (M)	10	2009 & 2012	2012-1969	13-41	Spring-Summer

* Locations that overlap for both species

Chemical analysis

Concentrations of elements were measured from the core to the proximal edge of the otolith using a Resonetics M-50-LR 193 nm Excimer laser ablation system (Resonetics, Nashua, New Hampshire, USA) coupled to an Agilent 7700cx quadrupole ICP-MS (Agilent Technologies, Santa Clara, California, USA) at Adelaide Microscopy, Adelaide, South Australia, Australia. The laser ran at a scan speed of 3µm/s with a frequency of 10Hz using a 29µm diameter to ablate a continuous laser transect across the otolith. This transect measured the concentrations of ^{55}Mn (1000 ms), ^{43}Ca (30 ms), ^{44}Ca (30 ms) and ^{115}In (100 ms). Measures of Ca were used to ratio elements to Ca, and In was used to confirm otolith material was constantly ablated. Background levels of elements in the ablation chamber were measured 30s prior to each ablation. Mn:Ca has been previously used to describe hypoxic occurrence in otoliths of marine fish and as such was the main element of interest in this study (Limburg *et al.* 2015).

Drift and precision of the instrument was compared to a reference standard (NIST 612; National Institute of Standards and Technology, Gaithersburg, Maryland, USA) after every 10 samples when multiple samples were on a single slide, or between each slide when slides only contained one or two samples. A reference carbonate standard (MACS 3; U.S. Geological Survey, Denver, Colorado, USA) was also measured at the beginning and end of each laser session. Precision calculated as the mean coefficients of variation (CV) of repeated measures for the reference NIST 612 standard and the MACS 3 standard was <1% for all elements. All raw data were processed using GLITTER software (www.glitter-gemoc.com) to distinguish background and otolith element mass counts. Excel was then used to further process the data and ratio element concentrations to Ca. Elevated levels of manganese were characterised by multi-point spikes >5µmol/mol Mn:Ca (multi-point defined as gradually increasing and decreasing data values around a central point) before being considered as elevated levels. The basis of this designation was derived from observations of the raw data before smoothing, where spikes were not obvious <5µmol/mol Mn:Ca and could have been affected by instrumental spikes. After observation of the raw data, the data were smoothed where data values ≥15µmol/mol Mn:Ca were subjected to averaging using the 6 data points around the value, this also reduced data noise. Data values exceeding ≥15µmol/mol Mn:Ca had to meet the

multi-point spike rule and remain after smoothing before being designated as an elevated spike.

Annual growth estimation

Images of ablated otoliths were taken under diascopic polarized light using a Nikon Eclipse LV_{100} POL A1r HD Petrographic Microscope (Adelaide Microscopy, Adelaide, South Australia, Australia). Otolith images were used to determine age and measure width of growth increments. These measurements were made using the program Nikon NIS-Elements D (magnification 2.5x) by measuring from the core to the edge on the proximal side of the otolith along the ablated transect. Growth increments were marked and measured in μm.

We used established aging methods for Murray cod (Anderson *et al.* 1992), and golden perch (Anderson *et al.* 1992), to estimate age of each fish at the time of death. Two independent readers assigned ages to each sample. Where the two counts differed, the primary author made a third count this always matched one of the other counts and was taken as the age. We assigned annual growth increments a year relative to the date of capture. As the laser was operated in a time resolved mode, we used the scan speed (μm/s) to convert each time dated element:Ca data point (s) to a distance measurement (μm). Using this method we could align growth increment measures to the element:Ca data using the ablated transect and otolith edge as reference points to assign appropriate years.

Archival data

We used Trove, a database of the National Library of Australia, to examine digitised newspaper articles among the years encompassed by our otolith collection 1935-2015. Searches were limited to references linked to the Murray Darling Basin, Australia across three states where otoliths were collected; South Australia, SA, Victoria, VIC, and New South Wales, NSW. General search terms included species common names and variations of terms related to mortality (for full list see Table 3), that might highlight a hypoxic event. Articles which could be associated directly with hypoxic events were categorised as *Hypoxic references* (n=14), while articles that were possibly linked to a hypoxic event but did not provide enough detail to conclusively determine hypoxic impacts were categorised based on the authors discretion as

Potential references (n=30). Trove is continuing to add digitised material and as such articles after the 1970s have yet to be fully archived, which means our data only represented the years between the 1940-1970s accurately. We used annual flow data gathered from the Murray Darling Basin Authority website (www.mdba.gov.au, under no known copyright restrictions) to produce a graph of annual flow for a site near where our otolith samples were collected (Figure 4). We examined data from the Corowa river gauge, along the main river channel, that spanned the years encompassed by our otolith collection 1935-2015. The data allowed us to compare annual flow with element:Ca spikes in otoliths and flood and drought records.

RESULTS

Table 2: Details of golden perch (M, Modern = 13 samples; H, Historic = 36 samples) and Murray cod (M = 47 samples; H = 44 samples) otolith samples where manganese was elevated in concentration. Shown are collection locations and state; total number of fish collected and total fish with manganese indicators found along ablated transect; year range of collected fish with manganese indicators; and total number of fish with manganese indicators found during flood and drought years and within the first year of growth.

State	Location	# fish collected	# fish w/spikes	Year Range of fish w/Mn	#fish w/spike in flood	#fish w/spike in drought	Spike in 1st Year
Golden perch							
NSW	Molongolo Reach (M)	2	1	2012-2007	-	1	1
NSW	Lake Ginnindera (M)	3	2	2005-2000	-	-	2
NSW	Barmah (H)*	12	9	1952-1945	9	4	9
VIC	Boundary Bend (H)*	12	11	1949-1940	-	11+	10
NSW	Moorna Woolshed, Wentworth (H)*	12	10	1954-1945	1	2+	9
SA	Swan Reach (M)	8	3	2014-2009	2	1+	3
Murray cod							
NSW	Yerrabi Pond (M)	5	1	2014-2011	1	-	1
VIC	Bundalong (H)	5	2	1953-1947	-	-	2
NSW /VIC	Yarrawonga Weir (H)	5	3	1953-1943	-	1	2
VIC	Murray/Ovens river junction (H)	9	7	1953-1942	-	-	3
VIC/ NSW	Moira Lakes/Barmah (H)*	3	-	-	-	-	-
VIC	Boundary Bend (H)*	13	5	1949-1942	-	5+	4
NSW /VIC	Moorna Woolshed, Wentworth/Lock 8 (H)*	9	3	1954-1947	-	-	2
SA	Berri (M)	10	1	2011-1994	1	-	-
SA	Lock 4 / Lock 3-4 (M)	3	1	2012-1977	1	1+	1
SA	Loxton (M)	10	3	2011-1992	3	-	-
SA	Lock 3 (M)	3	1	2009-1976	1+	1+	1
SA	Lock 2 (M)	6	1	2009-1994	1+	1+	1
SA	Lock 1 (M)	10	5	2012-1969	2	4+	3

*Locations that overlap for both species

+ Multiple years with Mn indicators for individual fish under either flood or drought conditions

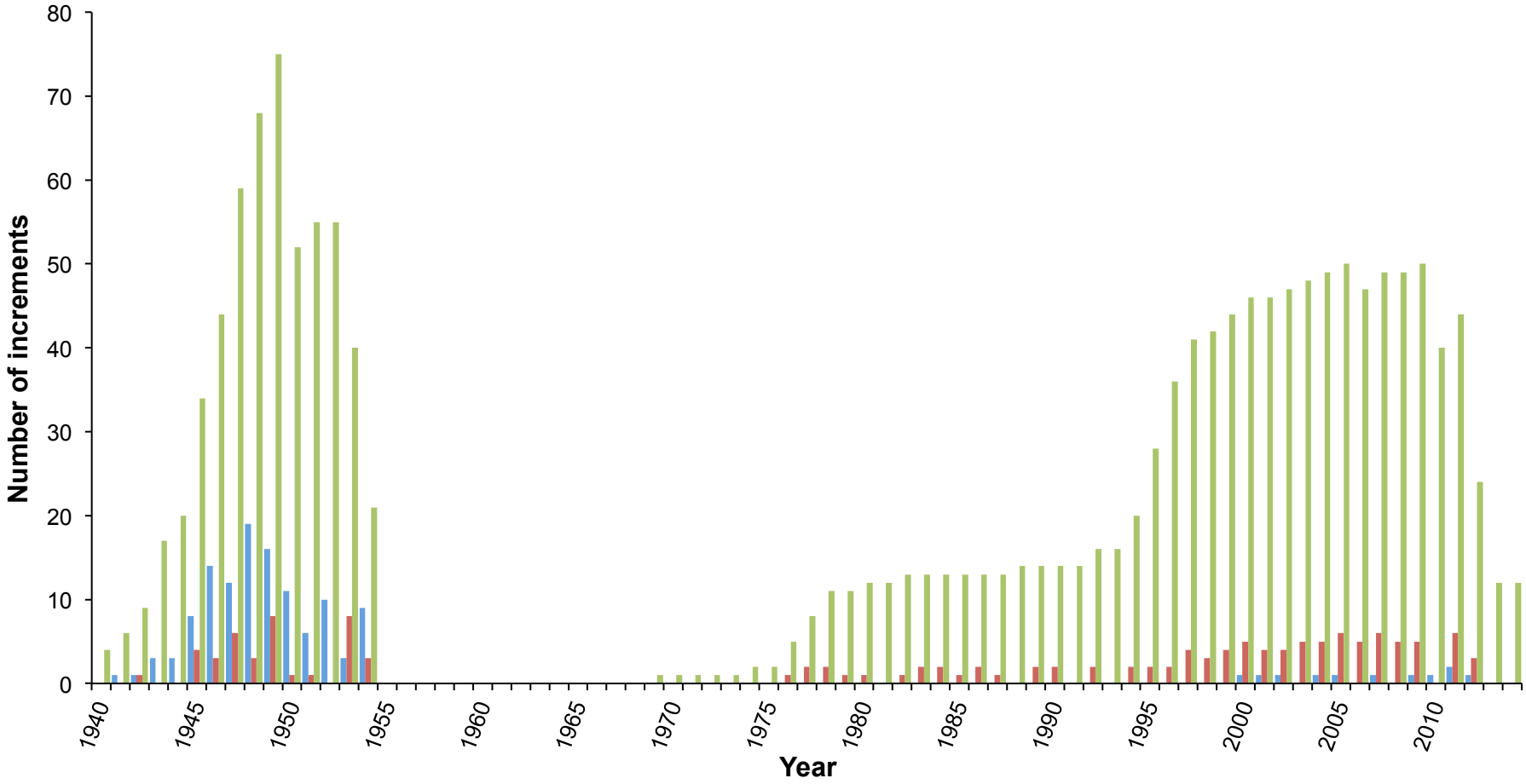


Figure 3. Number of otolith increment measurements representing each growth year for both species (*green columns*). Also shown are number of otolith increment measurements for each species with elevated levels of manganese, as a possible indicator of hypoxia (*blue columns* golden perch; *red columns* Murray cod).

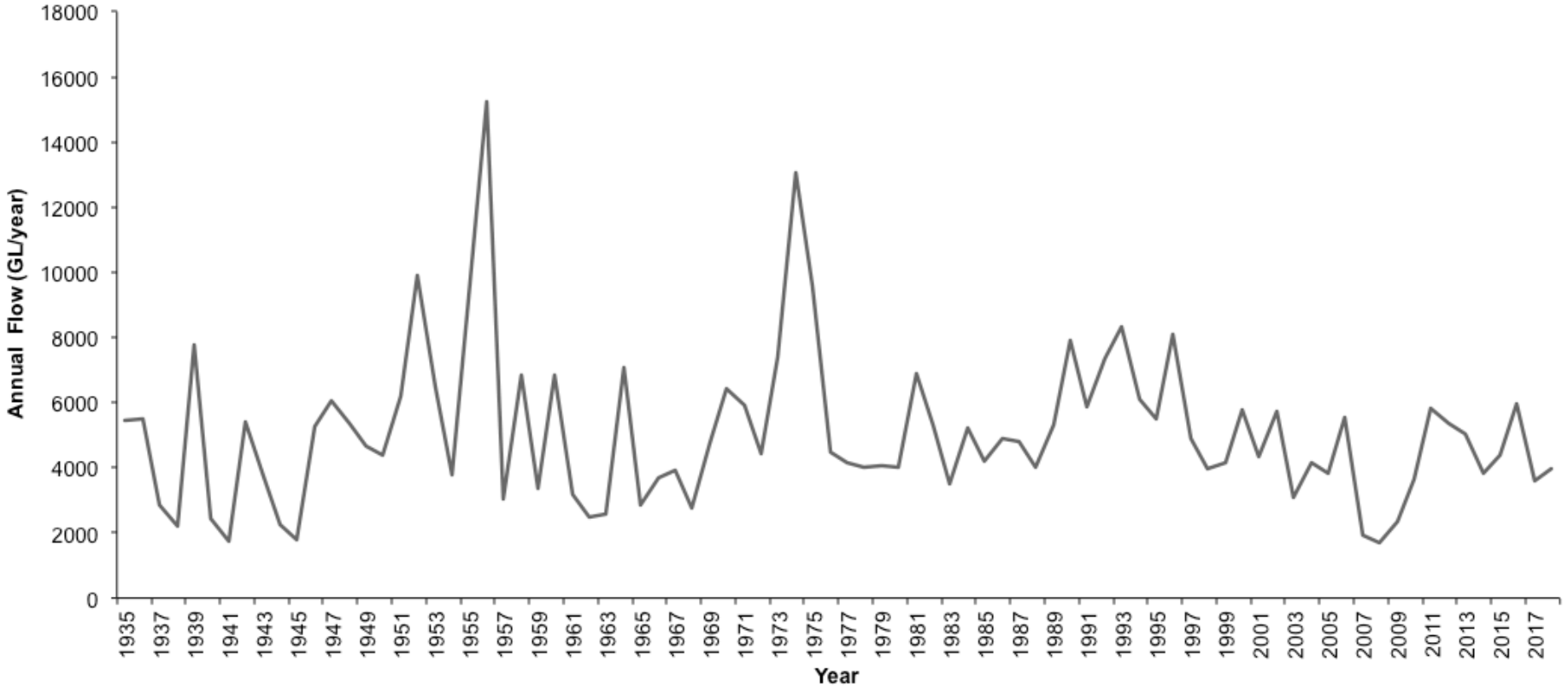


Figure 4: Annual River Murray flows through the Corowa river gauge spanning the years encompassed by our otolith samples (data was adapted from the Murray Darling Basin Authority, www.mdba.gov.au, no known copyright restrictions).

Otolith chemistry

Otolith growth increments spanned almost 60 years including 1935 to 2014 for Murray cod and 1925 to 2014 for golden perch (with some years excluded see Figure 3). Seventy three percent of golden perch otoliths (36 of 49 fish) had elevated levels of manganese in at least one year of growth (Table 2) suggesting they had experienced low oxygen conditions at some point in their life. Fewer Murray cod otoliths (36%; 33 of 91 fish) showed elevated levels of manganese (Table 2). Samples of golden perch with indicators of manganese spanned years 1999-2014 and 1940-1954 (Figure 3). Samples of Murray cod with indicators of manganese spanned years 1969-2014 and 1942-1954 (Figure 3). Both modern and historic samples of both species showed evidence of increased manganese concentrations (Figure 3 and 5).

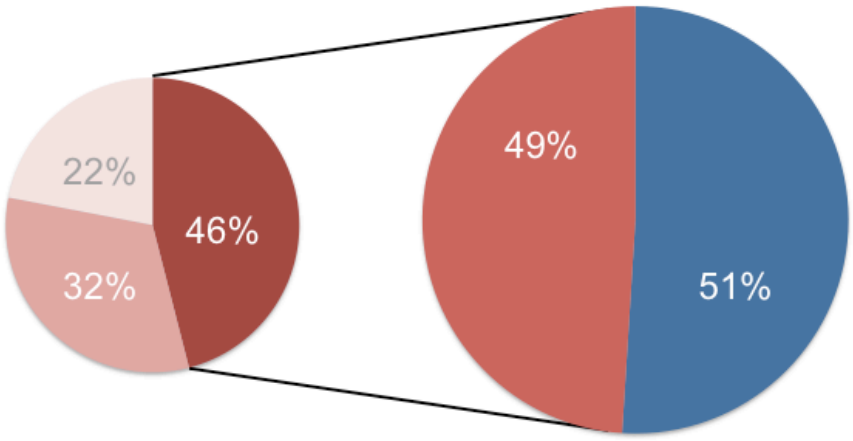


Figure 5: Pie chart of otoliths with manganese as indicators of hypoxia found in any year of growth spanning al years 1935-2014. The large pie chart shows the difference in otoliths with elevated manganese (red) and those without elevated manganese spikes (blue). The smaller pie chart shows the relation of manganese spikes to major droughts (dark red), major floods (red) and those without relation to an environmental disturbance. Drought and flooding events were validated in digitised newspaper records, and with annual flow data.

High manganese concentrations were not found between years not represented by our samples for Murray cod this was between 1954 and 1975 (15 years) and for golden perch this was between 1954 and 1999 (45 years, Table 2 & Figure 3). Elevated manganese was most often found in either the first year of growth (Table 2, Figures 6, 7 & 8) or the final year of growth (see Figures 6, 7 & 8). Manganese also appeared to be associated with flood and drought events (Figure 5). Elevated manganese was linked to major floods in 22 of the 69 fish, and was linked to major drought conditions in 32 of the 69 fish (Table 2, Figure 5). Flood years with the highest proportion of manganese occurred following the millennium drought in the Murray Darling Basin (2011; 66% of samples), and 1952 (29% of samples). Drought years with the highest proportion of manganese spikes occurred in 1945 and 1946 (70 and 68% of samples respectively). Additional samples where elevated manganese was detected occurred between 2006 and 2009, years that were also affected by the millennium drought (see Figure 7). Spikes in manganese, in relation to the timing of droughts and floods, were further supported by descents and peaks in annual flow fate (Figure 4).

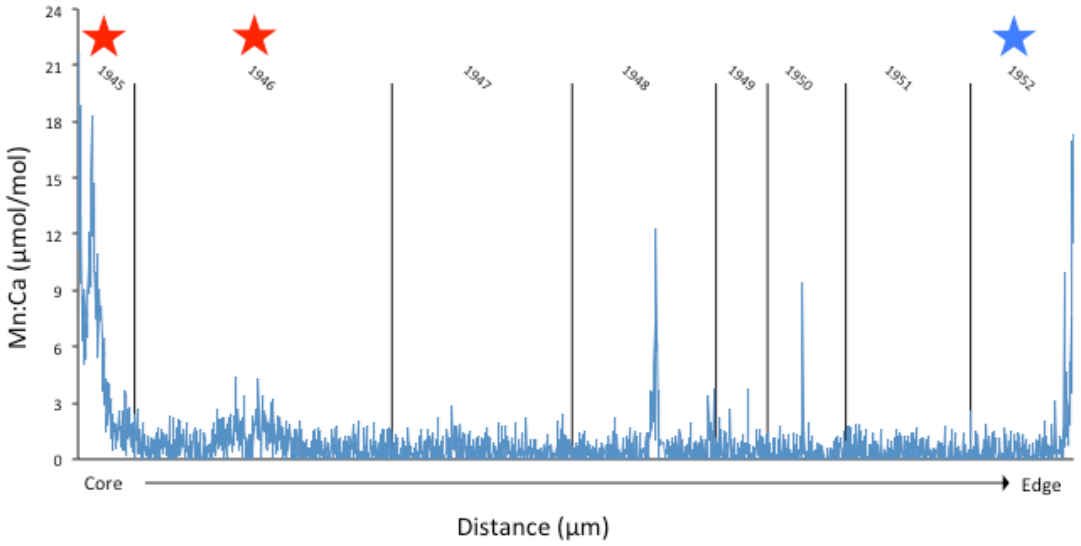


Figure 6. Manganese in an individual golden perch otolith from the historic Barmah collection, age7+ fish. Stars above graphs indicate years with major droughts (red star) and floods (blue star). Black vertical lines show edges of growth increments.

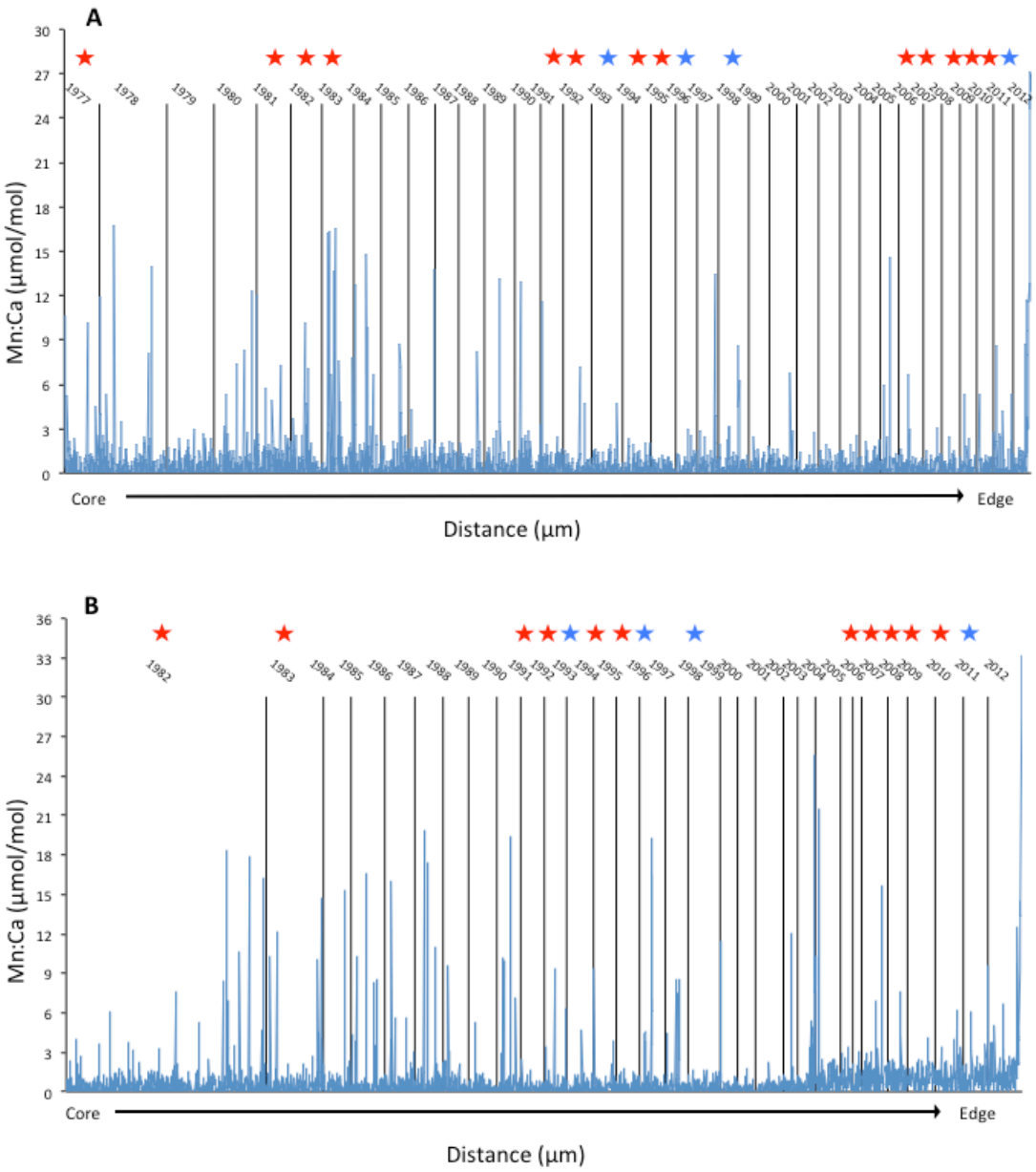
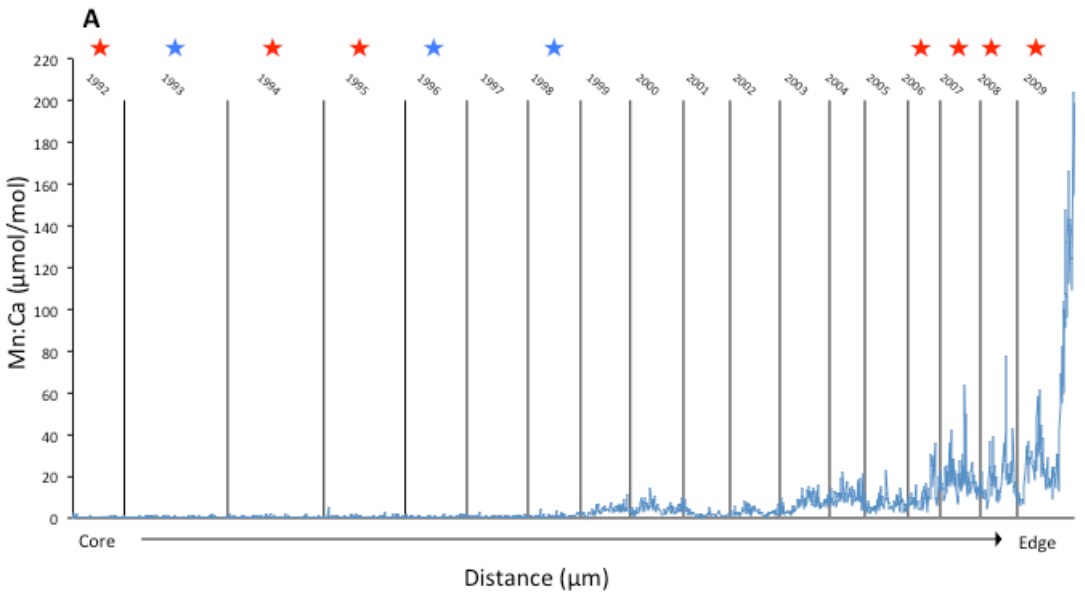
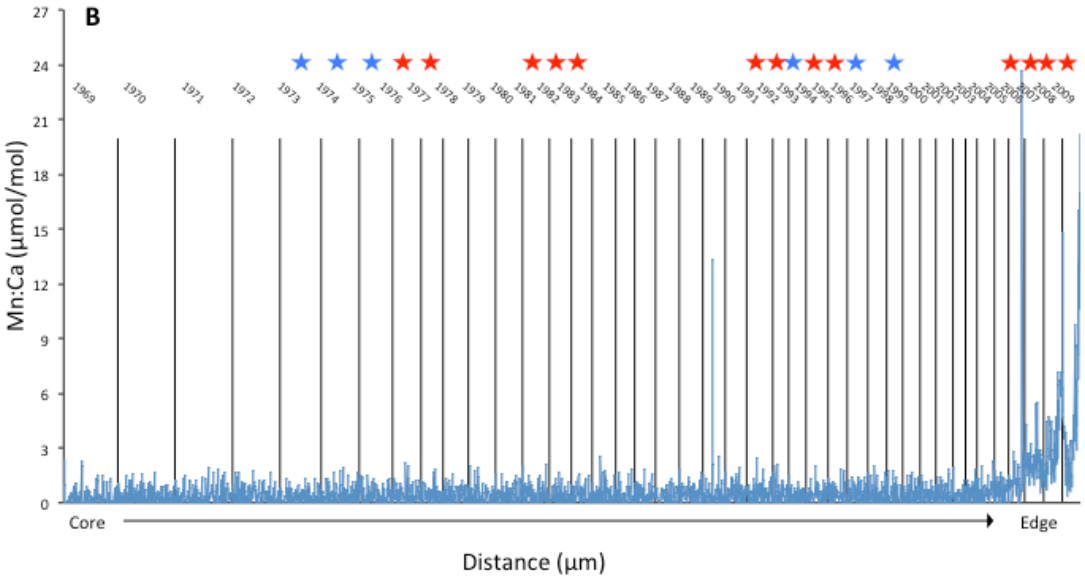


Figure 7. Manganese in otoliths from the modern Murray cod A) age 36+ fish and B) age 31+ fish. Stars above graphs indicate years with major droughts (red star) and floods (blue star). Black vertical lines show edges of growth increments.


A
Mn:Ca (µmol/mol)
Core
Edge
Distance (µm)


B
Mn:Ca (µmol/mol)
Core
Edge
Distance (µm)

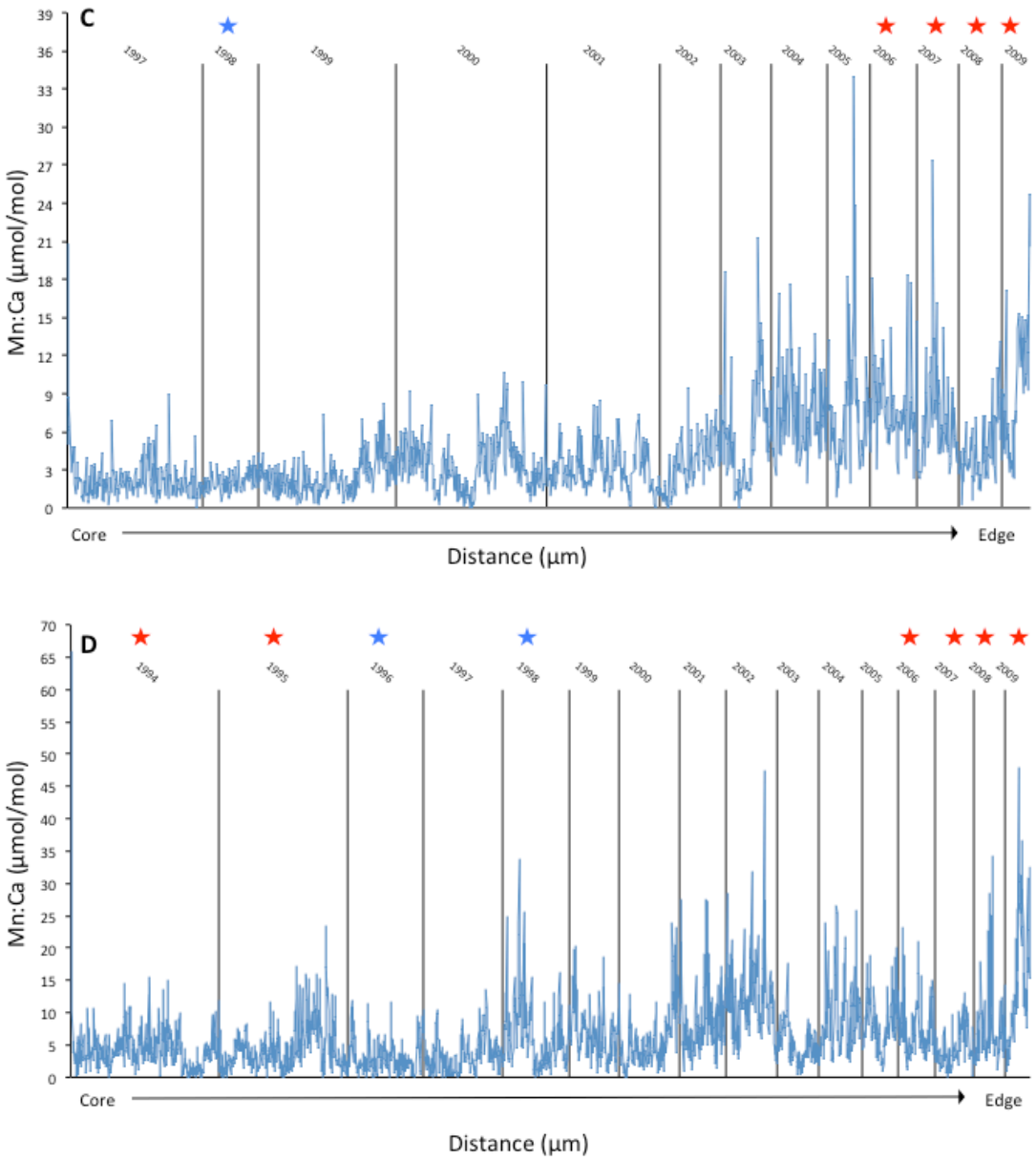


Figure 8. Manganese in otoliths from modern Murray cod A) age 18+ fish, B) age 41+ fish, C) age 13+ fish and D) age 16+ fish. Stars above graphs indicate years with major droughts (red star) and floods (blue star). Black vertical lines show edges of growth increments.

Newspaper archival and anecdotal references

A total of 14 archival and anecdotal records (CE 1939-1952) could be confirmed as occurrences of hypoxia in the Murray River, Australia across three states (Table 3). For example, the mention of dead fish and the colour of water was clear evidence of a hypoxic blackwater event:

> "For some weeks past fish in the Broken River and its tributaries have been dying, and the appearance of dead fish in the Broken River has been most noticeable. They were so numerous near the old weir on the property of Mr Percy Trewin, that he commenced to remove them and it presented an odd spot with 20 to 40 fish ranging from 10 lbs to 40 lbs. Mystery surrounds their death, but the colour of the water these days is enough to kill anything" (*Benalla Ensign, VIC,* 27 February 1942, colour of the water would be linked to hypoxic blackwater).

Another 30 records (CE 1939-1993) referenced or alluded to environmental disturbances (e.g. blackwater, drought, flood, stagnant water and hot temperatures), which may be linked to hypoxia; however, the articles did not have enough detail to clearly indicate a hypoxic event. No records used the terminology hypoxia and very few were linked to blackwater (an environmental term describing the tea stained colour of water, often present under low oxygen conditions due to leaching of organic matter, see also Table 3). Records categorised under *Hypoxic references* were recorded most often in warmer months (57% of records) at the end of the austral summer; however, those categorised as *Potential references* were recorded more in cooler months (53%). However, the cooler month bias of *Potential* records was driven by a single event that occurred in early March in 1951 which was initially thought to be caused by an outbreak of myxomatosis virus, however, this was later shown not to be the case and the event had all the markers of a hypoxic event (e.g. late summer, high mortalities, stagnant water also see SUPP Information).

> "The Superintendent of State Fisheries (Mr. T. C. Roughley) who visited Leeton some time ago to investigate reports that Murray cod were breeding in the irrigation canals evidently believes that there is something 'fishy' about the co-incidental deaths of hundreds of Murray cod and perch and large numbers of rabbits in the same areas of northern Victoria where myxomatosis has been introduced by the scientists. Mr Roughley states that both the Murray cod and perch are extremely hardy and he can think of no natural conditions that would have brought about their death. It is claimed that rabbits blinded by mxyomatosis are falling into rivers and creeks and being eaten by fish" (*The Murrumbidgee Irrigator, NSW,* 22 March 1951).

> "A scientist from Sydney University has reported that myxomatosis, which has been successful in killing rabbits, had not affected fauna in the Murray River Valley, chairman of the Taronga Park Trust (Mr. Hallstrom) said. The scientist Dr. A. Bollinger, is considered Australia's leading authority on marsupials. Mr. Hallstrom sent Dr. Bollinger to the Murray River Valley,

> where it was reported fish, birds, dogs, foxes and kangaroos were dying from the mosquito-borne disease. Mr. Hallstrom said Dr. Bollinger proved the reports false." (*Barrier Daily Truth, NSW*, 13 March 1951).
>
> "The Chairman of the C.S.I.R.O. (Dr. Clunies Ross) said tests had shown that myxomatosis would not affect any animal except the European wild hare, from which Australian rabbits were descended. He added: "There is no evidence to support that myxomatosis is killing kangaroos, foxes, birds and crayfish. The idea is fantastic."…A C.S.I.R.O. research worker, in Canberra early this year inoculated himself with myxomatosis virus. He showed "absolutely no reaction" a C.S.I.R.O. report said." (*The Daily Telegraph, NSW*, 2 March 1951).

Despite the large number of records found using our search terms very few could be related to hypoxic events, likely due to a poor understanding of hypoxia in the mid 1900s (see supplementary information for record specifics and Table 3).

Table 3. Table showing number of references linked to Trove archival digitised records and our search terms for each state and all states combined. Articles that could be directly associated with hypoxic events are listed under *Hyp. ref.* (n=14) and articles that were possibly linked to hypoxic events but did not have enough information to conclusively state they were a hypoxic event were listed under *Pot. ref.* (n=30). For each state the total number of records found and reviewed for hypoxic references are listed as *#Rec*. and then combined across all states in *Total Rec*. Records are sourced from articles and digitised newspapers; in addition, records were refined to cover the years 1939-2015 which encompassed the otolith samples.

Search Term	**NSW**			**SA**			**VIC**			**All States**			
	Hyp. Ref.	*Pot. Ref.*	*# Rec.*	*Hyp. Ref.*	*Pot. Ref.*	*# Rec.*	*Hyp. Ref.*	*Pot. Ref.*	*# Rec.*	*Hyp. Ref.*	*Pot. Ref.*	*Total Ref.*	*Total Rec.*
Fish low oxygen	0	0	202	0	0	44	0	0	49	0	0	0	295
Fish suffocation	0	2	328	0	0	77	1	0	103	1	2	3	508
Hypoxia	0	0	17	0	0	11	0	0	35	0	0	0	63
Blackwater	1	1	133	1	3	21	2	0	24	4	4	8	178
Blackwater river	1	1	133	1	3	21	1	0	24	3	4	7	178
Murray cod blackwater	0	1	3	1	1	5	1	1	1	2	3	5	9
Murray cod mortality	1	3	19	0	1	8	1	3	24	2	7	9	51
Murray cod death	4	9	283	0	1	114	1	1	84	5	11	16	481
Murray cod die off	1	8	81	0	0	43	1	0	18	2	8	10	142
Murray cod	4	12	3060	4	4	1591	1	4	1476	9	20	29	6127
Golden perch blackwater	0	1	322	0	1	86	0	0	113	0	2	2	521
Golden perch mortality	0	0	46	0	0	9	0	0	17	0	0	0	72
Golden perch death	1	1	257	0	0	67	0	0	98	1	1	2	422
Golden perch die off	0	1	131	0	0	27	0	0	56	0	1	1	214
Golden perch	1	1	1587	0	1	453	0	0	719	1	2	3	2759
Callop blackwater	0	0	7	0	1	42	0	0	1	0	1	1	50
Callop mortality	0	0	0	0	0	4	0	0	0	0	0	0	4
Callop death	0	0	8	0	0	18	0	0	2	0	0	0	28
Callop die off	0	0	3	0	0	14	0	0	2	0	0	0	19
Callop	0	0	321	0	4	565	0	0	89	0	4	4	975
Yellowbelly blackwater	0	0	49	0	0	3	0	0	8	0	0	0	60
Yellowbelly mortality	0	1	4	0	0	0	0	0	0	0	1	1	4
Yellowbelly death	1	0	45	0	0	3	0	0	4	1	0	1	52
Yellowbelly die off	0	1	15	0	0	0	0	0	3	0	1	1	18
Yellowbelly	1	1	278	0	0	21	0	0	31	1	1	2	330
River fish death	7	11	4024	0	1	742	2	1	741	9	13	22	5507
River fish mortality	3	4	250	0	1	62	1	4	81	4	9	13	393
River fish die off	4	10	925	0	0	233	1	1	247	5	11	16	1405
Oxygen depletion	0	0	65	0	0	31	0	0	22	0	0	0	118

DISCUSSION

Developing a baseline of hypoxic occurrence in rivers has been problematic, particularly during years before modern records were kept. However, trace amounts of manganese in the otoliths of fish were successfully linked to years with known hypoxic events in this study. Most importantly manganese's usefulness is limited more by adequate exposure of species to a hypoxic event than being present in only specific species within a system. Trace amounts of manganese found in historic collections of otoliths could be related to annual flow data from the main river channel, and qualitative records of environmental disturbances often linked to hypoxic events (drought and floods). The application of this method to larger collections of historic and archaeological otoliths could allow reconstructions of a hypoxia timeline, and has application across other environments and species.

Around half our samples had elevated spikes of manganese in the otoliths. Of these, 32% could be related to major flooding events and a further 46% were related to major droughts. These trends suggest manganese could be linked to environmental conditions like hypoxia, as drought and floods often exacerbate hypoxia. Some years in the otoliths were better represented than others for otoliths (2006-2011, 1952 and 1946-45), and of those there was some overlap in the years 1952 and between 1945 and 1946 with qualitative records of hypoxic events. Additionally elevated manganese during the final year of growth in many of our samples suggests fish died from hypoxic conditions. Spikes in manganese observed in years not linked to major drought, floods or hypoxic events collected could be caused by other smaller localised hypoxic events not recorded or observed.

Manganese spikes were also observed during the first years of growth in many samples. Corroborating previous evidence of maternal transfer of manganese to the embryo (Brophy *et al.* 2004, Ruttenberg *et al.* 2005, Limburg *et al.* 2015). In this study 78% of samples had elevated manganese in the first year of growth, suggesting maternal influence; these samples were not counted as hypoxic occurrences. Environmental influences likely contributed to elevated manganese in later years, likely a result of encountering elevated levels of dissolved manganese as part of hypoxic waters (Limburg *et al.* 2015).

Retrospective tracking of whether fish encounter hypoxic waters using trace amounts of manganese in otoliths has shown some promising results in marine systems (Limburg *et al.* 2011, Mohan *et al.* 2014, Limburg *et al.* 2015). Quantities of manganese may even be linked to the intensity of hypoxic events (Limburg *et al.* 2011). For example, manganese concentrations in our study varied among individual fish even within the same year. If manganese concentrations could discriminate the intensity of an event this may provide a further dimension to retrospective tracking of hypoxia. For example, during the Millennium drought manganese spikes ranged from 25µmol/mol to >200µmol/mol of Mn:Ca among different individuals. Increased manganese in otoliths has previously been attributed to natural variation and the ephemeral nature of hypoxia (Limburg *et al.* 2015). Additionally, very low levels of dissolved oxygen (<1 mg L^{-1}) may be required to produce the necessary redox conditions to allow the flux of Mn^{2+} into the water column (Limburg *et al.* 2015).

Like many rivers and lakes drought, flooding and hypoxic blackwater events are part of the natural cycle in the Murray Darling Basin (Small *et al.* 2014, Haidvogl *et al.* 2015, Jenny *et al.* 2016). Many native fish have adapted to hypoxic conditions, suggesting that survival is plausible and is likely to be recorded in the trace elemental chemistry of the otolith when encountered (Small *et al.* 2014). Whitworth *et al.* (2012), found that water temperature at time of floodplain inundation was critical in controlling dissolved oxygen concentrations, with higher water temperatures decreasing dissolved oxygen availability. In the case of the worst incidence of hypoxia during the Millennium drought in the Murray Daring Basin (2010-2011), flooding occurred during late spring in an area that historically experiences winter rainfall dominance, thus explaining the severity of the event. Fish in the wild may even be able to avoid hypoxic events by seeking refuge habitat or moving out of the affected area to avoid lethal conditions (i.e. not being present in the affected region thereby not laying down otolith material that reflects high Mn), possibly explaining the portion of samples with no recorded elevated spikes of manganese throughout their life (Limburg *et al.* 2015). Regardless, the data collected in this study is promising and shows that manganese is a useful indicator of hypoxic events in freshwater when used in tandem with other data sources.

Collecting information from multiple data sources is useful as it can extend the period over which environmental changes can be observed (Disspain *et al.* 2018). However,

different datasets, be those archaeological and historic collections of otoliths or archival and anecdotal references require careful interpretation (Disspain *et al.* 2018). For example, many data sources may be bias by targeted fishing effort toward larger specimens our 'trophy/icon' species, therefore only representing a portion of the total population (Disspain *et al.* 2018). We found that when hypoxic events were recorded in Trove they were largely misunderstood. In many instances, the scientific principles governing hypoxic occurrence were not recognised, which often resulted in misleading interpretations of events. Many of the records included indicators of a hypoxic event, e.g. hot temperatures, stagnant water, coloured water and fish gasping or acting dopily, however, the link to a hypoxic or low oxygen event was never drawn. Accounts of hypoxic events using the specific terminology are rare, as the phenomenon was not understood until much later (Sheldon and Walker 1989). However, the indicators for a hypoxic event can still be found and used to corroborate chemical otolith analyses and further extend hypoxic records, as we show herein.

Freshwater fish have been impacted by numerous threats with the most significant being through anthropogenic interference (Haidvogl *et al.* 2015, Alleway *et al.* 2016, Jenny *et al.* 2016). The majority of research utilising archival references agree that human influences have dramatically changed historical baselines in marine and freshwater systems (Rosenberg *et al.* 2005, Thurstan *et al.* 2015, Alleway *et al.* 2016), with few exceptions (Jones *et al.* 2016). For example, European colonisation drove a global baseline change to preferential land use transformations altering vegetation, and species composition particularly in inland waterways (Alleway *et al.* 2016). Herein we have shown how multiple datasets can contribute to improving our understanding of historic hypoxia and validating more rigorous scientific tests such as chemical analysis. Yet there remains a paucity of studies utilising qualitative information to reconstruct historical references (but see, Carder and Crock 2012, Haidvogl *et al.* 2015, Thurstan *et al.* 2015, Alleway *et al.* 2016). Often there is a disconnect between the management of fish and their habitats (Koehn 2015). However, fish, water and their habitats are intrinsically intertwined, especially historical accounts where knowledge was limited at the time. Furthermore, without historical baselines to refer to we can underestimate changes to communities and ecosystems when setting restoration targets (Rosenberg *et al.* 2005, Haidvogl *et al.* 2015, Alleway *et al.* 2016).

Hypoxia is a natural phenomena that occurs worldwide in aquatic systems. However given existing anthropogenic pressures and future climatic predictions, conservation and mitigation measures will be crucial to control the frequency, duration, spatial extent and severity of its occurrence. (Small *et al.* 2014, Koehn 2015). A key challenge for restoration and rehabilitation is the shifting baseline. Without fully comprehending the conditions prior to adjustment, how can restoration reflect history? To this end, combining multiple data sources can inform managers of historical environmental baselines that can be used to assess rehabilitation efforts. The dearth of information on hypoxia historic baseline conditions globally hinders our ability to predict future changes to biodiversity and ecosystems and recommend achievable and sustainable management objectives. This study demonstrates this concept by using otoliths as proxies for environmental disturbances like hypoxia retrospectively over many years. Otolith manganese appears to be an effective tracer of hypoxia; particularly as elevated levels could be linked to major known flood, drought and hypoxic events in the system. With the potential to describe the severity of an event, although this requires rigorous validation as much of the data suggesting this currently comes from wild caught fish. Combining quantitative records and otolith manganese as a proxy of hypoxia could reveal long-term trends, re-establish a baseline of hypoxic events, and be used to predict future occurrences.

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SUPPLEMENTARY INFORMATION

Continued on following pages.

SUPP Table 1: Historic newspaper articles that could be directly linked to hypoxic events that occurred from 1935-2015 (*Hypoxic. Ref*; n=14). Articles were found using search terms (see Table 3) in archival digitised records on Trove, a search database of the National Library of Australia.

Article Identifier	Harvard Citation	State	Species	Ref. to	Title	Date	Actual Location	Actual text
http://nla.gov.au/nla.news-article130249648	1949 'PASSING BY', *News (Adelaide, SA : 1923 - 1954)*, 6 April, p. 5. , viewed 26 Jul 2018, http://nla.gov.au/nla.news-article130249648	SA	Murray Cod	hypoxia	Passing By - Giant Cod	6/04/49	Moorook	Mr. Harry Dadleff SA secretary of the Federated Engine drivers and Firemen's Association, showed me today a picture of a Murray cod... The way the giant was caught was unfortunately, not spectacular. There was no grim, long battle. Mr Dadleff and some friends saw the cod floating on the river, evidently drugged by tannin picked up by the water when it overflowed on the timbered swamps. They simply got alongside it and gaffed it.
http://nla.gov.au/nla.news-article205961507	1939 'STREAM CLOSURES SUGGESTED', The Age (Melbourne, Vic. : 1854 - 1954), 3 February, p. 8. , viewed 27 Jul 2018, http://nla.gov.au/nla.news-article205961507	VIC	Murray Cod	likely hypoxia	Vary The Season	3/02/39	Barmah	Extensive losses of Murray cod through the shrinkage of anabranches of the Murray especially in the Barmah district, is reported by fisheries officials. It was noted that almost without exception the fish found dead along the streams were in remarkably good condition, which suggests that there has been no lack of natural food.
http://nla.gov.au/nla.news-article119383358	1951 'Strange Fish Mortality', The Braidwood Review and District Advocate (NSW : 1915 - 1954), 6 February, p. 1. , viewed 29 Jul 2018, http://nla.gov.au/nla.news-article119383358	NSW	Murray cod, shrimp, crayfish	likely hypoxia	Strange fish mortality	6/02/51	Darling River	The Darling River today reeks of the smell of dead fish, that are coming down in vast numbers, some of them Murray cod weighing up to 2lb. Near the edges of the river are thousands of apparently dazed or sick shrimps and crayfish, upon which the fish appear to be feasting. Wire nets are being used to secure big hauls of tooth fish and crustaceans, one party netting 125 fish, in one haul. (People are being warned not to drink the water without first boiling it. Various opinions have been put forward to account for the dead fish, which, in many cases, have their stomachs and mouths full of undigested shrimps. The death of so many fish will mean that years must elapse before they breed sufficiently to replace the losses. No authoritative explanation has been given for the death of the fish or the appearance and conditions of the shrimps and crayfish.)

http://nla.gov.au/nla.news-article131340493	1951 'DARLING RIVER SMELLS OF DEAD FISH', The Dubbo Liberal and Macquarie Advocate (NSW : 1894 - 1954), 30 January, p. 1. , viewed 31 Jul 2018, http://nla.gov.au/nla.news-article131340493	NSW	Murray cod, shrimp, crayfish	likely hypoxia	Darling River Smells of Dead Fish	30/01/51	Darling river	Today, the Darling River smells of dead fish, which are coming down in vast quantities, some of them Murray cod weighing up to 201lb. Near the edges of the river are thousands of apparently dazed or sick shrimps and crayfish, upon which the fish appear to be grazing. Wire nets are being used to secure record hauls of both fish and crustaceans, one party netting 125 fish in one haul. People are being warned not to drink the water from the river without first boiling it. Varied opinions have been put forward to account for the dead fish, which, in many cases, have their stomachs and mouths full of undigested shrimps. The death of so many fish will mean years must elapse before they breed sufficiently to replace losses. No authoritative explanation has been given for the death of the fish or the appearance and condition of the shrimps and crayfish.
http://nla.gov.au/nla.news-article139813605	1939 'DOINGS IN DIFFERENT DISTRICTS.', The Riverine Grazier (Hay, NSW : 1873 - 1954), 3 January, p. 1. , viewed 31 Jul 2018, http://nla.gov.au/nla.news-article139813605	NSW	Murray cod	possible hypoxia and drought	Doings in different districts	3/01/39	Billabong Creek	A Jerilderie telegram states that there is a serious water shortage in the Billabong Creek, and the town supply is becoming very limited. Murray cod and other fish are dying in great numbers, and the council has employed men to pull the dead fish out of the creek and burn them. It is estimated that over a thousand have been removed from the creek in close proximity to where the town water supply is pumped.
http://nla.gov.au/nla.news-article156129542	1951 'BIG COO CAUGHT BY UNUSUAL METHOD', The Murrumbidgee Irrigator (Leeton, NSW : 1915 - 1954), 9 January, p. 4. , viewed 31 Jul 2018, http://nla.gov.au/nla.news-article156129542	NSW	Murray Cod	possible hypoxia	Big Cod Caught By Unusual Method	9/01/51	Murrumbidgee	There are more ways of catching big fish than with net or line and sinker. Messers. E. N. Southwell and F. W. Gaylord of Wee Jasper were drifting along a quiet reach of the Murrumbidgee river between Gudagai and Wagga when they spotted an 85 pounder Murray cod apparently sleeping in about two feet of water. Mr. Southwell jumped out of the boat and stabbed it in the head with a six inch blade knife. The fish immediately came to life, and while its head was being held down threshed the water with its tail, churning up mud and sending the spray flying. The knife slipped out of the fish's head, and it swam under the boat. Mr. Southwell pounced on it again, and stuck one hand in its gills. The fish closed its gills with vicelike grip while Mr. Southwell sat down in the water with his heels dug in the mud trying to get the fish on to dry land. With Mr. Gaylords assistance a cord was passed through the fishes gills and out through its mouth and they were then able to drag it up on to the bank.

http://nla.gov.au/nla.news-article56061352	1949 'They're catching cod without hooks now', *The Mail (Adelaide, SA : 1912 - 1954)*, 13 August, p. 1. , viewed 01 Aug 2018, http://nla.gov.au/nla.news-article56061352	SA	Murray cod	possible hypoxia	They're catching cod without hooks now	13/08/49	Murray Bridge	Manager of Poltalloch Plains Station (Mr. Jones) noticed swirling in the shallow water of a nearby swamp area. On investigation he found a 90lb cod floundering in the water. He waded in, caught the fish in his arms, and with difficulty brought it to the shore.
http://nla.gov.au/nla.news-article130814185	1952 '£15 C.O.D. for 92-lb. K.O.'d cod', News (Adelaide, SA : 1923 - 1954), 8 July, p. 6. , viewed 01 Aug 2018, http://nla.gov.au/nla.news-article130814185	SA	Murray cod	possible hypoxia	K.O.'d cod	8/07/52	Wentworth weir	Mr Oscar Matson, 53, of Wentworth caught a 92lb Murray cod yesterday that didn't even try to get away. From the window of his shack on the Murray just below Wentworth weir, Mr. Matson saw the cod in the middle of the stream. He raced to his boat and rowed after it, puzzled. The fish obviously wasn't dead - it seemed to be sleepin, so he hauled it in with a landing net.
http://nla.gov.au/nla.news-article130811215	1952 'K.O.'d cod at 2/6 lb.', News (Adelaide, SA : 1923 - 1954), 10 July, p. 20. , viewed 01 Aug 2018, http://nla.gov.au/nla.news-article130811215	SA	Murray cod	possible hypoxia	K.O.'d cod	10/08/52	Wentworth weir	This is the big one that didn't even try to get away. A Wentworth man found the fish, a 92lb Murray cod, floating apparently stunned just below Wentworth weir. (Picture included in article, fish in picture appears emaciated)
http://nla.gov.au/nla.news-article139909838	1951 'AUSTRALIANA', The World's News (Sydney, NSW : 1901 - 1955), 13 January, p. 23. , viewed 01 Aug 2018, http://nla.gov.au/nla.news-article139909838	NSW	perch	possible hypoxia	Fish without hooks	13/01/51	Murray	The par (WN, 23/9/50) re catching fish without hooks reminds me of another method by which we caught big bags when we were youngsters. When dams and waterholes in the creeks became low in the summer time we would wade in (often up to our necks) and stir up the mud. The fish (mostly tench and perch) unable to breathe in the thick muddy water, would rise to the top and we would grab them. Another big haul I got was in an old mining cut down around which a bushfire had roared. The first heavy rains washed down the ashes and silt which thickened the water too much for the fish; perch were poppong their noses up everywhere. I caught 65.

http://nla.gov.au/nla.news-article65554236	1942 '"The ODD SPOT"', Benalla Ensign (Vic. : 1938 - 1954), 27 February, p. 2. , viewed 04 Aug 2018, http://nla.gov.au/nla.news-article65554236	VIC	unspecified	probable hypoxia	The Odd Spot	27/02/42	Broken River near Shepparton	For some weeks past fish in the Broken River and its tributaries have been dying, and the appearance of dead fish in the Broken River has been most noticeable. They were so numerous near the old weir on the property of Mr Percy Trewin, that he commenced to remove them and it presented an odd spot with 20 to 40 fish ranging from 10 lbs to 40 lbs. Mystery surrounds their death, but the colour of the water these days is enough to kill anything.
http://nla.gov.au/nla.news-article141166862	1951 'Puzzling Fish Mortality', Barrier Daily Truth (Broken Hill, NSW : 1908; 1941 - 1954), 24 August, p. 1. , viewed 06 Aug 2018, http://nla.gov.au/nla.news-article141166862	NSW	unspecified	probable hypoxia	Puzzling Fish Mortality	24/08/51	Broken Hill	Obscure but persistent reports continue to come in from stations in the river and lake districts of fish dying in unnaturally large numbers. Several weeks ago it was reported that large numbers of dead fish had been seen in the shallow pools left by residual floodwaters. It now appears that bodies of small fish have been found in the lakes proper and even in running river water. Experts have been investigating the mystery fish deaths but it is not yet know whether they have (unfinished in publication).
http://nla.gov.au/nla.news-article130455847	1939 'DARLINGTON POINT', Narrandera Argus and Riverina Advertiser (NSW : 1893 - 1953), 13 January, p. 3. , viewed 07 Aug 2018, http://nla.gov.au/nla.news-article130455847	NSW	unspecified	possible hypoxia	The River	13/01/39	Murray river	The state of the river is causing grave concern to residents of towns along its banks who depend upon it for their water supply. Weeds are choking the river and retarding the flow of water, and as the level of the river recedes decaying vegetable matter becomes exposed to the hot rays of the sun which pollutes the water and gives off a nasty stench. Fish are dying in hundreds, presumably from the heat of the water and the slime which clogs their gills and large numbers of young and old crayfish have been seen leaving the water and crawling up the bank to die.

http://nla.gov.au/nla.news-article225458392	1951 'GASPING FOR AIR', Weekly Times (Melbourne, Vic. : 1869 - 1954), 7 March, p. 28. , viewed 07 Aug 2018, http://nla.gov.au/nla.news-article225458392	VIC	unspecified	possible hypoxia	Gasping for air	7/03/51	Murray	GASPING FOR AIR When oxygen was out of the water, by too many fish breathing it either in an artificial bowl or natural dam, the fish die of suffocation. Hence the poor fish swam in a concentration of warm and muddy water.. (section too difficult to decifer)... "one day during the record dry spell when I was bringing in the cows I noticed a number of cranes about our large water hole. I went over to see what the matter was and saw more than 100 gold and silver fish with their mouths just above the surface of the water apparently gasping for breath. Floating dead on the top were about 30 fish."

SUPP Table 2: Historic newspaper articles that were possibly linked to hypoxic events that occurred from 1935-2015, but did not have enough information to conclusively state they were hypoxic, (*Potential. ref.*; n=30). Articles were found using search terms (for specifics see Table 3) in archival digitised records on Trove, a search database of the National Library of Australia among the years encompassed by our otolith collection 1935-2015.

Article Identifier	Harvard Citation	State	Species	Ref to	Title	Date	Actual Location	Text
http://nla.gov.au/nla.news-article131934678	1939 'Topics of Interest To Anglers', *News (Adelaide, SA : 1923 - 1954)*, 11 May, p. 25. , viewed 27 Jul 2018, http://nla.gov.au/nla.news-article131934678	SA	Murray cod	fishing	Topics of Interest to Anglers	11/05/39	Murray	Although big cod are being taken by the professionals along the Murray, the water is still muddy, and angling is slow.
http://nla.gov.au/nla.news-article186109813	1939 'Decreasing Murray Cod', Shepparton Advertiser (Vic. : 1914 - 1953), 18 August, p. 6. , viewed 27 Jul 2018, http://nla.gov.au/nla.news-article186109813	VIC	Murray Cod	plea for legislation to stop species extinction	Decreasing Murray Cod	18/08/39	Murray - Borough	In an endeavour to induce the early passing og legislation to prevent the exploitation of Murray cod to the extent that extinction is imminent… a continuing lessening of the quantity of fish and the killing off of large numbers of breeding fish, and it is now obvious that the natural increase is not nearly sufficient to keep pace with the mortality over recent years.
http://nla.gov.au/nla.news-article164489254	1939 'TONS OF FISH KILLED BY DRY WEATHER', Record (Emerald Hill, Vic. : 1881 - 1954), 14 January, p. 5. , viewed 27 Jul 2018, http://nla.gov.au/nla.news-article164489254	VIC	Murray cod	serious drought leading to lots of fish die offs not necessarily hypoxia though	Tons of fish killed by dry weather	14/01/39	Gunbower creek	So seriously has the continued dry weather affected Victorian streams that few of the many local fishermen who preferred the solitude of the country rivers over the Christmas holidays were able to report results commensurate with the devotion expended…Unless heavy rains fall in the near future, there is no determining the extent of loss which may accrue amongst fish in the streams - particularly north of the Dividing Ranges. One instance certifies the heavy mortality to fish life. At Jerilderie during the week, following the excessive heat, one narrow stream, almost dry, yielded over three tons of dead and dying fish. At Lake Wendouree, trout are dying in hundreds, and elsewhere the position is as acute.

http://nla.gov.au/nla.news-article116198416	1939 'THOUSANDS OF FISH DIE', The Riverine Herald (Echuca, Vic. : Moama, NSW : 1869 - 1954; 1998 - 1999), 19 May, p. 2. , viewed 29 Jul 2018, http://nla.gov.au/nla.news-article116198416	NSW	trout and Murray cod	fish die from ash and charcoal, fires and floods	Thousands of fish die	19/05/39	Albury, Kiewa	…almost all the fish in the Kiewa were killed in the recent floods following the devastating bush fires. The effect was to carry into the stream vast quantities of charcoal and ashes, which resulted in heavy mortality… Hundreds of brown trout from nine to 14 inches long were lying dead on the beaches between Keighan and Morgans brideges over the Kiewa, and Murray cod and redfin suffered the same fate as the trout. The size and number of the cod was a revelation. On one beach just above Kieghans bridge 40 cod weighing from two to 30lbs were lying dead. Fish up to 60lbs were found in the mud below Keighans bridge.
http://nla.gov.au/nla.news-article144239746	1939 'ACCLIMATISATION SOCIETY', Daily Advertiser (Wagga Wagga, NSW : 1911 - 1954), 8 December, p. 3. , viewed 31 Jul 2018, http://nla.gov.au/nla.news-article144239746	NSW	Murray cod, trout, redfin, perch	Murray cod population depleted in Murray, lack of food	Acclimatisation Society	8/12/39	Lake Albert, Murray River	Mr L. H. Shaw, president of the Wagga Acclimatisation Society, on the experimental work of the society in connection with the propagation of Murray cod and other fish life… He said that the depletion of cod could be clearly demonstrated by the fact that only a small number of small Murray cod were caught nowadays, whereas a few years ago they were regarded as nuisances by fishermen. Many factors' here combined to cause this depletion, he said, and the otustanding feature seemed to be that the food supply for Murray cod had outgrown the demand and had become the enemy of the cod in the resultant vicious cycle.
http://nla.gov.au/nla.news-article142545950	1939 'Fishing Closure.', Western Herald (Bourke, NSW : 1887 - 1970), 13 October, p. 4. , viewed 31 Jul 2018, http://nla.gov.au/nla.news-article142545950	NSW	Murray cod	fishing closure of Murray cod for conservation	Fishing Closure	13/10/39	Murray River	In reply to your communication of 11th August, regarding the question of prohibition of the sale of Murray Cod…'It would be an error to assume that such depletion of cod and perch as may have occurred is attributable solely to the operations of Commercial fishermen for there are many other possible, or even probable causes of depletion (1) The drying-up of water courses in time of drought with a consequent heavy mortality among fish. (2) Pollution of streams by drainage, particularly of ash etc., after bushfires and in some districts by drainage from agricultural lands. (3) Fishing in close season - the employment of illegal methods. (4) The taking of undersize fish.'

http://nla.gov.au/nla.news-article219738979	1948 'COUNTRY NEWS', Lithgow Mercury (NSW : 1898 - 1954), 18 October, p. 3. (CITY EDITION), viewed 31 Jul 2018, http://nla.gov.au/nla.news-article219738979	NSW	Murray cod	short on 94lbs catch, short on illegal fishing of MC	Country News	18/10/48	Murray River	On Tuesday last Mr J Neale of Cobram, landed a 94lb Murray cod. The fish, which was taken from the Murray River at Cobrawonga, about three miles from Cobram, was 55in long and 43in around the girth, and when being cleaned a 3lb cod was found inside the fish. Mr Neale said a 4lb yellowbelly was used for bait and the fish was landed pratically without a struggle....Large-scale illegal fishing in the Murray River threatened to exterminate the Murray Cod, Mr A. D. Butcher, Chief Inspector of Fisheries and Game, said on Thursday. Both Victoria and NS Wales were concerned at the increasing shortage of cod, and had appointed inspectors to make careful investigations along the Murray to detect illegal methods of catching large numbers of fish. Dynamite was sometimes used when rivers were running low. Some years ago an amatuer fisherman could throw a line into the Murray almost anywhere and get a good haul. Today he was lucky to catch a few fish over a wide area of river.
http://nla.gov.au/nla.news-article47521777	1953 'COUNTRY SECTION', The Advertiser (Adelaide, SA : 1931 - 1954), 21 January, p. 5. , viewed 31 Jul 2018, http://nla.gov.au/nla.news-article47521777	SA	Murray Cod	cod die mysteriously, likely salt content	Mystery Death of Cod	21/01/53	Lake Bonney	Barmera, Jan 20. Residents of the district are bewildered and concerned at the large number of Murray Cod being washed up on the shores of Lake Bonney. It is estimated that during the past 10 days about 100 fish have been found dead. Many were over 40lb, the smallest being 15lb, and all were very fat. In 1951 the salt content of Lake Bonney was 160gains to the gallon. Last year, before the flood water entered, it was 120 and dropped to 61 in September. Today it is 74. The fish seem to survive in high salt contents and the change of water is considered a likely cause of the deaths. The River Murray at Cobdogia is at present registering 4 to 5 grains.
http://nla.gov.au/nla.news-article73173653	1945 'ANGLING', The Horsham Times (Vic. : 1882 - 1954), 1 June, p. 5. , viewed 31 Jul 2018, http://nla.gov.au/nla.news-article73173653	VIC	Murray Cod	fish death caused by salt?	Angling	1/06/45	Wimmera	Bill was telling me recently he was at Jeparit and saw the carcasses of three Murray cod weighing 16, 18 and 22lb which had been taken out of the Wimmera near Jeparit, the cause of death being salty water. The club has been unfortunate with their efforts with Murray cod owing to the dryness of the seasons and the breaking away of the weir.

http://nla.gov.au/nla.news-article236353728	1954 'MYSTERIOUS DEATH OF FISH', The Corowa Free Press (NSW : 1875 - 1954), 10 December, p. 5. , viewed 31 Jul 2018, http://nla.gov.au/nla.news-article236353728	NSW	trout, english perch, redfin, Murray cod	fish dying from unknown pollutant not necessarily hypoxia	Mysterious Death of Fish	10/12/54	Ballarat, Murray	The Victorian Fisheries and Game Dept has issued a warning against people eating fish from Lake Learmouth near Ballarat as a rare germ has killed hundereds of rainbow trout and English perch in the lake. Hundreds of dead and dying fish have been floating on the surface of the lake and biologists from Melbourne University and the Dept. who have been attempting to identify the germ for several days are baffled. This has led residents along the Murray to wonder if something similar has taken place in that river, for since the fishing season opened on December 1, anglers have been struck by the almost complete absence of redfin which hitherto were very plentiful. Although there have been reports of several nice catches of cod, the elusive 'reddie' seems to have deserted the waters in the vicinity of Corowa, at any rate, and we have heard it said that even during the winter, very few redfin were caught in nets. Another curious thing noticed is that there are hundreds of small fish about 11/2 in long drifiting about in the water, singly, which seems to suggest that they are not being molested by bigger fish chasing them for food. Hitherto these small fish were seen in fast-travelling shoals upstream near the banks. It would be interesting to know what the explanation of the small fish is.
http://nla.gov.au/nla.news-article119384166	1951 'FARMERS WORRIED', The Braidwood Review and District Advocate (NSW : 1915 - 1954), 13 March, p. 4. , viewed 31 Jul 2018, http://nla.gov.au/nla.news-article119384166	NSW	Murray cod and perch	fish dying enmass perhaps it is myxie related	Farmers Worried	13/03/51	Murray Valley	Farmers worried myxomatosis causes havoc in Murray Valley. Fish, birds, dogs die. Reports from the Murray Valley areas, where rabbits are dying in millions from myxomatosis, say that fish, birds, dogs, foxes and kangaroos are dying from the disease. Farmers who are watching for any sign of the disease among their stock, have little faith in assertations that it will not spread beyond rabbits, says a report. (Experts have said there is no conection between the rabbit-destroying myxomatosis and the brain disease, encaphalitis). Murray cod and perch are dying in hundreds in Northern Victoria. It is claimed that rabbits, blinded by myxomatosis, are falling into rivers and watercourses and being eaten by fish...The Superintendent of State Fisheries in NSW (Mr T. C. Roughley) said that both the Murray cod and perch were extremely hardy and he could think of no natural conditions that would have brought about their death. An official of the C.S.I.R.O. Fisheries Division at Cronulla said it was extremely unlikely that the rabbit virus was responsible for the death of the fish.

http://nla.gov.au/nla.news-article156130411	1951 'IT LOOKS FISHY', The Murrumbidgee Irrigator (Leeton, NSW : 1915 - 1954), 22 March, p. 3. , viewed 31 Jul 2018, http://nla.gov.au/nla.news-article156130411	NSW	Murray cod and perch	fish dying enmass perhaps it is myxie related	It Looks Fishy	22/03/51	Leeton	The Superintendent of State Fisheries (Mr. T. C. Roughley) who visited Leeton some time ago to investigate reports that Murray cod were breeding in the irrigation canals evidently believes that there is something 'fishy' about the co-incidental deaths of hundreds of Murray cod and perch and large numbers of rabbits in the same areas of northern Victoria where myxomatosis has been introduced by the scientists. Mr Roughley states that both the Murray cod and perch are extremely hardy and he can think of no natural conditions that would have brough about their death. It is claimed that rabbits blinded by mxyomatosis are falling into rivers and creeks and being eaten by fish.
http://nla.gov.au/nla.news-article248641500	1951 'Biologist for virus area', The Daily Telegraph (Sydney, NSW : 1931 - 1954), 2 March, p. 7. , viewed 31 Jul 2018, http://nla.gov.au/nla.news-article248641500	NSW	Murray cod and perch	fish dying enmass perhaps it is myxie related	Biologist for virus area	2/03/51	Sydney	A Sydney University biologist will tour Murray River areas next week to see if myxomatosis has killed animals other than rabbits… Mr Hallstrom and the biologist last night both asked that the biologist's name not be published. 'I wish to be able to make a quiet, private, impartial survey', the biologist said. *(Linked to above)*
http://nla.gov.au/nla.news-article195304777	1951 'Doctors Inquire Into Spread Of Brain Disease', Daily Examiner (Grafton, NSW : 1915 - 1954), 2 March, p. 1. , viewed 31 Jul 2018, http://nla.gov.au/nla.news-article195304777	NSW	Murray cod and perch	fish dying enmass perhaps it is myxie related	Doctors Inquire Into Spread of Brain Disease	2/03/51	Murray Valley	Three doctors from Melbourne have been sent to Mildura to investigate the spread of the brain disease, encephalitis. Meanwhile, reports from the Murray Valley areas, where rabbits are dying in millions from myxomatosis, today say that fish, birds, dogs, foxes and kangaroos are dying from the disease...Dr. Caldwell would investigate the mode of transmission of the disease... (C.S.I.R.O. experts have said there is no connection between the rabbit destroying myxomatosis and encephalitis).In the Murray Valley areas, Murray cod and perch are dying in hundreds in flooded water courses and swamps in Northern Victoria. It is claimed that rabbits, blinded by myxomatosis, are falling into rivers and watercourses and being eaten by fish... Although the best medical minds had been studying encephalitis for some time, they were no nearer to finding either cause or cure.

http://nla.gov.au/nla.news-article141165168	1951 'MELB. DOCTORS TO STUDY BRAIN DISEASE', Barrier Daily Truth (Broken Hill, NSW : 1908; 1941 - 1954), 2 March, p. 1. , viewed 31 Jul 2018, http://nla.gov.au/nla.news-article141165168	NSW	Murray cod and perch	fish dying enmass perhaps it is myxie related	Melb. Doctors to study brain disease	2/03/51	Murray Valley	*Small variations to articles above (article numbers 119384166, 156130411, 248641500 and 195304777). No new information added to description.*
http://nla.gov.au/nla.news-article96554577	1951 'THREE DOCTORS SENT TO MILDURA TO INVESTIGATE BRAIN DISEASE', Northern Star (Lismore, NSW : 1876 - 1954), 2 March, p. 5. , viewed 31 Jul 2018, http://nla.gov.au/nla.news-article96554577	NSW	Murray cod and perch	fish dying enmass perhaps it is myxie related	Three doctors sent to mildura to investigate brain disease	2/03/51	Murray Valley	*Small variations to articles above (article numbers 119384166, 156130411, 248641500, 195304777 and 141165168).* …Mr. Casey said today the myxomatosis virus had never been tested on humans, but observations had been made on the effect of the virus on animals such as monkeys, which showed similar symptoms to disease as did humans..."Leading authorities on the virus have stated clearly there is no danger to humans in myxomatosis".
http://nla.gov.au/nla.news-article133996248	1951 'Myxomatosis Plague Animals In Victoria', Illawarra Daily Mercury (Wollongong, NSW : 1950 - 1954), 2 March, p. 3. , viewed 31 Jul 2018, http://nla.gov.au/nla.news-article133996248	NSW	Murray cod and perch	fish dying enmass perhaps it is myxie related	Myxomatosis Plague Animals in Victoria	2/03/51	Murray Valley	*Small variations to articles above (article numbers 119384166, 156130411, 248641500, 195304777, 141165168 and 96554577).*

http://nla.gov.au/nla.news-article156123847	1952 'Around the Riverina', The Murrumbidgee Irrigator (Leeton, NSW : 1915 - 1954), 26 August, p. 4. , viewed 31 Jul 2018, http://nla.gov.au/nla.news-article156123847	NSW	Murray cod and perch	historic reference and mention of 1952 flood	Around the Riverina	26/08/52	Murrumbidgee	In the 1930's it used to be commonly said in the Riverina 'we never get the floods in the Murrumbidgee like we used to, because of the fact that weirs have been built along the river and that is also the reason why the Murray cod, the Golden Perch, and the Catfish have practically vanished from their old haunts.' It is true that tip to the early thrities the Murray Cod, the Yellow Belly (or Golden Perch) and the Catfish were very much more numerous in the Murrumbidgee than they have ever been since that period. In those days there seemed to be some good grounds for believing that the construction of weirs along the Murrumbidgee had lessened the frequency of floods by holding the water back inplaces, and making it bank up more deeply in billabongs, as well as thinning out the indigenous fishes...There is no doubt that the absence of periodical flooding plays a big part in depleting the stocks of fish in the river, but that is not the only factor.
http://nla.gov.au/nla.news-article187690827	1951 'Thousands of Fish Dying in Border Rivers', Warwick Daily News (Qld. : 1919 - 1954), 16 August, p. 1. , viewed 31 Jul 2018, http://nla.gov.au/nla.news-article187690827	QLD	fish	fish dying enmass perhaps it is myxie related	Thousands of fish dying in border rivers	16/08/51	QLD	A mysterious recurrent epidemic may be causing the death of thousands of fish in South Queensland border rivers. Dead fish are floating in the Macintyre Rvier at Goondiwindi, and they are dying in thousands in the Culgoa River a Dirranbandi. The State Icthyologist, (Mr T. C. Marshall) yesterday said that similar 'epidemics' had been reproted from every four to six years. They stopped as suddenly as they started, he said. The last mass fatalityof a similar kind was in the Thompson River at Longreach...Cause of epidemics were unknown, despite intensive investigations Mr. Marshall said. They generally occurred in the winter months, and extreme cold was a possible cause. The epidemics have ben occurring over many years. A Goondiwindi message on Tuesday said that myxomatosis was suggested as the cause of the fish mortality. The message said that attempts were being made to establish whether the fish had become infected by eating virus-infected rabbits. *Related to myxomatosis cases, reference to these fish die off's occurring every 4 to 6 years, suggests it is more natural and could be linked with hypoxia.*

http://nla.gov.au/nla.news-article141160332	1951 'Myxomatosis Not Killing Other Animals', Barrier Daily Truth (Broken Hill, NSW : 1908; 1941 - 1954), 13 March, p. 1. , viewed 15 Dec 2018, http://nla.gov.au/nla.news-article141160332	NSW	fish	scientist disproves myxie killing other animals	Myxomatosis Not Killing Other Animals	13/03/51	Sydney	A scientist from Sydney University has reported that myxomatosis, which has been successful in killing rabbits, had not affected fauna in the Murray River Valley, chairman of the Taronga Park Trust (Mr. Hallstrom) said. The scientist Dr. A. Bollinger, is considered Australia's leading authority on marsupials. Mr. Hallstrom sent Dr. Bollinger to the Murray River Valley, where it was reported fish, birds, dogs, foxes and kangaroos were dying from the mosquito-borne disease. Mr. Hallstrom said Dr. Bollinger proved the reports false.
http://nla.gov.au/nla.news-article248641495	1951 'Brain, rabbit viruses not linked', The Daily Telegraph (Sydney, NSW : 1931 - 1954), 2 March, p. 7. , viewed 31 Jul 2018, http://nla.gov.au/nla.news-article248641495	NSW	Murray cod and perch	fish dying enmass perhaps it is myxie related	Brain, rabbit viruses not linked	2/03/51	Murray Valley	Proved by tests, say scientists Melbourne, Thurs. - The brain disease encephalitis has no connection with the rabbit killing disease myxomatosis…Tests in Australia and overseas had proved that there was no connection between the diseases...The Chairman of the C.S.I.R.O. (Dr. Clunies Ross) said tests had shown that myxomatosis would not affect any animal except the European wild hare, from which Australian rabbits were descended. He added: "There is no evidence to support that myxomatosis is killing kangaroos, foxes, birds and crayfish. The idea is fantastic."…A C.S.I.R.O. research worker, in Canberra early this year inoculated himself with myxomatosis virus. He showed "absolutely no reaction" a C.S.I.R.O. report said." *Disproving validity of idea that myxomatosis is related to fish deaths.*
http://nla.gov.au/nla.news-article187067952	1977 'Closed season on Murray cod', Victor Harbour Times (SA : 1932 - 1986), 23 November, p. 9. , viewed 01 Aug 2018, http://nla.gov.au/nla.news-article187067952	SA	Murray cod and golden perch	ref with specifc number of reduction in catch attributed to environmental change	Closed season on Murray Cod	23/11/77	Murray River	Mr. Kirkegaard said that the Murray cod population might be slowly recovering but it was too soon to say whether the fish would ever again become common in the river. From 1958-59 to 1975-76 the commercial catch fell from 140000kg to 4700kg. It is believed that this has been due to ecological changes in the river.

http://nla.gov.au/nla.news-article131551338	1940 'Big Catches By Murray Fishermen', News (Adelaide, SA : 1923 - 1954), 25 January, p. 19. , viewed 01 Aug 2018, http://nla.gov.au/nla.news-article131551338	SA	Murray cod	ref to dwindling numbers of cod due to drought	Big Catches By Murray Fisherman	25/01/40	Murray River	The quantity of Murray cod caught this season is twice as much as last year. The amount of callop caught is three times as much as the last season. This is chiefly due to the recent Murray floods, which brought the fish down in large numbers. Dwindling numbers of Murray cod have been reported in Victoria recently, due to droughts and other causes, but fisherman in South Australia are experiencing the biggest catches they have had for years.
http://nla.gov.au/nla.news-article36042776	1952 'PLENTY OF FISH IN DARLING', Burra Record (SA : 1878 - 1954), 5 February, p. 7. , viewed 01 Aug 2018, http://nla.gov.au/nla.news-article36042776	SA	Golden perch	ref to fish dying overcrowding	Plenty of fish in darling	5/02/52	Broken Hill	Fish in the Darling River have been breeding well and the stream below the weir is just swarming with perch, silver bream and black bream. Other fish in the stream are of a gold colour. Visitors from Broken Hill who were at Wilcannia over the weekend said that there were millions of small fish in the river, and others had been washed up on the banks and had died.
http://nla.gov.au/nla.news-article187367527	1969 'River Murray Reach Fishery', Victor Harbour Times (SA : 1932 - 1986), 17 January, p. 6. , viewed 01 Aug 2018, http://nla.gov.au/nla.news-article187367527	NSW	callop and others	ref to the murray as the first australian fishery and conditions good and bad for fish	River Murray Reach Fishery	17/01/69	Murray River	The River Murray fishery is one of the earliest established fisheries in South Australia. Prior to white settlement it was fished by the Aboriginies. The native species on which the fishery depends have adapted their spawning cycle to make use of the extremely productive conditions which occur in the river during and following flood. Good fishing years have always been associated with the extensive floods, and fishing has been poor during cycles of low river. In early days, droughts and floods caused extreme variations in catches. However, the introduction of locks and dams along the Murray drastically altered the environment of this system by reducing the size and duration of floods (apart from severe ones like the 1955 flood).

http://nla.gov.au/nla.news-article48282843	1953 'Tuna Haul Is Record', The Advertiser (Adelaide, SA : 1931 - 1954), 17 February, p. 1. , viewed 02 Aug 2018, http://nla.gov.au/nla.news-article48282843	SA	callop and bream	pic of good haul	Tuna Haul is Record	17/02/53	Murray River	The picture at right shows one of the good, hauls of River Murray fish, mainly callop and bream, made since a sudden drop last week in the rivers level after the most prolonged flood on record. It shows Mr. Les Hale, a Renmark fish merchant weighing a good catch by a fisherman.
http://nla.gov.au/nla.news-article205961961	1939 'ARARAT', The Age (Melbourne, Vic. : 1854 - 1954), 20 February, p. 14. , viewed 04 Aug 2018, http://nla.gov.au/nla.news-article205961961	VIC	perch	possible hypoxia	Ararat	20/02/39	Mildura	Heavy bags of perch and eels have been obtained at Lake Kiora since the water in the lake has receded. Three sacks were taken to Mildura and large numbers of fish had been found dead on the shore.
http://nla.gov.au/nla.news-article73018483	1939 'FEARS FOR RIVER FISH.', The Horsham Times (Vic. : 1882 - 1954), 13 January, p. 4. , viewed 06 Aug 2018, http://nla.gov.au/nla.news-article73018483	VIC	unspecified	pre-hypoxia from bushfires	Fears for river fish	13/01/39	Murray River	The Chief Inspector of Fisheries and Game (Mr. F. Lewis) has fears for the thousands of river fish in Victoria. He said that unless immediate relief was afforded from the intensity of the State-wide heat an exceptionally heavy mortality rate among fish could be expected. The only hope for many would be that they might find cool, deep holes and escape the effects of the heat. *(from bushfires)*

http://nla.gov.au/nla.news-article102660610	1946 'MUDDY WATER KILLING FISH', The Independent (Deniliquin, NSW : 1901 - 1946), 7 March, p. 3. , viewed 07 Aug 2018, http://nla.gov.au/nla.news-article102660610	NSW	unspecified	muddy water suffocating fish after flood	Muddy Water Killing Fish	7/03/46	Murrumbidgee	The sight of a fish caught in the river about Narrandera is now almost a novelty. Amateur fishermen have been trying in vain to hook enough for a meal. However, since the heavy rain which fell on the Murrumbidgee watershed about a fortnight ago reports have been recieved that muddy water resulting there has caused numbers of fish in the river to become suffocated and rise to the surface, where they have been caught by the peole along the river. Last week reports were recieved from upriver centers that fish were dying from suffocation, and this week a number of fair sized fish were taken from the river near Narandera. They were in good condition.
http://nla.gov.au/nla.news-article229472976	1939 'HEAT KILLS PLOUGHMAN', The Sun (Sydney, NSW : 1910 - 1954), 10 January, p. 2. (LATE FINAL EXTRA), viewed 07 Aug 2018, http://nla.gov.au/nla.news-article229472976	NSW	unspecified	heatwave kill	Heat Kills Ploughman	10/01/39	Murrumbidgee	Hundreds of dead fish are floating down the Murrumbidgee, having been suffocated in the hot shallows. The river water is warm, even in the deeper holes, todays temperature was 110.9 at noon.

CHAPTER SIX

GENERAL DISCUSSION

Aquatic systems worldwide are increasingly affected by hypoxia (Diaz and Rosenberg 2008, Collingsworth *et al.* 2010, Limburg *et al.* 2018). These systems are at the precipice of disrepair with intervention often required to assist recovery of faunal populations (Collingsworth *et al.* 2010, Jenny *et al.* 2016). Understanding the location and timing of hypoxic events, as well as how aquatic species respond, is crucial for predicting future hypoxic effects under a changing climate (Diaz and Rosenberg 2008). The hypoxia problem has generated great interest in predicting organism responses in marine ecosystems, but there is a paucity of studies in freshwater. Throughout this thesis, I have investigated the physiological impact of hypoxic exposure on freshwater fish, and tracked hypoxic events through the study of their otoliths (ear stones of fish).

In the first two data chapters, I exposed fish to prolonged hypoxic or normoxic conditions combined with different temperatures. Levels were set to those found at the upper limits of the fishes' thermal range where physiological hypoxic effects were likely to be exacerbated. Disparity in resistance to hypoxia occurred among the three study-species: Murray cod, golden perch and silver perch. Additionally, I showed that fish acclimated after exposure to sub-lethal levels of hypoxia; yet, a threshold was reached after prolonged exposure.

In the last two data chapters I used the otoliths from golden perch and Murray cod from chapters 2 and 3, to provide new data describing hypoxic occurrence in freshwater systems. *In situ* monitoring of oxygen levels in many systems is generally lacking, and where it does exist, records only span a few decades. Otolith carbonate records are well-preserved and readily accessible natural tags that can indicate environmental change. Otoliths provide a cost-effective mechanism to examine environmental change, notably where it is logistically difficult for rigorous collection

of data in both space and time. In chapter 4, I used controlled laboratory conditions to explore different elemental proxies in otoliths for freshwater hypoxic conditions, exploring a possible physiological connection to its occurrence. In chapter 5, I investigated the otoliths of wild caught fish of species from the same system and related manganese to hypoxia, along with major droughts and floods; that were likely to create hypoxic conditions using qualitative data from digitised newspaper articles.

In this final chapter, I discuss the main findings of my studies and provide suggestions for future research.

Physiological Considerations

Species-specific responses to hypoxic exposure were observed during the physiological tests. Mortality rates among the three native species tested were significantly different. Silver perch were unable to tolerate long-term hypoxic exposure, whereas Murray cod and golden perch survived the full experimental period. On a global-scale the tolerance observed in Murray cod and golden perch was similar to those of other lowland river fishes (Small *et al.* 2014). My estimates of hypoxic tolerance are likely to be a 'best case' response, where only temperature and oxygen conditions in the water were changed. However, hypoxic blackwater can be created and intensified by numerous stressors; in particular large quantities of dissolved organic carbon (DOC) from leaf litter and changes to pH (McMaster and Bond 2008, McCarthy *et al.* 2014). For example, in a study investigating simulated blackwater, small reductions in pH and elevated DOC exacerbated hypoxia driven mortalities (Small *et al.* 2014). My study exposed fish to sub-lethal hypoxia for longer durations than other studies, addressing responses to gradual and lingering hypoxia (Collins *et al.* 2013, Small *et al.* 2014). Notwithstanding, my results may still underestimate the effects of hypoxia in the wild, as long-term exposure was limited to sub-lethal levels, particularly for Murray cod and golden perch.

My study highlights the variable tolerance of native Australian species to hypoxia. Thresholds, including but not limited to those reported here, could be used for a precautionary hypoxia monitoring and warning system to prevent fish kills and maintain river health (Small *et al.* 2014). Although wild fish may be able to avoid hypoxic waters, significant barriers to connectivity in contemporary waterways can create risks of inescapable hypoxic exposure (Watts *et al.* 2018). Further, the risk of hypoxia-driven mortality is highly temperature dependent, with fish kills more common during periods of higher water temperature (Small *et al.* 2014). Mitigating these combined risks is crucial to the survival and proliferation of native species.

Whilst we found species-specific tolerances among sympatric native species, the methods used to determine species tolerance to hypoxia can vary significantly among studies (Collins *et al.* 2013, Eliason and Farrell 2016, Farrell 2016). The most obvious difference is where studies compare resting respirometry (measuring responses to environmental variables while fish are at rest), with swimming respirometry (measuring responses to environmental variables while fish are exercising, Farrell 2016). Both methods have merit, although some species respond better to one method over the other; this often relates to lifestyle, i.e. active swimmers respond better to swim respirometry versus benthic dwellers that perform better under resting respirometry (Farrell *et al.* 2009, Roche *et al.* 2013, Farrell 2016). Additionally, when testing hypoxic exposure the method of reducing oxygen in tanks varies greatly among studies (Roche *et al.* 2013). Future research would benefit from a unified method for testing and creating hypoxic conditions.

Otolith Considerations

Otolith chemistry represents an unparalleled natural tag of population structure, connectivity and a retrospective tracer of environmental change (Sturrock *et al.* 2015, Izzo *et al.* 2016). Nevertheless, physiological controls on elemental uptake and otolith formation have again been under scrutiny in recent literature (Sturrock *et al.* 2015, Grammer *et al.* 2017, Limburg *et al.* 2018), after initial investigations in the 1990s (Kalish 1989, Kalish 1992, Kalish 1993). Changes in organism metabolic rate, growth and reproduction complicate the mechanisms of incorporation of trace elements onto the otolith (Sturrock *et al.* 2015). Whether otolith elemental composition tracks the ambient environment (i.e. minimal physiological influence) or rather, reflects

physiological processes that coincide predictably with environmental change is still being debated (Sturrock *et al.* 2015), but likely depends on the element (Izzo *et al.* 2016, Thomas *et al.* 2017). In laboratory experiments I was unable to link elevated levels of manganese or magnesium in otoliths to hypoxic conditions or to metabolic rate. Understanding the contribution of physiological controls on otolith composition, will establish the correct interpretation for modelling element uptake in relation to environmental monitoring and reconstruction.

Manganese in otoliths is considered to be under physiological control, suggesting that while fish are under hypoxic stress this element may be incorporated more readily (Limburg *et al.* 2011, Limburg *et al.* 2015). I showed manganese could be used to retrospectively trace possible occurrences of hypoxic conditions by using field collected samples of otoliths and was linked to large-scale drought and floods that either precede hypoxic events or exacerbate them. Researchers have also shown that other physiologically controlled trace elements can be used to retrospectively identify spawning (Sturrock *et al.* 2015). Magnesium and zinc are two such elements that have been posited as tracers of environmental hypoxia (Sturrock *et al.* 2015, Limburg *et al.* 2018). My controlled laboratory experiment was unable to validate the use of magnesium or zinc as a proxy for hypoxic conditions potentially due to the lack of hypoxic conditions extreme enough to cause a physiological reaction. Phosphorous, is another element that may show some promise as a tracer of hypoxia, particularly in inland waters as it is closely related to high levels of DOC from plant material (McMaster and Bond 2008). Recently high phosphorus loads in the water were linked to the spread of lacustrine hypoxia, with timing of phosphorus load increases matching hypoxic escalation (Jenny *et al.* 2016).

Alternative aquatic fauna hard parts could also be used to track hypoxia, such as, scales, fin rays, vertebrae, scutes, eye lenses and beyond (Gillanders 2001, Izzo *et al.* 2016, Carlson *et al.* 2017, Tzadik *et al.* 2017). Concentrations of trace elements present in calcified structures of organisms (e.g. from corals, foraminifera, molluscs to fish hard parts; otoliths) have received attention across a wide range of disciplines including chemistry, palaeontology and ecology (Sturrock *et al.* 2015, Izzo *et al.* 2016). Studies validating the use of alternative hard parts have a number of benefits, 1) possible non-lethal sampling, useful for species of conservation concern, 2) allow sampling from species that do not have otoliths, for example, elasmobranchs, 3) may

provide complementary chemical information to validate environmental variation when elemental signatures differ from otoliths, and 4) may provide alternative chemical records, compared to otoliths, at a higher resolution for a more reliable sampling method to record environmental change (Izzo *et al.* 2016, Walther *et al.* 2017).

Historical Baselines

Understanding the environmental histories of aquatic ecosystems can enhance their value as a source of food (e.g. records of harvest) and energy (e.g. records of flow, Haidvogl *et al.* 2015). To date, studies of this nature for both freshwater and marine ecosystems have been limited to the Northern Hemisphere (Haidvogl *et al.* 2015, Izzo *et al.* 2016). Furthermore, our understanding of environmental change and its drivers has been limited by ecological data sets that rarely extend beyond a few decades (Thurstan *et al.* 2015). Archival records, however, can be used to extend ecological datasets to establish and improve historical baselines (Haidvogl *et al.* 2015, Thurstan *et al.* 2015, Alleway *et al.* 2016). The temporal gaps in our understanding of hypoxia occurrence are significant, and create uncertainties when considering restoration (Thurstan *et al.* 2015). Herein, I showed that increased otolith manganese in different years could indicate hypoxic events dating beyond conventional records. Digitised newspaper articles further corroborated the hypoxic occurrences found in the otoliths. Elevated manganese in otoliths could also be linked to major flooding and drought events in the river; events that exacerbate hypoxia. However, despite the increase in digitised resources and spread of information on a global scale, less conventional archival sources remain underexploited (e.g. records of precipitation, flow, photographs, menus, artwork, sediment cores and anecdotal references, Haidvogl *et al.* 2015, Thurstan *et al.* 2015).

In contrast to other aquatic biota, fish have been selectively taken from aquatic environments for millennia (Haidvogl *et al.* 2015, Izzo *et al.* 2016). While often records focus on trophy species, and those of commercial interest, they represent an unparalleled resource for environmental reconstructions (Thurstan *et al.* 2015, Disspain *et al.* 2018). Archaeological and historical remains (sourced from scientific collections, museums and human settlements e.g. castles, taverns, waste deposits and middens) allow investigations of historical baselines on longer timespans and larger

spatial scales, yet few examples of these studies exist (for further examples see Carder and Crock 2012, Haidvogl *et al.* 2015, Disspain *et al.* 2018). Generally speaking, these studies have linked declines in trophic level and mean length to fishing pressure, overexploitation and potential environmental disturbances that can eradicate species from the population (Thurstan *et al.* 2015, Alleway *et al.* 2016, Izzo *et al.* 2016). Fisheries data, while covering a far shorter timespan can also illuminate population shifts (Haidvogl *et al.* 2015, Alleway *et al.* 2016). For instance, the imposition of fishing regulations and shifts in commercial species from riverine to marine available at markets can indicate declines in species due to overexploitation (Haidvogl *et al.* 2015, Alleway *et al.* 2016). Effective conservation will require baseline information on both current and long-term patterns (Carder and Crock 2012). Historical data commonly show that the magnitude of change to our environments from anthropogenic influences is far higher than conventional predictions and contemporary data suggest (for a thorough review see Thurstan *et al.* 2015).

Management Implications

This thesis highlights the damaging effects of hypoxia on fish, and the necessity in tracking when and where hypoxia occurs. However, despite a growing interest in hypoxia and its effects, recovery and restoration efforts thus far have been relatively unsuccessful (Diaz and Rosenberg 2008, Vaquer-Sunyer and Duarte 2008, Jenny *et al.* 2016). I identified two areas of critical concern to improve management objectives in the future.

First, physiological requirements of key native species are either not represented at all, or tested over short-term timescales; resulting in misrepresentation of true tolerance and long-term impacts. Consequences of hypoxic exposure on organisms can manifest at the individual level as changes to feeding, ventilation or endocrine functions, growth, reproduction, disease resistance and mortality (Pollock *et al.* 2007, Small *et al.* 2014, Eliason and Farrell 2016). At the population and community level these changes result in broad scale losses of biomass, habitat alterations, altered migrations and mass mortalities (Breitburg *et al.* 2009, Poertner and Lannig 2009, Collins *et al.* 2016). Maintaining the health and biodiversity of native species is crucial to ecosystem recovery. Herein I identified thresholds of tolerance for three sympatric species, as well as identifying how duration of exposure influences hypoxic

tolerance as a cumulative stressor. Although sometimes disparate, the physiological requirements of species can be used to improve restoration goals. Data such as this, or similar, can then be incorporated into existing ecological models to improve management of hypoxic conditions and recovery efforts. Some instances of this type of management reform exist already (Baumgartner *et al.* 2017, Watts *et al.* 2018). With data on physiological tolerances to common stressors like hypoxia, but also pollution, becoming more common, ecological modelling and thus ecosystem restoration could be greatly enhanced.

Second, failure to consider historical baselines of hypoxic occurrence results in misrepresentation of ecosystems and in less ambitious targets for recovery (Thurstan *et al.* 2015, Jenny *et al.* 2016). Routine habitat monitoring was rare prior to the 1980s (Alleway *et al.* 2016, Jenny *et al.* 2016). This paucity of data complicates our ability to distinguish degraded natural habitats (Disspain *et al.* 2011, Thurstan *et al.* 2015, Izzo *et al.* 2016). Furthermore, if managers are unable to recognise the extent of change, they may be less likely to support conservation and recovery efforts (Koehn 2015, Thurstan *et al.* 2015, Koehn 2016). Creating a narrative through the use of historical data can also be effective as a management tool. It can aid in engaging communities (e.g. through community science) and can even alter scientific and public perception of the condition of the natural environment today (Thurstan *et al.* 2015). In turn, this can help unify conservation efforts at a number of levels (e.g. public sector, stakeholders, community and management, Thurstan *et al.* 2015). Restoration of hypoxic waters may be difficult without the collection of long-term datasets to properly inform management targets. In Europe and North America, restoration efforts to repair hypoxic affected waters began in the 1980s in Europe and North America, but with little improvement evident to date (Diaz and Rosenberg 2011, Jenny *et al.* 2016). Persistence of hypoxic conditions has been attributed to nutrients remaining in the watershed and climate change that exacerbates hypoxic conditions (Diaz and Rosenberg 2011, Jenny *et al.* 2016).

Historical datasets should also be incorporated into contemporary assessments and modern decision-making frameworks. For instance, the International Union for Conservation of Nature (IUCN) decline criteria, a key species risk assessment framework, is limited to species data from 10 years or three generations, whichever is longer (Thurstan *et al.* 2015). As such, species classification can change from

critically endangered to endangered based on the timeframe of assessment, even though conservation status may not be improved (Thurstan *et al.* 2015). Short-term criteria such as these proliferate the problem of shifting conservation baselines.

FUTURE DIRECTIONS

Measures of metabolic rate after prolonged hypoxic or normoxic exposure and measures of the chemical profiles of otoliths, both from experimental tests and field-collected samples, formed the basis of my research. This approach demonstrated the usefulness of combining data sets from different disciplines to detect physiological responses to environmental change, the influence of physiology on chemical incorporation in otoliths and the application of combined data sets to track hypoxia retrospectively. I now outline future research directions that would enable progression in the field of hypoxic research, ultimately allowing researchers to effectively implement findings to reduce the consequences of hypoxic incidences.

In freshwater systems hypoxic conditions are often associated with high levels of dissolved organic carbon (DOC; McMaster and Bond 2008, Watts *et al.* 2018). While DOC is crucial to the health of river systems, the long-term accumulation of plant material can promote a rapid increase of DOC into a system when flooded (King *et al.* 2012, Whitworth *et al.* 2012). Higher temperatures can increase the solubility of some forms of plant carbon, further elevating DOC concentrations and microbial respiration, and consequently the risk of hypoxic conditions (Whitworth *et al.* 2012). For example, a hypoxic blackwater event (following the Millennium drought in Australia), was characterised by high levels of DOC, which were leached from a large amount of accumulated plant material in the inundated floodplains and dry river channels, and led to mass mortalities of fish and other aquatic organisms (King *et al.* 2012). However, despite the simultaneous nature of elevated DOC and hypoxia in waterways there is limited research on the combined effects of these stressors (McMaster and Bond 2008). Elevated DOC increases the risks of hypoxic events to aquatic fauna, yet measures of physiological responses to this stressor, or attempts to trace its predominance through time using otolith chemistry, are generally lacking. Future research on hypoxic influences would benefit from investigation of DOC, as

far as physiological responses and otolith chemistry manipulated under experimental conditions using leaf litter.

Validation of existing proxies is crucial for moving forward. Manganese was successful as a tracer of environmental hypoxia in otoliths of wild caught fish but not in those from the controlled experiment. Sediment, not included in my experimental tanks, may be required to provide the necessary conditions for redox reactions, which is crucial for manganese flux to occur (Limburg *et al.* 2015). Future experiments should investigate mechanisms of manganese uptake in otoliths as physiological controls may not be all that is required for incorporation.

Expanding the proxies for tracing hypoxic exposure will also be key to developing realistic baselines for setting restoration and conservation goals in the future. For studies focusing on otolith chemical analysis, this will mean expanding the suite of chemicals that can reliably trace hypoxic exposure (e.g. magnesium, phosphorus, iron and zinc may have potential). Utilising unconventional methods can add to existing environmental histories (e.g. qualitative data, or data collected for other purposes; precipitation, fishing logs). Engaging the public in community science, such as rebuilding wetlands, re-snagging efforts and tag re-capture records from recreational fishers, could increase our awareness of ongoing change in ecosystems through community fed records. In particular, monitoring fish populations could expand our knowledge of less iconic, non-commercial or invasive species, for which there is often limited data (e.g. community monitoring of European carp catches). Further research needs to continue to investigate new proxies of long-term data with predictable patterns of hypoxic influence (e.g. sediment cores, foraminifera shells and tree rings).

Interdisciplinary studies combining data from multiple sources will be vital for future research and conservation efforts (Haidvogl *et al.* 2015, Thurstan *et al.* 2015, Izzo *et al.* 2016). It is particularly important for providing otherwise unattainable information for systems difficult to monitor; such as rivers, mangroves and estuaries to name a few (Izzo *et al.* 2016). Interdisciplinary research that embraces unconventional data sourced from ecologists, scientists, historians and archaeologists, as well as the general population (community fed research), can reveal long-term changes to ecosystems and climate (Haidvogl *et al.* 2015, Izzo *et al.* 2016). Additionally, interdisciplinary studies can reveal trends in the extent of human

impacts on ecosystems (Carder and Crock 2012, Haidvogl *et al.* 2015, Thurstan *et al.* 2015). For example, research looking at the hypoxic histories of sediments in lacustrine systems revealed anthropogenic influences had a greater impact on increasing hypoxic conditions globally than climate change (Jenny *et al.* 2016). In another study, air temperature and precipitation were used as proxies to infer current and future shifts in species distribution (Haidvogl *et al.* 2015). Integration of otolith and fisheries data have been successfully used to assess recovery strategies for Murray cod, including evidence of improved survival and persistence to historical age class structure, albeit at a reduced size (Disspain *et al.* 2012). In this instance declines in overall size of the species were attributed to a combination of factors; predation by humans, competition for resources with invasive species, and environmental degradation (Disspain *et al.* 2012). In each of these examples, utilising multiple data sources was critical to interpreting environmental changes. This focus on interdisciplinary research will continue to improve and support management strategies for restoration, conservation and adaptation to global change in the future (Haidvogl *et al.* 2015).

Finally, predictions of the ecological implications of hypoxia using data from multiple sources be it physiological, chemical or historical datasets, should be incorporated into current management frameworks. Increasing numbers of studies are investigating environmental shifts on expansive timescales, yet few ever scale up the effects investigated and make the connection to a management framework (Izzo *et al.* 2016, Carlson *et al.* 2017). Persistence of hypoxia globally suggests a weak resilience of ecosystems, with inland systems showing greater vulnerability to hypoxia than marine waters (Jenny *et al.* 2016). Comparative studies can reveal commonalities and differences in population, community and ecosystem changes after hypoxic exposure. Additionally, models can be used to scale up experimental investigations on stressors like hypoxia and evaluate population-level and ecosystem-level impacts allowing otherwise unattainable predictions of future effects of exposure to stressors like sub-lethal hypoxia. A recent study used individual based models to examine long-term hypoxia effects on reproduction, growth and mortality of fish, predicting small population losses after one hundred years of mild hypoxia, versus a 19% reduction in population abundance under severe hypoxia (Rose *et al.* 2018). However, without research into stressors such as hypoxia being implemented effectively into a

management framework, there will be few applied benefits of the research. Research that supports a greater connection between science and management is required (Thurstan *et al.* 2015, Izzo *et al.* 2016).

CONCLUSION

Historical baselines of natural events like hypoxia tend to be closely intertwined with the effects of past climate changes and anthropogenic pressures like altered land-use and habitat degradation. In turn, elucidating the exact cause of increasing hypoxic conditions on a global scale is difficult. Throughout this thesis I focused on two related areas to aid in reducing the damaging effects of hypoxia in freshwater systems; the physiological thresholds of fish (organisms under high risk of large scale mortalities during hypoxic exposure), and using otoliths to retrospectively trace the occurrence of hypoxia in freshwater. Significant challenges face aquatic fauna when threatened with hypoxic flows from both natural and anthropogenic sources. While natural occurrences are inevitable, a better understanding of the tolerance thresholds for our native species would allow water managers in the future to reduce the risks associated with hypoxic blackwater events. It is necessary that we understand the long-term implication of hypoxic events on fish health and at different spatial scales (e.g. small to large scale events). This project has helped determine acceptable levels of environmental dissolved oxygen for minimum impact to fish health.

All aquatic systems have undergone extensive change due to human activities and changing climates. Consequently, systems are studied and managed relative to a shifted baseline. The reconstruction of past hypoxic events will provide insight into high and low risk environmental conditions, and potentially some key environmental parameters that lead to or exacerbate hypoxic conditions. In the longer-term, the findings from this thesis can contribute to reducing the risks to fish health, providing data for water resource planning and the development of appropriate adaptation strategies for the changing environmental conditions. This research could also be potentially utilised to determine a national standard for dissolved oxygen levels for water allocation similar to other countries such as Canada and the USA. Furthermore, the techniques of this study can be adapted for use in other environments, such as marine systems, where hypoxia is an increasing issue (Pollock *et al.* 2007).

Hypoxia is complex and affects organisms differently depending on behaviour, physiology and other environmental factors. In this thesis, I used multiple methods to determine the effects of hypoxia on native fish from the Murray river. The greatest realisation herein was that one method alone could not describe all the effects of hypoxia. In the future complementary approaches will provide more information to support conservation and restoration efforts. To this end, this study describes the long-term effects of hypoxia, from both a physiological and chemical basis (otoliths to track hypoxia) and generates vital information to aid in effective management of these conditions to improve the overall health of organisms through to the entire ecosystem.

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