Comparative hypoxia responses and oxygen sensing in galaxiid fishes

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Abstract

Aquatic ecosystems are inherently characterised by limited oxygen availability and fluctuations in dissolved oxygen concentration, and therefore, environmental hypoxia (i.e. low oxygen conditions) is common. However, in recent decades, the frequency and global expansion of hypoxic environments have increased significantly, owing to intensified anthropogenic impacts on the natural environment.

Fish depend on environmental oxygen for oxidative metabolism. In the absence of oxygen, alternative anaerobic mechanisms are in place to maintain energy metabolism. Efficiency and productivity of anaerobic metabolism are, however, comparatively low and may become rapidly insufficient in light of high physiological and metabolic oxygen demand. Correspondingly, substrates used in the oxygen-independent metabolism often are limited, and are converted into metabolites detrimental for the organism. When anaerobic mechanisms fail to meet the energy demand and lethal levels of anaerobic by-products have accumulated, hypoxic death is initiated. In this context, a variety of adaptive hypoxia-response strategies and hypoxia-sensitivities are established in fish, in dependence on species-specific oxygen demand and life strategies, thereby potentially creating very specific environmental niches occupied by distinct species. Accordingly, fish may demonstrate distinct habitat requirements and preferences in relation to the oxygen environment, potentially reflecting species-specific hypoxia sensitivities and response capacities, that may ultimately initiate adaptive radiation. In this context, a range of studies has been undertaken previously in a variety of species, however these are difficult to evaluate in a comparative context due to pronounced taxonomic differences between study

species. New Zealand's galaxiids, however, include species which occupy a wide range of environments. They are closely related and may demonstrate adaptive radiation to particular environments. The three related galaxiid species inanga (*Galaxias maculatus*), banded kokopu (*Galaxias fasciatus*) and black mudfish (*Neochanna diversus*) demonstrate minimised phylogenetic distances, yet exhibit specialised habitat requirements with distinctly different oxygen environments. Thus, it was the overarching goal of this research to establish whether these differences are based upon species-specific oxygen sensitivities and unique hypoxia response strategies and mechanisms, by utilising a novel, comparative combination of behavioural, physiological, molecular and gill morphological studies.

To study whether the three closely related galaxiid species exhibit unique behavioural responses upon encountering a hypoxic environment, the ability to sense progressive hypoxia, as well as behavioural responses and hypoxia avoidance thresholds were investigated in a hypoxia-normoxia choice chamber. Inanga demonstrated avoidance of mild hypoxia (< 5.9 mg L^{-1}) and increased the frequency of visits into both the hypoxic and normoxic sides of the choice chamber in severe hypoxia (at dissolved oxygen concentration below 3.6 mg L^{-1} for hypoxic side and below 1.9 mg L^{-1} for normoxic side). Banded kokopu responded to progressive hypoxia primarily with an increased frequency of aquatic surface respiration and an elevated swimming speed (body lengths (BL) s^{-1}). Banded kokopu was less averse to hypoxia than inanga, as they displayed a lower hypoxia avoidance threshold and demonstrated horizontal migration from severe hypoxia (< 2.5 mg L^{-1}). By contrast, no avoidance of, or other distinct behavioural response to hypoxia was observed in black mudfish. In conclusion,

the three species exhibit unique behavioural responses upon encountering a hypoxic environment which demonstrates not only distinct behavioural response strategies towards hypoxia, but also indicates species-specific hypoxia sensitivities.

To investigate whether these distinct hypoxia sensitivities and behavioural responses are based on different metabolic oxygen demands and oxygen consumption profiles, intermittent-flow respirometry was utilised to investigate routine metabolic rates at normoxia, mild and severe hypoxia. Inanga demonstrated oxygen consumption rates similar to banded kokopu, while black mudfish was lower. Inanga and banded kokopu maintained normoxic oxygen consumption in mild hypoxia and exhibited distinct critical oxygen concentrations (C_{crit}), below which oxygen consumption rates declined, identifying these species as oxyregulators. Black mudfish was revealed to be an oxyregulator as well, but no C_{crit} could be ascertained in this study, possible due to insufficiently severe levels of hypoxia. Inanga displayed the greatest hypoxia sensitivity, reflected in a C_{crit} of 5.0 ± 0.4 mg L^{-1}, while banded kokopu was slightly more tolerant to hypoxia with a C_{crit} of 4.3 ± 0.1 mg L^{-1}. In conclusion, the three species exhibit distinct oxygen consumption profiles and different critical dissolved oxygen concentrations.

To examine whether swimming speed, as a measure of metabolic oxygen demand, and gill morphology are affected by prolonged hypoxia and whether responses to hypoxia are mediated by oxygen sensing neuroepithelial cells (NECs) and by the transcription protein hypoxia inducible factor 1 (HIF-1), the effect of inescapable moderate hypoxia at 5 mg L^{-1} dissolved oxygen concentration (without access to the water surface) on these parameters was

investigated. Swimming speed decreased significantly from 21.6 ± 2.6 to 7.4 ± 2.0 BL min^{-1} in inanga, while no change was seen in banded kokopu and black mudfish. All three species presented NECs in the gill epithelia; however, hypoxia exposure did not elicit gill morphological adaptations or changes in NEC- and HIF-1 alpha density in any species. Gill morphological traits in black mudfish (i.e. wider respiratory lamellae than in inanga and banded kokopu) indicated an adaptation to emersion and aestivation, frequently observed in this species. HIF-1 alpha protein stabilisation at normoxic conditions indicated distinct differences between mammalian and piscine HIF-1 alpha pathway and hypoxia-inducible gene transcription. In conclusion, the three species exhibit specific adjustments of their swimming speed as a measure of metabolic oxygen demand, however, they do not alter their gill morphology in response to prolonged moderate hypoxia. Furthermore, responses to hypoxia are not mediated by changes in the density of NECs or HIF-1-positive cells.

Overall, the findings from this thesis demonstrate that all three species are capable of managing hypoxic environments. In this context, they utilise distinct behavioural response strategies in progressive, escapable hypoxia as well as in prolonged, inescapable hypoxia, and they exhibit different oxygen consumption profiles and hypoxia sensitivities, possibly owing to specific metabolic oxygen demands and distinct behavioural and physiological response capacities. Therefore, the distinct habitat preferences of these galaxiid species are potentially a result of adaptive radiation in the context of the oxygen environment. These observations are relevant in regard to the anthropogenically caused increased expansion of hypoxic environments, affecting the natural habitats of inanga and, potentially, banded kokopu.

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12:12D	12 h light – 12 h dark – photoperiodic cycle
5–HT	serotonin (5-hydroxytryptamine)
ABC	avidin–biotin complex
Af	South Africa
ANOVA	analysis of variance
ASR	aquatic surface respiration
ATP	adenosine triphosphate
b	body mass scaling coefficient
BL	body lengths
BL min^{-1}	body lengths per minute
BL s^{-1}	body lengths per second
ΔC_{wO2}	change in dissolved oxygen concentration
Δt	difference in time
DAB	3,3’-diaminobenzidine tetrahydrochloride
DO	dissolved oxygen
EDTA	ethylenediaminetetraacetic acid
F	F–value: Critical measure of significance in a statistical test
F	gill primary filament

G	*Galaxias*
Gl	*Galaxiella*
H_2O_2	hydrogen peroxide
HIF-1	hypoxia inducible factor 1 protein
HIF-1 α/ HIF-1 alpha	alpha subunit of hypoxia inducible factor-1 protein dimer
HIF-1 β/ HIF-1 beta	beta subunit of hypoxia inducible factor-1 protein dimer
HSD	honest significant difference-aspect in Tukey's statistical test environment
ILCM	interlamellar cell mass
L	secondary respiratory lamellae
log	natural logarithm of a number
MAD	median absolute deviation
M_f	body wet mass
MMR	maximum metabolic rate
$\dot{M}O_2$	oxygen consumption
n	number of variables
N	*Neochanna*
NC	New Caledonia

NEC	neuroepithelial cells
NZ	New Zealand
P	P–value: Measure of significance in a statistical test
P	*Paragalaxias*
C_{crit}	critical oxygen concentration
pH	measure of acidity/basicity
post hoc	statistical examination, following analysis of variance
RMR	routine metabolic rate
rRNA	ribosomal ribonucleic acid
SA	South America and Falkland Islands
SE	standard error
SMR	standard metabolic rate
TBS	[Tris(hydroxymethyl)aminomethane]-buffered saline
Triton X –100	polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether
V_f	fish volume
V_r	respirometry chamber volume
x,y–position	horizontal and vertical coordinates of fish positional data in a two–dimensional video tracking environment

1 General introduction

1.1 Respiration in aquatic environments

All organisms depending on aerobic respiration and oxidative metabolism for energy production are confronted with the availability of environmental oxygen as a critical and limiting factor for habitat selection and survival. While terrestrial organisms typically inhabit environments with readily accessible and abundant oxygen supply, aquatic organisms occupy environments with comparatively low oxygen availability (Kramer, 1987). Density and viscosity of water is considerable higher than in air which limits concentration and rate of diffusion of oxygen in aquatic environments (Graham, 1990).

Oxygen available to meet the oxygen demand of all aquatic organisms originates from the atmospheric environment and from photosynthetic phytoplankton and vascular plants (Diaz, 2001), but oxygen solubility is dependent on water temperature and salinity (Graham, 1990) (Figure 1.1).

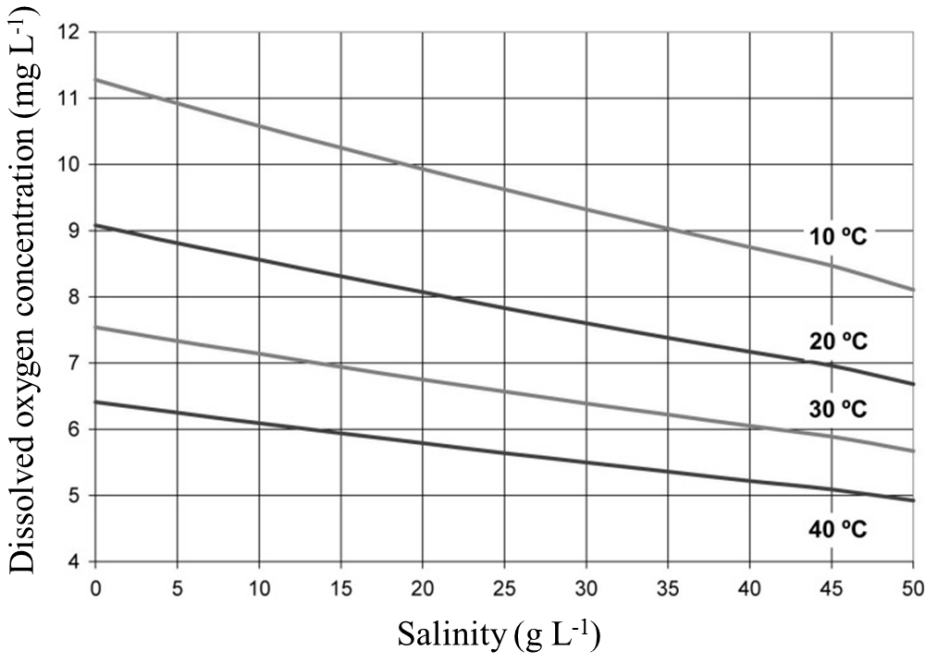


Figure 1.1: Relationship between dissolved oxygen concentration (mg L^{-1}) in water and salinity (g L^{-1}) at different water temperatures. Oxygen solubility and hence dissolved oxygen concentration decreases with increasing temperature and salinity. Modified from Lay *et al.* (2010).

1.1.1 Natural and anthropogenic causes of aquatic hypoxia

Aquatic ecosystems are naturally susceptible to diel, seasonal and spatial shifts in dissolved oxygen (DO) concentration, which can lead to decreased DO concentration (hypoxia) or complete depletion of oxygen (anoxia) in freshwater, estuarine and marine habitats (Diaz & Breitburg, 2009; Friedrich *et al.*, 2014). In this context, photosynthetic oxygen supply has been shown to depend on the availability of light and the magnitude of algal populations and aquatic vegetation (Davies-Colley *et al.*, 2013), thus the DO concentration does vary with daily sun-light regime and seasonal changes in temperature (Graham, 1990), insolation and algae, as well as vascular plant growth (Burnett, 1997; Davies-Colley *et al.*, 2013). Similarly, re-aeration of aquatic environments by atmospheric diffusion

depends on water temperature, turbulence and mixing, thus re-aeration declines with decreasing water flow velocity and turbulence, and increasing water depth and temperature (Davies-Colley *et al.*, 2013). While oxygen-rich surface water is typically vertically mixed down to bottom waters (Diaz, 2001), this process is affected by wind, rain-fall and water temperature, therefore the vertical DO concentration profile of a water column can be seasonally variable with temporarily low DO concentration in bottom waters (Diaz & Breitburg, 2009). Enclosed and semi-enclosed aquatic habitats like lakes and estuaries are often characterised by reduced mixing that can lead to stratification and depletion of oxygen in the hypolimnion to the point of anoxia (Figure 1.2). Ice cover or extensive amounts of floating vegetation may exacerbate hypoxic or anoxic conditions by inhibiting re-aeration with atmospheric oxygen (Kramer, 1987; Friedrich *et al.*, 2014). In freshwater ecosystems, natural hypoxic conditions therefore appear to develop predominantly in lentic (still) habitats, as lotic (flowing) environments are characterised by moving water masses and mixing.

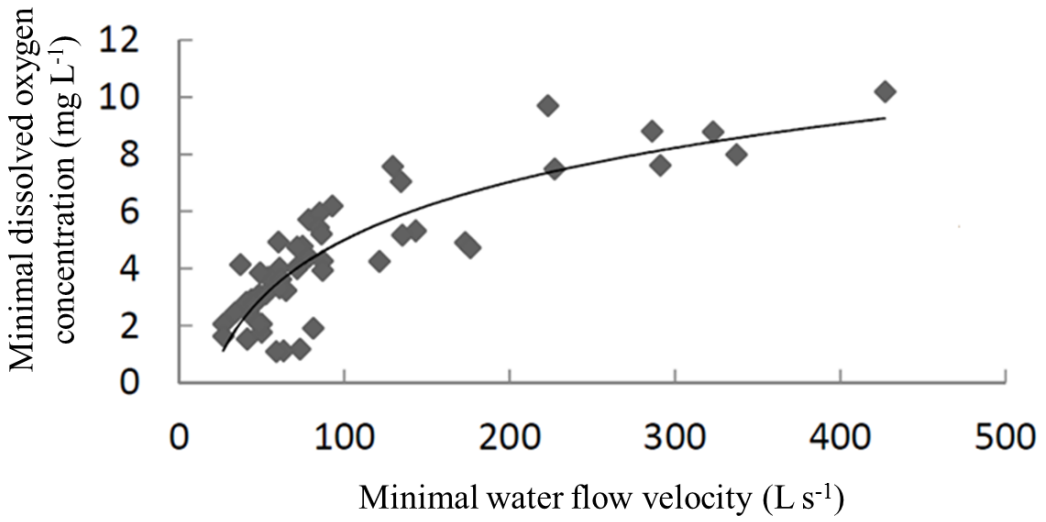


Figure 1.2: Decrease of DO concentration (mg L^{-1}) with declining instream water flow velocity (L s^{-1}). Measurements were taken from Waitangi Stream (Auckland, New Zealand) over a period from 2008 to 2011. From Davies-Colley *et al.* (2013).

Globally, increasing anthropogenic impact on the natural environment has amplified the occurrence of hypoxia and anoxia in freshwater, estuarine and marine ecosystems through elevated nutrient discharges, particularly nitrogen and phosphorus, as well as excess discharge of organic matter into streams, lakes and estuaries (Diaz, 2001; Diaz & Breitburg, 2009). Anthropogenic increase in aquatic nutrient levels originates from land run-off, which is primarily driven by land–uses, such as intensive farming, application of fertilizers and discharge of domestic and industrial wastewater. Deforestation, removal of riparian zones, and draining of swamps and wetlands intensify eutrophication (Wu, 2002; Hamill & McBride, 2003; Landman *et al.*, 2005). Excess nutrient input drives eutrophication of aquatic ecosystems as it leads to elevated primary production with an amplified growth of photosynthetic algae which is often referred to as algae blooms (Paerl *et al.*, 1998; Diaz, 2001; Friedrich *et al.*, 2014). Following

this temporary increase in phytoplankton photosynthesis and DO concentration in surface waters, oxygen re-supply through photosynthesis decreases as algae die and excess organic matter that exceeds uptake from the established food chain settles in bottom waters where it fuels microbial respiration. Microbial decomposition of organic matter intensifies uptake of dissolved oxygen and when this process consumes more oxygen than can be re-supplied through photosynthesis, re-aeration and mixing, hypoxic or anoxic conditions arise. This is particularly common in bottom habitats of stratified water bodies where the DO concentration may decrease below the minimum value necessary to sustain most aquatic organisms (Diaz, 2001; Davies-Colley *et al*., 2013; Friedrich *et al*., 2014). At times this condition is accompanied by a high proportion of bacterioplankton in the aquatic community which often results in a further increase in biological and chemical oxygen demand of the ecosystem (Jonas, 1997). Similarly, an overabundance of phytoplankton and floating as well as submerged vascular plants has been shown to elevate diurnal variations in DO concentration to potentially extreme levels of fluctuations. In this context, maximum concentration during the day may rise above the habitat's physical concentration level and minimum DO concentration during the night potentially reach near-zero values, resulting in hypoxic or anoxic conditions (McDowell & Wilcock, 2008). Likewise, hydrological alterations of water bodies such as hydroelectric dams and river flow diversions are affecting water quality and hence aquatic DO concentration (Paerl *et al.,* 1998), especially when oxygen-poor hypolimnetic water from an artificially stratified lake is being discharged down-stream of a hydroelectric dam or when modification of streams results in distinctly decreased water flow velocities.

Concurrent with globally occurring anthropogenic degradation of aquatic environments, New Zealand's aquatic ecosystems are also showing degradation. Hypoxic or anoxic freshwater environments in New Zealand are commonly found in warm, un-shaded, slow-flowing and often structurally altered lowland ecosystems with a distinct abundance of algae and aquatic plants (Davies-Colley *et al.*, 2013). Further environments prone to low DO concentration are downstream sections of hydroelectric dams, stream regions with upwelled oxygen-depleted groundwater, and downstream regions of high-organic content pollution sources such as intensive farming and fertilizer use as well as grazing cattle pasture (Wilcock *et al.*, 1995; Hamill & McBride, 2003; Larned *et al.*, 2004; McDowell & Wilcock, 2008; Davies-Colley *et al.*, 2013). Thus hypoxic environments are common throughout New Zealand especially in small lowland streams in the Waikato and Auckland region but also in the Southland region where dairy farming is widely prevalent (Wilcock *et al.*, 1995; Wilcock *et al.*, 1998; Wilcock *et al.*, 1999; Hamill & McBride, 2003; Landman *et al.*, 2005) and in a number of New Zealand lakes, particularly in the Rotorua region during the summer period (Landman *et al.*, 2005; Verburg *et al.*, 2010). Similarly, decreased aquatic DO concentration arising from industrial waste water discharge is also an issue in New Zealand, as has been reported for the Tarawera River, Bay of Plenty, downstream of a pulp and paper mill (Landman *et al.*, 2005).

1.1.2 Piscine respiration and adaptation to hypoxia

Hypoxia as a common occurrence in aquatic ecosystems has facilitated the development of various adaptive respiratory strategies, enabling fish to endure environmental oxygen fluctuation and reduction. The main respiratory organs in water-breathing fish are the gills, which are adapted to an efficient gas exchange

in an aquatic environment (Fernandes *et al.*, 2007). The bilaterally positioned organs are structured into four gill arches, with each arch supporting two sets of gill filaments from which the secondary respiratory lamellae extend (Moyes & Schulte, 2008), and are the primary site of gas exchange in adult fish (Evans *et al.*, 2005). Lamellae are characterised by a large surface area (Fernandes *et al.*, 2007) and lamellar blood flows in a counter–current direction to water flow across the lamellae (Perry & McDonald, 1993), and the diffusion distance for exchange of oxygen and carbon dioxide between water and blood is reduced (Fernandes *et al.*, 2007). Gill morphology, such as total respiratory surface area, has been shown to be adaptive in relation to species–specific oxygen demands and physicochemical habitat conditions (Hughes, 1984). Alternative to water respiration, air–breathing structures in adaptation to hypoxia have evolved in multiple fish groups (Hughes, 1984) and include, but are not restricted to, lungs, modified respiratory gas bladders, chambers or pouches in the head region, and organs along the alimentary system (Graham, 1997).

Water–respiration associated gill function in relation to fluctuating ambient DO concentrations occurs under nervous control (Randall, 1982), initiating circulatory (Bushnell & Brill, 1991) and respiratory (Randall, 1982; Balfour, 1999) adaptations, and maintaining adequate oxygen uptake to satisfy metabolic demand. However, this oxyregulatory strategy is limited by species–specific capacities, and continuously declining ambient DO concentration may elicit a progressive decline of oxygen uptake in dependence on decreasing ambient DO concentration, a process which is termed oxyconforming (Pörtner & Grieshaber, 1993). Consequently, a shift occurs from oxidative metabolism to anaerobic glycolysis, which is characterised by decreased efficiency and

productivity (Richards, 2009), and is often quickly exceeded by the oxygen demand of non-subsistence processes such as locomotor activity, digestion, growth or reproduction (Chabot *et al.*, 2016). The critical DO concentration at which the described shift occurs, is hereby indicative of the species–specific hypoxia sensitivity (Rogers *et al.*, 2016).

Capability of oxygen sensing is a prerequisite for processes that are maintaining oxygen uptake in fluctuating ambient DO concentrations. Neuroepithelial cells have been demonstrated as putative oxygen sensing cells in gill (Dunel-Erb *et al.*, 1982; Sundin *et al.*, 1998; Saltys *et al.*, 2006; Coolidge *et al.*, 2008) and skin epithelia (Saltys *et al.*, 2006; Regan *et al.*, 2011). These respond to decreasing ambient DO concentrations with the release of the neurotransmitter serotonin, presumably initiating hypoxia response pathways and adaptations (Jonz & Nurse, 2003) according to species–specific oxygen requirements. In this context, hypoxia tolerant species, such as goldfish (*Carassius auratus*, (Sollid *et al.*, 2003)) and crucian carp (*Carassius carassius*, (Mitrovic *et al.*, 2009)) reversibly increase their gill surface area via apoptosis of interlamellar cell mass (ILCM) that under normoxic conditions is highly accumulated between the respiratory lamellae, thereby markedly increasing the level of lamellae protrusion in long-term hypoxia. Distinctly different response pathways are initialised in emersion tolerant, scale-less fish species, such as New Zealand native galaxiids, which utilise aerial cutaneous respiration, thereby extracting more oxygen compared to respiration in hypoxic aquatic environments (Meredith, 1985; Urbina *et al.*, 2011). Other hypoxia response strategies include aquatic surface respiration (ASR), which involves skimming of water at the water/air interface (Kramer & McClure, 1982), and bubble respiration, where an

air bubble is stored in the buccal cavity and elevates the DO concentration of water, ventilated over the gills (McPhail, 1999). Alternatively, horizontal migration as a behavioural response may be triggered, as it has been shown in hypoxia–sensitive species, which are thereby avoiding the hypoxic habitat (Kramer, 1987; Poulsen *et al.*, 2011).

1.2 Model species

1.2.1 The Galaxiidae family

Comparative studies on sensitivity and responses to aquatic hypoxia require model species that typically inhabit ecosystems with distinct differences in the environmental DO concentration profile. However, ideally, they should exhibit only minor taxonomic differences. The Galaxiidae family (Order Osmeriformes) comprises eight genera and more than 50 distinct species (McDowall, 2006) of scale-less fish that are prevalent throughout the southern hemisphere including South Africa, South America, Falkland Islands, mainland Australia, Tasmania, New Caledonia and New Zealand (Figure 1.3). Galaxiids populate a wide range of environments, including rivers, lakes and swamps ranging in altitude from sea level to more than 1500 m above sea level, however are present within the same geographical distribution (i.e. geographical latitudes and longitudes) within the North Island of New Zealand (McDowall, 1990, 2010). Galaxiids are usually solitary species with few exceptions that aggregate in shoals, such as inanga (*Galaxias maculatus*) and banded kokopu (*Galaxias fasciatus*). While the life-span of many galaxiid species occur exclusively in freshwater habitats, several species are diadromous as they migrate to sea after hatching in freshwater and return back into freshwater habitats at early juvenile stages, commonly known as whitebait (McDowall, 1990).

From the Galaxiidae family, I have selected inanga (*Galaxias maculatus* Jenyns 1842), banded kokopu (*Galaxias fasciatus* Gray 1842) and black mudfish (*Neochanna diversus* Stokell 1949) as model species. These three species occur in similar latitude and altitude areas but they populate a diverse range of habitats in New Zealand's freshwater ecosystems (Figure 1.4) reflecting markedly differing

sensitivities to aquatic hypoxia (Dean & Richardson, 1999; McPhail, 1999; Landman *et al.*, 2005). Distinct ecological differences within the galaxiids in the context of habitat use may have been established through adaptive radiation. This process of species diversification is driven by the development of distinct morphological and / or physiological traits which permit utilisation of distinct resources (Schluter, 1996). This may have enabled some galaxiid species, such as the black mudfish, to withstand and utilize hypoxic environments while other species, like inanga and banded kokopu, may be restricted to normoxic habitats, which is potentially reflected in distinct hypoxia sensitivities. Adaptive radiation as a mechanism of species diversification has been established for multiple taxonomic fish groups, such as Antarctic notothenioids (Clarke & Johnston, 1996) and African cichlids (for a review see Seehausen, 2006). Similarly, studies have been done previously in New Zealand triplefin fishes, demonstrating that environmental differences may have elicited distinct physiological trait divergence and adaptation, which potentially enabled species expansion into new habitats (Hilton, 2010). In this context, it has previously been shown that the mudfish genus *Neochanna* of the Galaxiidae family presents distinct morphological and ecological adaptations, that enable the utilisation of swampy habitats (Waters & McDowall, 2005). The genus has been shown to aestivate, and demonstrates an evolutionary transition from a typically galaxiid appearance to an elongated, eel-like morphology featuring reduced or modified fins, long nostrils and small eyes, while diadromy is absent in all New Zealand mudfish species. However, to date no comprehensive and comparative studies on ecological, morphological or physiological differences in the context of hypoxia sensitivity

and habitat preferences within the galaxiids, which may be based upon adaptive radiation, have been undertaken.

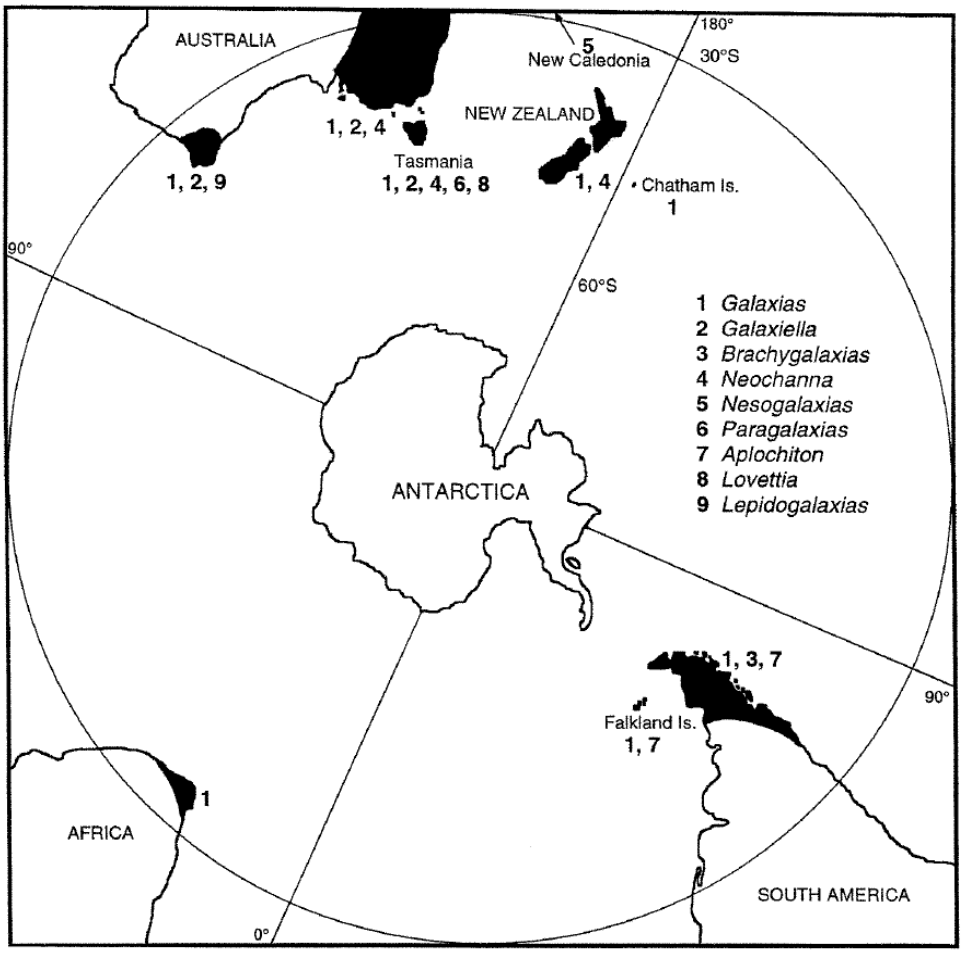


Figure 1.3: The Galaxiidae family demonstrates a wide geographic distribution in freshwater environments of the Southern hemisphere marked by black areas. Occurence of distinct genera is indicated by their specific number. From Waters *et al.* (2000).

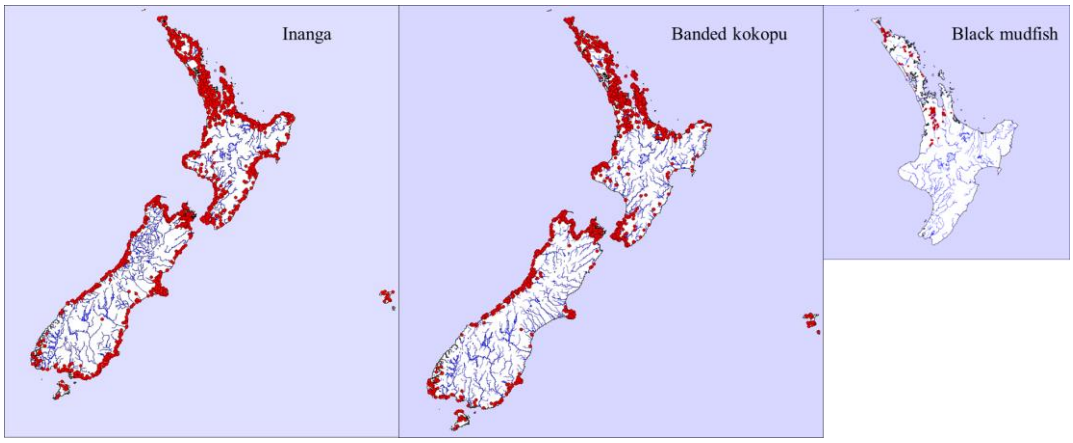


Figure 1.4: Geographical distribution of inanga (*Galaxias maculatus* Jenyns 1842), banded kokopu (*Galaxias fasciatus* Gray 1842) and black mudfish (*Neochanna diversus* Stokell 1949) that occurs at similar latitude and altitude regions in New Zealand. From (https://www.niwa.co.nz/freshwater-and-estuaries/nzffd/NIWA-fish-atlas/fish-species).

Comparative studies on different fish species are often compromised by substantial taxonomic differences possibly affecting the outcome of these studies when conclusions regarding adaptation mechanisms to distinct habitats are impaired by the specific phylogeny of the investigated species. By investigating these three closely related species of the Galaxiidae family, taxonomic differences should be minimised (Figure 1.5), leading to a comprehensive understanding of adaptive strategies to aquatic hypoxia and hence the effect of aquatic hypoxia on three of New Zealand's native fish species.

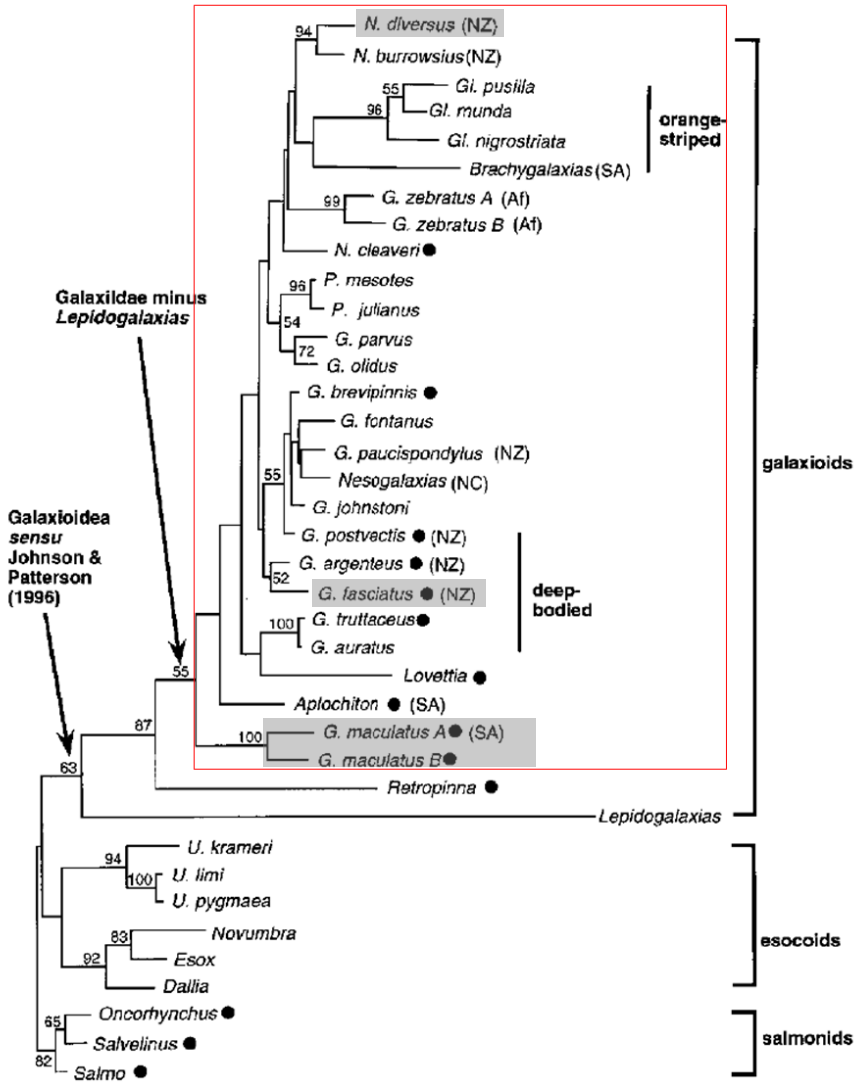


Figure 1.5: Phylogenetic relationships in the galaxioidea superfamily. Data are based on combined cytochrome b and 16S rRNA sequence analysis and maximum likelihood-ratio tests illustrating the phylogenetic degree between inanga (Galaxias maculatus Jenyns 1842), banded kokopu (Galaxias fasciatus Gray 1842) and black mudfish (Neochanna diversus Stokell 1949) of the Galaxiidae family. Galaxiids are marked by a red rectangle. Model species are marked by shaded boxes. Closed circles distinguish migratory from non-migratory species. When not specified otherwise, taxa originate from the Tasmania and Australia region. Applied abbreviations: G. = Galaxias, Gl. = Galaxiella, N. = Neochanna, P. = Paragalaxias, NZ = New Zealand, NC = New Caledonia, SA = South America and Falkland Islands and Af = South Africa. From Waters et al. (2000).

1.2.2 Inanga (*Galaxias maculatus* Jenyns 1842)

The inanga (Figure 1.6 A), a small diadromous fish native to New Zealand, is characterised as one of the most widely geographically distributed freshwater fish throughout the southern hemisphere, including New Zealand, Australia and South America (McDowall, 1990; Barbee *et al.*, 2011). At the onset of autumn spring tides, inanga migrate to estuarine habitats where eggs are deposited amongst flooded riparian vegetation, fertilized, and left to develop in humid air amongst vegetation after water levels have receded. Hatching occurs during inundation from subsequent spring tides followed by flood-driven migration of larvae to the sea (McDowall & Charteris, 2006). Given this specialisation in spawning behaviour, inanga are highly dependent on the presence of appropriate spawning habitats, which are threatened by anthropogenic eutrophication and removal of riparian vegetation accompanying deforestation and pasture development in New Zealand. After several weeks of growth in a marine environment, juveniles migrate back into freshwater habitats as one of the most common whitebait species attracted by increased freshwater outflow into coastal seas following river floods (McDowall, 1990). Habitat selection is also potentially affected by pheromone detection from adult galaxiids (Baker & Hicks, 2003). Inanga are commonly unable to overcome water falls or long sections of swift rapids due to limited climbing abilities. Therefore, they are usually not present in habitats far inland and at high altitude, however they are widely abundant in coastal and lowland rivers, streams, lakes and swamps around New Zealand (McDowall, 1990; Eikaas & McIntosh, 2006), whereupon they prefer habitats with cover from in-stream debris and overhanging bank vegetation (Richardson, 2002). Human alteration of migratory waterways such as installation

of weirs (Baker, 2003) or thermal discharge from riverine power stations exceeding their temperature tolerance (Simons, 1986; Boubée *et al.*, 1991; Shingles *et al.*, 2005), however, are potentially impeding upstream migration. Inanga inhabit a range of freshwater environments tolerating a wide spectrum of water clarity, temperature, pH and salinity (McDowall, 1990). While inanga are generally characterised as diadromous, land-locked and non-migrating populations have been described in small coastal lakes in New Zealand and overseas (McDowall, 1972; Barbee *et al.*, 2011; Barriga *et al.*, 2011).

The sensitivity of inanga to low dissolved oxygen concentration has been studied in laboratory experiments showing that inanga survived a dissolved oxygen concentration of 3 mg L^{-1} for 48 h, while exposure to 1 mg L^{-1} induced a 61% mortality in whitebait and 38% mortality in adults (Dean & Richardson, 1999). Accordingly, a further study showed a 50% mortality of inanga whitebait to 48 hours of exposure to 2.65 mg L^{-1} (Landman *et al.*, 2005). Recently, inanga has been shown to emerse from hypoxic water in a laboratory experiment at dissolved oxygen levels below 1.9 mg L^{-1} (Urbina *et al.*, 2011), most likely employing cutaneous and gill respiration in air. However, the duration of the experiment was relatively short (60 minutes) and a different study showed that inanga were able to survive emersion for less than one day (Meredith, 1985), making emersion behaviour an unlikely response strategy of inanga towards aquatic hypoxia.

1.2.3 Banded kokopu (*Galaxias fasciatus* Gray 1842)

Banded kokopu (Figure 1.6 B) is a medium sized, largely nocturnal galaxiid species endemic to and widely distributed in New Zealand. In similarity to inanga, this species has a diadromous life history as spawning occurs in

freshwater during autumn at spring tides amongst flooded stream banks (McDowall, 1990; Charteris *et al.*, 2003). Unlike inanga, however, banded kokopu are generally not displaying extended downstream migration to distinct spawning habitats as spawning appears to occur close to adult habitats (Hopkins, 1979). But contrary to Hopkins (1979), an isolated observation of banded kokopu eggs developing amongst inanga spawning sites suggests versatile spawning site requirements for banded kokopu (Mitchell, 1991). Hatched larvae are transported to the sea by river floods (Ots & Eldon, 1975) where they undergo pelagic development. Banded kokopu is one of five whitebait species, hence juveniles migrate back into freshwater habitats after several weeks at sea. Moving upstream banded kokopu are able to overcome vertical, rocky obstacles and therefore can be found throughout New Zealand from coastal waters at sea level to freshwater habitats far inland and upstream of waterfalls (Eikaas & McIntosh, 2006). Habitat selection may be affected by pheromones from adult banded kokopu as it has been demonstrated in laboratory experiments that migrating juveniles are attracted by adult banded kokopu pheromones (Baker & Montgomery, 2001; Baker & Hicks, 2003). They are most abundant in small clear and cold streams draining lowland podocarp forests that offer cover through forest canopy, large boulders or logs, or overhanging stream bank vegetation (McDowall, 1990; Eikaas *et al.*, 2005; Baker & Smith, 2007). Banded kokopu avoid habitats with elevated turbidity (Rowe *et al.*, 2000), which concurs with laboratory experiments that demonstrated a distinct sensitivity towards suspended sediments (Richardson *et al.*, 2001a). They have also been shown to exhibit distinct temperature sensitivity in laboratory experiments (Simons, 1986). In this context, preference for cool water temperatures may limit upstream recruitment of banded kokopu if it requires

passage through thermal water discharge from river power stations. Similarly, they are not abundant in streams without forest canopy or similar cover, as is common when forest and bush has been replaced by pasture and they are less abundant in streams from which wood has been removed. Woody debris in streams can create slow-flowing pools and cover to shelter fish from predation (Baillie *et al.*, 2013). Banded kokopu display distinct size-dependent differences in their nocturnal microhabitat selection with small fish preferring shallow pools and larger fish preferring deeper pools with decreased water velocities (Akbaripasand *et al.*, 2011). In similarity to inanga, this species also occurs in land-locked lake populations without diadromous life stages (McDowall, 1990; Tana & Hicks, 2012).

Previous studies have shown banded kokopu to survive emersion for more than one week (Meredith, 1985), while their whitebait stages demonstrated an increased sensitivity to aquatic hypoxia compared to inanga (Dean & Richardson, 1999). Specifically, they were able to survive 48 hours of exposure to 3 mg L^{-1} dissolved oxygen, however demonstrated 100% mortality after 12 h of exposure to 1 mg L^{-1}. In this context, banded kokopu demonstrated emersion behaviour at 1 mg L^{-1} dissolved oxygen. Here, banded kokopu were established as the most hypoxia sensitive species which is inconsistent with the outcome of a more recent study that demonstrated inanga as a hypoxia sensitive species, however did not include banded kokopu (Landman *et al.*, 2005). Therefore, further comparative research on responses towards hypoxia studying the same life stages of the two species is necessary to resolve the relative hypoxia sensitivities of inanga and banded kokopu.

1.2.4 Black mudfish (*Neochanna diversus* Stokell 1949)

Black mudfish (Figure 1.6 C) are small, partly nocturnal fish endemic to the North Island of New Zealand. They occur most abundantly in still or gently flowing waters of swampy lakes, drains and wetlands overgrown by vegetation and filled with forest litter such as twigs and leaves providing cover (McDowall, 1990; Hicks & Barrier, 1996). Habitat loss due to fragmentation and draining of extensive areas of wetland and swamp systems for agricultural use has greatly reduced black mudfish abundance (McDowall, 1990; Gleeson *et al.*, 1999; Willis & Ling, 2000). With seasonal receding water levels black mudfish aestivate in humid substrate and vegetation. During this process they produce a mucous layer on the skin which potentially inhibits desiccation, and utilize cutaneous and gill respiration while their metabolic rate is decreased, reducing energy consumption and nitrogenous waste production (Hicks & Barrier, 1996; Gleeson *et al.*, 1999; McPhail, 1999). In contrast to inanga and banded kokopu, this species is non-migratory and all life stages occur in freshwater habitats (McDowall, 1990; Willis & Ling, 2000), with spawning occurring at the start of the wet season in winter (Barrier & Hicks, 1994) by depositing and fertilizing eggs amongst aquatic vegetation (Perrie, 2004).

Mudfish habitats naturally become temporarily hypoxic or even anoxic (Dean, 1995) and dry out in the summer drought season but black mudfish are able to survive these conditions. They have been shown to gulp air at dissolved oxygen levels below 2.5 mg L^{-1}. During air breathing they rise to the surface and ingest an air bubble into the buccal cavity from which respiration occurs (McPhail, 1999). While black mudfish have been established as a hypoxia tolerant species (Dean, 1995), further research facilitating a comparative investigation

with other galaxiid species is of significance to reveal the behavioural and physiological basis for this hypoxia tolerance.

(A) Inanga

(B) Banded kokopu

(C) Black mudfish

Figure 1.6: Inanga [(A): *Galaxias maculatus* Jenyns 1842, © Paddy Ryan], banded kokopu [(B): *Galaxias fasciatus* Gray 1842, © Stephen Moore] and black mudfish [(C): *Neochanna diversus* Stokell 1949, © Rod Morris].

1.2.5 Significance and conservation status of model species

Adult inanga and banded kokopu are of traditional importance in the Maori fishing culture. Additionally, their juvenile life stages are of significance for recreational and economic aspects of the modern whitebait fishery in New Zealand as they represent two of the five species of which whitebait is composed.

By day, with rising or full tide, these galaxiid juveniles migrate into river estuaries in vast mixed shoals, at which point they are caught by scoop or set netting (Rowe *et al.*, 1992) and therefore provide one of the most expensive seafood products on the New Zealand and international market (McDowall, 1990). Migratory strategies are common among many of New Zealand's native freshwater fish species (McDowall, 1990). Hence, upstream migration of juvenile fish into adult habitats may be affected by the decreased water quality and low DO concentrations that are becoming increasingly widespread in New Zealand aquatic ecosystems (Maes *et al.*, 2007).

The three model species are categorized by distinct conservation status assessments. Inanga are currently evaluated as 'Least Concern', characterised by widespread and abundant populations (David *et al.*, 2014). However, extensive habitat loss and deterioration affecting rearing and spawning habitat, as well as whitebait fishery prior catchment regulations (McDowall, 1990) have reduced the distribution of inanga in New Zealand (David *et al.*, 2014). Furthermore, predation by introduced species, such as mosquitofish (*Gambusia affinis*; (Rowe *et al*., 2007)) and trout, salmon and char of the genera *Salmo, Oncorhynchus* and *Salvelinus*, which were introduced into New Zealand for sports fishing (McIntosh *et al.*, 2010), were shown to impact inanga populations. Similarly, banded kokopu has been assessed as 'Least Concern', with currently stable populations, which are widespread and abundantly represented in New Zealand freshwater environments (West *et al.*, 2014b). Previous significant historical declines in distribution and population abundance occurred, however, due to loss and degradation of adult and spawning habitats, especially in regards to native forest streams and wetlands, unregulated whitebait fishery and artificial barriers impeding migration

(McDowall, 1990). Furthermore, introduced invasive and predatory fish species have been shown to constitute issues due to predation on, and competition with banded kokopu as well (McIntosh *et al.*, 2010; West *et al.*, 2014b). Black mudfish, in contrast, have been classified as 'Endangered', with a currently decreasing population trend (West *et al.*, 2014a). Substantial historical population declines, as well as ongoing reduction, were primarily caused by habitat loss, pollution and sedimentation, with wetland drainage being the most significant threat. Since human colonisation of New Zealand, approximately 85-90% of wetlands have been destroyed, thus reducing black mudfish range to less than 10% of its former distribution, characterised by very few remaining and highly fragmented as well as relictual populations (West *et al.*, 2014a). The mosquitofish (*Gambusia affinis*) is of additional concern for black mudfish populations, as this introduced species has been shown to prey upon mudfish fry and juveniles (Barrier & Hicks, 1994), and is competing with adults for habitat and food (West *et al.*, 2014a).

1.2.6 Adequacy for hypoxia studies

All three species, inanga, banded kokopu, and black mudfish, are readily available in the Waikato region of the North Island of New Zealand and are easily maintained in an aquarium environment. Upon consideration of their conservation status, phylogenetics, distinct differences in habitat preferences and hypoxia sensitivities, as well as distinct response strategies to low dissolved oxygen concentration, these species constitute a highly suitable model for the comparative study of adaptive mechanisms to aquatic hypoxia. Consequently, results from this study may be applied toward biodiversity management not only in New Zealand,

but globally, protecting species with comparable life histories inhabiting similar freshwater habitats.

1.3 Experimental hypoxia environment

When studying the effect of aquatic hypoxia on fish in a laboratory environment it is essential to have precise control over the aquatic dissolved oxygen (DO) concentration ad libitum without affecting the biochemistry of the water and potentially eliciting fish responses unrelated to aquatic hypoxia. Therefore, it was crucial to design and construct a deoxygenation device that allows an efficient and accurate execution of hypoxia experiments while meeting the financial and technical parameters of this doctoral study.

1.3.1 Oxygen stripping using nitrogen

In previous studies on aquatic hypoxia, water has been deoxygenated most commonly by applying gaseous nitrogen, which efficiently displaces oxygen from the solution as it diffuses into the nitrogen gas bubbles and therefore decreases the DO concentration (Mount, 1961) without alteration of the biochemical properties of the water. This process is frequently carried out directly inside the experimental environment with fine-bubble air stones (Nilsson *et al.*, 1993; Dean & Richardson, 1999; Robb & Abrahams, 2003; Shimps *et al.*, 2005; Janssen *et al.*, 2010; Urbina *et al.*, 2011; McBryan *et al.*, 2016) or porous aeration tubing (Burleson *et al.*, 2001). This was not feasible, however, for the studies included in this thesis due to the large amounts of water that needed to be deoxygenated at a distinct rate or to specific DO concentration levels. Furthermore, nitrogen gas bubbles in the experimental environment could potentially alter the observed fish behaviour and obstruct video recordings of the fish responses towards hypoxia. Alternatively, nitrogen stripping is commonly carried out in a circulatory water system in which the DO concentration of the water is decreased or adjusted to the preferred level before entering the experimental environment (Breitburg, 1994;

Balfour, 1999; Domenici *et al.*, 2000; Richardson *et al.*, 2001b). Often these systems are equipped with a computerised control of the deoxygenation process and maintenance of a distinct DO concentration level, as continuous oxygen measurements provide feedback on the actual DO concentration of the water and hence the nitrogen flow is adjusted automatically avoiding excessive deoxygenation or reoxygenation of the water (Schurmann & Steffensen, 1994; Brady *et al.*, 2009; Herbert *et al.*, 2010; Cook *et al.*, 2011; Poulsen *et al.*, 2011; Tiedke *et al.*, 2014; Hedgpeth & Griffitt, 2016). Such control systems eliminate manual observation and control of the DO concentration level, and reduce experimental operating maintenance but a computerised DO concentration control system was beyond the resources of this study.

1.3.2 Vacuum degassing

An alternative method of water deoxygenation is the application of vacuum degassing. Water is directed through a partial vacuum that reduces the total gaseous pressure and decreases the oxygen partial pressure in the water lowering the DO concentration (Mount, 1961; Bejda *et al.*, 1987). Recently, this method has been advanced by utilizing electronic control over distinct DO concentrations in multiple experimental environments (Landman & Van den Heuvel, 2003).

1.3.3 Study device

Water deoxygenation with vacuum degassing is effective and cost efficient in long term studies, however, due to its elevated initial costs and engineering complexity the installation of a vacuum degassing system was beyond the technical and financial scope of this research. Hence, for the studies presented in Chapter 2 and Chapter 4 of this thesis, a novel circulatory water system was

devised and constructed, in which water was efficiently deoxygenated utilizing nitrogen stripping, whilst the financial costs related to nitrogen consumption remained within feasible limits (Figure 1.7).

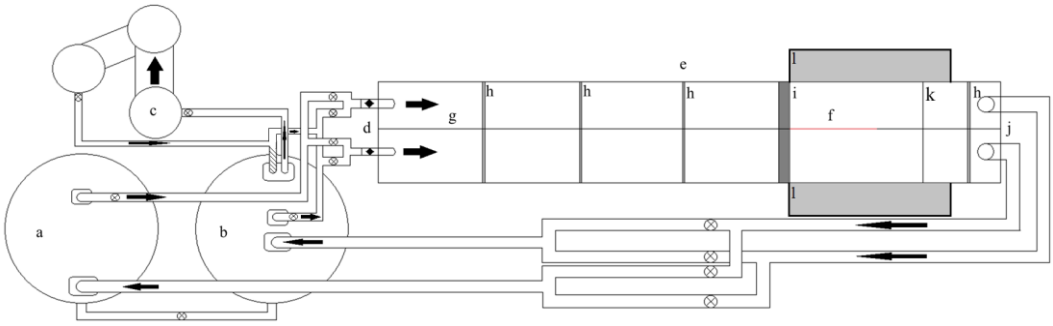


Figure 1.7: Circulatory water system and study device as seen from above, outlining normoxic (a) and hypoxic (b) water reservoirs, deoxygenation tower (c), study device (e) with flowmeter equipped inlets (d) and outlets (j). Deoxygenated water from the tower was either directed into the study device or into the hypoxic water reservoir (shaded pipe section). The study device consisted of a rectangular flow chamber with two sides divided by an acrylic sheet (g), facilitating laminar, non-mixing water flow in the test arena (f) due to the installation of light diffuser panels (h) and honeycomb-structured sheets with 3x3 mm perforations (i) even after removal of a 300 mm sections of the dividing acrylic sheet (red section of g). Mirrors (l) were installed adjacent to either side of the test arena at an approximate angle of 55° for evaluation of aquatic surface respiration (ASR). Ramps (k) were installed in the rear section of the study device maintaining an appropriate water level. Arrows indicate water flow direction and valves are marked by crossed circles.

Reservoirs for normoxic and hypoxic water respectively were set up and fitted with submerged recirculating water pumps. The normoxic water reservoir was also fitted with an external air pump. The reservoir for hypoxic water was connected to a three-columned interconnected deoxygenation tower (Figure 1.8). Each column had a flat circular gas diffuser fitted into the bottom section through which gaseous oxygen-free nitrogen from a commercial gas cylinder was introduced, deoxygenating water as it was flowing through the tower. Valves installed in the gas tubing enabled selective nitrogen bubbling of individual

towers. Alternatively, it was possible to oxygenate water passing through the tower when an external air pump replaced the nitrogen cylinder. To avoid accumulation of redundant gas in the top section of the tower, gas outlets were fitted into the lids of each column.

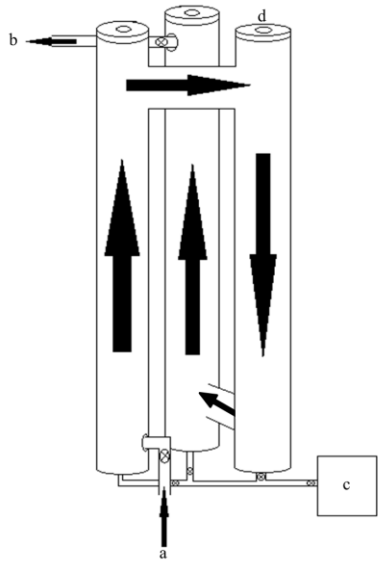


Figure 1.8: Three-columned interconnected deoxygenation tower as seen from the side depicting water inlet (a), water outlet (b), nitrogen or air supply (c) introduced into the bottom section of individual or all columns and gas outlets for discharge of redundant gas (d). Arrows indicate water flow direction and valves are marked by crossed circles.

The outlet from the deoxygenation tower was either connected to the hypoxic water reservoir or directly to the study device. The study device consisted of a rectangular flow chamber with two sides divided by a 2 mm transparent acrylic sheet. Water flow was kept laminar and non-mixing by installing light diffuser panels and honeycomb-structured sheets with 3x3 mm perforations, thus in the test arena of the study device measuring 280 x 600 mm in which

experiments were conducted, the two water streams would remain separated even after removal of a 300 mm section of the dividing acrylic sheet. To maintain a depth of 155 mm throughout chamber, ramps were installed in the rear section of the test arena. Installing valves into water pipes enabled direction of water from the normoxic reservoir, hypoxic reservoir or deoxygenation tower respectively into either side of the study device. Both water inlets entering the study device were fitted with flow meters enabling observation and manual adjustment of the water flow velocity on either side of the study device by modifying the openings of the appropriate valves. From the study device, water was recycled back into the reservoirs. As the recycling pipes were also fitted with valves, it was possible to direct water from either side of the chamber into either reservoir keeping normoxic and deoxygenated water separated within the entire circulatory system. To avoid differences in the chemical properties of water from the reservoirs possibly affecting the measured responses of the observed fish, the water reservoirs were connected by a pipe fitted with a valve, to enable mixing in pre-experimental fish acclimation procedures.

Pre-experimental dye tests were conducted to confirm that the two distinct water flows in the test arena of the study device remained laminar and non-mixing following removal of a length of 300 mm of the dividing barrier that would allow fish to move from one side of the chamber to the other (Figure 1.9).

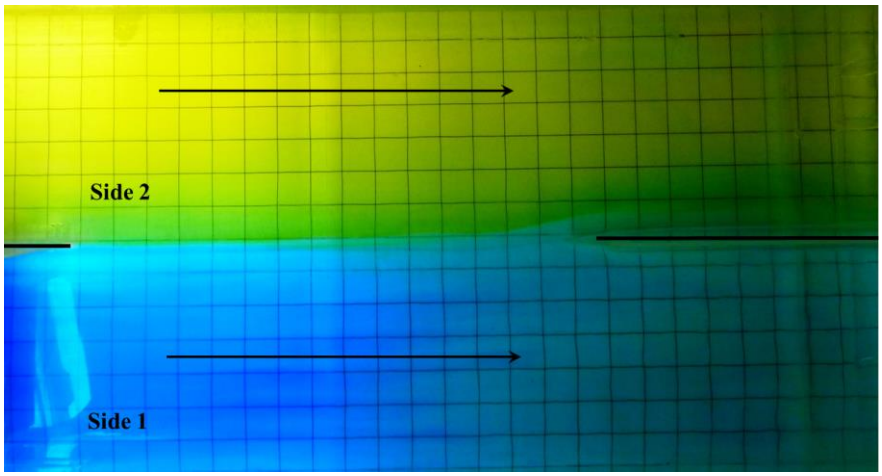


Figure 1.9: Pre-experimental dye test confirmed non-mixing, laminar water flows in the test arena. Position of the remaining separation sheet between both sides of the study device is marked by black bars.

With this system it was possible to efficiently produce water with continuously decreasing DO concentration or water of distinct DO concentration levels. Measurements of the actual water DO concentration were conducted throughout the process of the experiments, and adjustments of aquatic DO concentration were carried out manually by modifying the volume of nitrogen, which was bubbling through the deoxygenation tower, when applicable.

1.4 Thesis objectives

Previous studies have established specific hypoxia sensitivities and oxygen sensing mechanisms in fish, as well as adaptive radiation in the context of habitat utilisation. Furthermore, galaxiids have been shown to exhibit distinct differences in habitat preferences in relation to the oxygen environment. Therefore, it is the overarching goal of this thesis to establish whether the distinct habitat preferences of the closely related inanga, banded kokopu and black mudfish might stem from species-specific hypoxia sensitivities and unique response mechanisms towards hypoxia. To achieve this comprehensive comparison, a novel, integrative approach combining behavioural, physiological, molecular, and gill morphology studies on three closely related New Zealand native fish species was developed. This facilitated the testing of the following specific hypotheses:

1) The three species exhibit unique behavioural responses upon encountering a hypoxic environment;

2) Distinct differences in sensitivity and behavioural responses towards hypoxia are based on different metabolic oxygen demands and thus are reflected in distinct oxygen consumption profiles and critical DO concentrations; and

3) Responses to hypoxia are mediated by oxygen sensing neuroepithelial cells and by the transcription protein HIF 1. Furthermore, swimming behaviour, as a measure of activity and metabolic oxygen demand, and gill morphology undergo distinct adjustments during persistent hypoxia.

1.5 Thesis overview

This thesis is comprised of three research chapters (Chapter 2 – 4), which have been prepared as stand–alone documents for submission to peer–reviewed journals. These chapters therefore inherently contain repetitive aspects in introductory and methodological sections. All data from those chapters are based on my research ideas and findings, under the supervision of Associate Professor Nicholas Ling, Professor Brendan J. Hicks and Dr Pawel Olszewski.

The first chapter provides a general introduction into the subjects examined and discussed in this thesis.

In Chapter 2, the behavioural response of inanga, banded kokopu and black mudfish to progressive hypoxia were investigated in the presence of normoxic refuge and with permitted water surface access, which facilitated an assessment of distinct species–specific hypoxia sensitivities and responses (Thesis objective 1).

In Chapter 3, the effect of distinct hypoxic DO levels on oxygen consumption were investigated, which facilitated an assessment of the oxygen consumption strategy exhibited by each of the three species (Thesis objective 2).

In Chapter 4, the effect of prolonged, moderate and inescapable hypoxia without water surface access, on locomotor and gill morphological parameters were examined. Furthermore, the species–specific distribution of oxygen sensing NECs in gill epithelia was investigated and it was explored, whether hypoxia–induced responses are potentially elicited and controlled by adaptations in NEC and HIF-1 alpha protein densities (Thesis objective 3).

The final Chapter 5 provides a conclusive summary of the thesis findings and provides suggestions for future research.

2 Species–specific hypoxia avoidance threshold and behavioural responses to continuous progressive hypoxia

2.1 Abstract

Aquatic low oxygen (hypoxic-) environments are an increasingly common phenomenon encountered by freshwater fish species around the globe. Hypoxia sensitivity and avoidance responses vary greatly between different species depending on species-specific hypoxia tolerance, habitat preferences and life strategies. In a hypoxia-normoxia choice chamber, the ability of fish to sense progressive hypoxia, as well as their behavioural responses, such as hypoxia avoidance threshold were investigated in three galaxiid species; inanga (*Galaxias maculatus*), banded kokopu (*Galaxias fasciatus*) and black mudfish (*Neochanna diversus*). Inanga demonstrated avoidance of mild hypoxia (< 5.9 mg L^{-1}) and increased frequency of visits into both the hypoxic and normoxic sides of the choice chamber in more severe hypoxia (< 3.6 mg L^{-1} for hypoxic side and < 1.9 mg L^{-1} for normoxic side). Banded kokopu responded to progressive hypoxia primarily with an increased frequency of aquatic surface respiration as well as elevated swimming speed (BL s^{-1}) and displayed a lower hypoxia avoidance threshold than inanga, resulting in horizontal migration from more severe hypoxia (< 2.5 mg L^{-1}). By contrast, no avoidance of, or behavioural response to hypoxia was observed in black mudfish. These findings potentially reflect species-specific hypoxia sensitivities, accompanied by distinct behavioural mechanisms, especially in inanga and banded kokopu, reflecting the specialised habitats of these three species.

2.2 Introduction

Fish utilize oxidative metabolism as their primary means of energy production and therefore depend on environmental oxygen, which presents inherent challenges as oxygen availability in water is comparatively low (Kramer, 1987) and subjected to fluctuations in dissolved oxygen (DO) concentration (Graham, 1990).

2.2.1 Causes and effects of aquatic hypoxia

The occurrence of low-oxygen (hypoxic) or oxygen-absent (anoxic) environments is a natural phenomenon in aquatic ecosystems (Kramer, 1987) caused primarily by stratification, isolated bottom waters (Diaz, 2001), or in situations where biological oxygen consumption exceeds oxygen re-supply through photosynthesis and atmospheric diffusion (Friedrich *et al.*, 2014). Intensified anthropogenic impacts on the natural environment (Hamill & McBride, 2003; Landman *et al.*, 2005; McDowell & Wilcock, 2008), have been shown to globally increase the frequency and expansion of hypoxia events in recent decades (Diaz & Rosenberg, 2008; Rabalais *et al.*, 2010; Verburg *et al.*, 2010). Sublethal levels of aquatic hypoxia have been shown to elicit decreased food consumption and predation as well as reduced growth (Breitburg, 1994; Petersen & Pihl, 1995; Thetmeyer *et al.*, 1999; Brandt *et al.*, 2009), affect courtship (Sundin *et al.*, 2015) and impair reproduction (Wu *et al.*, 2003; Shang & Wu, 2004; Landry *et al.*, 2007; Thomas *et al.*, 2007) and disturb predator-avoidance (Robb & Abrahams, 2002; Killen *et al.*, 2012) as well as escape responses (Lefrançois *et al.*, 2005). Similarly, hypoxia modulates schooling behaviour (Moss & McFarland, 1970; Domenici *et al.*, 2000; Domenici *et al.*, 2002), elicits stress responses (Van Raaij *et al.*, 1996a; Van Raaij *et al.*, 1996b; Vianen *et al.*, 2001; Johansen *et al.*, 2006) and modifies fish

migration and distribution as well as species composition (Smale & Rabeni, 1995; Schurmann *et al.*, 1998; Ludsin *et al.*, 2009; Roberts *et al.*, 2009; Arend *et al.*, 2011; Froeschke & Stunz, 2011; Bunch *et al.*, 2015). Fish are able to detect and avoid hypoxic environments, a common response that has been documented in previous studies (Kramer, 1987; Breitburg, 1994; Claireaux *et al.*, 1995; Schurmann *et al.*, 1998; Eby & Crowder, 2002; Cook *et al.*, 2011; Herbert *et al.*, 2011; Poulsen *et al.*, 2011; Herbert *et al.*, 2012). In contrast, it has also been shown that several fish species can remain within or actively seek out hypoxic regions which has been hypothesized to enable predator avoidance (Shingles *et al.*, 2005) and non-competitive access to habitat and food sources (Kaartvedt *et al.*, 2009; Neuenfeldt *et al.*, 2009).

2.2.2 Piscine oxygen sensing and behavioural responses

The basis for distinct responses to hypoxic environments is the ability to sense low oxygen concentration which is effected by chemoreception in serotonergic neuroepithelial cells (NECs) located in the gill and epidermis. The receptors respond to decreased environmental oxygen tension with inhibition of membrane-bound potassium channels causing degranulation of cytoplasmic synaptic vesicles that are oriented towards associated nerve fibres. Consequently, the neurotransmitter serotonin is released and activates sensory neural pathways which coordinate physiological and behavioural responses to environmental hypoxia (Dunel-Erb *et al.*, 1982; Burleson & Smatresk, 1990b; Bailly *et al.*, 1992; Fritsche *et al.*, 1992; Gonzalez *et al.*, 1994; Sundin *et al.*, 1998; Lopez-Barneo *et al.*, 2001; Jonz & Nurse, 2006; Regan *et al.*, 2011; Porteus *et al.*, 2012).

Behavioural responses to aquatic hypoxia include horizontal and vertical migration when the dimension of the hypoxic area allows this strategy of

avoidance (Jones, 1952; Kramer, 1987; Pihl *et al.*, 1991; Breitburg, 1994; Schurmann *et al.*, 1998; Eby & Crowder, 2002; Skjæraasen *et al.*, 2008; Hasler *et al.*, 2009; Ludsin *et al.*, 2009; Stierhoff *et al.*, 2009). Further responses are aquatic surface respiration (ASR) involving the skimming of water at the water/air interface (Kramer & McClure, 1982; Shingles *et al.*, 2005; McNeil & Closs, 2007; Dwyer *et al.*, 2014), 'air gulping' at the water surface (McPhail, 1999) and emersion from the aquatic habitat (Regan *et al.*, 2011; Urbina *et al.*, 2011). Changes in swimming activity and speed (Moss & McFarland, 1970; Carlson & Parsons, 2001; Herbert & Steffensen, 2005; Lefrançois *et al.*, 2005; Herbert & Steffensen, 2006; Johansen *et al.*, 2006; Behrens & Steffensen, 2007; Fitzgibbon *et al.*, 2010; Herbert *et al.*, 2012; Cook *et al.*, 2014) have been observed as well as decreased activity reducing metabolic oxygen demand (Kramer, 1987; Herbert & Steffensen, 2005).

2.2.3 Hypoxia studies in choice devices

The majority of studies on responses to aquatic hypoxia thus far have been conducted in study devices facilitating inescapable hypoxic conditions precluding submerged avoidance behaviour. Recently more studies have been conducted in a choice chamber environment facilitating escapable aquatic hypoxia. In this context it has been shown that the oxygen concentration threshold eliciting hypoxia avoidance ranges from 1 mg L^{-1} for juvenile weakfish (*Cynoscion regalis*), pinfish (*Lagodon rhomboids*), croaker (*Micropogonias undulatus*) and menhaden (*Brevoortia tyrannus*) (Wannamaker & Rice, 2000; Stierhoff *et al.*, 2009; Froeschke & Stunz, 2011) to approximately 7.5 mg L^{-1} in rainbow trout (*Oncorhynchus mykiss*; (Poulsen *et al.*, 2011)). This avoidance threshold however is subject to variability as chronic hypoxia acclimation of juvenile snapper

(*Pagrus auratus*) to 4.4 – 5.2 mg L^{-1} has been shown to decrease the oxygen concentration at which hypoxia avoidance is initiated (Cook *et al.*, 2013). Moreover, for silver perch (*Bairdiella chrysoura*) the avoidance response has been shown to be size-dependent as small- and medium-sized fish avoided water of ≤ 1.9 mg L^{-1} oxygen concentration while larger fish showed no avoidance of hypoxic habitats (Hanke & Smith, 2011). Similarly, pinfish and croaker do avoid hypoxic habitats with DO concentrations of 1 mg L^{-1} (Froeschke & Stunz, 2011). However, if predators were present within the normoxia refuge, this avoidance response has been shown to be absent. Furthermore, the availability of a normoxia refuge habitat has been shown to influence the avoidance response in juvenile Atlantic cod (*Gadus morhua* L.) that continuously demonstrated excursions into progressive hypoxia if a normoxia refuge is presented. When oxygen concentration in both habitats were initially reduced, however, followed by a stepwise increase in oxygen concentration in the refuge habitat, cod reduced visit frequency into and residence time in the hypoxic habitat (Herbert *et al.*, 2011). To date, no generalised effect of escapable hypoxia on swimming speed is deductible even within the same species. Snapper and yellowtail kingfish (*Seriola lalandi*) previously have been shown not to adjust swimming speed in escapable hypoxia (Cook *et al.*, 2011; Cook & Herbert, 2012b), while a decrease in swimming speed has been elicited in Atlantic cod, (Skjæraasen *et al.*, 2008; Herbert *et al.*, 2011), but also in a further study on snapper (Cook & Herbert, 2012a). In contrast, an increase in swimming speed was initiated by Atlantic cod when exposed to lower temperatures (Skjæraasen *et al.*, 2008), and in rainbow trout from oxygen concentration of ≤ 3.8 mg L^{-1} (Poulsen *et al.*, 2011). In cape silverside (*Atherina breviceps*), swimming speed in hypoxia increased before hypoxic water with DO

levels of approximately 2.1 mg L^{-1} were avoided (Herbert *et al.*, 2012). However, in *A. breviceps*, no correlation between swimming speed and hypoxia avoidance response was derived. It is also notable that some fish species do not exhibit any hypoxia avoidance, such as mummichog (*Fundulus heteroclitus*) and yellowtail kingfish because neither appear to avoid hypoxic habitats with oxygen concentration as low as 1 mg L^{-1} and approximately 2 mg L^{-1} respectively (Wannamaker & Rice, 2000; Cook & Herbert, 2012b). The wide variability in hypoxia responses therefore necessitates more research on the effect of escapable hypoxia to further our understanding of behavioural and physiological mechanisms of hypoxia avoidance responses in an extensive range of species that inhabit widely differing ecosystems, especially in the context of the global hypoxic events.

2.2.4 Galaxiids

Inanga, banded kokopu and black mudfish are related species of the Galaxiidae family (Waters *et al.*, 2000), members of which inhabit a wide range of ecosystems in the southern hemisphere (McDowall, 2006). As such, they were selected as model species for this comparative environmental study since these three species are commonly found in freshwater ecosystems of New Zealand's North Island while they demonstrate rather specialised habitat requirements and populate environments with distinctly different oxygen concentration profiles. Banded kokopu is most commonly found in cool, covered forest streams, while inanga is abundant in shallow, slower flowing and uncovered streams frequently subjected to fluctuations in oxygen concentration. Black mudfish, however, inhabit slow flowing or still waters of wetlands, swamps or drains that regularly experience depletions in dissolved oxygen (McDowall, 1990). These distinct

differences in habitat preferences suggest discrete hypoxia sensitivities and tolerances, as well as different behavioural and physiological responses to hypoxia potentially exist within the one family where phylogenetic distances are minimised.

2.2.5 Study objectives

In order to investigate whether the markedly species-specific habitat preferences in inanga, banded kokopu and black mudfish are potentially associated with (and therefore potentially driven by differences in) their respective hypoxic behavioural sensitivity to escapable hypoxia, this study utilised a choice chamber apparatus and escapable progressive hypoxia environment similar to Poulsen *et al.* (2011) to determine the dissolved oxygen concentration eliciting avoidance responses in these species. Furthermore, this study examined how aquatic surface respiration and swimming speed are affected by escapable hypoxia. Finally, this study investigated whether distinct inter-species differences in hypoxia avoidance responses are correlated with the ecosystem types typically inhabited by the model species.

2.3 Methodology

2.3.1 Experimental fish

Adult inanga (body mass: 2.79 ± 0.21 g; total length: 69.5 ± 1.7 mm; data presented here and thereafter in mean ± SE) were purchased from a fish farm that provides natural habitat for upstream migrating whitebait at the conclusion of their marine larval development (Raglan EELS Ltd, Raglan, New Zealand). Immature, post-larval banded kokopu that are comparable in their physiology to adult fish (body mass: 5.79 ± 0.39 g; total length: 74.6 ± 1.7 mm) were caught by backpack-electrofishing from Puketirini stream in Huntly, New Zealand, and adult black mudfish (body mass: 2.21 ± 0.20 g; total length: 69.2 ± 2.0 mm) were caught with minnow traps from wetland and field drainage areas surrounding Hamilton and Huntly, Waikato, New Zealand. All fish were transported to the Aquatic Research Facility at the University of Waikato, Hamilton, New Zealand. Fish were acclimatised to laboratory conditions in the Aquatic Research Facility for at least four weeks prior to experiments, and kept in indoor tanks with dechlorinated tap water fitted with aquarium filters and supplementary aeration at a constant temperature of 16°C and a 12:12 light:dark photoperiodic cycle. To assist osmoregulation and reduce incidence of white spot disease, marine salt (Crystal Sea Marinemix, Marine Enterprises International) was added to inanga and banded kokopu tanks to a concentration of 3.5‰ and frequent water changes limited build-up of waste products. Black mudfish were housed in water of their respective habitat that was brought back to the facility with them, and therefore did not necessitate added salt. Fish were fed to satiation with frozen bloodworms every two days. All procedures and experiments followed the standard operating procedures for captive fish maintenance given by the University of Waikato, and

were approved by the University of Waikato Animal Ethics Committee (protocol # 844).

2.3.2 Experimental hypoxia environment and study device

A study device was constructed, which enabled fish to move without restrictions within two distinct water flows, thus facilitating studies on behavioural responses of fish presented with a choice between continuous progressive hypoxia and normoxia (Figure 2.1).

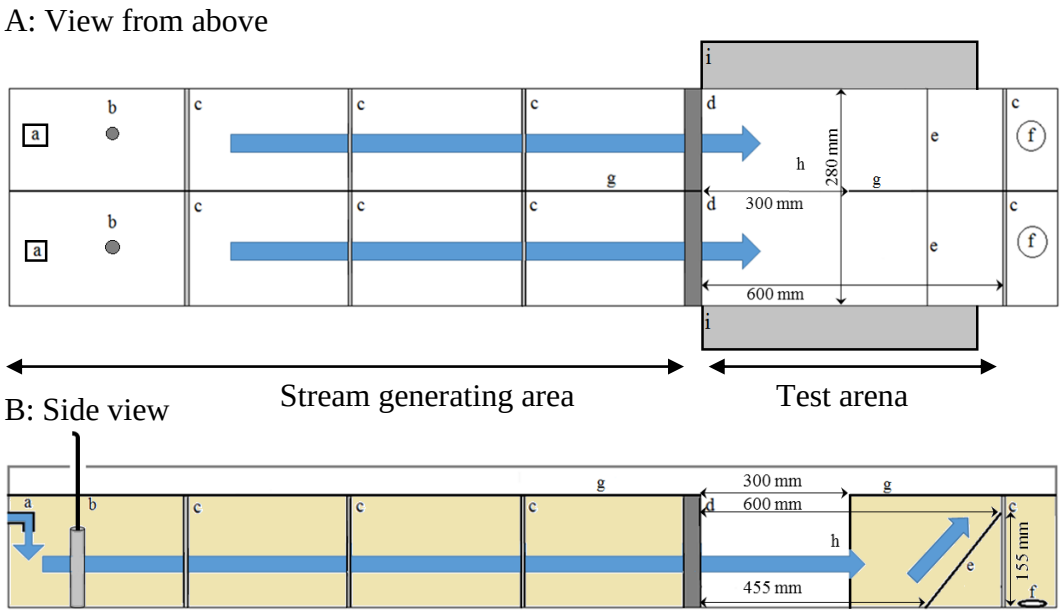


Figure 2.1: Choice chamber study device from above (A) and from the side (B) featuring water inlets each fitted with a flow meter (a), oxygen sensors (b), a 2 mm acrylic sheet dividing water flows (g), light diffuser panel sheets (c) and honeycomb-structured sheets (d) for laminar, non-mixing water flow in the test arena (h), ramps to maintain an appropriate water depth (e) and water outlets (f). Mirrors (i) were installed adjacent to either side of the test arena at an approximate angle of 55° for evaluation of vertical fish movements.

To generate two distinct water flows the study device was divided into two sides by a transparent acrylic sheet of 2 mm thickness, with the exception of the test arena where the two sides were kept unseparated over a length of 300 mm. Furthermore, the study device was fitted with light diffuser panel sheets and

honeycomb-structured sheets with 3x3 mm perforations to generate two distinct laminar and non-mixing water flows in the unseparated test arena, as was confirmed with dye tests prior to the experiments (Figure 2.2). The entire test arena measured 280 x 600 mm and ramps at the rear section maintained a water depth of 155 mm.

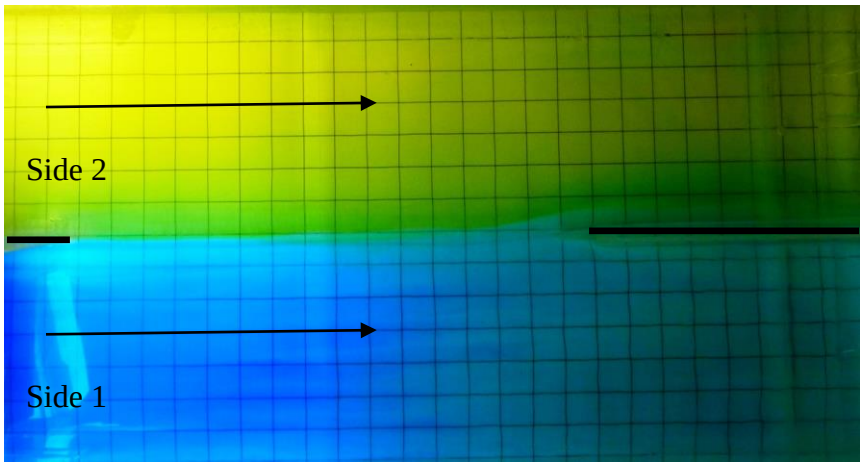


Figure 2.2: Dye tests prior to experiments confirmed the generation of two distinct laminar, non-mixing water flows in the test arena. Position of the wall separating the two water streams has been marked by black bars. Water flow direction is indicated by black arrows.

For normoxia, air-saturated dechlorinated tap water from a normoxic water reservoir tank fitted with a submerged recirculating water pump, and an external air pump with a submerged air stone, was pumped into the study device via the appropriate inlet. After flowing through the study device, normoxic water was recycled back into the normoxic reservoir. For continuous progressive hypoxia, dechlorinated tap water from a hypoxic water reservoir tank fitted with a submerged recirculating water pump was pumped into a three-column deoxygenation tower (Figure 1.8). The bottom of each column was fitted with a flat circular gas diffuser which was approximately in size the diameter of the column. As water flowed through the interconnected columns of the

deoxygenation tower it was continuously deoxygenated by bubbling gaseous oxygen-free nitrogen through the gas diffusers in the column. From the deoxygenation tower, hypoxic water flowed to the study device via the inlet on the appropriate side and was recycled back into the hypoxic reservoir (Figure 1.7). For pre-experimental acclimation and observation of the fish behaviour in normoxic water, an external air pump was connected to the gas diffusers to generate air-saturated water from both reservoir tanks. Flow rate sensors were installed in the inlet pipes of the study device and connected to digital flow meter units (Savant Electronics Inc.) for continuous flow rate measurements (L min^{-1}).

A digital video camera was mounted above the test arena to record the behavioural responses of the fish throughout the experiment, and a 2x2 cm grid was placed underneath the test arena for movement reference. On both sides of the test arena, mirrors were installed at an approximate angle of 55° for evaluation of aquatic surface respiration (ASR). The test arena was enclosed in black fabric to minimize fish disturbance during the experiments that may potentially affect the behavioural responses. All experiments were carried out as individual tests to avoid group behaviour affecting individual behavioural responses. Experiments were carried out in a temperature-controlled room where the experimental temperature was maintained between 17 and 18°C. At least 10 fish per species were tested (13 inanga, 10 banded kokopu and 12 black mudfish).

2.3.3 Experimental procedure

Fish were kept unfed for 48 h and were transferred to the test arena of the study device, where they were allowed to acclimatise to normoxic recirculating water for 17 h to account for the increase in metabolic rate due to handling, transition to

a new environment and isolation (Poulsen *et al.*, 2011; Urbina *et al.*, 2011). To prevent side preferences in the test arena due to potential chemical differences between the reservoir tanks and the deoxygenation tower, or due to different flow rates, all water in the device was mixed uniformly during the acclimatisation period.

Following acclimatisation, behaviour in normoxic water in both sides of the test arena was recorded and observed for 60 min as a behavioural reference and to determine the preferred side for each fish. The preferred side was subsequently allocated to continuous progressive hypoxia whereas the non-preferred side was allocated to normoxia (Table 2.1).

Table 2.1: Side preferences in the test arena during the behavioural reference period in inanga, banded kokopu and black mudfish.

Species	Preferences	
	Side 1	Side 2
Inanga	4	9
Banded kokopu	4	6
Black mudfish	5	7

Dissolved oxygen concentration and temperature of both sides were monitored continuously throughout the experiment using Clark-type electrochemical dissolved oxygen sensors connected to oxygen meters (YSI dissolved oxygen meter 57 and 50). Before each individual experiment the oxygen sensors were calibrated in 100% water saturated air. The oxygen meter monitoring the continuous progressive hypoxia side was connected to a chart recorder. During the experimental period the normoxic flow rate was matched to the hypoxic flow

rate to avoid any effects on the measured dissolved oxygen concentration or on fish behaviour due to different water flow rates. It was determined in pre-experimental tests that at an average water flow rate of 14 L min^{-1}, a time period of 3.5 min was required for the dissolved oxygen concentration in the rear section of the test arena to correspond to the dissolved oxygen concentration measured at the oxygen sensor probes. Flow rates were converted into water flow velocities to preclude the possibility of water boundary layers within the test arena during the experiments (Inanga: 10.9 ± 0.1 mm s^{-1}; banded kokopu: 10.9 ± 0.04 mm s^{-1}; black mudfish: 11.01 ± 0.05 mm s^{-1}).

Following the initial 60 min period for control behavioural observation, the dissolved oxygen concentration of the preferred side in the test arena was decreased over a period of 60 min whereas the dissolved oxygen concentration of the normoxic side remained above 90% saturation at all times during the experiment (Table 2.2).

Table 2.2: Decrease in dissolved oxygen concentration (%) in hypoxia and normoxia sides during 60 min progressive hypoxia choice chamber experiment. Values are mean ± SE for each species. $n_{inanga} = 13$, $n_{banded\ kokopu} = 10$, $n_{black\ mudfish} = 12$.

Species	Dissolved oxygen saturation (%)			
	Hypoxia side		Normoxia side	
	Start of experiment	End of experiment	Start of experiment	End of experiment
Inanga	95.0 ± 1.4	16.0 ± 0.7	95.0 ± 1.4	92.2 ± 1.5
Banded kokopu	95.0 ± 0.4	9.5 ± 1.2	95.0 ± 0.4	94.3 ± 0.5
Black mudfish	95.2 ± 1.0	11.5 ± 1.7	95.2 ± 1.0	94.1 ± 1.1

After 60 min of progressive hypoxia the experiment was concluded, and normoxic water was pumped through both sides of the study device. Care was taken during the experiments to reduce any movement and noise in close proximity to minimize any fish disturbances. After the conclusion of the experiments, fish were anaesthetised in water containing 20 mg L^{-1} benzocaine before body mass and total length measurement and then returned to their respective holding tanks for recovery. Each fish underwent the experimental procedure only once. During the experiment and the two-week post-experimental period no mortalities were recorded, and no fish lost equilibrium during the course of the experiments.

2.3.4 Data analyses

The observation period for behavioural reference and the experimental period both were subdivided into 20 three-minute periods. For each of these periods the average dissolved oxygen concentration (DO) was determined. Video recordings were transferred to a computer and manually analysed for each fish to determine residence time per side, number of visits per side as well as frequency and duration of aquatic surface respiration (ASR) for each time period and average DO. In order to analyse the video recordings for swimming speed for each period and average DO, an object tracking program was developed using MATLAB R2014b, which determined the x,y-position of the fish within the parameters of the test arena for every 0.5 seconds of the video recording and the position data sets were saved as an Excel file until further analysis (For a more detailed documentation of the tracking program, please see Appendix 2.1).

Statistical analyses were carried out using Microsoft Excel 2013 Data Analysis ToolPak. Average DO per period from all fish was determined for each

species. For each period and average DO, the following parameters were calculated: Proportion of time spent in progressive hypoxia or normoxia, average number of visits to the hypoxia and normoxia sides as well as average frequency and proportional duration of ASR. Furthermore, the position data from every fish were utilized to determine the average swimming speed in body lengths per second (BL s^{-1}) for each period and average DO concentration. In addition, generalised linear mixed effects (GLME) models were utilised in an R environment to test for significant effects of decreasing DO concentration on the aforementioned parameters. In this context, individual fish were allowed to have random intercepts, while average dissolved oxygen concentration was allowed random effects. Residual variation for parameters expressed in percentage (residence time and ASR duration) were considered as coming from a Normal distribution, while residual variation from count data parameters (side visits, ASR frequency, swimming speed) were treated as a Poisson distribution. Furthermore, two-stage change-point models were developed to highlight at what stage during the experimental decrease in ambient DO concentration a significant change in the parameters occurred.

2.4 Results

Continuous progressive DO decrease in the preferred side of the test arena elicited markedly different responses in inanga, banded kokopu and black mudfish.

2.4.1 Residence time

Progressive hypoxia in the preferred side of the test arena caused a decrease in proportional residence time per period in inanga and banded kokopu but not in black mudfish.

Inanga demonstrated a 50% decrease in residence time beginning at a DO concentration of 5.85 mg L^{-1}, 15 min after initiating progressive hypoxia in the preferred side of the test arena. Banded kokopu reduced their residence time on the preferred side by 19% beginning at a DO concentration of 2.34 mg L^{-1}, 42 min after initiating progressive hypoxia, albeit from a lower starting point which indicated a weaker preference for one side of the test arena. No significant change in mean proportional residence time occurred in black mudfish (Table 2.3).

In inanga, there was a significant effect of declining DO concentration on residence time, with each 3-minute decrease in DO concentration resulting in 6.4% less time spent in the hypoxic area, on average (GLME model: $\chi^2 = 9.53$, df = 1, $P < 0.01$). The mean proportion of residence time in the preferred side of the test arena amounted to 0.86 ± 0.02/period (mean ± SE) during 60 min normoxic control conditions at 9.34 ± 0.01 mg L^{-1} DO concentration. Residence time remained nearly unchanged with a mean of 0.89 ± 0.01/period during the first 15 min of hypoxia. At a DO of 5.85 ± 0.28 mg L^{-1} a significant decrease in residence time, as indicated by the two-stage change-point model, occurred over the course

of 15 min while the DO declined to 3.32 ± 0.21 mg L^{-1}. In the subsequent decrease of DO to 1.64 ± 0.08 mg L^{-1}, residence time did not decrease further but varied around a mean of 0.39 ± 0.02/period (Figure 2.3 A, Appendix 2.2).

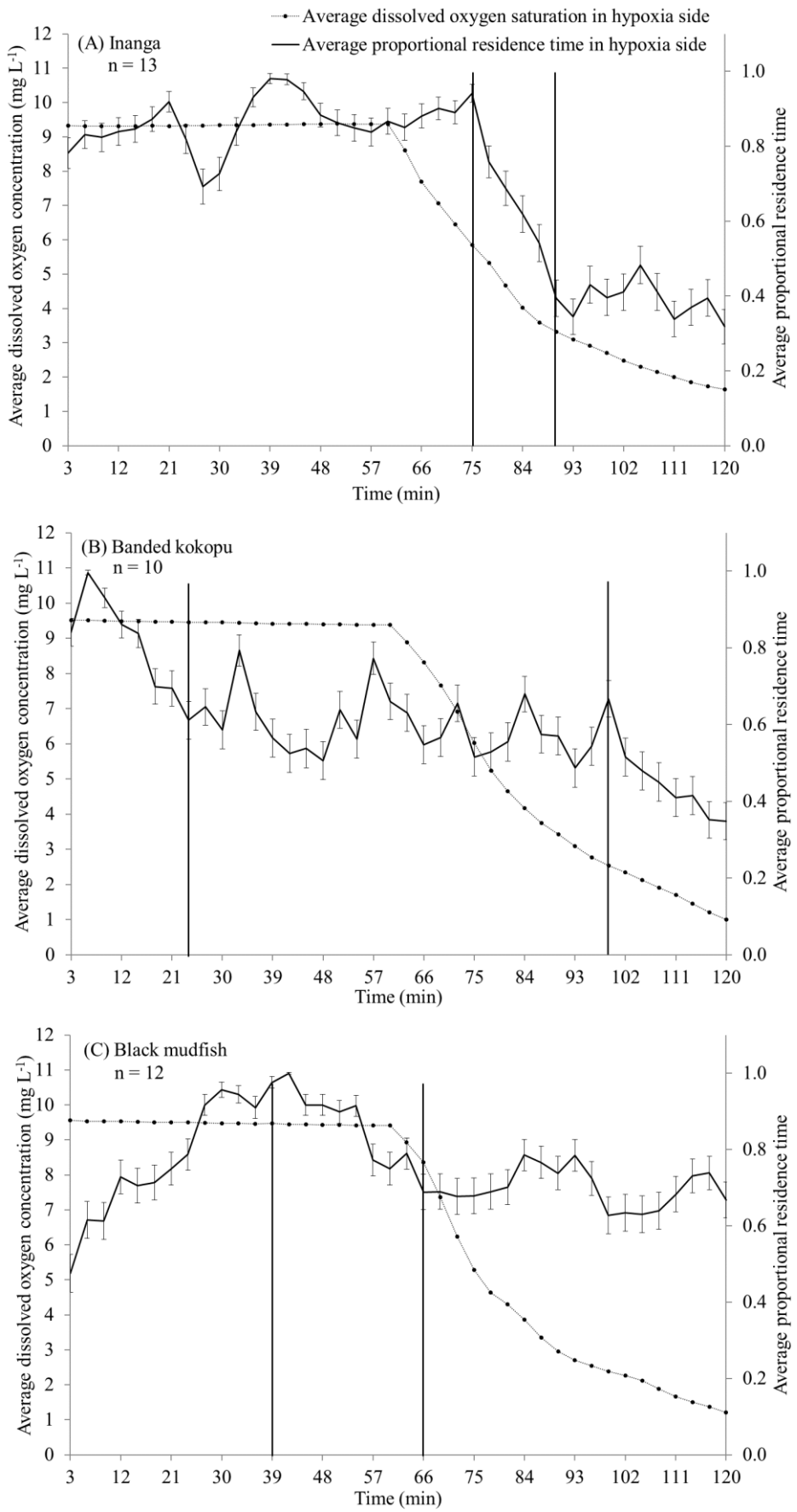
In banded kokopu, there was a significant effect of declining DO concentration on residence time, with each 3-minute decrease in DO concentration resulting in 2.9% less time spent in the hypoxic area, on average (GLME model: χ^2 = 8.08, df = 1, P < 0.01). The mean proportion of time spent in the preferred side of the test arena was 0.69 ± 0.03/period during 60 min normoxic control conditions at 9.44 ± 0.01 mg L^{-1} DO. Proportional residence time declined slightly to 0.57 ± 0.02/period during the first 39 min of hypoxia in the preferred side of the test arena. At a DO of 2.53 ± 0.15 mg L^{-1} the proportion of time spent in the preferred side decreased markedly to 0.35 ± 0.05/period within 21 min as indicated by the two-stage change-point model, while the DO declined to 1.01 ± 0.14 mg L^{-1} (Figure 2.3 B, Appendix 2.2).

In black mudfish, progressive hypoxia elicited no significant effect of declining DO concentration on residence time (GLME model: χ^2 = 1.29, df = 1, P > 0.05). During 60 min of normoxic control conditions at 9.48 ± 0.01 mg L^{-1} DO the mean proportional residence time constituted 0.81 ± 0.03/period. In progressive hypoxia with a DO as low as 1.21 ± 0.17 mg L^{-1} the residence time in hypoxia remained relatively steady, with a mean of 0.70 ± 0.01/period. During the normoxic control conditions, residence time underwent variations, displaying a distinct decrease in the proportion of time spent in the principally preferred side at 39 min of normoxic control observations. This decrease ceased at 66 min of the overall experiment (Figure 2.3 C, Appendix 2.2).

Table 2.3: Residence time parameters in the preferred side of the test arena (mean ± SE) in inanga, banded kokopu and black mudfish. As black mudfish did not demonstrate any change in preference, no values were available for this species (N/A). Normoxic control period = 60 min baseline observation.

Response parameters	Species		
	Inanga	Banded kokopu	Black mudfish
Residence time in preferred side during normoxic control period	0.86 ± 0.02	0.69 ± 0.03	0.81 ± 0.03
Residence time in preferred side during experimental period before decrease	0.89 ± 0.01	0.57 ± 0.02	N/A
Residence time in preferred side during experimental period after decrease	0.39 ± 0.02	0.35 ± 0.05	N/A
DO concentration during normoxic control period (mg L^{-1})	9.34 ± 0.01	9.44 ± 0.01	9.48 ± 0.01
DO concentration at which decrease in residence time occurred (mg L^{-1})	5.85 ± 0.28	2.53 ± 0.15	N/A
DO concentration at which decrease in residence time ceased (mg L^{-1})	3.32 ± 0.21	1.01 ± 0.14	N/A
Duration of experimental period until decrease in residence time started (min)	15	39	N/A

Figure 2.3: Average proportional residence time per three-minute period in inanga (A), banded kokopu (B) and black mudfish (C) during 60 min of normoxic control baseline and subsequent 60 min of acute progressive hypoxia (63 – 120 min) in a normoxia-hypoxia choice chamber. All data are presented as mean ± SE. Vertical black lines indicate significant changes in the residence time parameter (two-stage change-point model).


Average dissolved oxygen saturation in hypoxia side
Average proportional residence time in hypoxia side
(A) Inanga
n = 13
(B) Banded kokopu
n = 10
(C) Black mudfish
n = 12
Average dissolved oxygen concentration (mg L-1)
Average proportional residence time
Time (min)

2.4.2 Side visits

Progressive hypoxia caused an increase in the number of times fish of all three species, but especially inanga, switched between the two sides of the test arena. However, this effect was accompanied by pronounced variabilities and therefore no significant effect of decreasing DO concentration on the number of side visits was ascertained with the GLME model ($P > 0.05$). Significant changes in the number of side visits were, however, indicated by the two-stage change-point model.

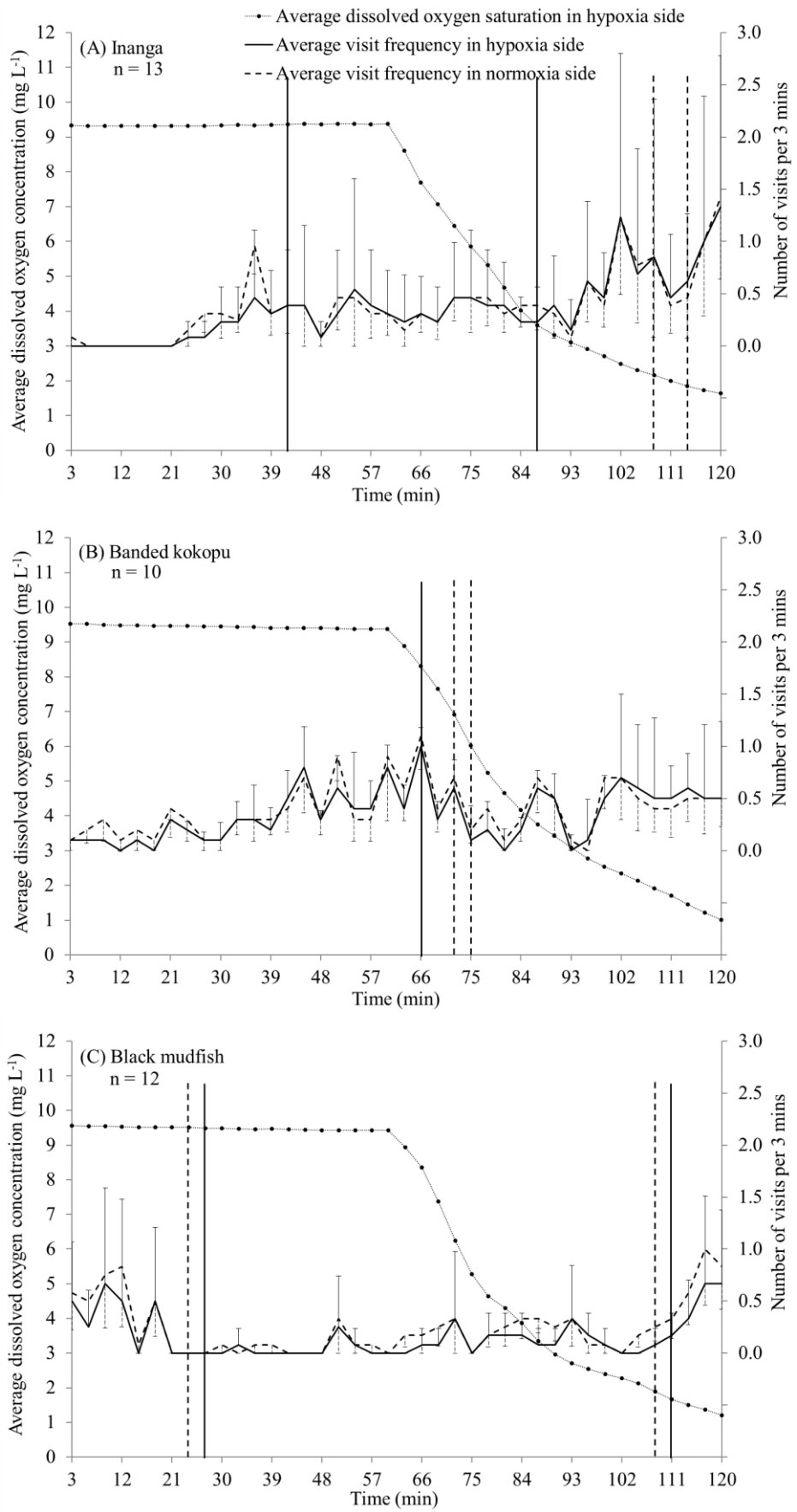
In inanga, the two-stage change-point model indicated an increase of the mean number of visits into the normoxic side at a DO of 1.85 mg L^{-1} from 0.5 to 1.4 visits/period, while it varied between 0.0 and 1.0 visits/period in the normoxic control baseline. Similarly, the mean number of visits into the hypoxic side increased at 3.6 mg L^{-1} and varied between 0.2 and 1.3/period as opposed to a variation between 0.0 and 0.5 visits/period observed in the normoxic control baseline (Figure 2.4 A, Appendix 2.3).

In banded kokopu the number of visits into the normoxic and hypoxic side were not distinctly different from observations of the normoxic control baseline. It is however notable that at a DO of 2.34 mg L^{-1} the number of visits into the normoxic side remained relatively steady between 0.4 and 0.7 visits/period, while the two-stage change-point model indicated a decrease of the number of side visits into normoxia after onset of decreasing ambient DO concentration at 6.91 mg L^{-1}, followed by an increase of the mean number of visits into the normoxic side to values approximating those observed during the normoxic control baseline at a DO of 6.03 mg L^{-1}. Similarly, the number of visits into the hypoxic side remained between 0.5 and 0.7 visits/period, with the two-stage change-point model

indicating an initial decrease of the number of side visits into normoxia after onset of decreasing ambient DO concentration at 8.31 mg L^{-1}, followed by an increase of the mean number of visits into the normoxic side to values approximating those observed during the normoxic control baseline at a DO of 6.03 mg L^{-1} (Figure 2.4 B, Appendix 2.3).

In black mudfish, the two-stage change-point model indicated an increase of the mean number of visits into the normoxic side from 0.3 to 1.0 visits/period at 1.89 mg L^{-1}. Likewise, the mean number of visits into the hypoxic side was indicated to increase from 0.2 to 0.7 visits/period at 1.66 mg L^{-1}. However, similar observations were made in the normoxic control baseline (Figure 2.4 C, Appendix 2.3).

Figure 2.4: Average frequency of visits per three-minute period in inanga (A), banded kokopu (B) and black mudfish (C) during 60 min of normoxic control baseline and subsequent 60 min of acute progressive hypoxia (63 – 120 min) in a hypoxia-normoxia choice chamber. Presented are average visit frequencies into preferred (hypoxic) and non-preferred (normoxic) side of the choice chamber. All data are presented as mean ± SE. Vertical black lines indicate significant changes in number of visits into the hypoxic side, and dashed black vertical lines indicate significant changes in number of visits into the normoxic side (two-stage change-point model), with the exception of banded kokopu at 75 min, where the dashed vertical line indicates significant changes in number of visits into both, normoxic side and hypoxic side.


Average dissolved oxygen saturation in hypoxia side
Average visit frequency in hypoxia side
Average visit frequency in normoxia side
(A) Inanga
n = 13
(B) Banded kokopu
n = 10
(C) Black mudfish
n = 12
Average dissolved oxygen concentration (mg L-1)
Number of visits per 3 mins
Time (min)
0 1 2 3 4 5 6 7 8 9 10 11 12
0.0 0.5 1.0 1.5 2.0 2.5 3.0
3 12 21 30 39 48 57 66 75 84 93 102 111 120

2.4.3 Aquatic surface respiration

Direct measurements of ASR were beyond the scope of this study, however visits to and breaking of the water surface were established as a measure for ASR behaviour, and are the basis for data, presented in this section. While this represents a confident indication, the possibility that fish may not have been performing ASR by skimming water of the water/air interface has to be acknowledged.

The ASR frequency per period was variable in both normoxic and hypoxic sides but also in the normoxic control baseline in all three species. A distinct increase of ASR frequency per period in the preferred, hypoxic side as well as the non-preferred, normoxic side was indicated by the two-stage change-point model in banded kokopu, but not in inanga and black mudfish (Figure 2.5). The significant increase of ASR frequency in banded kokopu, however, was not reflected by the GLME model, which showed no significant effect of DO concentration on the ASR frequency in inanga and banded kokopu (GLME model: $P > 0.05$). Moreover, the ASR frequency in black mudfish was significantly lower during the experimental period than in the normoxic control baseline (GLME model: $P < 0.05$ for hypoxic side and $P < 0.01$ for normoxic side).

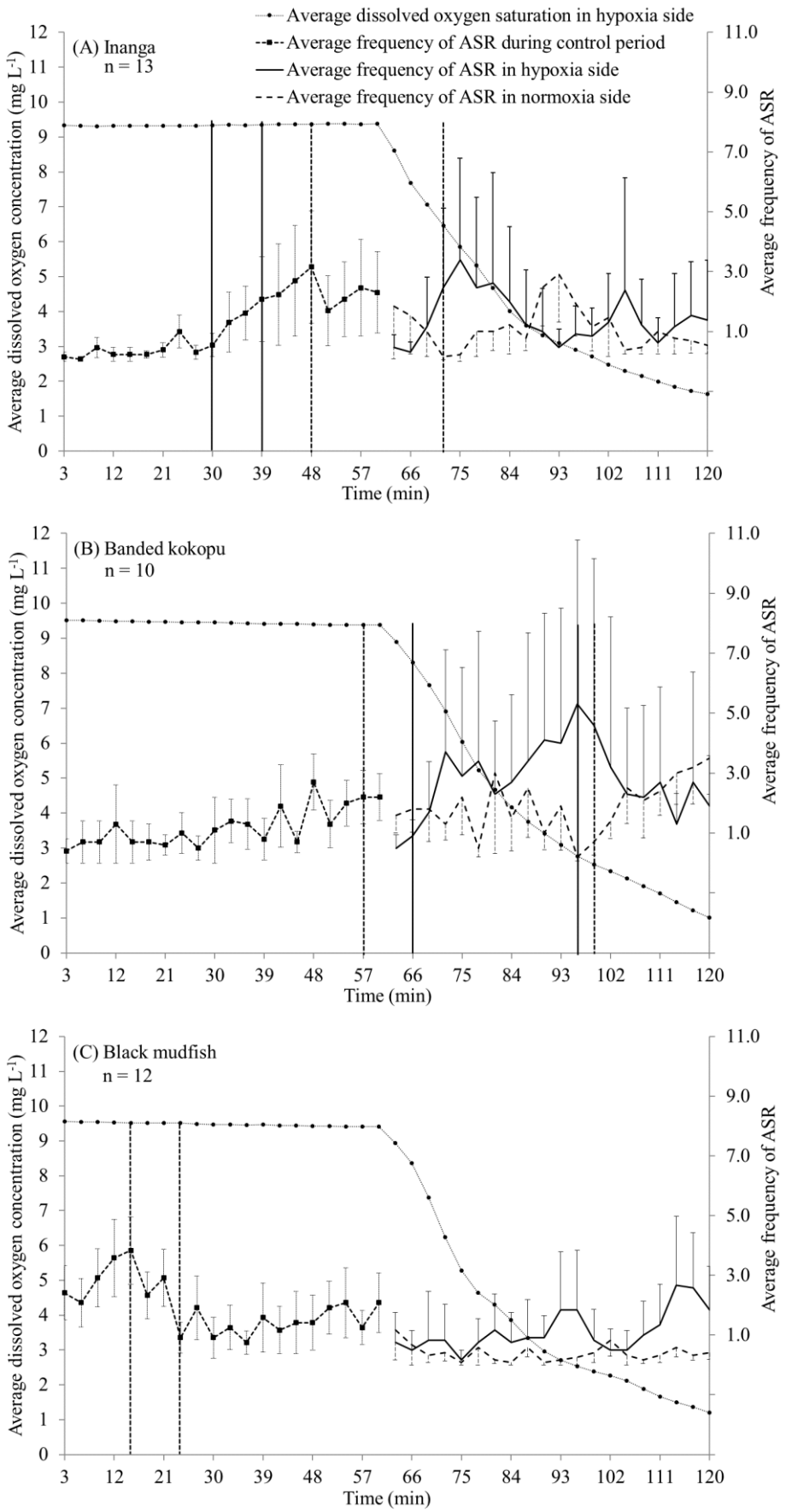
In inanga, the ASR frequency in the hypoxic side increased rather markedly from 0.3 to 3.4/period while DO changed from 7.69 to 5.85 mg L^{-1}. Thereafter the ASR frequency declined to 0.5/period, concurrent with the decreasing residence time in the hypoxia side, as DO dropped from 5.85 to 3.10 mg L^{-1}. A second increase in ASR frequency, displayed by those fish, that did not change their side preference, from 0.5 – 2.4/period was noted as DO changed

from 3.10 to 2.30 mg L^{-1}. Similarly, the ASR frequency in the normoxic side increased from 0.8 to 2.9/period, while DO decreased from 3.60 to 3.10 mg L^{-1}. However, due to pronounced variability and similar observations made from the normoxic control baseline these findings cannot be evaluated as significant (Figure 2.5 A, Appendix 2.4).

In banded kokopu the two-stage change-point model indicated an increase of ASR frequency in the hypoxic side from 0.9 to 5.3/period while DO changed from 8.31 to 2.77 mg L^{-1}. While subsequently the ASR frequency in the hypoxic side decreased to 1.3/period as DO declined to 1.01 mg L^{-1}, the ASR frequency in the normoxic side was shown to increase simultaneously from 0.7 to 3.5/period (Figure 2.5 B, Appendix 2.4).

In black mudfish a pronounced increase of ASR frequency from 0.3 to 2.7/period was observed as DO decreased from 2.21 to 1.50 mg L^{-1}, however this variation was smaller than observations from the normoxic control baseline where ASR frequency ranged from 0.8 to 3.8/period (Figure 2.5 C, Appendix 2.4).

Figure 2.5: Average frequency of aquatic surface respiration (ASR) per three-minute period in inanga (A), banded kokopu (B) and black mudfish (C) during 60 min of normoxic control baseline and subsequent 60 min of acute progressive hypoxia (63 – 120 min) in preferred (hypoxic) and non-preferred (normoxic) sides of a choice chamber. All data are presented as mean ± SE. Vertical black lines indicate significant changes in ASR frequency in hypoxic side, and dashed black vertical lines indicate significant changes in ASR frequency in normoxic side (two-stage change-point model), with the exception of black mudfish, where both dashed vertical lines indicate significant changes in ASR frequency in both, normoxic side and hypoxic side.


Average dissolved oxygen saturation in hypoxia side
Average frequency of ASR during control period
Average frequency of ASR in hypoxia side
Average frequency of ASR in normoxia side
(A) Inanga
n = 13
(B) Banded kokopu
n = 10
(C) Black mudfish
n = 12
Average dissolved oxygen concentration (mg L-1)
Average frequency of ASR
Time (min)

The average proportion of time spent performing ASR per period was variable in both, normoxic and hypoxic sides, but also in the normoxic control baseline in all three species. No distinct change in proportional duration of ASR was observed in inanga, ranging from 0.00 to 0.07 in both, the normoxic control baseline as well as in the hypoxic side, while it varied from 0.00 to 0.04 in the normoxic side (GLME model: $P > 0.05$; Figure 2.6 A, Appendix 2.5). Comparable observations were made in banded kokopu, where the proportional duration of ASR ranged from 0.00 to 0.13 in both, the normoxic control baseline as well as in the normoxic side, while it varied between 0.03 and 0.10 in the hypoxic side (GLME model: $P > 0.05$; Figure 2.6 B, Appendix 2.5). In contrast, black mudfish proportional duration of ASR was indicated by the two-stage change-point model to be markedly decreased during progressive hypoxia in comparison to the normoxic control baseline (GLME model: $P < 0.01$), as it varied between 0.03 and 0.22 in the normoxic control baseline, but ranged from 0.01 to 0.10 in the hypoxic side and from 0.00 and 0.06 in the normoxic side (Figure 2.6 C, Appendix 2.5).

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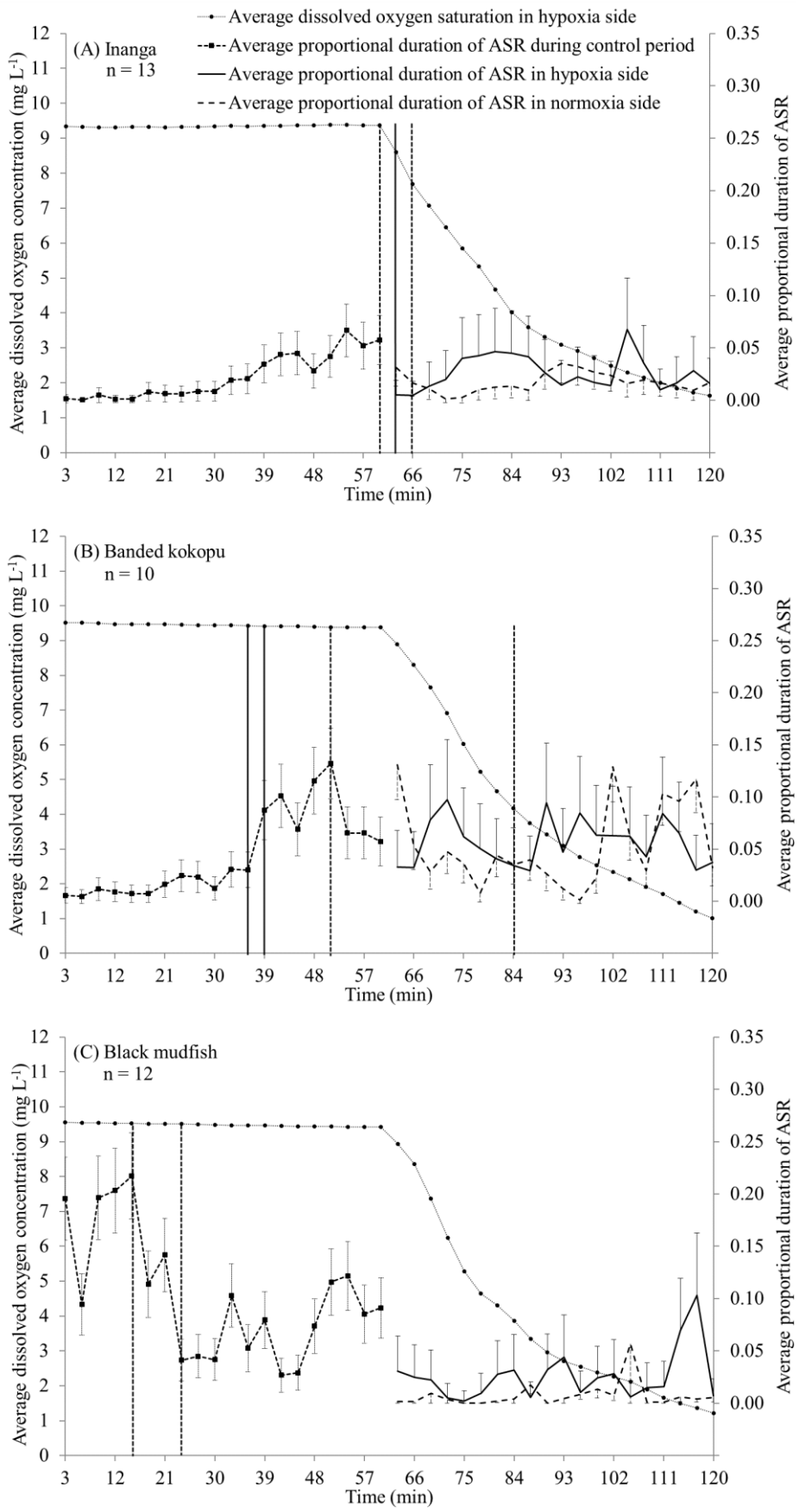
Figure 2.6: Average proportion of time spent performing aquatic surface respiration per three-minute period in inanga (A), banded kokopu (B) and black mudfish (C) during 60 min of normoxic control baseline and subsequent 60 min of acute progressive hypoxia in preferred (hypoxic) and non-preferred (normoxic) sides of a choice chamber. All data are presented as mean ± SE. Vertical black lines indicate significant changes in ASR duration in hypoxic side, and dashed black vertical lines indicate significant changes in ASR duration in normoxic side (two-stage change-point model), with the exception of inanga at 60 min and black mudfish at 15 and 24 min, where the dashed vertical lines indicate significant changes in ASR duration in both, normoxic side and hypoxic side.


Average dissolved oxygen saturation in hypoxia side
Average proportional duration of ASR during control period
Average proportional duration of ASR in hypoxia side
Average proportional duration of ASR in normoxia side
(A) Inanga
n = 13
(B) Banded kokopu
n = 10
(C) Black mudfish
n = 12
Average dissolved oxygen concentration (mg L-1)
Average proportional duration of ASR
Time (min)

2.4.4 Swimming speed

The average swimming speed (BL s^{-1}) per period was noticeably variable in both, normoxic and hypoxic sides, but also in the normoxic control baseline in all three species. No distinct changes in swimming speed were observed in inanga (GLME model: $P > 0.05$). In banded kokopu a notable increase in average swimming speed was observed in the hypoxic side of the test arena (GLME model: $P < 0.05$), which was also indicated for the normoxic side, although a significant difference could not be ascertained. In black mudfish, however, swimming speed during the trial was significantly lower than in the control period (GLME model: $P < 0.01$).

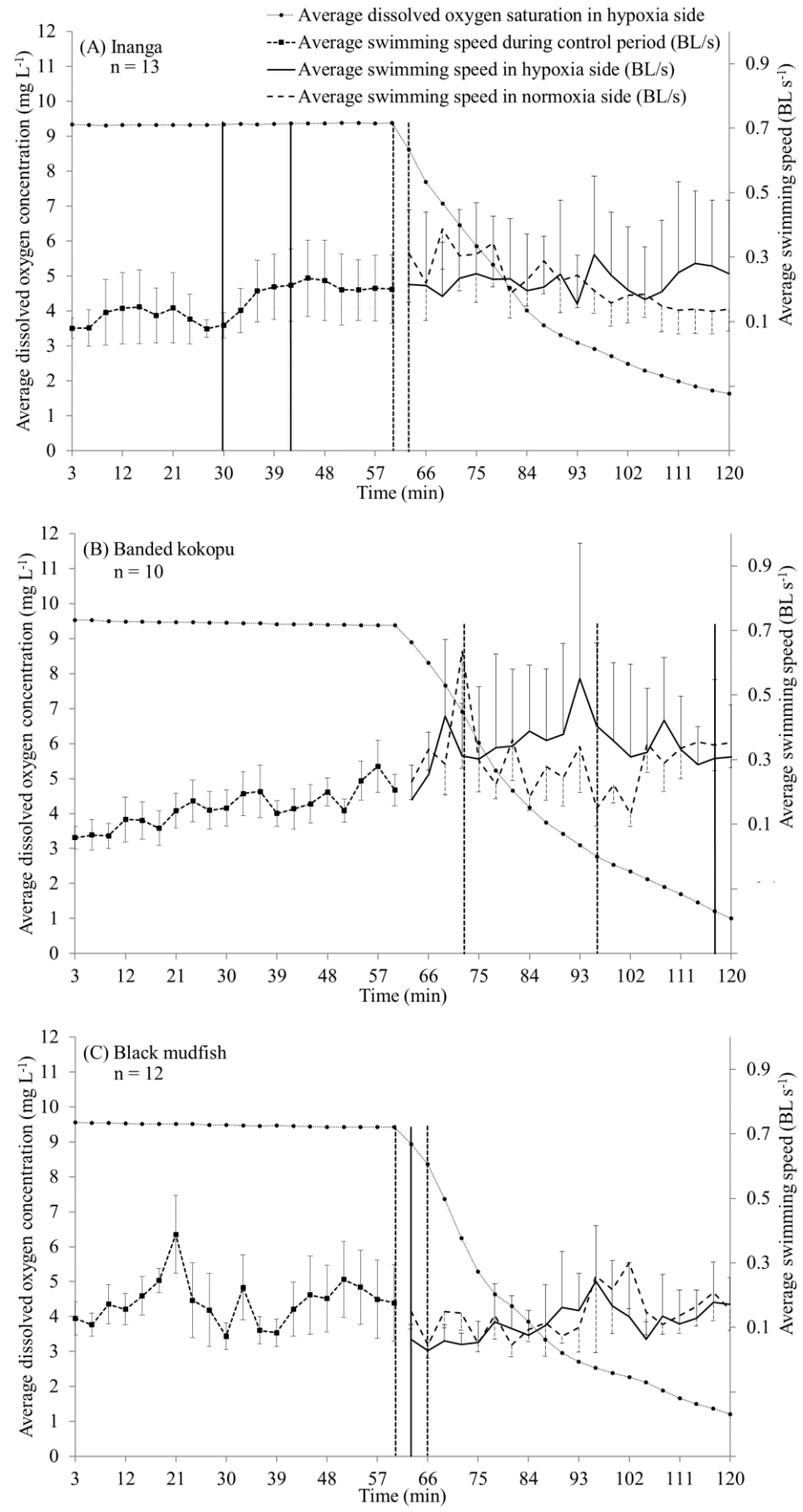
In inanga the average swimming speed in the normoxic control baseline ranged from 0.08 to 0.24 BL s^{-1}. In the hypoxic side it varied between 0.16 and 0.31 BL s^{-1}, while swimming speed ranged from 0.13 to 0.39 BL s^{-1} in the normoxic side. Therefore, no distinct effect of the declining ambient DO concentration on swimming speed was ascertained in the two-stage change-point model (Figure 2.7 A, Appendix 2.6).

In banded kokopu the average swimming speed in the normoxic control baseline ranged from 0.06 to 0.28 BL s^{-1}. Swimming speed in the hypoxic side increased from 0.18 to 0.44 BL s^{-1} as DO decreased from 8.89 to 7.66 mg L^{-1}, after which it remained between 0.30 and 0.55 BL s^{-1} while DO decreased further to 2.34 mg L^{-1}. In the normoxic side swimming speed increased from 0.23 to 0.64 BL s^{-1} as DO changed from 8.89 to 6.91 mg L^{-1}. Subsequently, swimming speed in the normoxic side decreased and varied between 0.15 and 0.36 BL s^{-1} until DO decreased to 2.77 mg L^{-1}. At this point, swimming speed in the normoxic side was

indicated by the two-stage change-point model to increase to 0.35 BL s^{-1} while DO was reduced to 1.01 mg L^{-1} (Figure 2.7 B, Appendix 2.6).

In black mudfish the average swimming speed in the normoxic control baseline ranged from 0.07 to 0.39 BL s^{-1}. In the hypoxic side it varied between 0.03 and 0.26 BL s^{-1}, while swimming speed ranged from 0.05 to 0.30 BL s^{-1} in the normoxic side and no distinct effect of the declining ambient DO concentration on swimming speed was ascertained in the two-stage change-point model (Figure 2.7 C, Appendix 2.6).

Figure 2.7: Average swimming speed in body lengths per second (BL s^{-1}) per three-minute period in inanga (A), banded kokopu (B) and black mudfish (C) during 60 min of normoxic control baseline and subsequent 60 min of acute progressive hypoxia (63 – 120 min) in preferred (hypoxic) and non-preferred (normoxic) sides of a choice chamber. All data are presented as mean ± SE. Vertical black lines indicate significant changes in swimming speed in hypoxic side, and dashed black vertical lines indicate significant changes in swimming speed in normoxic side (two-stage change-point model), with the exception of banded kokopu at 96 min and black mudfish at 60 min, where the dashed vertical lines indicate significant changes in swimming speed in both, normoxic side and hypoxic side.


Average dissolved oxygen saturation in hypoxia side
Average swimming speed during control period (BL/s)
Average swimming speed in hypoxia side (BL/s)
Average swimming speed in normoxia side (BL/s)
(A) Inanga
n = 13
(B) Banded kokopu
n = 10
(C) Black mudfish
n = 12
Average dissolved oxygen concentration (mg L-1)
Average swimming speed (BL s-1)
Time (min)
0 1 2 3 4 5 6 7 8 9 10 11 12
0.1 0.3 0.5 0.7 0.9
3 12 21 30 39 48 57 66 75 84 93 102 111 120

2.5 Discussion

2.5.1 Avoidance response towards decreasing DO

In response to a free choice between normoxia and acute progressive hypoxia, inanga and banded kokopu presented distinct hypoxia avoidance behaviour while black mudfish displayed no low oxygen avoidance, which is in accordance with black mudfish reportedly inhabiting hypoxic or anoxic environments (McDowall, 1990). No species demonstrated 100% avoidance of any low oxygen level (Figure 2.3), as residence time did not decline to 0% at any DO level, and no mortalities were recorded during the experiments as well as seven days post–experimental period, indicating that all three species are able to sustain acute progressive hypoxia for a limited time at the least.

While no change in residence time was elicited in black mudfish, inanga commenced avoiding decreasing oxygen concentrations via horizontal migration and thus decreased residence time at markedly higher dissolved oxygen concentrations (< 5.9 mg L^{-1}) than banded kokopu (< 2.5 mg L^{-1}) albeit displaying stronger initial side preference (Figure 2.3), suggesting greater hypoxia sensitivity and a higher hypoxia avoidance threshold compared to banded kokopu. Similar findings were presented by Herbert *et al.* (2012), who demonstrated a markedly higher hypoxia avoidance threshold in Cape silverside in comparison to New Zealand snapper, which was potentially caused by differences in body size and aggregation patterns. Cape silverside, like inanga, are small and active schooling fish characterised by an increased metabolic rate (Meredith, 1985) contrary to the larger and effectively solitary banded kokopu (McDowall, 1990). Inanga, however, have been described to encounter fluctuating oxygen concentration levels (Wilcock *et al.*, 1998), possibly lower than 1 mg L^{-1} (Chapman, 2003).

Concurrent with Cape silverside, the hypoxia avoidance in inanga may therefore not be caused by physiological challenges due to decreasing oxygen levels but possibly reflects an early behavioural response to mild hypoxia (Herbert *et al.*, 2012) in the presence of an oxygen refuge. In heterogeneous habitats, horizontal migration enables fish to seek normoxic shelter (Kramer, 1987), thereby avoiding detrimental physiological effects of decreased oxygen levels. This strategy has been described for several fish species at distinct oxygen concentrations. The oxygen-sensitive rainbow trout has been shown to decrease residence time in mild hypoxia from approximately 7.5 mg L^{-1} DO (Poulsen *et al.*, 2011). In contrast, juvenile weakfish have been shown to exhibit no avoidance until water was severely hypoxic at 1 mg L^{-1} DO (Stierhoff *et al.*, 2009), comparable to snapper which demonstrated a hypoxia avoidance threshold of approximately 1.3 mg L^{-1} (Cook *et al.*, 2011). Similar to black mudfish, Atlantic cod demonstrated no avoidance of DO levels as low as approximately 2.2 mg L^{-1} in the presence of steady oxygen refuge areas (Herbert *et al.*, 2011). Likewise, mummichog have been shown to not avoid water as severely hypoxic as 1 mg L^{-1} (Wannamaker & Rice, 2000). These combined findings suggest that species-specific hypoxia avoidance thresholds, if present, are remarkably variable between species and may reflect species-specific habitat preferences and physiological adaptations.

While inanga displayed an elevated hypoxia avoidance threshold in comparison to banded kokopu, acute hypoxia tolerance studies established juvenile banded kokopu to be more hypoxia sensitive than juvenile and adult inanga (Dean & Richardson, 1999). Hereby banded kokopu displayed 100% mortality after 12 h exposure to 1 mg L^{-1} dissolved oxygen concentration in contrast to 61% and 38% mortality in juvenile and adult inanga, respectively, after

48 h exposure to 1 mg L^{-1} (Dean & Richardson, 1999). These findings support the suggestion that the avoidance of mild hypoxia in inanga may not be caused by physiological challenges due to decreasing oxygen concentrations, as they have been shown to sustain more severe levels of hypoxia. However, the marked decrease of residence time in mild hypoxia does indicate a clear behavioural avoidance response towards mild hypoxia. A more recent study found a higher hypoxia sensitivity in juvenile inanga with a 50% mortality after 48 h exposure to 2.6 mg L^{-1} (Landman *et al.*, 2005). This study, however, prevented the possibility of aquatic surface respiration and emersion, which has been shown to be an important hypoxia avoidance strategy in inanga when confronted with inescapable hypoxia (Urbina *et al.*, 2011).

A decrease in residence time occurred at a markedly low DO level, in contrast to the distinct hypoxia sensitivity which has previously been established for banded kokopu (Dean & Richardson, 1999). This change in residence time was preceded by a distinct increase in ASR frequency in mild hypoxia of 8.3 mg L^{-1} DO. Concurrent with increasing residence time in normoxia from 2.5 mg L^{-1} DO, ASR frequency in normoxia increased as well, suggesting that the decreasing oxygen level is a strong initiator of surfacing, continuing even after horizontal migration into the normoxia side has removed hypoxia as an ASR trigger. Conversely, only a minor increase in hypoxia ASR frequency was demonstrated for inanga in comparison to the baseline period, while no distinct change in ASR frequency was observed in black mudfish. It is however notable that these ASR frequency findings were accompanied by pronounced variability, suggesting caution when interpreting these findings (Figure 2.5). Aquatic surface respiration as a behavioural response to hypoxic water conditions has been previously

established in several fish species (Kramer & McClure, 1982; Wannamaker & Rice, 2000), including inanga and banded kokopu (Dean & Richardson, 1999; Urbina *et al.*, 2011). Near-surface water is characterised by an elevated oxygen concentration (Kramer & McClure, 1982), thus respiration at the water surface improves oxygen uptake (Urbina *et al.*, 2011), but does however increase the risk of aerial predation (Kramer, 1987) and is energetically demanding, as it requires fish to maintain an upright position in the water column (Dean & Richardson, 1999). The minor increase in ASR frequency demonstrated for inanga suggests that with an oxygen refuge available, this species responds to hypoxia with horizontal migration rather than surfacing. This would explain the dissimilarity to findings from Urbina *et al.* (2011), where ASR was increasingly utilised by inanga in very low oxygen concentrations of circa 1.9 and 1.5 mg L^{-1}, when the experimental conditions did not offer an aquatic oxygen refuge. The increase in ASR frequency demonstrated by banded kokopu before reduced residence time in the hypoxia side, suggests surfacing as the primary hypoxia response strategy of this species and is followed by horizontal migration from more severe hypoxia. Related findings were demonstrated by Wannamaker and Rice (2000), where mummichogs did not avoid hypoxic oxygen levels as low as 1 mg L^{-1}, but instead increased the percentage of time spent performing ASR. While black mudfish displayed no distinct increase in ASR frequency, occasional surface trips were observed and may have been employed for bubble respiration. Black mudfish previously has been described to utilise this mechanism for improved oxygen uptake (McPhail, 1999). At the water surface an air bubble is stored in the buccal cavity after which the fish returns to the bottom and demonstrates very little activity until the bubble is expelled from the fish. It was, however, not possible to

explicitly verify this behavioural strategy in the experimental observations due to technical limitations of the camera device utilised.

Avoidance response of inanga towards mild hypoxia in this study contrasts with previous findings where inanga showed no avoidance of low oxygen water when offered a choice between 2 mg L^{-1} and 8.5 mg L^{-1} (Richardson *et al.*, 2001b). The pronounced dissimilarity may be due to distinct differences in experimental protocols. The experiments of Richardson *et al*. were conducted in groups of ten fish where shoaling behaviour may have been affecting individual response movements while the present study was conducted with single fish. On the other hand, shoaling in the gregarious damselfish (*Chromis viridis*) has been shown to decrease the minimum metabolic rate and reduce the physiological reaction to stress (Nadler *et al.*, 2016). Thus, shoaling in the experiments of Richardson *et al*. may have decreased the metabolic oxygen demand in individual fish. Moreover, fish were acclimated to the study fluvarium for five minutes by Richardson et al., whereas fish were acclimated to the test arena for 17 h in the present study, and it has been shown that handling and transfer to a new environment necessitates an extended acclimation period in which fish become settled and return to their natural behaviour (Urbina *et al.*, 2011). The disparity of the present findings from Richardson *et al.* (2001b) emphasises the necessity of comparable experimental protocols to enable comparative evaluation of hypoxia avoidance responses within the same species but also between different species.

In a recent study (Urbina *et al.*, 2011), groups of inanga were subjected to acute progressive hypoxia, and avoidance behaviour in the form of emersion from the hypoxic water was observed at very low levels of approximately 1.9 and 1.5 mg L^{-1} DO. Emersion behaviour imposes advantages in scale-less galaxiids due to

an improved oxygen uptake across the skin (Urbina *et al.*, 2011). At the same time it presents an increased risk of desiccation (McPhail, 1999), predation and collapse of gill structures reducing the gill surface area and thereby impairing gill gas exchange (Hughes & Morgan, 1973). Inanga has previously been classified as an emersion intolerant species lacking well developed aerial adaptations and therefore surviving emersion for less than 24 h, while banded kokopu and black mudfish were shown to survive emersion for more than one week (Meredith, 1985). Emersion as a hypoxia avoidance strategy in inanga may therefore be facilitated at more severe hypoxia levels than submerged horizontal migration responses presented in this study.

Remarkably, no increase in the duration of ASR performance was observed for any species (Figure 2.6), because this contradicts previous findings where the amount of time spent performing ASR was significantly increased at 1 mg L^{-1} DO in mummichogs (Wannamaker & Rice, 2000) and at approximately 1.9 and 1.5 mg L^{-1} DO in inanga (Urbina *et al.*, 2011). In this context, it has previously been shown that glass catfish (*Kryptopterus bicirrhis*), when exposed to hypoxia permitting ASR, utilise their secondary vascular system, which is comprised of predominantly clear circulatory vessels and superficial capillaries (Rummer *et al.*, 2014), thereby supporting gas transfer across the skin surface. As surfacing increases predation risk (Kramer, 1987), the unchanged ASR duration may be reflective of a mechanism allowing improved oxygen uptake by increased frequency of surfacing while minimising predation pressure via brief dwelling time at the surface. In this context, the experimental setup, specifically the installation of a camera above the test arena, may have inhibited an elevated duration of surface dwelling. Conversely, in black mudfish the duration of

proportional time spent performing ASR was markedly elevated in normoxia, prior to the initiation of progressive hypoxia. This may suggest that environmental factors other than low oxygen levels, such as visual cues trigger occupation of and hence respiration in surface water in black mudfish as well.

2.5.2 Side visit frequencies in the context of hypoxia avoidance

In inanga, hypoxia avoidance was accompanied by elevated frequencies of visits into both normoxia and hypoxia sides in comparison to the baseline period. This effect was however not observed in banded kokopu or black mudfish (Figure 2.4), which suggests a marked side preference established during the acclimatisation period in inanga that stimulates continued exploration of the low oxygen side even in increasingly severe hypoxia. Related findings were demonstrated in yellowtail kingfish which did not reduce residence time in escapable hypoxia with DO concentrations reduced to approximately 1.9 mg L^{-1} (Cook & Herbert, 2012b). Instead, this species continuously explored both normoxic and hypoxic areas. It has been shown previously that several fish species periodically utilise hypoxic environments for foraging (Claireaux *et al.*, 1995; Neuenfeldt *et al.*, 2009; Roberts *et al.*, 2009; Herbert *et al.*, 2011) or predator avoidance (Robb & Abrahams, 2002; Shingles *et al.*, 2005) when a normoxic refuge is available which presents an advantage over less hypoxia-tolerant species that are unable to utilise temporarily or permanent hypoxic environments for forage, predator avoidance or habitat, and accentuates the importance of oxygen refuge availability in the context of hypoxia avoidance responses.

2.5.3 Swimming speed adjustments

The hypoxia response of banded kokopu was accompanied by an increase in swimming speed in the normoxia and hypoxia side of the test chamber in comparison to baseline observations, although this observation was associated with considerable variation. In contrast, swimming speed remained completely unchanged in inanga and was decreased during escapable hypoxia in black mudfish (Figure 2.7). Previous studies presented increased swimming speeds in several fish species exposed to hypoxia and it was therefore established that elevated swimming speed is essential in facilitating fast hypoxia avoidance (Jones, 1952; Domenici *et al.*, 2000; Herbert & Steffensen, 2006). However, as discussed in Herbert *et al.* (2011) and Herbert *et al.* (2012), these studies were conducted utilising inescapable hypoxia. Recent findings from studies utilising escapable low oxygen conditions range from non-adjusted swimming speed in New Zealand snapper (Cook *et al.*, 2011) and yellowtail kingfish (Cook & Herbert, 2012b) to decreased swimming speed in Atlantic cod (Skjæraasen *et al.*, 2008). Increased swimming speeds were however reported as well, specifically for rainbow trout (Poulsen *et al.*, 2011) and Cape silverside (Herbert *et al.*, 2012). While Urbina *et al.* (2011) utilised hypoxia without an aquatic oxygen refuge, they interpreted the increase in swimming speed observed in inanga as a mechanism which may improve the effectiveness of ASR due to increased ventilation of oxygen-richer surface water, a strategy previously described by Soares *et al.* (2006). This is in agreement with the findings of this study, where an increase of swimming speed was only observed alongside an increased ASR frequency in banded kokopu.

2.6 Conclusions

Distinct species-specific responses to and avoidance of hypoxia were demonstrated by inanga, banded kokopu and black mudfish. While inanga and banded kokopu appeared to detect and respond to decreasing oxygen concentration, no distinct behavioural response was elicited in black mudfish. However, the hypoxia level applied may not have been severe enough to elicit distinct behavioural or physiological responses in the hypoxia-tolerant black mudfish.

Hypoxia responses in inanga and banded kokopu were characterised by species-specific strategies. Inanga primarily reduced residence time in hypoxic conditions and displayed horizontal migration from mild hypoxia, possibly elicited by elevated activity and increase metabolic rate, while banded kokopu initially increased ASR frequency and swimming speed with a secondary reduction in residence time in more severe hypoxia. These findings may suggest potential effects of increasing occurrences of hypoxic estuaries on the migratory pattern of the diadromous inanga. While inanga are known to frequently encounter hypoxic episodes in their natural habitat and are able to sustain hypoxia for multiple days, an early evasion response towards mild hypoxia may possibly interfere with upstream recruitment and habitat selection of juvenile inanga migrating from sea to freshwater.

The variability in observed responses to hypoxia from the present study as well as from previous work implies that hypoxia avoidance responses may depend on species-specific habitat preferences and life strategies, and corresponding physiological adaptations. Interpretation of hypoxia avoidance strategies must

therefore be carried out carefully in the context of effective environmental factors interacting with hypoxia response such as the availability of oxygen refuge, the presence of food or shelter and predation pressure.

The findings presented here are founded in species-specific hypoxia sensitivities, reflected in the specialised habitat requirements that can be observed in these three species and which are accompanied by distinct behavioural and physiological response mechanisms towards hypoxia.

3 Response of oxygen consumption to increasing hypoxia

3.1 Abstract

Freshwater fish frequently encounter low oxygen (hypoxic) environments and exhibit distinct species-specific differences in hypoxia sensitivity and response capabilities, which are often reflected in their respective habitat preferences. With intermittent-flow respirometry, routine oxygen consumption rates (RMR) at normoxia and the effect of distinct levels of mild and severe hypoxia on routine oxygen consumption rate were investigated in three galaxiid species; inanga (*Galaxias maculatus*), banded kokopu (*Galaxias fasciatus*) and black mudfish (*Neochanna diversus*). Inanga and banded kokopu demonstrated similar routine oxygen consumption rates, while these were lower in black mudfish. Inanga and banded kokopu maintained routine oxygen consumption rates in mild hypoxia and exhibited distinct critical oxygen concentrations (C_{crit}), below which routine oxygen consumption rate declined with environmental DO, identifying these species as oxyregulators. Black mudfish was indicated as an oxyregulator as well, however no C_{crit} could be ascertained in this study, possible due to insufficiently severe hypoxia levels applied. Inanga displayed the greatest hypoxia sensitivity, reflected in a C_{crit} of 5.0 ± 0.4 mg L^{-1}, while banded kokopu was more hypoxia tolerant with a C_{crit} of 4.3 ± 0.1 mg L^{-1}. These findings demonstrate species-specific oxygen demand and sensitivities to hypoxia, as well as distinct oxyregulatory capabilities, offering further explanation of the distinct habitat preferences observed in these three species.

3.2 Introduction

3.2.1 Respiration and oxygen consumption in fish

Aquatic respiration imposes intrinsic challenges based on naturally reduced oxygen availability and diffusion rate in water compared to air as a respiratory medium (Kramer, 1987; Graham, 1990). Thus, oxygen uptake from water adjacent to the respiratory surfaces forms an oxygen diffusion boundary layer and limits gas transfer across the respiratory surface (Graham, 1990). Environmental oxygen, however, is essential for aerobic metabolism in which high-energy molecules such as ATP (adenosine triphosphate) are generated and drive biosynthetical and active processes of the organism (Moyes & Schulte, 2006; Rogers *et al.*, 2016). Anaerobic metabolism is a much less energy efficient alternative for ATP production, however physiological processes such as locomotor activity, feeding and digestion, and growth and reproduction, particularly at higher temperatures, increase ATP-demand above that which can be supported by anaerobic production and hence results in a metabolic oxygen demand (Chabot *et al.*, 2016).

Piscine respiratory and circulatory structures are evolved to safeguard oxygen uptake and internal supply at normoxic as well as a range of hypoxic environmental oxygen concentration levels (Claireaux & Chabot, 2016). In this context, hypoxia tolerance in fish, indicated by a species-specific threshold in hypoxic dissolved oxygen concentration, at which oxygen uptake and aerobic metabolism ceases to be maintained, varies greatly (Rogers *et al.*, 2016). Hypoxia-tolerant species exhibiting low hypoxia thresholds may therefore temporarily or permanently inhabit or utilise hypoxic environments. In contrast,

species demonstrating elevated hypoxia thresholds and sensitivities are not able to withstand hypoxic environments.

3.2.2 Aquatic hypoxia and critical environmental oxygen tension

Fish are naturally confronted with varying environmental oxygen concentrations (Friedrich *et al.*, 2014). However, recent global expansion of hypoxic aquatic environments in context with anthropogenic impacts such as eutrophication due to intense land-use and introduction of organic waste from urban and industrial installations is of growing concern (Wilcock *et al.*, 1995; Diaz & Rosenberg, 2008; Rabalais *et al.*, 2010; Friedrich *et al.*, 2014). Such impacts may impose adverse effects on hypoxia-sensitive fish, therefore affecting survival, recruitment and community composition (Rogers *et al.*, 2016). This necessitates a better comprehension of piscine responses to environmental hypoxia by studying oxygen uptake in correlation with varying environmental oxygen concentration.

In hypoxic habitats, shifting energy production to non-oxygen demanding anaerobic glycolysis is no permanent solution due to reduced efficiency (Urbina *et al.*, 2012) and increased accumulation of metabolic waste products (Chabot *et al.*, 2016). Instead, most fish have developed diverse behavioural and physiological responses towards hypoxia. These responses facilitate maintained uptake and consumption of environmental oxygen to uphold aerobic metabolism over a range of environmental oxygen concentration levels, a strategy which is termed oxyregulation (Rogers *et al.*, 2016), and has been demonstrated for a range of invertebrates and vertebrates including fish (McKenzie *et al.*, 2007). In this context, reduction of locomotor activity as an oxygen demand adjusting strategy (Kramer, 1987) has been observed previously in several species (Carlson & Parsons, 2001; Herbert & Steffensen, 2005; Lefrançois *et al.*, 2005; Johansen *et*

al., 2006; Behrens & Steffensen, 2007; Cook *et al.*, 2014). In contrast, ram-ventilating fish have been shown to increase swimming speed in response to hypoxia, resulting in improved gill ventilation and oxygen uptake (Carlson & Parsons, 2001; Fitzgibbon *et al.*, 2010). Alternative strategies that improve oxygen uptake from hypoxic environments include respiratory adjustments such as increased ventilation rate (Balfour, 1999; Shingles *et al.*, 2005) and elevated stroke volume (Randall, 1982; Bushnell & Brill, 1991), as well as circulatory adaptations comprising decreased heart rate (Claireaux *et al.*, 1995), which often occurs in correspondence with increased heart stroke volume and elevated aortic blood pressure (Randall, 1982).

Alternative long-term hypoxia-response strategies involve gill morphological modulation increasing the respiratory surface area. In this context, interlamellar cell mass which is embedding the gill secondary lamellae at normoxia has been shown to be reduced via cell apoptosis in several fish species, including crucian carp (*Carassius carassius*; (Sollid *et al.*, 2003)), goldfish (*Carassius auratus*; (Mitrovic *et al.*, 2009)), Qinghai carp (*Gymnocypris przewalskii*; (Sollid & Nilsson, 2006)) and Atlantic stingray (*Dasyatis sabina*; (Dabruzzi & Bennett, 2013)). Similarly, fish have been shown to increase the concentration and oxygen affinity of the haemoglobin protein, which binds oxygen and thereby transports it via the bloodstream (Randall, 1982; Robb & Abrahams, 2003; Wu *et al.*, 2016). In the context of long-term adaptations to hypoxia, Reardon and Chapman (2010) demonstrated a marked capacity for developmental plasticity of hypoxia sensitivity in Egyptian mouth-brooder (*Pseudocrenilabrus multicolor*), which were reared at either normoxic or hypoxic

conditions and hence displayed a decrease in routine metabolic rate and hypoxia sensitivity when raised under hypoxic conditions.

Particularly in the context of acute hypoxia, oxygen uptake ceases to be sustained at certain levels of continuously reduced environmental oxygen concentration, despite oxyregulatory capacities fish may exhibit. Therefore, oxygen consumption declines with subsequently continuously reducing oxygen concentration which is known as oxyconforming, and at which point anaerobic glycolysis is utilised by the fish for energy metabolism. The ambient oxygen concentration at which a shift from oxyregulation to oxyconforming occurs is termed critical oxygen concentration C_{crit} (Pörtner & Grieshaber, 1993) and is utilized to assess hypoxia sensitivities because hypoxia tolerant species demonstrate a relatively low C_{crit} while hypoxia sensitive species are characterised by higher C_{crit} values (Urbina *et al*., 2012; Rogers *et al*., 2016).

There are only a few reported cases of fish not oxyregulating and oxyconforming at all exposed DO levels. In this scenario oxygen uptake declines continuously with decreasing environmental oxygen concentration and thus the fish does not demonstrate a C_{crit}. Recently, Urbina *et al*., (2012) characterised inanga (*Galaxias maculatus*), which inhabit shallow and often uncovered lowland habitats, frequently encountering hypoxic episodes, as general oxyconformers. In this context it was suggested that a substantial proportion of oxygen uptake occurs via cutaneous gas exchange. Similar observations have been made for South American fish species of the Gymnotiformes order inhabiting naturally hypoxic habitats (Rogers *et al.*, 2016). Likewise, white sturgeon (*Acipenser transmontanus*) has been described as an oxyconformer (Burggren & Randall, 1978). However, subsequent studies revealed oxyregulatory strategies in this

species and it has been argued that the initial study may have been affected by methodological artefacts (McKenzie *et al.*, 2007; Urbina *et al.*, 2012). This highlights the necessity of an appropriate respirometry chamber and experimental design for oxygen uptake and hypoxia sensitivity studies utilizing standardised methods for comparable data (Steffensen, 1989; Chabot *et al.*, 2016).

3.2.3 Respirometry in fish

Respirometry utilised in recent studies included closed, open (flow-through) and intermittent (stop-flow) modes of operation. Closed respirometry commonly leads to accumulation of carbon dioxide (hypercapnia) and metabolic waste products, (Steffensen, 1989), thereby not providing steady environmental conditions, and potentially affecting the oxygen demand (i.e. oxygen consumption rate) of the animal and thereby the oxygen uptake measurements. While hypercapnia and increased levels of metabolic waste are avoided in open respirometry, this method is associated with washout and mixing issues that also potentially disturb oxygen consumption rate determination (Steffensen, 1989). It has been previously proposed that intermittent-flow respirometry is the most appropriate experimental technique for oxygen consumption rate measurements, as it utilises combined features of both aforementioned methods, while reducing the associated common issues (Svendsen *et al.*, 2016b).

Respirometry is a useful and commonly adopted technique but there are a range of technical features that require careful consideration. For example, the size of respirometry chamber in relation to fish volume, as well as the time period over which measurements are made, have also recently been shown to affect oxygen consumption rate variation and therefore measurement error. While the respirometry chamber should be large enough not to elicit stress responses in the

fish due to confinement issues, an increased respirometer-fish volume ratio has been shown to result in elevated variation and measurement errors. In this context, fish mass to volume ratios (g:ml) suggested for optimal respirometry chamber size are between 1:20 and 1:100 for static respirometry chambers and up to 1:350 for highly active species exercised in swim tunnel respirometry (Clark *et al*., 2013). Likewise, a sufficient length of time for discrete oxygen measurements have been suggested to decrease errors in oxygen consumption rate determination (Svendsen *et al.*, 2016a). Also, the metabolic parameter of interest requires careful consideration. Standard metabolic rate (SMR) reflects the minimal oxygen uptake sustaining an inactive fish, while the routine metabolic rate (RMR) describes oxygen uptake in a relatively calm fish, but it may contain oxygen uptake attributed to small movements. Maximum metabolic rate (MMR), however, reflects an estimate of the maximum oxygen uptake generally obtained via swimming respirometry (Rogers *et al.*, 2016).

Thus, respirometry chamber and experimental design need to either preclude extensive movements and activity for SMR estimation, or enforce activity levels via exercise that are sufficiently high enough to derive MMR estimates. In this context, it has previously also been shown that MMR estimates may differ significantly when derived from different exercise protocols (Roche *et al.*, 2013; Rummer *et al.*, 2016). This emphasises the necessity for appropriate and, ideally, identical respirometry chamber and experimental design protocols when undertaking comparative respirometry studies.

Respirometry and C_{crit} determination has previously been utilized to comparatively investigate the hypoxia sensitivity of closely related species which can be found in habitats with different gradients of distinct environmental

parameters (for a comprehensive review see Richards, 2011). In this context, it has been shown by (Hilton *et al.*, 2008) that two intertidal triplefin fish species present distinct tolerances toward hypoxia and temperature change, thereby facilitating associated differences in the habitat choice of these species. Similarly, Mandic *et al.* (2009) demonstrated a correlation between hypoxia tolerance (measured as C_{crit}) and environmental distribution of closely related sculpin species between intertidal and subtidal zones or freshwater habitats. To date, comparable studies have not yet been carried out with closely related species of the galaxiid family to verify whether their distinct habitat selectivity in the context of environmental oxygen is based upon distinct metabolic oxygen demand and critical oxygen concentration.

3.2.4 Galaxiids

Inanga, banded kokopu (*Galaxias fasciatus*) and black mudfish (*Neochanna diversus*) are closely related freshwater fish species of the Galaxiidae family (Waters *et al.*, 2000) inhabiting a range of distinct oxygen concentration environments in the North Island of New Zealand (McDowall, 2006) in accordance with their respective specialised habitat requirements. As such they are readily available and utilisable for environmental studies in a laboratory setting. Furthermore they represent a distinct scale of habitat preferences in terms of oxygen concentration, with banded kokopu primarily found in cool, canopy-covered forest streams, while inanga are often found in shallow, uncovered, slow flowing streams and estuaries, frequently encountering hypoxic episodes (Urbina *et al.*, 2011). Black mudfish on the other hand inhabit swampy wetlands and drains, commonly becoming hypoxic or even anoxic during summer months or drying up, during which black mudfish survive by aestivation (McDowall, 1990).

These species therefore represent an outstanding comparative model to investigate whether their respective hypoxia sensitivity and oxygen uptake profile in progressive hypoxia reflect distinct adaptive responses to hypoxia consistent with their respective preferred habitat while our findings are largely unimpaired by evolutionary and phylogenetic differences. This study furthermore broadens our understanding on how tolerant these native species are towards hypoxia and thus how progressive occurrences of aquatic hypoxia may affect some freshwater fish communities in New Zealand.

3.2.5 Study objectives

In this study, oxygen consumption rate and hypoxia sensitivity were investigated for inanga, banded kokopu and black mudfish by determining the oxygen uptake profile and routine metabolic rate at normoxic and declining environmental oxygen concentration. Intermittent-flow respirometry allowing small movements in a static environment was utilised at normoxia and five levels of environmental hypoxia. Exposing fish to progressive hypoxia in a decreasing order of ambient oxygen concentration levels as well as maintaining fish in a closed respirometry chamber depleting ambient oxygen concentration until loss of equilibrium may constitute experimental artefacts possibly affecting the oxygen uptake rates, therefore distinct ambient oxygen concentration levels were tested in non-decreasing order in intermittent respirometry.

3.3 Methodology

3.3.1 Experimental fish

Adult inanga (body mass: 3.22 ± 0.30 g; total length: 70.6 ± 2.0 mm; data presented here and thereafter in mean ± SE) were purchased from a fish farm that provides natural habitat for upstream migrating whitebait at the conclusion of their marine larval development (Raglan EELS Ltd, Raglan, New Zealand). Immature, post-larval banded kokopu that are comparable in their physiology to adult fish (body mass: 5.40 ± 0.32 g; total length: 73.6 ± 1.6 mm) were caught by backpack-electrofishing from Puketirini Stream in Huntly, New Zealand, and adult black mudfish (body mass: 2.32 ± 0.15 g; total length: 70.8 ± 2.2 mm) were caught with minnow traps from wetland and field drainage areas surrounding Hamilton and Huntly, Waikato, New Zealand. All fish were transported to the Aquatic Research Facility at the University of Waikato, Hamilton, New Zealand. Fish were acclimatised to laboratory conditions in the Aquatic Research Facility for at least two weeks prior to experiments and kept in indoor tanks with dechlorinated tap water fitted with aquarium filters and supplementary aeration at a constant temperature of 16°C and a 12:12 light:dark photoperiodic cycle. To assist osmoregulation and reduce incidence of white spot disease, marine salt (Crystal Sea Marinemix, Marine Enterprises International) was added to inanga and banded kokopu tanks to a concentration of 3.5‰ and frequent water changes limited build-up of waste products. Black mudfish were housed in water of their respective habitat that was brought back to the facility with them, and therefore did not necessitate added salt. All fish were fed to satiation with frozen bloodworms every two days. All procedures and experiments followed the standard operating procedures for captive fish maintenance by the University of

Waikato and were approved by the University of Waikato Animal Ethics Committee (protocol # 844).

3.3.2 Experimental procedure

Chambers (0.8 L) fitted with optical oxygen sensors (oxygen sensor spots at the inside of the chamber, in connection with fibre-optic probe at the outside, PreSens Precision Sensing GmbH, Germany) were immersed in an ambient water bath of dechlorinated tap water and partially covered with black fabric to minimize fish disturbance during the intermittent flow respirometry experiments. Recirculating water pumps were used to flush the chambers with ambient water. The dissolved oxygen concentration in the ambient water bath was adjusted via air stones using an external air pump or gaseous oxygen-free nitrogen. Experiments were carried out in a temperature-controlled room where the experimental temperature was maintained between 16 and 18°C. Fish had been starved 48 h prior to acclimatisation to minimize any adverse effect of specific dynamic action on oxygen consumption rate measurements (Urbina *et al*., 2012; Clark *et al*., 2013). Twelve fish per species were placed individually into respiratory chambers and allowed to habituate to normoxic recirculating water for 12 h. Before each set of experiments, a non-oxygen consuming, optical oxygen meter was calibrated to 0% and 100% oxygen concentration using sodium dithionite saturated water and water saturated air respectively. The dissolved oxygen (DO) concentration levels tested were 10.6 ± 0.03 (= normoxia), 7.2 ± 0.05, 5.8 ± 0.04, 4.8 ± 0.02, 3.5 ± 0.01 and 2.5 ± 0.01 mg L^{-1}. To avoid any effects from a decreasing order of DO levels on oxygen consumption rate, the DO level sequence was altered to 10.6, 4.8, 7.2, 2.5, 5.8 and 3.5 mg L^{-1}. Chambers were flushed with ambient water in which a distinct DO level was being set, which took 20 to 71 min depending on

the severity of hypoxia generated. Water recirculation was stopped when the DO inside the chambers reached the desired level. Fish movements were considered sufficient to keep water in the chambers mixed. DO inside the chambers was directly measured 16 times in each cycle, every two minutes over a period of 30 min using fibre-optic oxygen sensor probes. The decline in dissolved oxygen concentration in each cycle was between 5.4 ± 0.8 and 11.4 ± 1.5% in inanga, between 6.0 ± 0.6 and 12.3 ± 1.9% in banded kokopu, and between 3.4 ± 0.4 and 8.7 ± 1.4% in black mudfish. Subsequently, normoxic water recirculation was re-established and once normoxic conditions were restored inside the chambers, a 30 min recovery period was allowed before adjusting the dissolved oxygen concentration in the ambient water and chambers to the subsequent DO level. Re-establishing normoxic water conditions in the chambers took between 17 and 32 min. Care was taken to minimize any fish disturbances when taking oxygen measurements, utilizing the recirculating water pumps and adjusting the DO level. After the conclusion of the experiments, fish were individually anaesthetised in water containing 20 mg L^{-1} benzocaine before body mass and total length measurement and then returned to their respective holding tanks for recovery. Two mortalities occurred during these experiments as one fish became trapped in the outlet of its respiratory chamber and one fish died during the habituation period due to unknown causes. In the two-week post-experimental period no mortalities were recorded. To account for bacterial oxygen consumption and potential changes in dissolved oxygen concentration measurements that were unrelated to fish oxygen consumption, each respirometry chamber underwent two repeated control experiments without fish. From these eight control experiments, the mean difference in dissolved oxygen concentration (Δ DO_{con}) between

beginning and conclusion of the experiments was determined for each ambient DO level. Subsequently, these Δ DO_{con} values were subtracted from each calculated difference in DO concentration for each individual fish at each DO level, respectively. Across all six DO levels tested, Δ DO_{con} (mean ± SE) was 0.011 ± 0.014 mg L^{-1}.

The water mass from each chamber was determined for calculation of respirometry chamber volume (Appendix 3.1). The respirometer volume : fish mass ratio was 282.2 ± 25.3 for inanga, 156.4 ± 11.6 for banded kokopu, and 360.3 ± 19.6 for black mudfish.

3.3.3 Data analyses

Individual fish and control oxygen measurements were plotted against time for linear regression analysis at each ambient DO concentration level. Measurements were censored excluding distinctly outlying data points. Regression equations were utilized to calculate the change in dissolved oxygen concentration in the respirometry chamber. To account for changes in DO concentration unrelated to fish consumption (i.e. bacterial oxygen consumption), mean control DO change was subtracted from the change in DO concentration for each individual fish.

Following Clark *et al*., (2013) for intermittent respirometry, individual mass-specific oxygen consumption rate at each DO concentration level ($\dot{M}O_2$) was calculated as:

$$\dot{M}O_2 = [(V_r - V_f) \times \Delta C_{wO2}] / (\Delta t \times M_f),$$

in which V_r is the respirometry chamber volume (L), V_f is the fish volume (L), ΔC_{wO2} is the change in dissolved oxygen concentration in the respirometry

chamber (mg L^{-1}), Δt is the time over which ΔC_{wO2} is calculated (h) and M_f is the fish wet mass (g). Fish volume was hereby equated with fish mass (kg), under the assumption that fish were neutrally buoyant, thereby exhibiting the same density as the water (1.0 kg L^{-1}; (Svendsen *et al.*, 2016b)). For each species and ambient DO concentration level, the individual oxygen consumption rate values were averaged and trimmed, excluding negative values as they were assumed to be measurement errors (n = 1) and the top 25% of values to account for excessively active fish potentially affecting the oxygen consumption rate measurements.

To account for body mass effects on the oxygen uptake, and to facilitate inter-species comparisons, the mass-independent oxygen consumption rate was determined as:

$$\dot{M}O_2 = [(V_r - V_f) \times \Delta C_{wO2}] / [\Delta t \times (M_f{}^b)],$$

in which ^{b} is a mass-scaling coefficient of 0.67. This scaling measure has previously been established in the context of comparisons of oxygen consumption rate between quiescent and similarly shaped species of different sizes (Hopkins & Cech, 1994; Meloni *et al.*, 2002).

In recent studies, prolonged habituation of 10-12 hr (Behrens & Steffensen, 2007) and 40 hr (Cook *et al.*, 2011) to the respirometry chamber at normoxia, accompanied by regular automated DO concentration measurements, preceded the hypoxia respiratory experiments. Upon utilising the quantile method (Chabot *et al.*, 2016), this approach allows an accurate estimation of SMR in fish. However, as DO concentration measurements were done manually in the present study, this approach, albeit potentially more accurate than calculating C_{crit} from RMR estimates, which are potentially more susceptible to be affected by changes

in swimming activity, was beyond the scope of this research. In this context, it was considered more appropriate to determine critical oxygen concentration from each individual fish and to subsequently calculate a species specific C_{crit}, in contrast to calculating C_{crit} from the mean oxygen consumption rates at each DO level, as has predominantly been done in previous studies (e.g. Cook *et al*., 2013 and Dwyer *et al*., 2014). The critical ambient oxygen concentration C_{crit} at which oxyregulation ceases and oxyconforming respiration commences was calculated individually for each fish that demonstrated a clear breakpoint between oxyregulation and oxyconforming. In this context, individual mass-specific as well as mass-independent oxygen consumption rates were plotted against the ambient oxygen concentration levels that were applied (Figure 3.1). From all $\dot{M}O_2$ values (at normoxia and decreasing DO levels) that did not reflect a continuous decline in oxygen uptake, an average value for oxygen uptake was derived, and visualized in the plot by a horizontal line. To all $\dot{M}O_2$ data points falling below the aforementioned average oxyconforming $\dot{M}O_2$, a least squares linear regression was applied. The resulting regression equation was utilized to calculate the intercept point with the average oxyregulatory $\dot{M}O_2$ value, which reflects C_{crit} for this specific fish (McKenzie *et al.,* 2003). For each species a mean C_{crit} was calculated from all individually determined C_{crit} values.

While the critical breakpoint at which a shift from oxyregulation to oxyconforming occurs has typically been denoted as critical oxygen pressure limit P_{crit} in the majority of previous publications, this measure will be denoted as critical oxygen concentration C_{crit} in this thesis. Species specific hypoxia tolerance thresholds from the outcome of this research will thereby be directly applicable for environmental management agencies, where any recent guidelines feature

oxygen measurements as dissolved oxygen concentration in mg L^{-1}. Furthermore, the measure of dissolved oxygen concentration in mg L^{-1} includes the effect of temperature, salinity and barometric pressure on oxygen solubility and hence dissolved oxygen concentration, while these parameters have to be denoted separately for any oxygen measurements taken as oxygen partial pressure in mmHg or kPa.

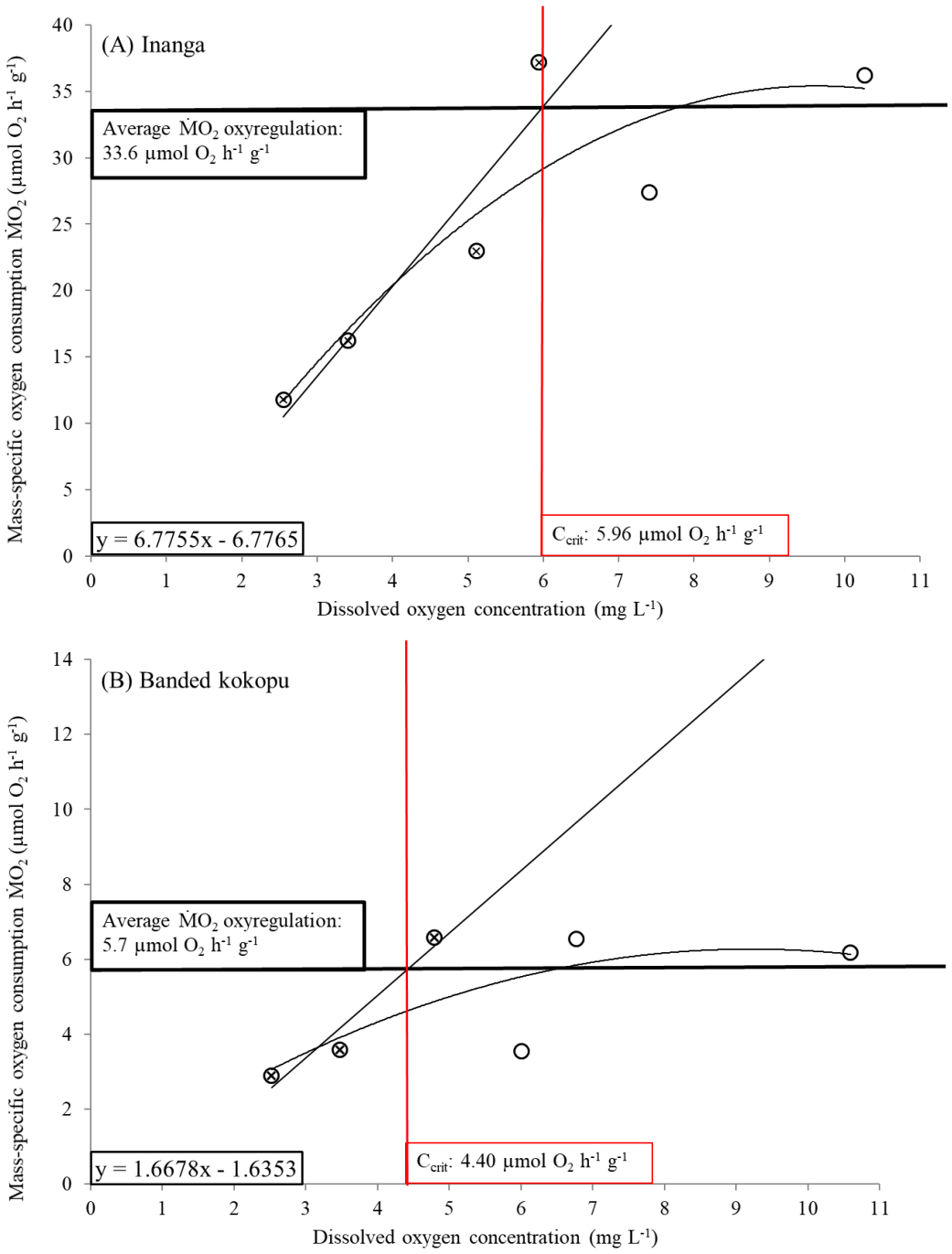


Figure 3.1: Examples of Ccrit determination method utilising least squares linear regression on ṀO2 measurements of individual fish for inanga (A) and banded kokopu (B). Curved line denotes principal oxygen uptake across all DO levels tested. White circles reflect oxyregulatory ṀO2, cross-filled circles demonstrate oxyconforming ṀO2. Thick black line marks average oxyregulatory ṀO2. Thin black line marks least squares regression line for all ṀO2 of oxyconforming oxygen uptake. Red line marks breakpoint (Ccrit) between oxyregulation and oxyconforming.

3.3.4 Statistical analyses

Statistical analyses were carried out using Statistica v.12.0 (StatSoft, USA) and Microsoft Excel 2013 Data Analysis ToolPak. All data were tested for normality using Shapiro-Wilk W test, Kolmogorov-Smirnov test for normality and Lilliefors p test. Data were subsequently evaluated for significant differences using one-way analysis of variance (ANOVA) and post hoc Tukey's HSD or Tukey's HSD for unequal n respectively in dependence on sample numbers. Data were considered significantly different with a P-value < 0.05.

3.4 Results

Fish did not appear to be distressed by the experimental procedures. It was observed that they were alternating sporadically between periods of swimming and resting within their respirometry chamber independently of the stage of the experiment. The observations are therefore regarded as estimates of routine metabolic rates derived from the obtained oxygen consumption rate measurements.

In decreasing, yet mildly hypoxic ambient oxygen concentration levels, inanga and banded kokopu maintained their respective oxygen consumption rate (oxyregulation), while both species oxyconformed at more severely hypoxic concentrations. The oxygen consumption pattern of black mudfish, however, was more reflective of this species being a strict oxyregulator without any noticeable decline in oxygen uptake rate.

3.4.1 Mass-specific oxygen consumption

Mass-specific oxygen consumption rate ($\dot{M}O_2$) in inanga did not differ significantly between ambient oxygen concentration levels of 10.6, 7.2 and 5.8 mg L^{-1} (Tukey's HSD for unequal n: $P > 0.05$, Appendix 3.2, pooled mean ± SE: 14.7 ± 0.6 µmol O_2 h^{-1} g^{-1}). Oxygen consumption rate decreased continuously at levels of 4.8, 3.5 and 2.5 mg L^{-1} to 11.9 ± 0.7, 9.7 ± 0.5 and 7.7 ± 0.9 µmol O_2 h^{-1} g^{-1}, respectively, constituting 78.0, 63.3 and 50.7% of normoxic $\dot{M}O_2$. Of these, $\dot{M}O_2$ at 3.5 and 2.5 mg L^{-1} were significantly lower than at 10.6, 7.2 and 5.8 mg L^{-1} (ANOVA: $F_{5,51} = 9.6021$, $P < 0.0001$, Tukey's HSD for unequal n: $P \leq 0.01$, Appendix 3.2, Figure 3.2 A).

Mass-specific $\dot{M}O_2$ in banded kokopu was not statistically different between ambient oxygen concentration levels of 10.6, 7.2, 5.8 and 4.8 mg L^{-1}

(Tukey's HSD for unequal n: $P > 0.05$, Appendix 3.3, pooled mean ± SE: 9.2 ± 0.7 µmol O_2 h^{-1} g^{-1}). Oxygen consumption rate decreased continuously with decreasing ambient oxygen concentration levels of 3.5 and 2.5 mg L^{-1} to 6.2 ± 0.3 and 4.7 ± 0.7 µmol O_2 h^{-1} g^{-1} respectively, which constituted 64.4 and 48.9% of normoxic $\dot{M}O_2$. Oxygen consumption rate at 2.5 mg L^{-1} ambient oxygen concentration was significantly lower from $\dot{M}O_2$ at 7.2 mg L^{-1} (ANOVA: $F_{5,49}$ = 4.3814, P = 0.0022, Tukey's HSD for unequal n: $P < 0.05$, Appendix 3.3, Figure 3.2 B).

Oxygen consumption rate in black mudfish was not significantly different between any ambient oxygen concentration levels (ANOVA: $F_{5,46}$ = 1.1244, P = 0.3609). Between 10.6 and 7.2 mg L^{-1} $\dot{M}O_2$ decreased from 11.3 ± 2.0 to 6.8 ± 1.7 µmol O_2 h^{-1} g^{-1}. At 7.2, 5.8, 4.8 and 3.5 mg L^{-1} no distinct change in $\dot{M}O_2$ occurred (pooled mean ± SE: 7.6 ± 0.8 µmol O_2 h^{-1} g^{-1}, 67.6% of normoxic oxygen consumption rate). At the lowest ambient oxygen concentration $\dot{M}O_2$ decreased to 6.1 ± 1.4 µmol O_2 h^{-1} g^{-1} (54.0% of normoxic $\dot{M}O_2$; Figure 3.2 C).

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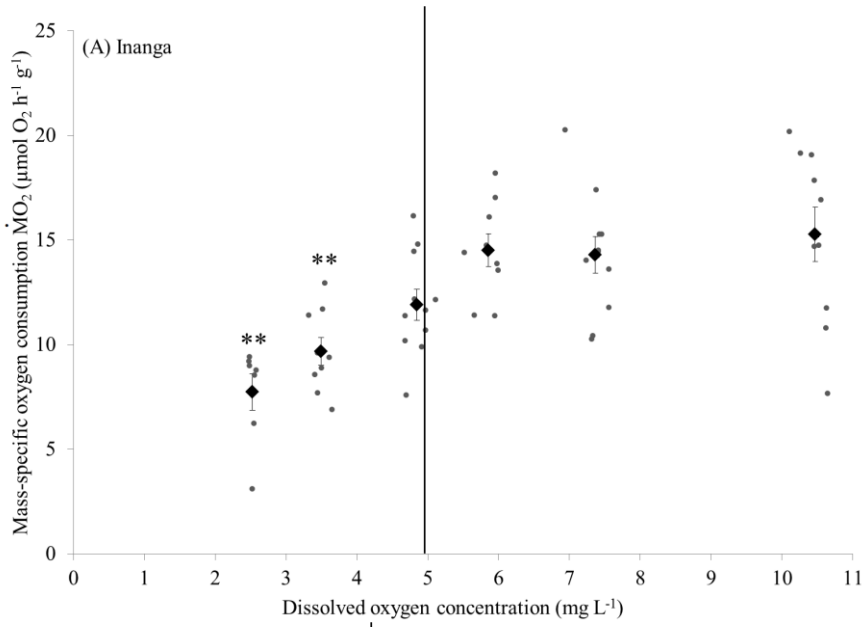
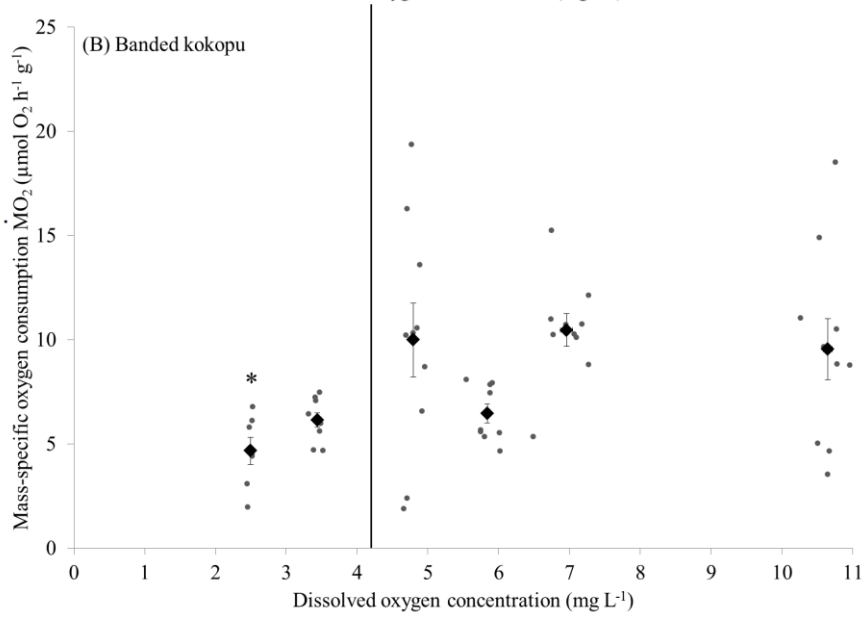
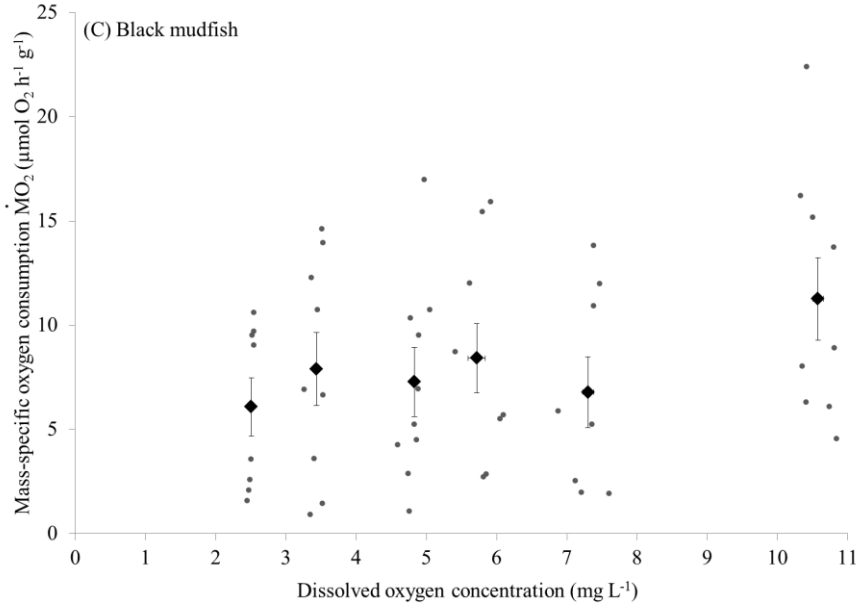
Figure 3.2: Mass-specific oxygen consumption rate $\dot{M}O_2$ (µmol O_2 h^{-1} g^{-1}) of inanga (A), banded kokopu (B) and black mudfish (C) in intermittent-flow respirometry at decreasing dissolved oxygen concentrations with individual measurements (grey circles) and mean oxygen consumption rate at each dissolved oxygen concentration level (black rectangulars). Vertical error bars are standard error of mean $\dot{M}O_2$ and horizontal error bars are standard error of mean dissolved oxygen concentration level. Statistical significant differences between normoxic and distinct hypoxic $\dot{M}O_2$ are marked by * ($P < 0.05$) and by ** ($P < 0.01$). Vertical black line indicates mean critical ambient oxygen concentration (C_{crit}) at which oxygen consumption rate changed from oxyregulation to oxyconform respiration.


(A) Inanga
Mass-specific oxygen consumption $\dot{M}O_2$ (µmol O_2 h^{-1} g^{-1})
Dissolved oxygen concentration (mg L^{-1})
**
**


(B) Banded kokopu
Mass-specific oxygen consumption $\dot{M}O_2$ (µmol O_2 h^{-1} g^{-1})
Dissolved oxygen concentration (mg L^{-1})
*


(C) Black mudfish
Mass-specific oxygen consumption $\dot{M}O_2$ (µmol O_2 h^{-1} g^{-1})
Dissolved oxygen concentration (mg L^{-1})

Mass-specific oxygen consumption rate in banded kokopu was significantly lower than in inanga at 10.6, 7.2 and 5.8 mg L^{-1} ambient oxygen concentration ($ANOVA_{10.55\ mg\ L^{-1}}$: $F_{2,26} = 3.5433$, $P = 0.0436$; $ANOVA_{7.23\ mg\ L^{-1}}$: $F_{2,26} = 11.316$, $P = 0.0003$; $ANOVA_{5.81\ mg\ L^{-1}}$: $F_{2,24} = 14.748$, $P < 0.0001$; Tukey's HSD: $P < 0.05$, Appendix 3.5). Similarly, mass-specific $\dot{M}O_2$ in black mudfish was significantly lower than in inanga at 7.2 and 5.8 mg L^{-1} ambient oxygen concentration (Tukey's HSD: $P < 0.01$, Appendix 3.5). Oxygen consumption rate was not significantly different between banded kokopu and black mudfish at any ambient oxygen concentration level (Tukey's HSD: $P > 0.05$, Appendix 3.5). At lower ambient oxygen concentration levels of 4.8, 3.5 and 2.5 mg L^{-1} oxygen consumption rate was not significantly different between any species ($ANOVA_{4.84\ mg\ L^{-1}}$: $F_{2,27} = 2.6749$, $P = 0.0871$; $ANOVA_{3.46\ mg\ L^{-1}}$: $F_{2,24} = 2.5634$, $P = 0.0980$; $ANOVA_{2.51\ mg\ L^{-1}}$: $F_{2,19} = 1.9676$, $P = 0.1673$, Figure 3.3).

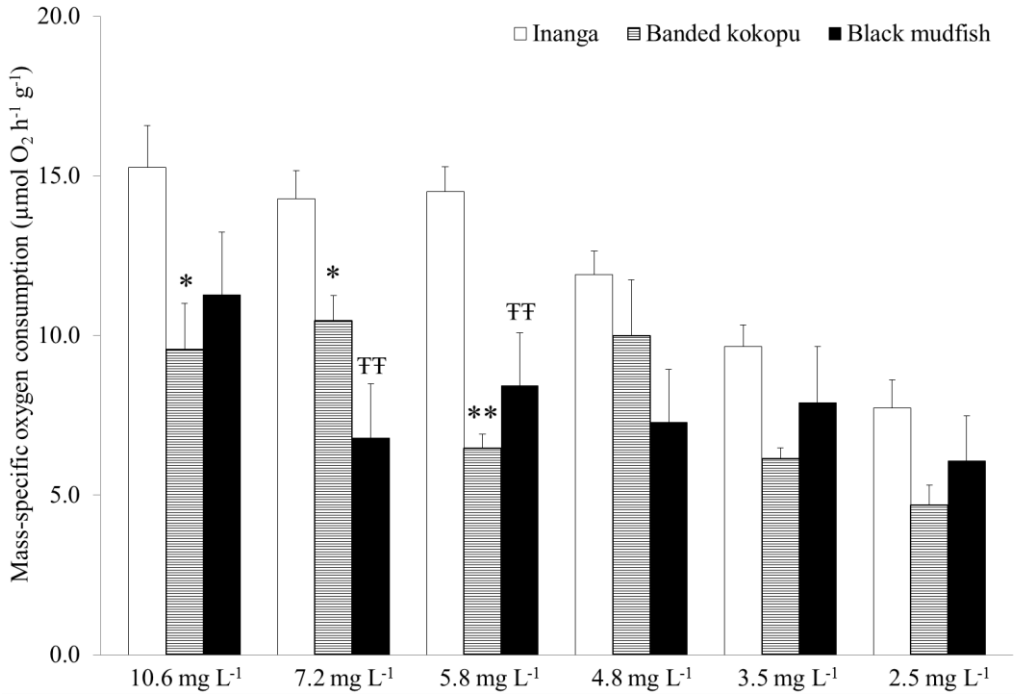


Figure 3.3: Comparative mass-specific oxygen consumption rate $\dot{M}O_2$ (µmol O_2 h^{-1} g^{-1}) in inanga (white), banded kokopu (grey) and black mudfish (black) at six distinct ambient oxygen concentration levels, presented as mean ± SE. Statistical significant differences between inanga and banded kokopu are indicated by * ($P < 0.05$) and ** ($P < 0.01$). Statistical significant differences between inanga and black mudfish are indicated by Ŧ ($P < 0.05$) and ŦŦ ($P < 0.01$).

3.4.2 Mass-independent oxygen consumption

The relationship between log-transformed total oxygen consumption rate and log-transformed wet mass was investigated at normoxic ambient oxygen concentration (10.6 mg L^{-1}). Mass-specific $\dot{M}O_2$ in inanga increased with increasing body mass, displaying a positive relationship between fish mass and oxygen consumption rate, thus larger fish exhibited higher oxygen consumption rate values than smaller fish (Figure 3.4 A). Banded kokopu and black mudfish showed a negative relationship between fish mass and oxygen consumption rate, as $\dot{M}O_2$ decreased with increasing body mass. Correspondingly, in these two species smaller fish presented higher oxygen consumption rate values than larger fish (Figure 3.4 B, C).

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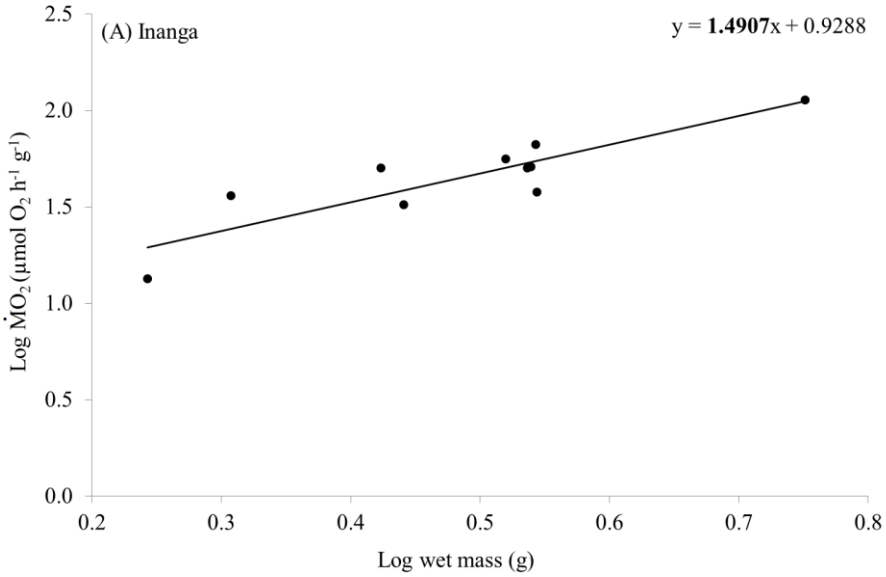
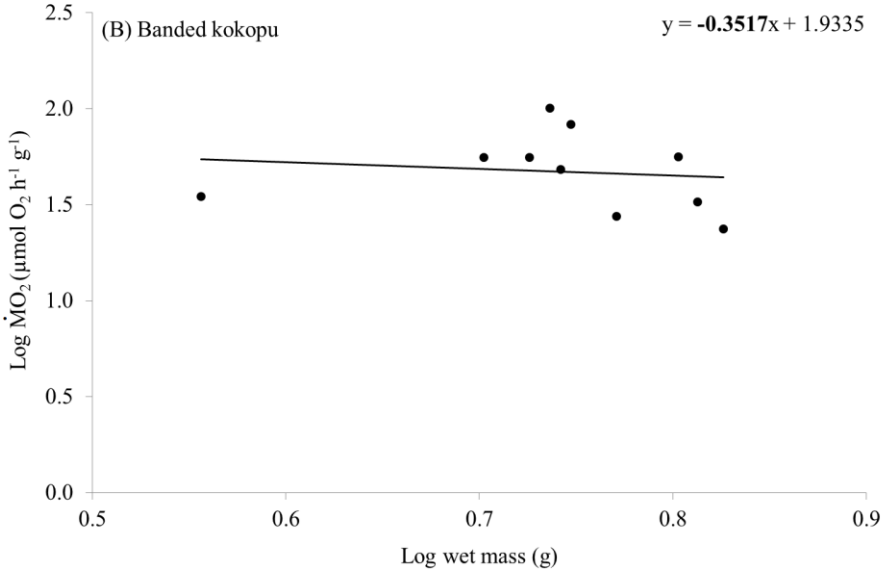
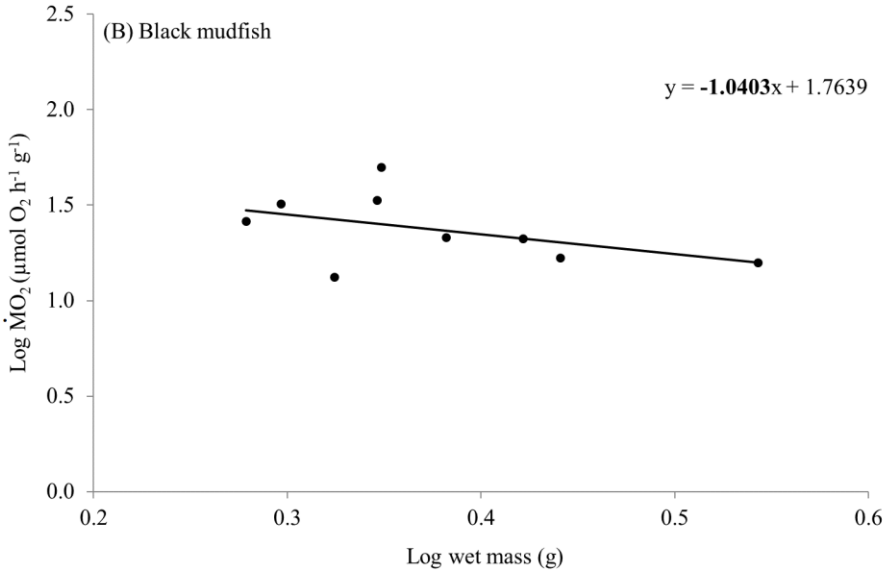
Figure 3.4: Least squares linear regression of the relationship between log-transformed total oxygen consumption rate (μmol O_2 h^{-1}) and log-transformed wet mass (g) in inanga (A; n = 10), banded kokopu (B; n = 10) and black mudfish (C; n = 9).


(A) Inanga
y = **1.4907**x + 0.9288
Log $\dot{M}O_2$ (µmol O_2 h^{-1} g^{-1})
Log wet mass (g)
2.5
2.0
1.5
1.0
0.5
0.0
0.2
0.3
0.4
0.5
0.6
0.7
0.8


(B) Banded kokopu
y = **-0.3517**x + 1.9335
Log $\dot{M}O_2$ (µmol O_2 h^{-1} g^{-1})
Log wet mass (g)
2.5
2.0
1.5
1.0
0.5
0.0
0.5
0.6
0.7
0.8
0.9


(B) Black mudfish
y = **-1.0403**x + 1.7639
Log $\dot{M}O_2$ (µmol O_2 h^{-1} g^{-1})
Log wet mass (g)
2.5
2.0
1.5
1.0
0.5
0.0
0.2
0.3
0.4
0.5
0.6

The relationship between total oxygen consumption rate and body mass reflected distinct variabilities between inanga and both, banded kokopu and black mudfish (Figure 3.4). Consequently, species-specific body mass scaling coefficients derived from distinct least square linear regression equations did produce markedly unrealistic results for species-comparative mass-independent oxygen consumption rate. Thus, a standardised body mass scaling coefficient of 0.67 (Heusner, 1985; Hopkins & Cech, 1994) was applied to oxygen consumption rate data from all three species.

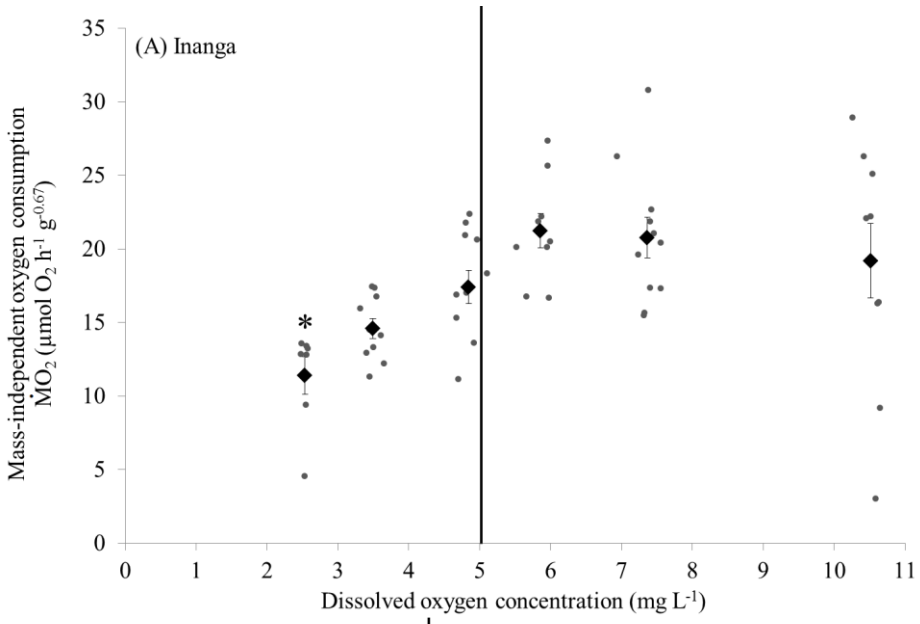
Mass-independent oxygen consumption rate in inanga did not differ significantly between ambient oxygen concentration levels of 10.6, 7.2 and 5.8 mg L^{-1} (Tukey's HSD for unequal n: $P > 0.05$, Appendix 3.6, pooled mean ± SE: 20.4 ± 1.0 µmol O_2 h^{-1} $g^{-0.67}$). Oxygen consumption rate appeared to decrease continuously at levels of 4.8, 3.5 and 2.5 mg L^{-1} to 17.4 ± 1.1, 14.6 ± 0.7 and 11.3 ± 1.3 µmol O_2 h^{-1} $g^{-0.67}$ respectively, constituting 90.7, 76.0 and 59.3% of normoxic $\dot{M}O_2$. Oxygen consumption rate at 3.5 mg L^{-1} was significantly lower than at 5.8 mg L^{-1}, while oxygen consumption rate at 2.5 mg L^{-1} was significantly lower than at 10.6, 7.2 and 5.8 mg L^{-1} (ANOVA: $F_{5,52} = 5.5559$, $P = 0.0004$, Tukey's HSD for unequal n: $P < 0.05$, Appendix 3.6, Figure 3.5 A).

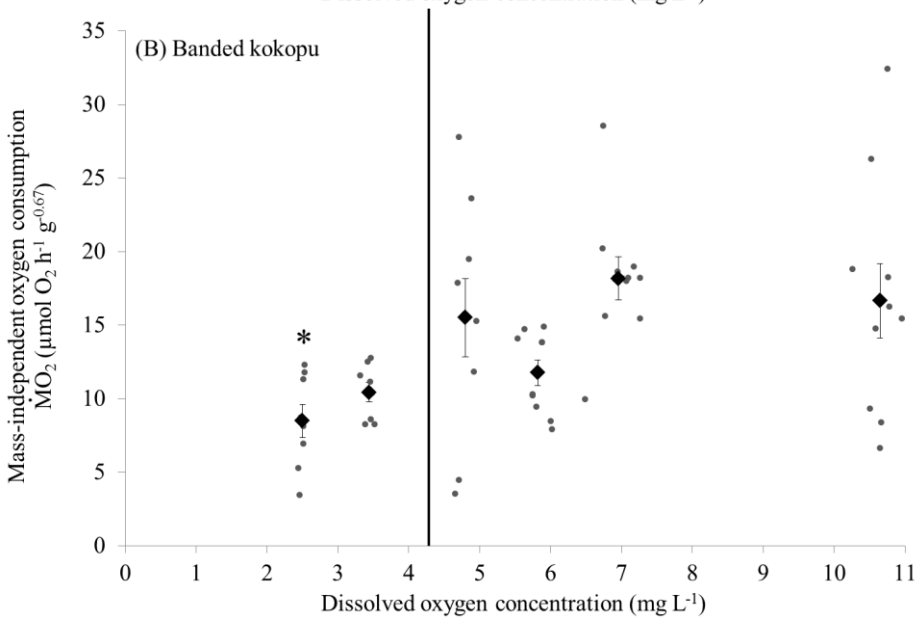
In banded kokopu, no distinct decrease in mass-independent $\dot{M}O_2$ occurred between 10.6 and 7.2 mg L^{-1} DO concentration (Tukey's HSD for unequal n: $P > 0.05$, Appendix 3.7, pooled mean ± SE: 17.4 ± 1.4 µmol O_2 h^{-1} $g^{-0.67}$). At 5.8 mg L^{-1}, $\dot{M}O_2$ decreased to 11.8 ± 0.9 µmol O_2 h^{-1} $g^{-0.67}$, which constituted 70.6% of normoxic $\dot{M}O_2$. This was followed by an increase to 15.5 ± 2.7 µmol O_2 h^{-1} $g^{-0.67}$ at 4.8 mg^{-1} DO. At 3.5 and 2.5 mg L^{-1}, $\dot{M}O_2$ declined continuously to 10.4 ± 0.7 and 8.5 ± 1.1 µmol O_2 h^{-1} $g^{-0.67}$, respectively, which constituted 62.6 and 51.0% of

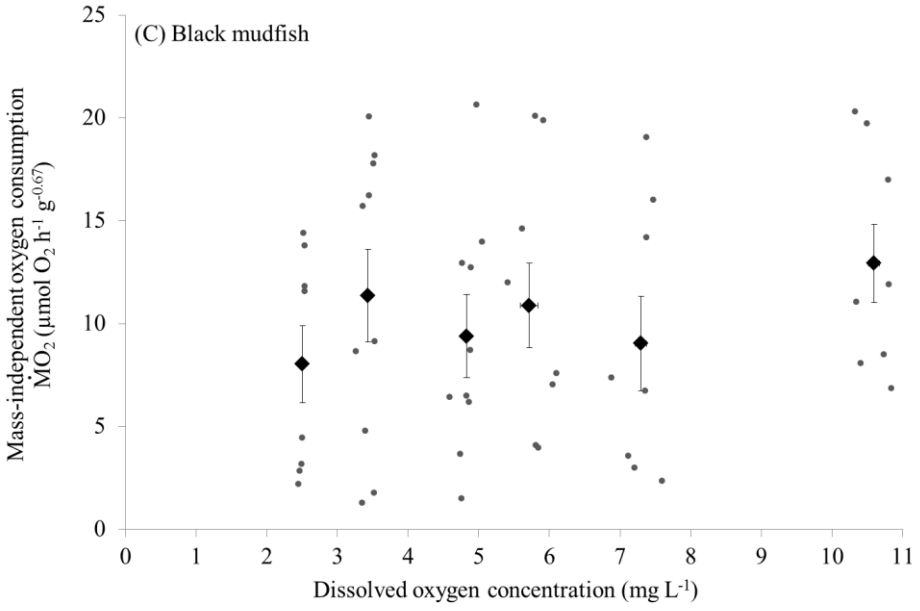
normoxic $\dot{M}O_2$. Oxygen consumption rate at 2.5 mg L^{-1} was hereby significantly lower than at 10.6 and 7.2 mg L^{-1} (ANOVA: $F_{5,49} = 4.4464$, $P = 0.0020$, Tukey's HSD for unequal n: $P < 0.05$, Appendix 3.7, Figure 3.5 B).

Mass-independent oxygen consumption rate in black mudfish was not significantly different between any ambient oxygen concentration levels (ANOVA: $F_{5,46} = 0.6893$, $P = 0.6341$). Between 10.6 and 7.2 mg L^{-1}, $\dot{M}O_2$ decreased from 12.9 ± 1.9 to 9.0 ± 2.3 µmol O_2 h^{-1} $g^{-0.67}$. At ambient oxygen concentration levels of 7.2, 5.8, 4.8, 3.5 and 2.5 mg L^{-1} no distinct change in $\dot{M}O_2$ occurred (pooled mean ± SE: 9.8 ± 0.9 µmol O_2 h^{-1} $g^{-0.67}$), 76.1% of normoxic oxygen consumption rate (Figure 3.5 C).

Figure 3.5: Mass-independent oxygen consumption rate $\dot{M}O_2$ (µmol O_2 h^{-1} $g^{-0.67}$) of inanga (A), banded kokopu (B) and black mudfish (C) in intermittent-flow respirometry at decreasing ambient dissolved oxygen concentrations with individual measurements (grey) and mean oxygen consumption rate at each dissolved oxygen concentration level (black rectangles). Vertical error bars are standard error of mean $\dot{M}O_2$ and horizontal error bars are standard error of dissolved oxygen concentration level. Statistical significant differences between normoxic and distinct hypoxic $\dot{M}O_2$ are marked by * ($P < 0.05$). Vertical black line indicates mean critical ambient oxygen concentration (C_{crit}) at which oxygen consumption shifts from oxyregulation to oxyconform respiration.


(A) Inanga
Mass-independent oxygen consumption
MO_2 (µmol O_2 h^{-1} $g^{-0.67}$)
Dissolved oxygen concentration (mg L^{-1})
*


(B) Banded kokopu
Mass-independent oxygen consumption
MO_2 (µmol O_2 h^{-1} $g^{-0.67}$)
Dissolved oxygen concentration (mg L^{-1})
*


(C) Black mudfish
Mass-independent oxygen consumption
MO_2 (µmol O_2 h^{-1} $g^{-0.67}$)
Dissolved oxygen concentration (mg L^{-1})

No inter-species differences in mass-independent oxygen consumption rates were observed at 10.2, 3.5 and 2.5 mg L^{-1} ($ANOVA_{10.6\ mg\ L^{-1}}$: $F_{2,25} = 1.6085$, $P = 0.2202$; $ANOVA_{3.5\ mg\ L^{-1}}$: $F_{2,25} = 2.1287$, $P = 0.1401$; $ANOVA_{2.5\ mg\ L^{-1}}$: $F_{2,20} = 1.4482$, $P = 0.2586$). Mass-independent oxygen consumption rate in banded kokopu was significantly lower than the oxygen consumption rate exhibited by inanga at 5.8 mg L^{-1} DO (Tukey's HSD for unequal n: $P = 0.0004$). Oxygen consumption rate in black mudfish, however, was distinctly lower than in inanga at 7.2, 5.8 and 4.8 mg L^{-1} (Tukey's HSD for unequal n: $P_{7.2\ mg\ L^{-1}} = 0.0005$; $P_{5.8\ mg\ L^{-1}} = 0.0002$; $P_{4.8\ mg\ L^{-1}} = 0.0244$). Similarly, banded kokopu exhibited significantly higher mass-independent oxygen consumption rate than black mudfish at 7.2 mg L^{-1} (Tukey's HSD for unequal n: $P = 0.0046$, Appendix 3.9, Figure 3.6).

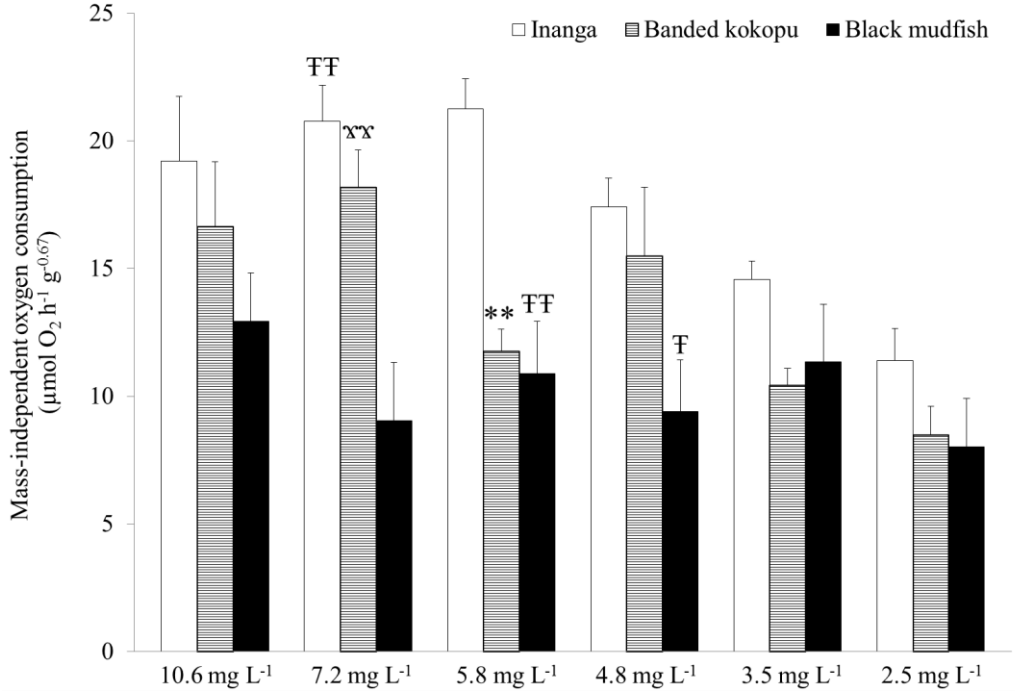


Figure 3.6: Comparative mass-independent oxygen consumption rate $\dot{M}O_2$ (µmol O_2 h^{-1} $g^{-0.67}$) in inanga (white), banded kokopu (grey) and black mudfish (black) at six distinct ambient oxygen concentration levels, presented as mean ± SE. Statistically significant differences between inanga and banded kokopu are indicated by ** ($P < 0.01$), between inanga and black mudfish by Ŧ ($P < 0.05$) and ŦŦ ($P < 0.01$), and between banded kokopu and black mudfish by ϫϫ ($P < 0.01$).

3.4.3 Critical oxygen concentration

Mass-specific and mass-independent oxygen consumption rates in inanga and banded kokopu revealed oxyregulatory mechanisms at elevated ambient dissolved oxygen concentration levels and oxyconforming respiration at lower levels (Figure 3.2 and Figure 3.5). The critical ambient oxygen concentration at which $\dot{M}O_2$ started to decline with decreasing ambient oxygen concentration (C_{crit}) was not statistically different between inanga (n = 9) and banded kokopu (n = 5; ANOVA: $F_{1,12}$ = 1.8795, P = 0.1955), at 5.0 ± 0.4 and 4.3 ± 0.1 mg L^{-1}, respectively. The black mudfish oxygen consumption pattern did not facilitate any C_{crit} calculation.

3.5 Discussion

3.5.1 Oxyregulator or oxyconformer?

All three species, inanga, banded kokopu and black mudfish, demonstrated oxyregulatory responses. In mild hypoxia, inanga and banded kokopu exhibited sustained oxygen consumption rates which approximated normoxic observations. With further decreasing ambient DO levels, oxygen consumption rate ceased to be maintained and declined in dependence of the ambient DO level. These observations characterise both species as oxyregulators with distinct critical ambient DO concentrations (C_{crit}) at which the oxygen uptake strategy shifted from oxyregulation to oxyconformation (Figure 3.2 A, B; (Pörtner & Grieshaber, 1993)), a mechanism commonly observed in teleost species. The oxygen consumption pattern observed in black mudfish however did not permit a definite classification of this species. Here, the oxygen consumption rate demonstrated a small initial decrease and fluctuated around a steady level thereafter for all subsequent DO concentrations tested. As the respirometry chamber did not restrict fish movement, the initial decrease in oxygen consumption rate may have occurred in coherence with a decrease in activity, reducing the metabolic rate and hence oxygen demand. Similar strategies have previously been observed in Florida smoothhound shark (*Mustelus norrisi*; (Carlson & Parsons, 2001)), Atlantic cod (*Gadus morhua*; (Herbert & Steffensen, 2005; Johansen *et al.*, 2006)), golden grey mullet (*Liza aurata*; (Lefrançois *et al.*, 2005)), lesser sandeel, (*Ammodytes tobianus*; (Behrens & Steffensen, 2007)) and striped surfperch (*Embiotoca lateralis*; (Cook *et al.*, 2014)). The maintenance of oxygen consumption at mild and low levels of hypoxia is suggestive of a pronounced hypoxia tolerance and an oxyregulatory strategy in black mudfish. In this context

it is possible, that the DO levels, at which oxygen consumption rate was tested in this study, were not severe enough to elicit a shift from oxyregulation to oxyconformation at a distinct C_{crit}. Future investigation of metabolic rate and oxygen uptake in black mudfish during more severe hypoxia levels would therefore be of great value in furthering our comprehension of the physiological mechanisms enabling black mudfish to withstand markedly hypoxic environments (Figure 3.2 C).

Inanga mass-specific oxygen consumption rate at normoxia and distinct hypoxic DO levels were comparable with, albeit slightly elevated in comparison to, Urbina *et al*. (2012). This may have been caused by the difference in experimental temperature (16 - 18°C versus 14°C in Urbina *et al*. (2012)), or by different metabolic demands of the fish studied. The observations on the oxygen consumption strategy in inanga are, however, in stark contrast to recent findings from Urbina *et al*. (2012) and Urbina and Glover (2013), where inanga displayed a distinct oxyconforming strategy, characterised by a continuous decrease in oxygen consumption rate with declining ambient DO concentration. The sample number of both fish and DO levels, was smaller in the present study, which may have resulted in such distinctly different observations. More likely however, the order of distinct DO levels at which oxygen consumption rate was established, may have elicited the different findings. Contrary to Urbina *et al*. (2012), DO levels were not applied in decreasing, but in pseudo-randomised order, to avoid an adjustment in metabolic demand and hence reduction of oxygen consumption rate in response to a declining order of DO levels. This underlines the importance of comparable, standardised experimental protocols for comparative studies on responses to environmental variables in fish. Furthermore, inanga have been

shown previously to respond to decreasing ambient DO concentrations with an elevated opercula frequency (Urbina *et al.*, 2011), a mechanism that increases the ventilation of the gill epithelia in oxyregulating species (Randall & Shelton, 1963) and thereby maintains the amount of oxygen absorbed at the gills. The increased ventilation is hereby maintained during decreasing ambient DO concentration levels until a critically low ambient DO concentration (C_{crit}) level is reached, at which point the cost for increased ventilation exceeds the benefit of additional oxygen absorbed, and a shift to oxyconformation occurs (Fritsche & Nilsson, 1993). In this context, it has been shown that sustained hyperventilation may be accompanied by respiratory alkalosis (Perry & Gilmour, 2006). The partial pressure of CO_2 in the blood decreases in hyperventilating fish during hypoxia, therefore, a decrease in respiratory rate may be induced to re-establish the blood acid-base balance (Ern & Esbaugh, 2016). However, the aforementioned decrease in blood-CO_2 due to hyperventilation is a minor effect, which may not elicit significant adjustments in respiration patterns.

3.5.2 Species-specific oxygen consumption and C_{crit}

Oxyregulation is a common strategy among teleost species (Rogers *et al.*, 2016), which enables a maintained oxygen uptake in changing environmental DO concentrations. Below a distinct critical threshold in environmental DO level (C_{crit}), however, oxygen uptake declines with decreasing DO concentration, which has been shown to correspond with a shift from aerobic to anaerobic metabolism (Pörtner & Grieshaber, 1993). Species-specific C_{crit} has therefore been utilised as a measure of oxygen sensitivity in numerous fish species (Rogers *et al.*, 2016).

The critical ambient DO concentration C_{crit}, at which oxyregulatory mechanisms ceased and oxygen consumption rate declined, was 5.0 ± 0.4 mg L^{-1} for inanga and 4.3 ± 0.1 mg L^{-1} for banded kokopu. This is in agreement with previous observations on behavioural responses to hypoxia and the hypoxia avoidance threshold (Chapter 2), where inanga was found to initiate reduced residence time and horizontal migration into a normoxic refuge at mild hypoxia below 5.9 mg L^{-1}, just above their specific C_{crit}. These findings suggest that inanga is a markedly hypoxia-sensitive species, with little physiological response mechanisms developed in adaptation to hypoxia, while capacity for swimming activity and metabolic rate, hence oxygen demand and consumption, are greater in inanga than in banded kokopu and black mudfish (Meredith, 1985), thereby eliciting the previously observed, early behavioural avoidance response. Inanga oxygen consumption rate, however, was similar to that exhibited by banded kokopu at all ambient DO levels of this study, except at 5.8 mg L^{-1}, where banded kokopu oxygen uptake was significantly lower than in inanga. This similarity indicates comparable oxygen demand and routine metabolic rate between these two species. In contrast, black mudfish oxygen uptake was lower than in inanga and banded kokopu at distinct DO levels, which suggests a lower routine metabolic rate, and thus a lower energy and oxygen demand in this species (Figure 3.3 and Figure 3.6). Banded kokopu C_{crit} of 4.3 mg L^{-1} was lower than in inanga, classifying them as a more hypoxia-tolerant species. The C_{crit} was, however, well above the previously observed hypoxia avoidance threshold of 2.3 mg L^{-1} (Chapter 2). Similar observations have been made in Atlantic cod (*Gadus morhua* L. (Herbert *et al.*, 2011)) and New Zealand snapper (*Pagrus auratus* (Cook & Herbert, 2012a)), which did not limit excursions into a progressively

hypoxic habitat with DO levels well below their respective C_{crit}, albeit with the onset of anaerobic metabolism eliciting physiological stress, as seen with an accumulation of anaerobic end products.

The suggestion that black mudfish is a highly hypoxia-tolerant, oxyregulating fish species, whose C_{crit} could not be ascertained within the scope of this study, is supported by preceding observations as well, where black mudfish demonstrated no behavioural response to, or avoidance of, hypoxic DO levels of as low as 1.2 mg L^{-1} (Chapter 2).

Comparable species-specific observations of distinct differences in critical DO concentration and hypoxia sensitivity have previously been seen in several cichlid and elephant fish species, which exhibited distinctly lower C_{crit} levels and hence increased hypoxia tolerance, when inhabiting swamp habitats in contrast to those found in lakes (Chapman *et al.*, 2002). Similar results were obtained for Egyptian mouthbrooder, which exhibited a decreased resting metabolic rate and reduced C_{crit}, when raised under hypoxic conditions, which indicated developmental plasticity in hypoxia tolerance within the same species (Reardon & Chapman, 2010).

It has been previously shown that hypoxia sensitivity and C_{crit} may depend on fish size, as large oscar (*Astronotus ocellatus*) were able to maintain oxyregulation to a lower ambient DO level than smaller fish (Sloman *et al.*, 2005). In contrast, it has been demonstrated for largemouth bass (*Micropterus salmoides*), that smaller fish are utilising more hypoxic water than larger fish (Burleson *et al.*, 2001). A more recent comprehensive review, however, has established that body size on its own has little or no impact on the ability for

oxygen uptake under hypoxic conditions (Nilsson & Östlund-Nilsson, 2008). Size-related differences in oxygen uptake abilities within one species are therefore proposed to reflect distinct life strategies or habitat preferences. Nonetheless, an advantage was detected for large fish during anaerobic glycolysis during severe hypoxia, as these have a larger reservoir of glycogen and a lower mass-specific metabolic rate, thereby reaching lethal levels of accumulated anaerobic end products later than smaller fish. Fish in the present study were, nevertheless, selected from similar size ranges within and between the species, to minimise any size-related effect on hypoxia sensitivity observed.

3.5.3 Dependence of oxygen consumption on body mass and scaling exponent

When studying oxygen consumption rate and performing inter-species comparison to elucidate species-specific differences in hypoxia sensitivity and response mechanisms, it is essential to consider the effect of body mass on metabolic rate and hence on oxygen consumption rate, especially when the fish studied represent a wide separation in body size. Metabolic demand and oxygen consumption rate have been shown to increase with body mass for a range of species from different phyla (Zeuthen, 1953; White *et al.*, 2006), including teleosts (Caulton, 1978; Clarke & Johnston, 1999; Urbina & Glover, 2013). Numerous studies on teleost species have been undertaken to investigate the dependence of oxygen consumption rate on various environmental factors, such as temperature (Caulton, 1978; Hopkins & Cech, 1994; Clarke & Johnston, 1999), salinity (Meloni *et al.*, 2002; Cao & Wang, 2015) and dissolved oxygen concentration (Burggren & Randall, 1978; Fischer *et al*., 1992; Urbina *et al*., 2012). Frequently the oxygen consumption rate was expressed as mass-

independent observation by applying a mass scaling coefficient, which most adequately expressed the relationship between oxygen consumption rate and body mass. The application of species-specific mass scaling coefficients, derived from this experimental setting would have been ideal for mass-correction of oxygen consumption rate. The specific relationships between body mass and total oxygen consumption rate, however, yielded pronounced variability between the three species, possibly due to the limited sample number and small size range of fish utilised in this study. Therefore, mass-independent oxygen consumption rate calculations produced nonsensical data, inappropriate for further analysis and a literature-derived scaling coefficient was utilised. In this context, a standard mass scaling exponent that enables comparative studies on species of different sizes, but similar body shapes, has been previously established by Heusner (1985) and was subsequently utilised in comparative respirometry studies (Hopkins & Cech, 1994; Meloni *et al*., 2002).

The positive relationship between oxygen consumption rate and body mass in inanga (Figure 3.4 A) was similar to findings from Urbina and Glover (2013), where larger fish consumed more oxygen than smaller at a range of ambient DO levels. These findings are also in agreement with the positive body mass - metabolic rate relationship from 138 studies on 69 teleost species, compiled to an extensive database by Clarke and Johnston (1999), albeit the specific inanga scaling coefficient of 1.49 resides above the range of scaling exponents derived in the aforementioned survey study. In contrast, the relationship between body mass and oxygen consumption rate was negative for banded kokopu and black mudfish, with larger fish exhibiting lower oxygen uptake than smaller fish. The relationship may have been affected by different

activity levels of individual fishes, as the respirometry chamber did not impede fish movements. Thus, smaller banded kokopu and black mudfish potentially may have been more active than larger during the cause of this experiment, thereby increasing their metabolic rate and oxygen consumption rate. In addition, the limited sample number and narrow body mass range of fish utilised in this study, may have elicited an increased sensitivity for potential outliers as well.

3.5.4 Potential errors in oxygen consumption rate measurements

Detrimental effects on oxygen consumption rate measurements inherent to closed and open respirometry were reduced in this study by utilising intermittent-flow respirometry (Steffensen, 1989; Svendsen *et al.*, 2016b), providing respirometer water changes and pronounced resting periods in flow-through normoxia between respirometric measurements at different DO levels. Furthermore, specific ambient DO levels were not established by leaving the fish in the closed chamber to deplete DO concentration to a certain level, but by manually adjusting the DO concentration in the ambient water. DO levels were hereby not applied in sequential, decreasing order, but in a pseudo-randomised way.

Recent work has established the estimated measurement error of oxygen uptake as a function of measurement time period and respirometer volume : fish mass ratio in the common roach (*Rutilus rutilus*, (Svendsen *et al*., 2016a). In this context, the measurement error decreases with smaller respirometer volume : fish mass ratio, and with longer measurement time period. Applied to this study with a 30 minute measurement period and a respirometer volume : fish mass ratio of 1:282 for inanga, 1:156 for banded kokopu, and 1:360 for black mudfish, the estimated measurement error may be assumed to be as low as 1 - 2% for inanga and banded kokopu, and 2 - 4% for black mudfish.

The respirometer volume : fish mass ratios exceeded a previously suggested optimal ratio range between 1:20 and 1:100 (Clark *et al.*, 2013) and no water circulation mechanisms was installed within the respirometry chambers. However, a highly sensitive oxygen measuring system, which did not consume oxygen throughout the measurement process itself, was utilised. Therefore, declines in dissolved oxygen concentration occurred steadily and were not erratic, which demonstrates that no overestimated or insufficiently resolved measurements of oxygen uptake were taken despite the larger respirometer volume : fish mass ratios. Furthermore, the oxygen uptake levels determined in inanga were comparable to those demonstrated previously by (Urbina *et al.*, 2012), where smaller respirometer volume : fish mass ratios had been utilised.

3.6 Conclusions

Inanga and banded kokopu were characterised as oxyregulators, while black mudfish was indicated as a strict oxyregulator. Oxygen consumption rates and hypoxia sensitivity as well as ability to respond to hypoxia with physiological mechanisms, however, were markedly different. Inanga and banded kokopu mostly displayed comparable oxygen consumption rates, indicative of a similar metabolic rate and oxygen demand. Nonetheless, inanga demonstrated a comparatively high C_{crit}. Banded kokopu, on the other hand, was a less hypoxia-sensitive species with a lower C_{crit}. In this context, fish adapted to hypoxic environments often display not only a low C_{crit}, but also a decreased P50 reflecting the oxygen partial pressure of the blood, at which the hemoglobin is 50% saturated with oxygen. Thus, fish with lower P50 values exhibit haemoglobin with an increased affinity for binding oxygen (Moyes & Schulte, 2006), thereby increasing the amount of oxygen extracted from the hypoxic environments. It was, however, beyond the scope of this research to investigate these parameters. Oxygen consumption rates in black mudfish suggest a comparatively lower routine metabolic rate, and no C_{crit} could be ascertained, possible due to not testing more severe levels of hypxoxia. These findings provide further evidence that inanga, banded kokopu and black mudfish exhibit distinct hypoxia sensitivities, mediated by species-specific response mechanisms to hypoxia, and offers explanation for their distinct differences in habitat requirements and preferences.

4 Responses to moderate hypoxia: Elicited by molecular adaptations?

4.1 Abstract

Freshwater environments inherently feature low-oxygen (hypoxic) episodes, yet fish display distinct species-specific hypoxia sensitivities and response mechanisms, which often can be observed to be reflected within specific habitat requirements and life history strategies. By acclimation to hypoxia for 96 h, omitting surface access and oxygen refuge, the effect of moderate hypoxia on swimming behaviour as a measure of metabolic oxygen demand, gill morphological plasticity, oxygen sensing neuroepithelial cell (NEC)- and hypoxia inducible factor 1 (HIF-1) alpha density was investigated in three related galaxiid species; inanga (*Galaxias maculatu*s), banded kokopu (*Galaxias fasciatus*) and black mudfish (*Neochanna diversus*). Swimming speed decreased significantly from 21.6 ± 2.6 to 7.4 ± 2.0 BL min^{-1} in inanga, while no change was seen in banded kokopu and black mudfish. All three species presented NECs in the gill epithelia; however, hypoxia exposure did not elicit gill morphological adaptations or changes in NEC- and HIF-1 alpha density in any species, while comparatively wider respiratory lamellae in black mudfish indicated an adaptation to emersion and aestivation frequently observed in this species. HIF-1 alpha protein stabilisation at normoxic conditions indicated distinct differences between mammalian and piscine HIF-1 alpha pathway and hypoxia-inducible gene transcription. These findings demonstrate distinct differences in hypoxia sensitivity and response between inanga, banded kokopu and black mudfish, however preclude the mediation of hypoxia responses via adaptations of NEC- and HIF-1 alpha density.

4.2 Introduction

4.2.1 Aquatic hypoxia and piscine metabolism

In contrast to terrestrial animals, water-breathing fish utilise a respiratory medium which provides limited oxygen by comparison, due to the physico-chemcial principles of decreased oxygen solubility and diffusion rate in water (Graham, 1990). In addition, aquatic ecosystems are naturally subjected to fluctuations in dissolved oxygen (DO) concentrations, elicited by an increase in biological oxygen consumption which exceeds photosynthetic and atmospheric oxygen re-supply (Friedrich *et al.*, 2014) or by water-body stratification (Diaz, 2001), resulting in low-oxygen (hypoxic) or oxygen-absent (anoxic) environments (Kramer, 1987). Therefore fish commonly encounter environments with decreased DO concentrations (Kramer, 1987), more so since global anthropogenic impacts are causing an expansion, and more frequent occurrence, of hypoxic or anoxic habitats (Diaz & Rosenberg, 2008; Rabalais *et al.*, 2010).

Oxidative metabolism, fuelled by aerobic respiration, is the primary energy production strategy in fish (Moyes & Schulte, 2006). While anaerobic metabolism is a principle alternative for energy production, especially in the context of reduced environmental oxygen availability, its efficiency is markedly decreased (Richards, 2009) and rapidly exceeded by highly energy-consuming physiological processes, especially at increased temperatures (Chabot *et al.*, 2016). Furthermore, anaerobic fermentation of glycogen produces detrimental metabolites such as protons and lactate, necessitating metabolic decomposition and physiological recovery (Vianen *et al.*, 2001; Cook & Herbert, 2012b). Accumulation of waste products at toxic levels and ATP production below levels necessary to sustain metabolic processes may induce necrotic cell-, and

ultimately, hypoxic death (Richards, 2009). Fish therefore exhibit a wide range of responses to hypoxia, ensuring a physiological level of oxygen uptake at varying environmental DO concentrations.

4.2.2 Piscine hypoxia responses and their sensory and nervous control

Behavioural responses include vertical and horizontal migration to avoid spatially limited hypoxic zones (Kramer, 1987; Pihl *et al.*, 1991; Breitburg, 1994; Eby & Crowder, 2002; Ludsin *et al.*, 2009) and utilisation of oxygen-rich water masses (e.g. the water/air interface using aquatic surface respiration (Kramer & McClure, 1982; Shingles *et al.*, 2005; Dwyer *et al.*, 2014)), 'bubble respiration' (McPhail, 1999), facultative air breathing (Johansen, 1970; Meredith, 1985; Kramer, 1987) and emersion (Sayer, 2005; Regan *et al.*, 2011; Urbina *et al.*, 2011). Additionally, fish commonly respond to hypoxia with distinct changes in swimming activity and speed. However, no generalist strategy has emerged, as reduced swimming speed (Nilsson *et al.*, 1993; Skjæraasen *et al.*, 2008; Herbert *et al.*, 2011; Cook & Herbert, 2012a), as well as increased swimming speed (Dizon, 1977; Bushnell & Brill, 1991; Skjæraasen *et al.*, 2008; Poulsen *et al.*, 2011) have been observed, while some fish demonstrated no swimming speed adjustments (Dizon, 1977; Herbert *et al.*, 2011; Cook & Herbert, 2012b). This implies an intricate correlation with additional environmental factors such as temperature, as well as species-specific life-strategies and hypoxia tolerances, and even the availability of normoxic refuge.

Physiological responses maintaining oxygen uptake from hypoxic environments include circulatory adaptations such as bradycardia (decreased heart rate; (Bushnell & Brill, 1991; Claireaux *et al.*, 1995; Shingles *et al.*, 2005)) in combination with increased stroke volume and aortic blood pressure (Randall,

1982). In addition, respiratory adjustments may occur, initiating an increase in breathing rate (Balfour, 1999; Shingles *et al.*, 2005), which is often observed as increased opercula-beat frequencies (Urbina *et al.*, 2011), as well as elevated ventilation stroke volumes (Randall, 1982; Bushnell & Brill, 1991). Further adjustments include an increase in haemoglobin concentration and haemoglobin-oxygen affinity (Randall, 1982; Robb & Abrahams, 2003; Wu *et al.*, 2016).

Gill-morphological adaptations to hypoxia commonly involve apoptotic reduction of interlamellar cell mass which results in an increased respiratory surface area, potentially at the expense of elevated osmoregulatory costs (Nilsson, 2007) and which has been observed in numerous fish species, such as crucian carp (*Carassius carassius*; (Sollid *et al.*, 2003)), goldfish (*Carassius auratus*; (Mitrovic *et al.*, 2009)), Qinghai carp (*Gymnocypris przewalskii*; (Sollid & Nilsson, 2006)) and Atlantic stingray (*Dasyatis sabina*; (Dabruzzi & Bennett, 2013)). Other adaptations include raised lamellae and lamellar enlargement, reduced thickness of the blood-water barrier, and an increased gill vascular space/lamellar epithelium ratio (Laurent & Perry, 1991).

Physiological and behavioural adjustments to hypoxia have been demonstrated to be under nervous control (Randall, 1982; Burleson & Smatresk, 1990a; Florindo *et al.*, 2006), elicited by distinct, oxygen-sensitive chemoreceptive cells, responding to internal (blood) and external (water) changes in dissolved oxygen concentrations (Smatresk, 1990; Burleson & Smatresk, 1990b). Previously, external oxygen sensing cells have been identified as serotonergic neuroepithelial cells (NECs) located in the gill (Dunel-Erb *et al.*, 1982; Sundin *et al.*, 1998; Jonz & Nurse, 2006) and epidermis (Jonz & Nurse, 2006; Rombough, 2010; Regan *et al.*, 2011). In response to decreasing

environmental DO concentrations, inhibited membrane-bound potassium channels of these receptors cause a degranulation of cytoplasmatic synaptic vesicles, which are oriented towards associated nerve fibres. Thus, the neurotransmitter serotonin is released, initiating hypoxia response pathways (Jonz & Nurse, 2003, 2006), one of which is the stabilisation of the heterodimeric transcription factor hypoxia inducible factor 1 (HIF-1). In mammalia, the HIF-1α gene-subunit has been shown to be continuously expressed, while the associated protein is constantly degraded in the normoxic cytoplasm (Semenza, 2001). Upon cellular hypoxia, the alpha (α)-protein is stabilised and dimerises with the β-protein in the nucleus, where the activated HIF-1 protein controls the transcription of numerous hypoxia-response genes involved in adaptive mechanisms ((Lee & Percy, 2011) and, for a review, see Wu (2002)). While it has been shown that different oxygen sensitivities in fish are not reflected in variations of the HIF-1α gene-sequence (Rytkönen *et al.*, 2007), hypoxia acclimation has demonstrably elicited an increase in HIF-1α expression in common carp (*Cyprinus carpio*; (Randall *et al.*, 2004)) and longjaw mudsucker (*Gillichthys mirabilis*; (Gracey *et al.*, 2001). In contrast, rainbow trout and chinook salmon (*Oncorhynchus tshawytscha*) cells have been shown to exhibit elevated HIF-1α accumulation already at DO levels common in venous blood under normoxic environmental conditions (Soitamo *et al.*, 2001).

4.2.3 Galaxiids

Inanga (*Galaxias maculatus*), banded kokopu (*Galaxias fasciatus*) and black mudfish (*Neochanna diversus*) are related fish species of the Galaxiidae family (Waters *et al.*, 2000), which exhibit distinct oxygen requirements and preferences, reflected in the occupancy of distinct freshwater environments in the North Island

of New Zealand (McDowall, 1990). In this context inanga are commonly observed in shallow, uncovered and slowly flowing lowland streams and estuaries, where they frequently encounter DO concentration fluctuations. Banded kokopu on the other hand are most commonly encountered in cool, covered forest streams, while black mudfish can be found in slowly flowing or still drains and wetlands with a tendency for hypoxia or anoxia and habitat-desiccation in the warm season, during which mudfish have been shown to aestivate (McDowall, 1990). The three species therefore exhibit distinct hypoxia sensitivities (Chapter 2) and may thus display different behavioural and morphological responses to hypoxia, while phylogenetically induced response variances are minimised.

4.2.4 Study objectives

As inanga, banded kokopu and black mudfish inhabit distinctly different environments in the context of DO concentrations and fluctuations, this study investigated if the three species exhibit different behavioural and gill-morphological adaptations to prolonged, inescapable hypoxia acclimation without access to the water surface or normoxia refuge. The study further examined the presence and hypoxia-induced variability of oxygen sensing NEC-density in the gill epithelium and whether behavioural hypoxia responses (e.g. swimming speed as measure of metabolic oxygen demand) are mediated by acclimation adjustments of HIF-1α in cells of the gill epithelium.

4.3 Methodology

4.3.1 Experimental fish

Adult inanga (body mass: 2.57 ± 0.19 g; total length: 69.0 ± 1.6 mm; data presented here and thereafter in mean ± SE) and immature, post-larval banded kokopu that are comparable in their physiology to adult fish (body mass: 4.40 ± 0.59 g; total length: 65.0 ± 3.3 mm) were purchased from a fish farm that provides natural habitat for upstream migrating whitebait at the conclusion of their marine larval development (Raglan EELS Ltd, Raglan, New Zealand). Adult black mudfish (body mass: 1.39 ± 0.07 g, total length 61.5 ± 0.9 mm) were caught with minnow traps from field drainage areas north-east of Hamilton in Waikato, New Zealand. All fish were transported to the Aquatic Research Facility at the University of Waikato, Hamilton, New Zealand. Fish were acclimatised to laboratory conditions in the Aquatic Research Facility for at least four weeks prior to experiments and kept in indoor tanks with dechlorinated tap water fitted with aquarium filters and supplementary aeration at a constant temperature of 16°C and a 12:12 light:dark photoperiodic cycle. To assist osmoregulation and reduce incidence of white spot disease, marine salt (Crystal Sea Marinemix, Marine Enterprises International) was added to inanga and banded kokopu tanks to a concentration of 3.5‰ and frequent water changes limited build-up of waste products. Black mudfish were housed in water of their respective habitat that was brought back to the facility with them, and therefore did not necessitate added salt. All fish were fed to satiation with frozen bloodworms every other day. All procedures and experiments followed the standard operating procedures for captive fish maintenance by the University of Waikato and were approved by the University of Waikato Animal Ethics Committee (protocol # 843).

4.3.2 Experimental device

To enable acclimation of fish to hypoxia while a control group is simultaneously exposed to identical, but normoxic conditions, a study device was constructed consisting of two separate compartments (Figure 4.1).

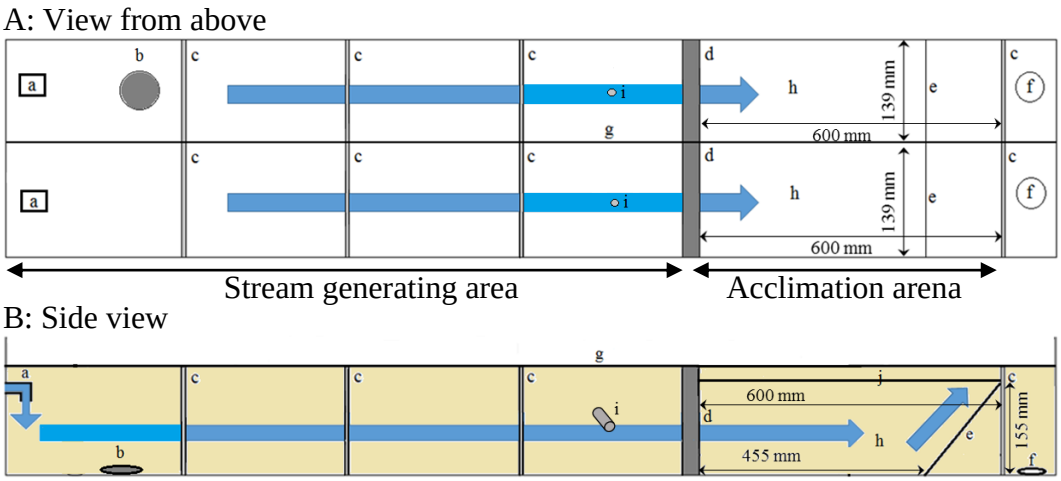


Figure 4.1: Hypoxia acclimation device as seen from above (A) and from the side (B) featuring two distinct compartments each equipped with an inlet (a), light diffuser panel sheets (c) and a honeycomb-structured sheet (d) for laminar water flow in the test arena (h), a 2 mm acrylic sheet separating the two compartments (g), a ramp to maintain an appropriate water level (e), an outlet (f) and an optical oxygen sensor (i). Both acclimation arenas are covered with a transparent acrylic sheet to inhibit aquatic surface respiration and emersion (j). The hypoxic side is furthermore equipped with a flat air stone (b) to supply gaseous oxygen-free nitrogen into the water for maintaining a hypoxic environment.

To achieve distinctly different oxygen concentrations in the acclimation arena, the study device was subdivided into two equal compartments by a transparent acrylic sheet of 2 mm thickness. Both compartments of the study device were fitted with light diffuser panel sheets and a honeycomb-structured sheet with 3x3 mm perforations to generate a laminar water flow in the acclimation arenas. Each arena measured 139 x 600 mm and ramps at the rear

section maintained a water depth of 155 mm. Both acclimation arenas were covered with a transparent acrylic sheet to inhibit aquatic surface respiration and emersion.

To maintain normoxia, air-saturated dechlorinated tap water from a normoxic water reservoir tank, fitted with a submerged recirculating water pump and an external air pump with a submerged air stone, was pumped into the study device via the appropriate inlet. After flowing through the study device, normoxic water was recycled back into the normoxic reservoir. To generate and maintain moderate hypoxia of 5 mg L^{-1} dissolved oxygen concentration, dechlorinated tap water from a hypoxic water reservoir tank fitted with submerged recirculating water pumps was pumped into a three-columned interconnected deoxygenation tower (Figure 1.8). The bottom of each column was fitted with a flat cylindrical gas diffuser approximating in size the diameter of the column. As water was flowing through the interconnected columns of the deoxygenation tower it was deoxygenated through the use of gaseous oxygen-free nitrogen that was led through each column via the gas diffusers. From the deoxygenation tower hypoxic water was flowing through the study device by entering via the inlet on the appropriate side and was recycled back into the hypoxic reservoir (Figure 1.7). When the required (DO) level was reached, the deoxygenation tower was disengaged from the study device and hypoxic water was recirculated from the hypoxic reservoir tank. Gaseous oxygen-free nitrogen at low flow rate was led into the hypoxic compartment through a flat air stone to compensate re-oxygenation occurring as water flowed from the ramp into the outlet.

A digital video camera was mounted above the test arena to record the behavioural responses of the fish throughout the experiment and a 2x2 cm grid

was placed underneath the test arena for movement reference. The acclimation arena was enclosed in black fabric to minimize fish disturbance during the experiments that may potentially affect the behavioural responses.

4.3.3 Experimental procedures

All experiments were carried out in groups of five fish. For each species 10 fish were exposed to 96 h of normoxia and moderate hypoxia at a DO concentration level of 5 mg L^{-1}, respectively. Experiments were carried out in a temperature-controlled room where the experimental temperature was maintained between 16 and 18.5°C. Temperature and DO concentration of both compartments were continuously monitored (min^{-1} measurements) with optical oxygen sensors placed directly before the acclimation arenas (Table 4.1). Before each experiment, optical oxygen meters were calibrated to 0% and 100% oxygen concentration using sodium dithionite saturated water and water saturated air, respectively.

Fish were kept unfed 24 h prior acclimatisation and were transferred to the acclimation arena of the study device where they were allowed to acclimatise to normoxic recirculating water for 17 h to account for the increase in metabolic rate due to handling and transition to a new environment (Poulsen *et al.*, 2011; Urbina *et al.*, 2011).

Prior to changing the DO concentration, fish were video recorded for 30 min and the resulting outcome of analysed swimming behaviour served as a reference for changes of the swimming behaviour during the course of the acclimation experiment. Subsequently the DO concentration in one compartment was decreased to and maintained at 5 mg L^{-1} for 96 h moderate hypoxia acclimation. 30 min video recordings were taken every 24 h of exposure. Two mortalities occurred during the acclimation period due to unknown reasons. Those

fish were removed either several hours before or immediately after video recordings had been prepared to allow for sufficient recovery of the remaining fish from interference thus minimizing any adverse effects on the behaviour recorded.

At the conclusion of the experiments, fish were individually euthanised in normoxic or hypoxic water containing 100 mg L^{-1} benzocaine. Subsequently body mass and total length measurements were taken before gill baskets were removed and fixed in 0.1% picric acid and 4% paraformaldehyde in 0.1 M phosphate buffer for 48 h at 4°C. Then gill baskets were transferred into Tris-buffered saline (TBS; 6.06 g Trizma hydrochloride, 1.39 g Trizma Base and 8.76 g sodium chloride in 1 L deionised water; pH 7.2) and kept at 4°C until further use.

An exploratory study was conducted exposing five inanga to a more severe hypoxia level of 3 mg L^{-1} DO concentration and normoxia, respectively, for 48 h following the same experimental procedure as in the aforementioned acclimation to moderate hypoxia.

Table 4.1: Dissolved oxygen concentration and temperature profiles applied in the study (means ± SE). Data are from minute-by-minute measurements throughout the 96 and 48 h acclimation experiments.

Species	Dissolved oxygen (mg L^{-1})		Temperature (°C)	
	Hypoxia side	Normoxia side	Hypoxia side	Normoxia side
Moderate hypoxia				
Inanga	4.9 ± 0.002	9.6 ± 0.001	17.5 ± 0.002	17.7 ± 0.001
Banded kokopu	5.2 ± 0.003	9.8 ± 0.001	18.0 ± 0.002	18.1 ± 0.002
Black mudfish	5.1 ± 0.001	10.1 ± 0.002	16.4 ± 0.005	16.5 ± 0.006
Severe hypoxia				
Inanga	3.2 ± 0.006	9.5 ± 0.001	18.4 ± 0.002	18.5 ± 0.002

4.3.4 Gill tissue processing

Gill tissue was prepared for paraffin embedding using standard techniques. From moderate hypoxia acclimation the second left gill arch was separated from inanga and banded kokopu gill baskets and the first left gill arch was separated from black mudfish gill baskets. From severe hypoxia acclimation the first left gill arch was separated from the inanga gill baskets. Gill arches were decalcified in 0.1 M EDTA in TBS for at least 7 days on a rocker table at 4 – 6°C. Subsequently gill arches were transferred into TBS and kept at 4°C until further processing. In preparation for paraffin embedding, gill arches were dehydrated in increasing concentrations of ethanol. Subsequently gill tissue was incubated in xylene, xylene–paraffin and paraffin before embedding into paraffin wax. 5 µm longitudinal sections were prepared for immunohistochemistry and gill morphology evaluation using a microtome. All sections were mounted on gelatine-coated glass slides and kept at 4°C until further use.

4.3.5 Immunohistochemistry

The serotonin (5-HT) content of oxygen sensing NECs and the HIF-1α immunoreactivity of the gill tissue were determined by standard methodology with minor modifications described in Olszewski et al. (2010). Sections were de-paraffinised in xylene and rehydrated in decreasing concentrations of ethanol. Subsequently sections were rinsed in TBS and incubated in 10% methanol and 3% hydrogen peroxide (H_2O_2) in TBS for 10 min to increase permeabilisation of antibodies and remove endogenous peroxidase activity, followed by incubation in either primary polyclonal anti-serotonin antibody (1:1000) made in rabbit (Sigma-Aldrich, Germany) or primary polyclonal anti-HIF1α antibody (1:1000) made in goat (Santa Cruz Biotechnology; Thermo Fisher Scientific, New Zealand) for 48 h

at 4 – 6°C in a humidity chamber. Subsequently gill sections were incubated in goat anti-rabbit and rabbit anti-goat secondary antibody (1:400; Vector Laboratories, Inc., USA) respectively, for 2 h at room temperature in a humidity chamber. Afterwards sections were incubated in avidin-biotin complex (ABC, 1:800; Vector Laboratories, Inc., USA) for 2 h at room temperature in a humidity chamber. For visualisation of antigen-antibody complex, serotonin-stained sections were incubated in 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich, Germany) and 0.01% H_2O_2 in TBS, whereas HIF-1α-stained sections were incubated in 0.05% DAB, 0.01% H_2O_2 and 0.5% nickel ammonium sulfate in TBS. Sections were stored in TBS at 4°C until continuing procedures. Each incubation period was followed by multiple rinses in TBS. Antibodies and avidin-biotin complex were diluted in 0.25% gelatine and 0.5% Triton X-100 in TBS. Serotonin-stained sections were dehydrated in increasing concentrations of ethanol and cleared in xylene before embedding with Entellan® (Merck Millipore, New Zealand). HIF-1α-stained sections were lightly counter-stained with eosin (Alcoholic Eosin Y 515, Surgipath®, Leica Biosystems, Germany) for 30 s before differentiation in increasing concentrations of ethanol, clearing in xylene and embedding in Entellan®. Subsequently slides were stored in a dark, cool place until analysis utilizing light microscopy.

For a preliminary evaluation of 5-HT immunoreactivity and NEC distribution, gill arches were dissected from three fish per species acclimated to laboratory holding conditions for at least two weeks and euthanised in 100 mg L^{-1} benzocaine. After fixation, NECs were detected in whole–mount gill tissue preparations following the aforementioned methods. Specifically, samples were rinsed in TBS and incubated in 10% methanol and 3% hydrogen peroxide (H_2O_2)

in TBS for 10 min, followed by incubation in rabbit primary polyclonal anti-serotonin antibody (1:10,000) for 24 h at 4 – 6°C in a humidity chamber. Subsequently tissue was incubated in goat anti-rabbit secondary antibody followed by incubation in avidin-biotin complex (ABC, 1:800) in a humidity chamber for 1 h at room temperature respectively. For visualisation of antigen-antibody complex, samples were incubated in 0.05% 3,3'-DAB and 0.01% H_2O_2 in TBS. Thereafter gill arches were dried onto gelatine-coated glass slides, dehydrated in ethanol and cleared in xylene before embedding in Entellan®. Slides were stored in a dark, cool place until evaluation with light microscopy.

4.3.6 Histology

For enhanced differentiation of gill tissue margins for morphological evaluation gill sections were stained in hematoxylin (Gill II Hematoxylin, Surgipath®, Leica Biosystems, Germany) and eosin following standard procedures. After de-paraffinisation in xylene and rehydration in decreasing concentrations of ethanol, sections were incubated in hematoxylin for 5 min before rinsing in tap water. Subsequently, sections were rinsed in Scott's tap water for 2 min before incubation in eosin for 10 min. After another rinse in tap water, sections were dehydrated in increasing concentrations of ethanol and cleared in xylene before embedding in Entellan®. Slides were stored in a dark, cool place until evaluation with light microscopy. Morphometric analyses were conducted from photographs prepared with a Leica-DMRE-Microscope and attached Olympus-DP70-Camera. In this context five randomly chosen gill filaments per fish were analysed for filament length (mm), number of secondary lamellae per mm filament, interlamellar distance (µm), lamellar height (µm) and lamellar width (µm). From the moderate hypoxia acclimation experiment, six fish were analysed from each

normoxic control and moderate hypoxic group. From the severe hypoxia acclimation experiment, three fish were analysed from each normoxic control and severe hypoxic group.

4.3.7 Digital video analysis

Video footage was transferred to a computer and from the 30 min recordings, nine one-minute sections were selected for analysis (Figure 4.2) as pre-analytical power analysis using GPower 3.1.9.2 (Heinrich Heine University, Düsseldorf, Germany) showed that 9 x 1 min blocks were statistically more powerful than 3 x 3 min blocks. The sections were manually analysed for the swimming behaviour of each fish. The VLC 2.2.1 media player (VideoLan Organization) program window depicting the video recording was underlayed by a Microsoft Paint document on which each distinct fish position within the established margins of the test arena were recorded throughout the analysed video sections. Fish position data were transferred to and saved as an Excel file until further analysis.

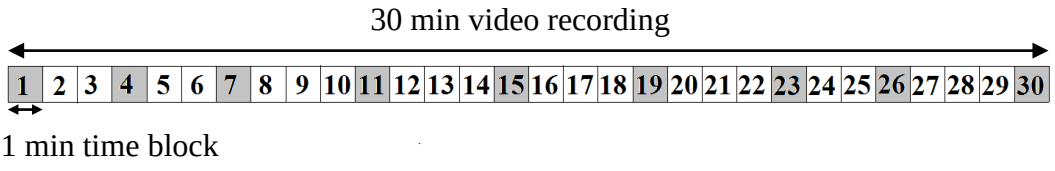


Figure 4.2: Schematic representation of analysis of swimming behaviour video footage. Each 30 min video recording was subdivided into 30 one-minute time blocks of which nine blocks (marked in grey) were selected for analysis.

4.3.8 Data analyses

For each fish the swimming speed was calculated as body lengths per min (BL min^{-1}) and averaged over all analysed one-minute time blocks for each video recording.

Gill morphometric measurements from five filaments were averaged per fish and subsequently per group.

NEC analysis was carried out by manually counting the number of 5-HT immunoreactive cells in three filaments that were randomly chosen from the proximal, median and distal sections of the gill arch from each fish. For each arch the number of filaments was counted as well and NEC density per gill arch was calculated as average number of NECs from three filaments, multiplied by the number of filaments per arch. HIF-1α-positive nuclei and cytoplasm immunoreactivity and density per gill arch were determined concordantly.

4.3.9 Statistical analysis

Statistical analyses were carried out using Statistica v.12.0 (StatSoft, USA) and Microsoft Excel 2013 Data Analysis ToolPak. All data were tested for normality using Kolmogorov-Smirnov test for normality. Parametric data were subsequently evaluated for significant differences using one-way as well as factorial analysis of variance (ANOVA) and post hoc Tukey's HSD or Tukey's HSD for unequal n respectively in dependence on sample numbers. Nonparametric data were analysed utilizing Kruskal-Wallis analysis of variance (ANOVA) and post hoc multiple comparison of mean ranks analysis as well as Kolmogorov-Smirnov two-sample test and post hoc Mann-Whitney U test. Data were considered significantly different with a P-value < 0.05.

4.4 Results

Inanga, banded kokopu and black mudfish displayed different responses in their respective swimming behaviour and alteration of cellular HIF1α content, while 96 h exposure to inescapable hypoxia effected no changes in gill morphology, gill morphometrics and NEC density within each of the three species.

4.4.1 Swimming behaviour

While the study was conducted in groups of five and four fish, no shoal effect on the swimming behaviour was observed during the experimental period. As such, it was frequently observed that distinct fish within the same group were actively swimming, while other fish were resting.

Moderate hypoxia

Swimming speed in inanga did not change significantly within the control acclimation in normoxic water and 96 h of normoxic control acclimation (18.4 ± 1.2 and 19.9 ± 4.6 BL min^{-1}, respectively (P = 0.9821) and was not different between the control and subsequent hypoxic group at 0 h (18.4 ± 1.2 and 21.6 ± 2.6 BL min^{-1} respectively, P = 0.8685). In the moderate hypoxia group swimming speed decreased significantly from 21.6 ± 2.6 at 0 h to 7.4 ± 2.0 BL min^{-1} after 96 h of acclimation (P = 0.0049) and was therefore significantly lower than in the normoxic control group at 96 h as well (P = 0.0216; Figure 4.3 A).

Similarly, swimming speed within the normoxic control did not change significantly between 0 and 96 h in banded kokopu (16.8 ± 4.7 and 8.7 ± 1.1 BL min^{-1}, respectively, P = 0.4499). Furthermore, swimming speed was not different between normoxic control and subsequently moderate hypoxic groups at 0 h (16.8 ± 4.7 and 20.4 ± 5.1 BL min^{-1}, respectively, P = 0.9220). Acclimation to 96 h of

moderate hypoxia did not elicit a change in swimming speed (20.4 ± 5.1 BL min^{-1} at 0 h and 20.3 ± 3.4 BL min^{-1} at 96 h, P = 0.9999). However, swimming speed at 96 h was significant different between normoxic control and moderate hypoxic groups (8.1 ± 1.1 and 20.3 ± 3.4 BL min^{-1}, respectively, P = 0.0061; Figure 4.3 B).

Black mudfish control swimming speed varied between the exposure periods from 9.0 ± 2.0 at 0 h to 16.5 ± 2.3 BL min^{-1} at 96 h (P = 0.0416). Swimming speed was however not significantly different between control and hypoxic groups at 0 h (9.0 ± 2.0 and 6.0 ± 1.7 BL min^{-1}, respectively, P = 0.6943). Swimming speed in the hypoxic group increased significantly between 0 and 96 h from 6.0 ± 1.7 to 18.7 ± 1.8 BL min^{-1} (P = 0.0004), however as swimming speed increased in the normoxic control group as well, swimming speed in both groups were comparable at 96 h acclimation (P = 0.8499; Figure 4.3 C).

In all three species, especially in inanga and banded kokopu, distinct variability in swimming speed was observed within groups receiving the same, normoxic control treatment, and across all five time points (0, 24, 48, 72, 96 h of acclimation; Figure 4.4).

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Figure 4.3: The effect of 96 h moderate hypoxia acclimation (5 mg L^{-1} DO concentration) on swimming speed in (A) inanga, (B) banded kokopu and (C) black mudfish. Data are mean ± SE. BL: Body lengths. Significant differences between normoxic control and moderate hypoxic groups are indicated by 'a'. Significant differences between 0 and 24, 48, 72, or 96 h in both treatment groups are indicated by * ($P < 0.05$) or ** ($P < 0.01$).

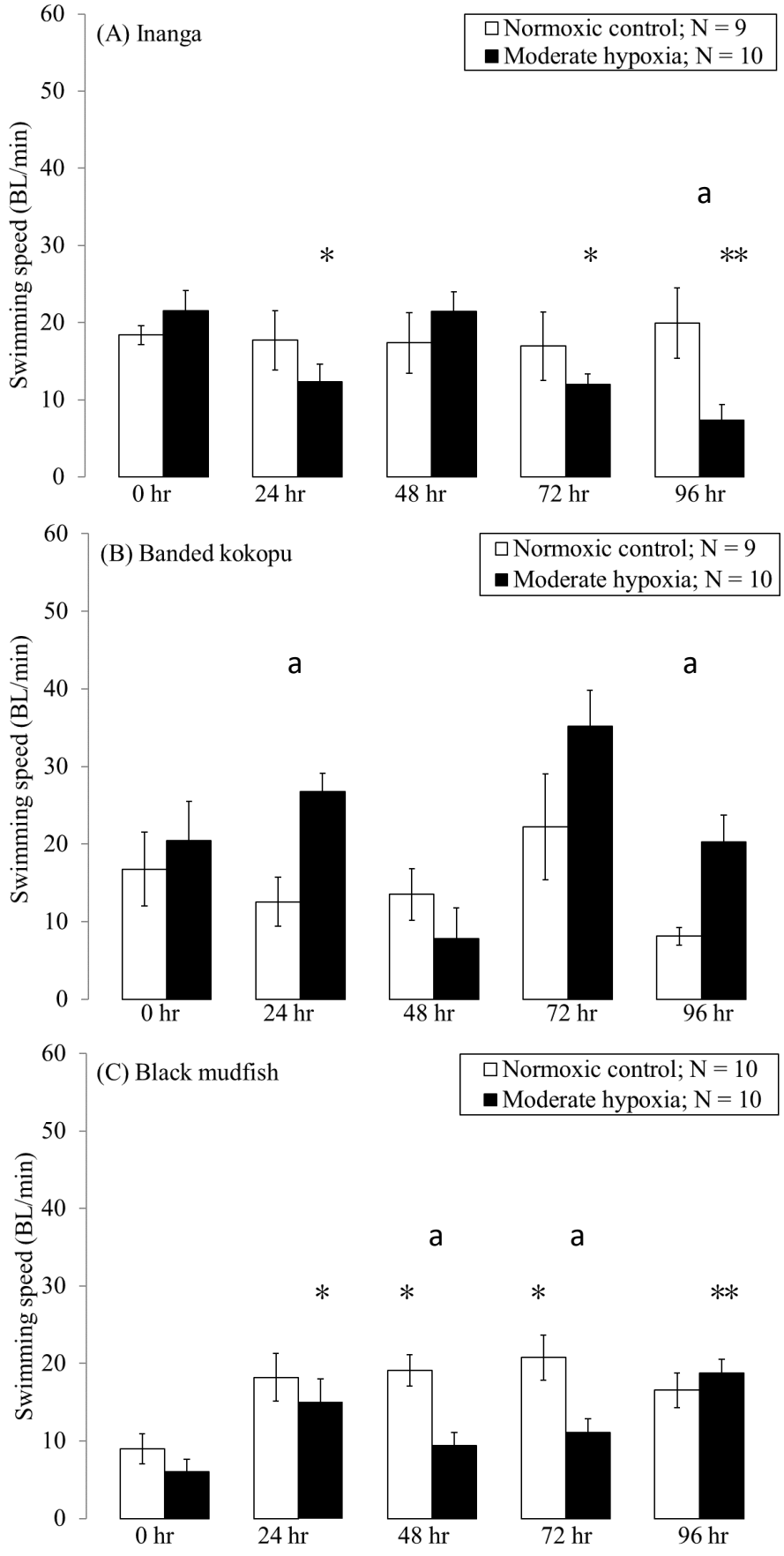
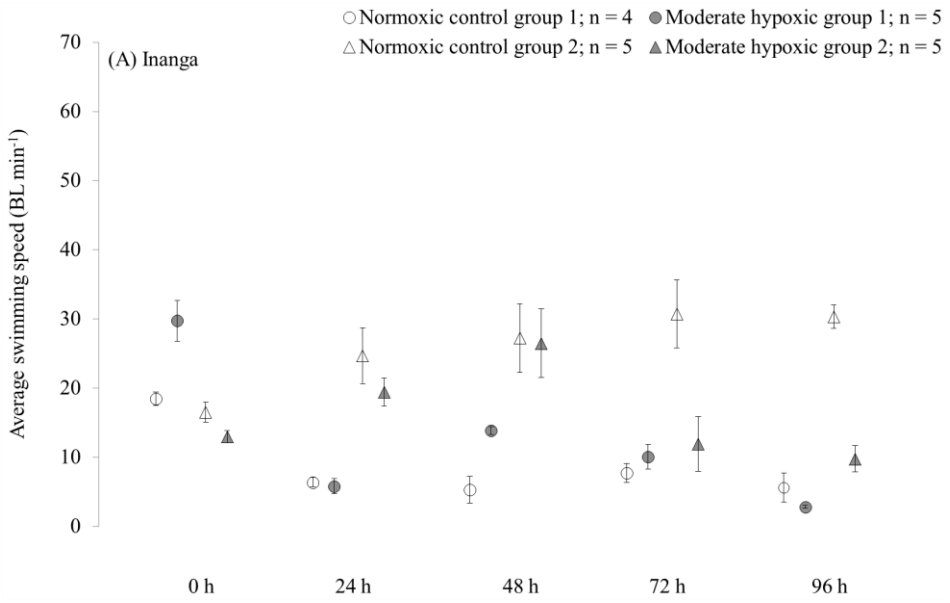
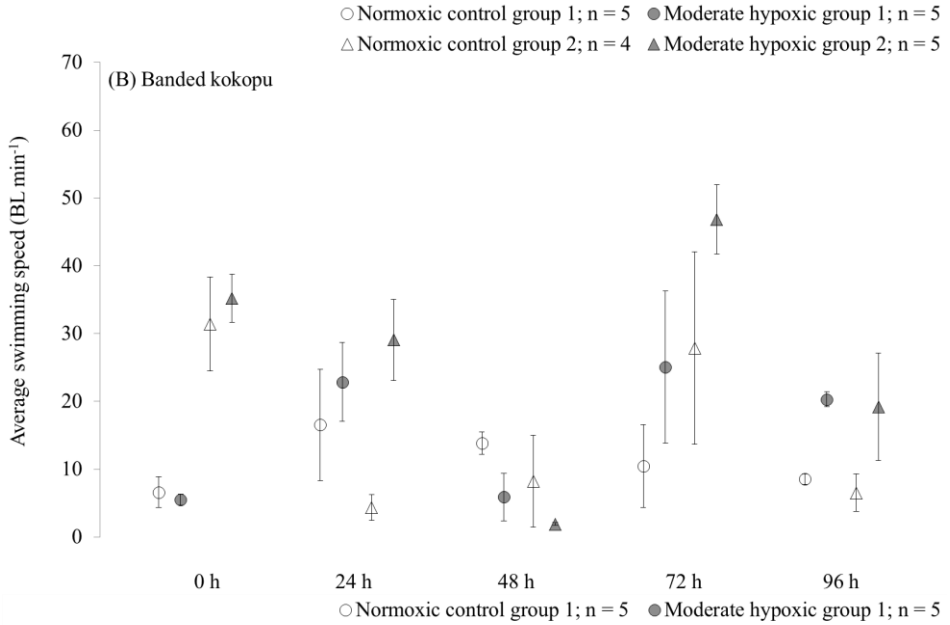
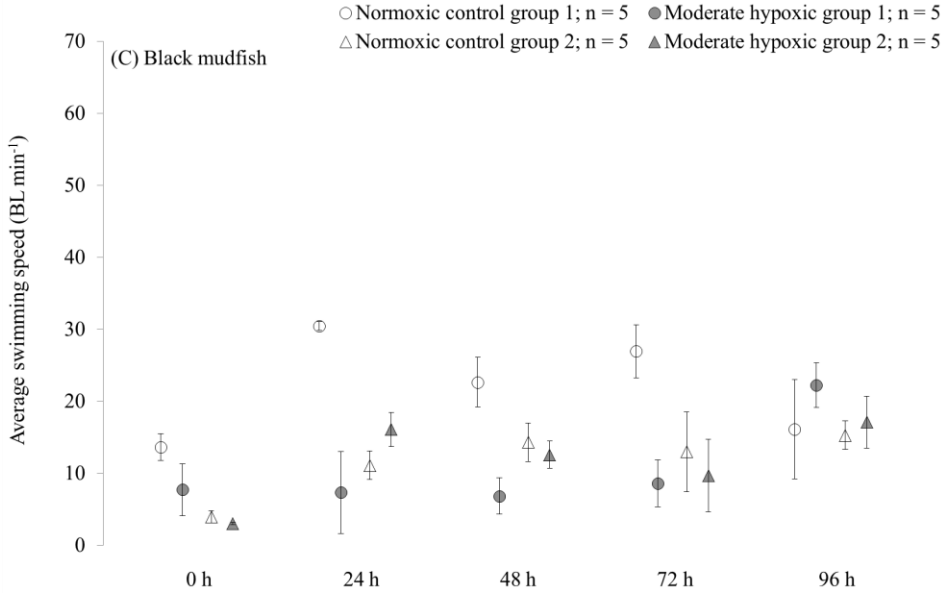

(A) Inanga
Normoxic control; N = 9
Moderate hypoxia; N = 10
Swimming speed (BL/min)
60
50
40
30
20
10
0
a
*
*
**
0 hr
24 hr
48 hr
72 hr
96 hr
(B) Banded kokopu
Normoxic control; N = 9
Moderate hypoxia; N = 10
Swimming speed (BL/min)
a
a
0 hr
24 hr
48 hr
72 hr
96 hr
(C) Black mudfish
Normoxic control; N = 10
Moderate hypoxia; N = 10
Swimming speed (BL/min)
a
a
*
*
*
**
0 hr
24 hr
48 hr
72 hr
96 hr

Figure 4.4: The effect of moderate hypoxia acclimation (5 mg L^{-1} DO concentration) on swimming speed in (A) inanga, (B) banded kokopu and (C) black mudfish. Data are median ± median absolute deviation (MAD), displayed separately for each group of fish tested, in order to indicate inter-group variation. BL: Body lengths.


Normoxic control group 1; n = 4
Moderate hypoxic group 1; n = 5
Normoxic control group 2; n = 5
Moderate hypoxic group 2; n = 5
(A) Inanga
Average swimming speed (BL min-1)
0
10
20
30
40
50
60
70
0 h
24 h
48 h
72 h
96 h


Normoxic control group 1; n = 5
Moderate hypoxic group 1; n = 5
Normoxic control group 2; n = 4
Moderate hypoxic group 2; n = 5
(B) Banded kokopu
Average swimming speed (BL min-1)
0
10
20
30
40
50
60
70
0 h
24 h
48 h
72 h
96 h


Normoxic control group 1; n = 5
Moderate hypoxic group 1; n = 5
Normoxic control group 2; n = 5
Moderate hypoxic group 2; n = 5
(C) Black mudfish
Average swimming speed (BL min-1)
0
10
20
30
40
50
60
70
0 h
24 h
48 h
72 h
96 h

Severe hypoxia

Inanga swimming speed did not change significantly within the normoxic control group between 0 and 48 h of acclimation (1.8 ± 0.4 to 1.9 ± 0.8 BL min^{-1}, P = 0.9996) and was not different between control and subsequent hypoxic group at 0 h (1.8 ± 0.4 and 1.6 ± 0.4 BL min^{-1} respectively, P = 0.9982). In the hypoxia group, swimming speed increased markedly from 1.6 ± 0.4 BL min^{-1} at 0 h to 14.7 ± 1.1 BL min^{-1} at 48 h of acclimation (P = 0.0002), thereby significantly elevated in comparison to the normoxic control group at 48 h acclimation (P = 0.0002; Figure 4.5).

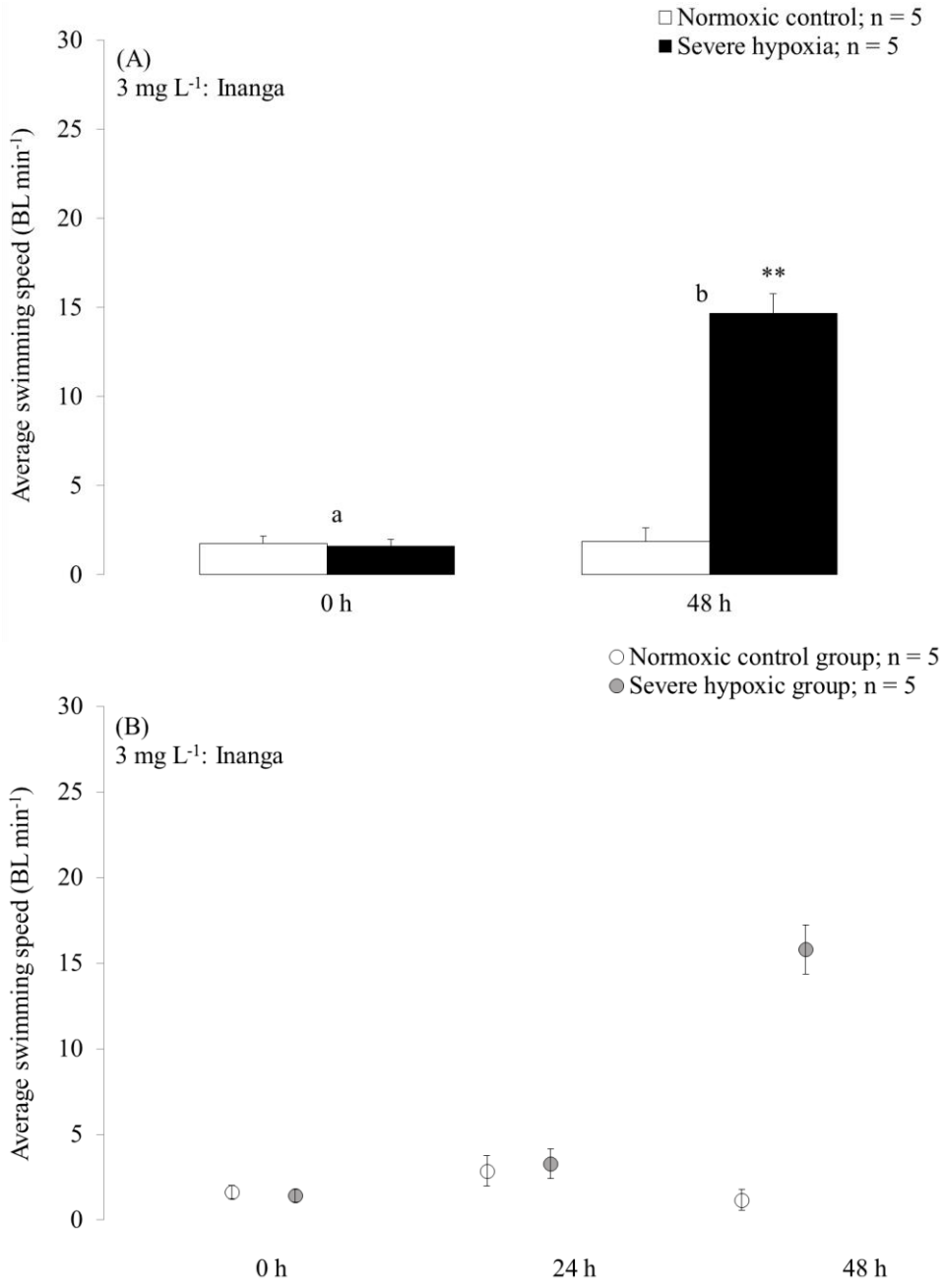


Figure 4.5: The effect of 48 h severe hypoxia acclimation (3 mg L-1 DO concentration) on swimming speed in inanga. Data are mean ± SE (A) and median ± median absolute deviation (MAD; B). BL: Body lengths. Significant differences between normoxic control and severe hypoxic group are indicated by 'b'. Non-different normoxic control and severe hypoxic group are marked by 'a'. Significant differences between 0 and 48 h in severe hypoxia are indicated by ** ($P < 0.01$).

4.4.2 Gill morphology

The morphological structure of the gills in inanga, banded kokopu and black mudfish are characterised by decidedly comparable features. In all three species the secondary respiratory lamellae are fully protruding from the gill filament with no apparent interlamellar cell mass (ILCM) between distinct lamellae (Figure 4.6, A1 - C1). Accordingly, no changes in gill morphology were observed after acclimation to moderate hypoxia (5 mg L^{-1} DO concentration) for 96 h or to severe hypoxia (3 mg L^{-1} DO concentration) for 48 h (Figure 4.6).

Acclimation to moderate or severe hypoxia did not elicit gill morphometric variations in any of the three species ($P > 0.05$). However, morphometric measurements from normoxic control groups revealed that banded kokopu displayed distinctly longer gill filaments relative to total body length than inanga and black mudfish ($P_{inanga} = 0.0148$; $P_{black\ mudfish} = 0.0016$), while black mudfish secondary lamellae were significantly wider than in inanga and banded kokopu ($P_{inanga} = 0.0004$; $P_{banded\ kokopu} = 0.0076$). Gill morphometric parameters are presented in Table 4.2.

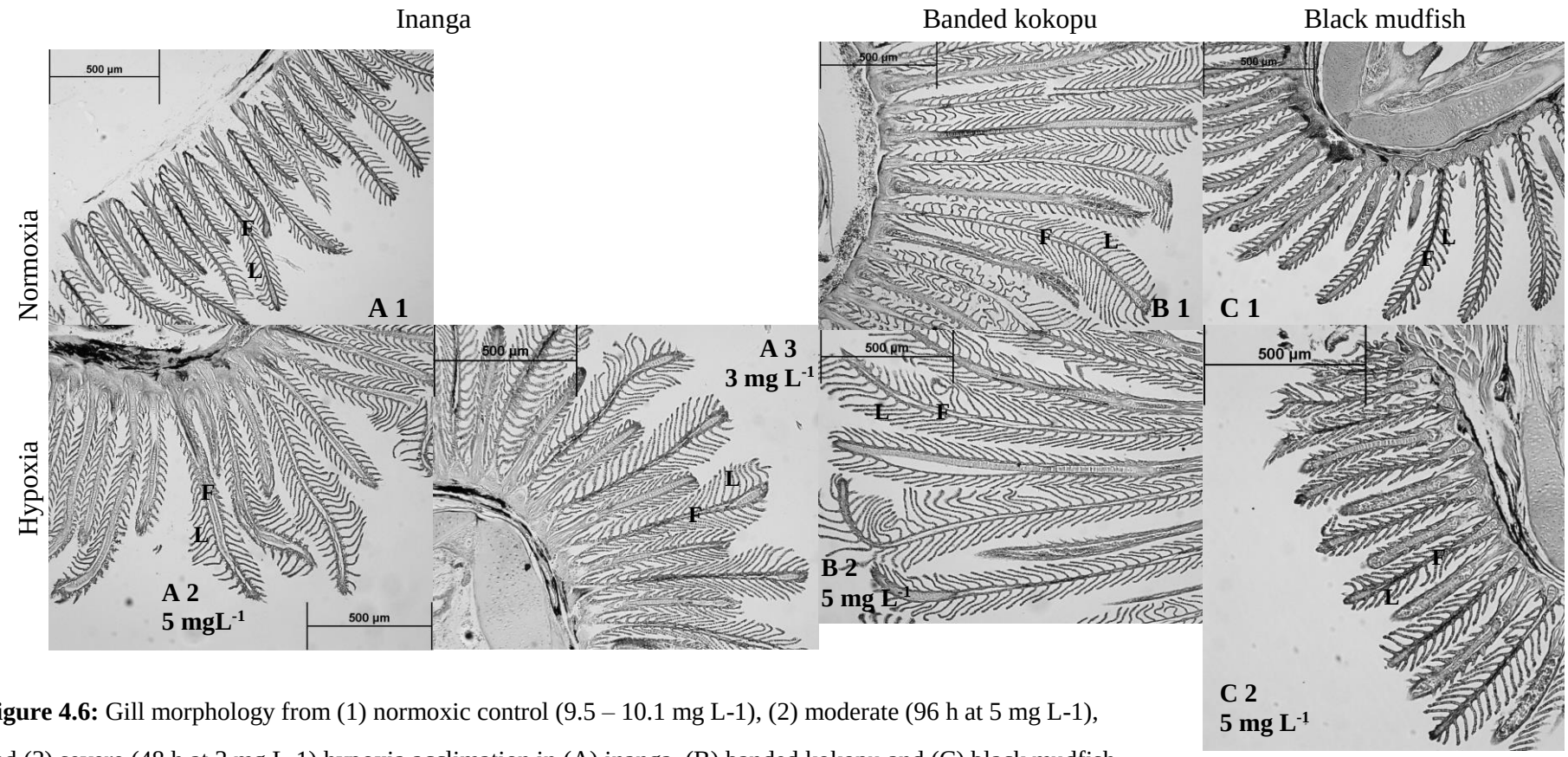


Figure 4.6: Gill morphology from (1) normoxic control (9.5 – 10.1 mg L-1), (2) moderate (96 h at 5 mg L-1), and (3) severe (48 h at 3 mg L-1) hypoxia acclimation in (A) inanga, (B) banded kokopu and (C) black mudfish. F: Filament, L: Lamellae. Magnification: 40. Bars: 500 µm.

Table 4.2: Effect of moderate (5 mg L^{-1} DO concentration) hypoxia on gill morphometrics in inanga, banded kokopu and black mudfish and effect of severe (3 mg L^{-1} DO concentration) hypoxia on gill morphometrics in inanga. Data are mean ± SE. $n_{\text{normoxic control/moderate hypoxia}} = 6$ for all species. $n_{\text{normoxic control/severe hypoxia}}$ in inanga = 3. No significant differences in any gill morphometric measure between normoxic control and hypoxia for any species. Significant differences in relative filament length between banded kokopu and inanga are marked by [a] ($P < 0.05$), between banded kokopu and black mudfish by [b] ($P < 0.01$). Significant differences in lamellar width between black mudfish and inanga are marked by [c] ($P < 0.01$), between black mudfish and banded kokopu by [d] ($P < 0.01$). Fil L: Filament length. TL: Total fish length.

Species	Treatment	Filament length relative to fish size (µm Fil L/mm TL)	Number 2° lamellae per mm filament	Interlamellar distance (µm)	Lamellar height (µm)	Lamellar width (µm)
Inanga	Normoxic control	16.3 ± 1.6[a]	49.9 ± 1.5	35.6 ± 1.2	107.7 ± 10.0	6.2 ± 0.2[c]
	Moderate hypoxia	15.2 ± 1.1	48.7 ± 1.5	36.2 ± 1.2	105.1 ± 7.7	6.8 ± 0.2
	Normoxic control	13.4 ± 0.4	53.1 ± 2.1	33.6 ± 1.5	90.1 ± 6.5	6.0 ± 0.03
	Severe hypoxia	13.7 ± 1.5	56.2 ± 1.0	30.7 ± 0.5	94.7 ± 10.1	6.3 ± 0.1
Banded kokopu	Normoxic control	21.1 ± 0.8	50.9 ± 1.8	33.5 ± 1.6	125.7 ± 5.8	6.6 ± 0.1[d]
	Moderate hypoxia	18.6 ± 1.8	54.7 ± 5.7	33.4 ± 3.0	117.3 ± 10.2	6.4 ± 0.1
Black mudfish	Normoxic control	14.6 ± 0.4[b]	48.9 ± 0.7	35.4 ± 0.6	101.1 ± 5.0	7.3 ± 0.1
	Moderate hypoxia	14.0 ± 0.5	49.5 ± 2.1	35.8 ± 1.5	107.4 ± 4.1	7.1 ± 0.2

4.4.3 Neuroepithelial cells

Whole-mounted gills from inanga, banded kokopu and black mudfish showed positive immunoreactivity for 5-HT cells. In inanga and black mudfish NECs were located in sequence parallel to the longitudinal axis of the gill primary filaments with decisively higher concentration in the distal filament tip while sparsely represented in proximal filament areas. NECs were also detected within the secondary respiratory lamellae, at times in close proximity to the external environment (Figure 4.7 A and C). In banded kokopu NECs were scattered throughout the whole length of the filament and within the secondary lamellae Figure 4.7 B 1), where they were especially concentrated at the proximal edge of the lamellae exposed to the external environment (Figure 4.7 B 2).

Density of NECs did not change significantly in response to moderate hypoxia in inanga ($F_{1,13} = 0.0050$, $P = 0.9446$) and banded kokopu ($F_{1,12} = 0.0071$, $P = 0.9341$, Figure 4.8) or in response to severe hypoxia in inanga ($F_{1,6} = 0.0000$, P = 0.9971, Figure 4.9). While NEC immunoreactivity was positively verified in black mudfish whole-mount gill preparations (Figure 4.7 C), positive serotonergic immunoreactivity was not obtainable in sectioned gill preparations, thereby excluding black mudfish from NEC density analysis.

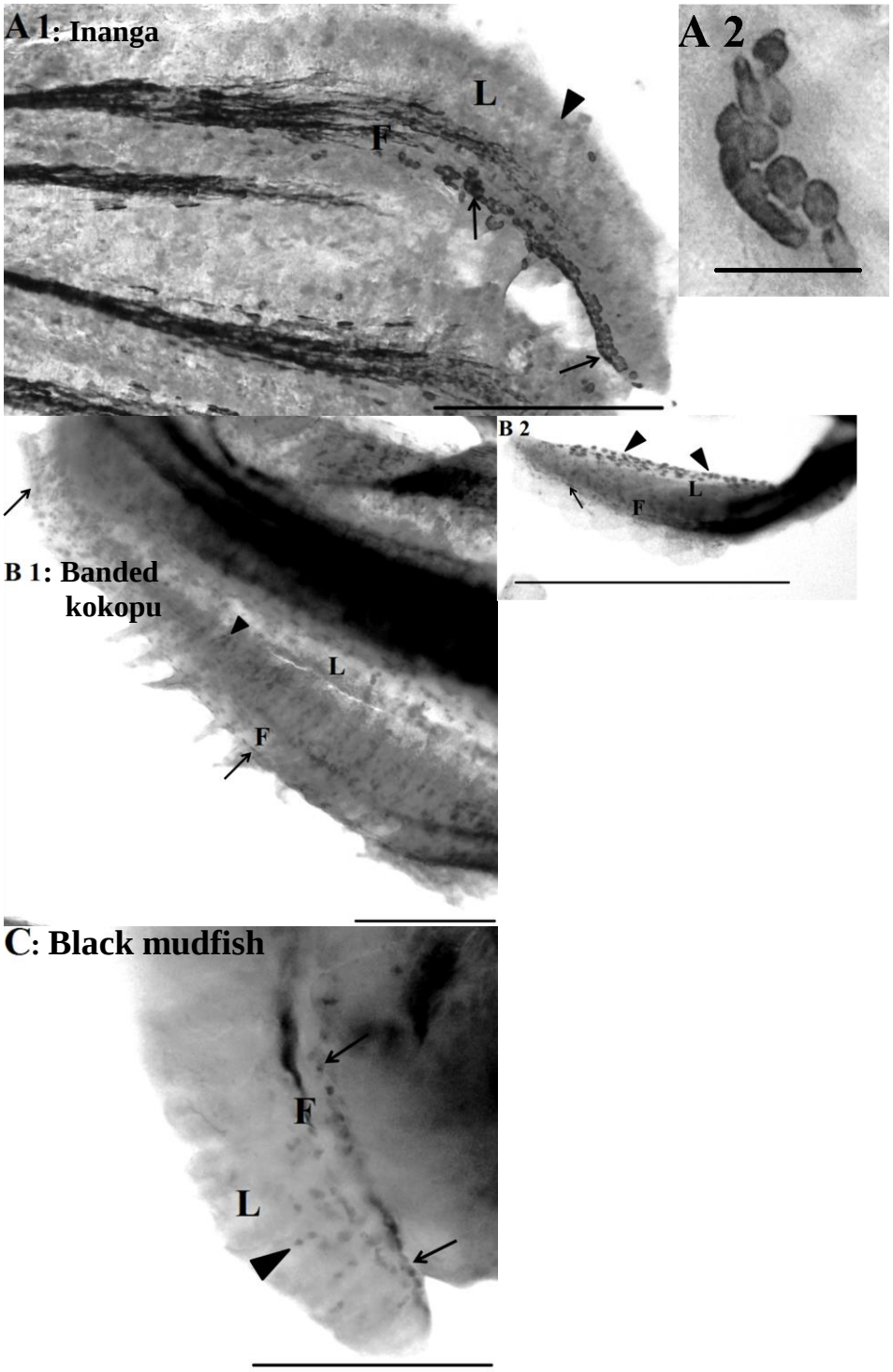


Figure 4.7: Distribution of darkly stained 5-HT-positive NECs (A 2) along gill filament (F, marked by arrow) and within lamellae (L, marked by closed arrow-head) in inanga (A), banded kokopu (B) and black mudfish (C). Scale bars: (A 1, B 1) 200 µm, (A 2) 25 µm, (B 2, C) 100 µm.

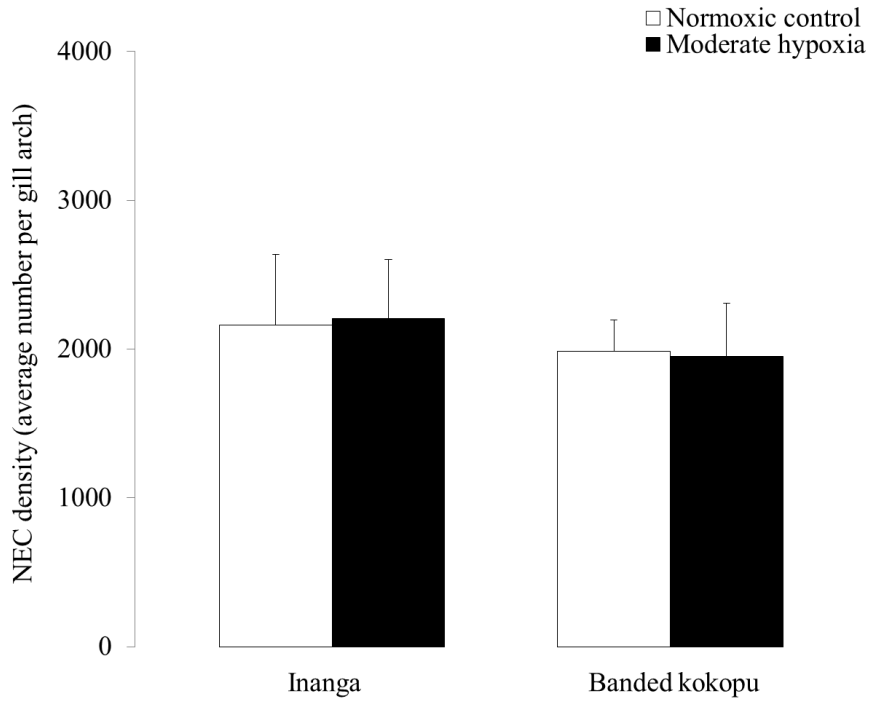


Figure 4.8: Effect of 96 h moderate hypoxia acclimation (5 mg L^{-1} DO concentration) on NEC density in inanga and banded kokopu. Data are average number of NEC per gill arch, presented as mean ± SE.

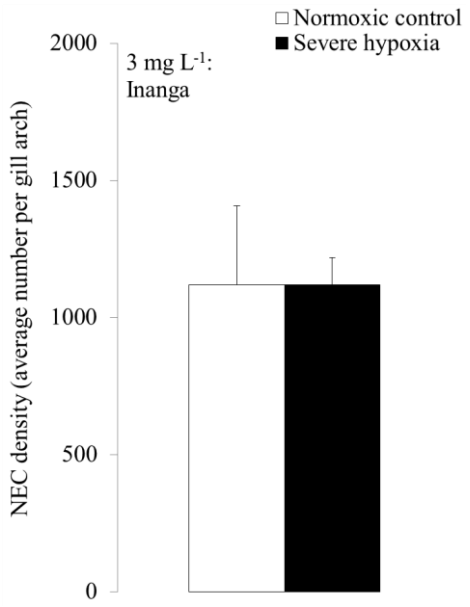


Figure 4.9: Effect of 48 h severe hypoxia acclimation (3 mg L^{-1} DO concentration) on NEC density in inanga. Data are average number of NEC per gill arch, presented as mean ± SE (n = 4 each).

4.4.4 Hypoxia inducible factor

Gill sections of inanga, banded kokopu and black mudfish exhibited positive immunoreactivity for HIF-1α in nuclei and cytoplasm (Figure 4.10). In inanga the protein was mainly distributed along lamellae and the edge of the filament parallel to the longitudinal filament axis. Similarly, HIF-1α was found especially along the outer edge of the filament however only infrequently along the lamellae in banded kokopu. Conversely, HIF-1α immunoreactivity was sparse along filament edge and lamellae in black mudfish.

The total density of HIF-1α and the density of HIF1α positive nuclei was not significantly increased in the hypoxia groups of 96 h moderate exposure in inanga, banded kokopu and black mudfish in comparison to the control groups. Cytoplasmic HIF-1α density, however was significantly increased in inanga ($F_{1,13}$ = 10.2950, P = 0.0069) and banded kokopu ($F_{1,15}$ = 4.6202, P = 0.0483; Figure 4.11).

Total, cytoplasmic and nuclei HIF-1α density was not distinctly different after exposure to 48 h of severe hypoxia in inanga (Figure 4.12).

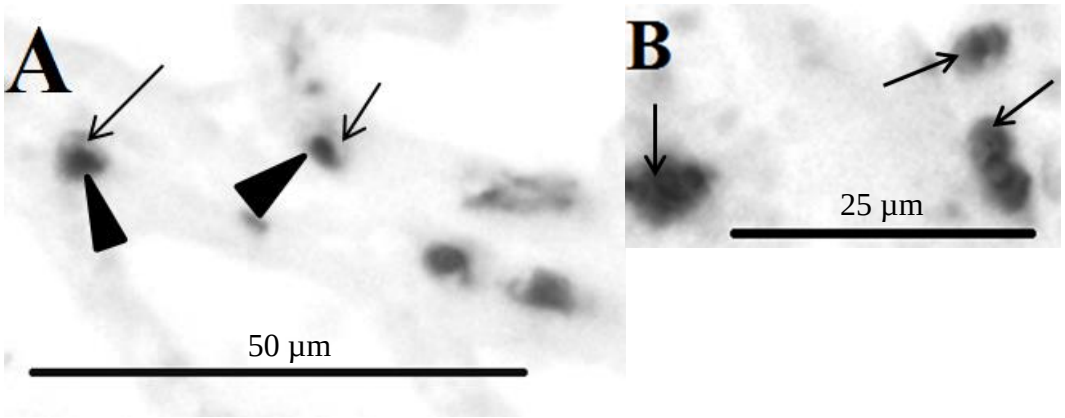


Figure 4.10: HIF-1α immunoreactivity in inanga gill epithelium. HIF-1α positive nuclei (A, closed arrow-heads) and cytoplasm (B, arrows), as well as HIF-1α-inert cytoplasm (A, arrows). Scale bars: (A) 50 µm, (B) 25 µm.

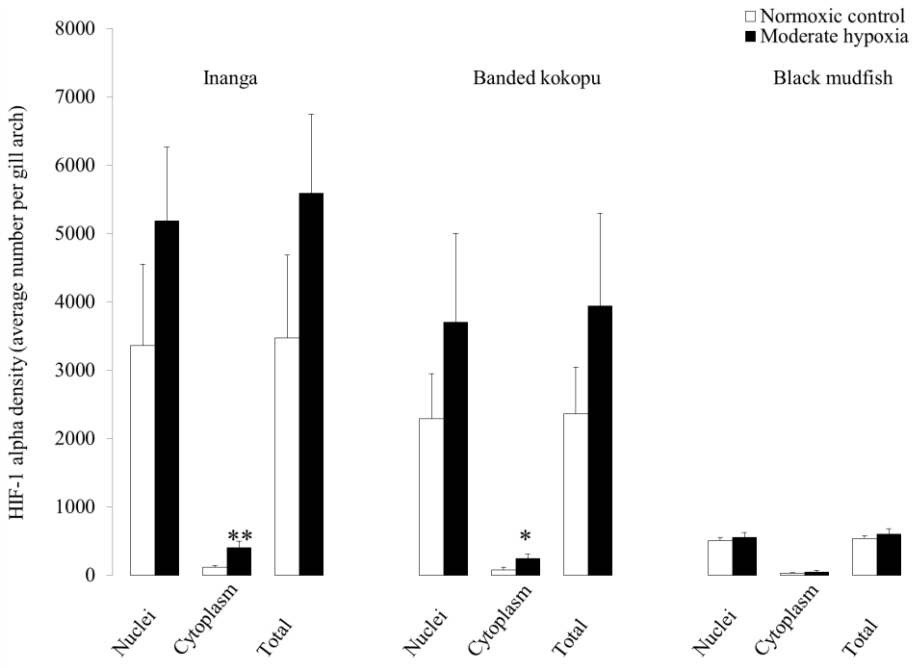


Figure 4.11: Effect of moderate hypoxia acclimation (5 mg L^{-1} DO concentration) on density of HIF-1 alpha positive nuclei and cytoplasm in inanga, banded kokopu and black mudfish. Data are average number of HIF-1 alpha positive cell structures per gill arch, presented as mean ± SE. Significant differences in density between normoxic control and moderate hypoxic groups are indicated by * ($P < 0.05$) and ** ($P < 0.01$).

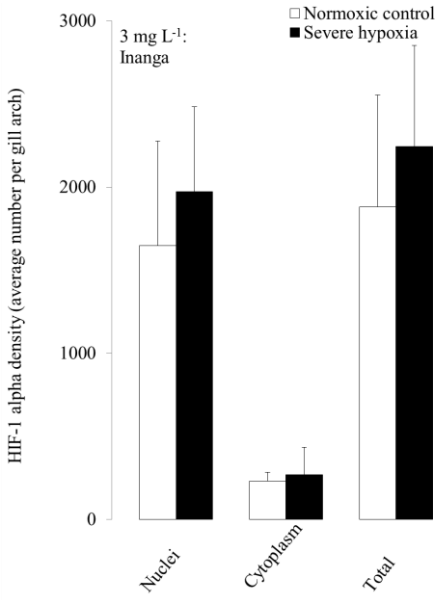


Figure 4.12: Effect of severe hypoxia acclimation (3 mg L^{-1} DO concentration) density of HIF-1 alpha positive nuclei and cytoplasm in inanga. Data are average number of HIF-1 alpha positive cell structures per gill arch, presented as mean ± SE ($n_{normoxic\ control} = 5$; $n_{severe\ hypoxia} = 4$).

4.5 Discussion

4.5.1 Swimming speed

Moderate, inescapable hypoxia acclimation for 96 h elicited a decrease in swimming speed in inanga, while no change was effected in banded kokopu, and swimming speed increased in black mudfish (Figure 4.3). This inter-species variability is in agreement with the marked variety of swimming speed-specific responses to hypoxia demonstrated by previous studies. Inanga is an active, pelagic schooling species and previous studies show that such species typically respond to hypoxia with an initial increase in swimming speed, especially when the onset of hypoxia is rapid and progressive (Domenici *et al.*, 2000; Herbert & Steffensen, 2006; Johansen *et al.*, 2006) or a normoxic refuge is provided (Poulsen *et al.*, 2011; Urbina *et al.*, 2011), which therefore has been suggested as a hypoxia avoidance strategy. Swimming speed, however, has been shown to decrease in sustained hypoxia in Atlantic cod (Schurmann & Steffensen, 1994; Skjæraasen *et al.*, 2008), lesser sandeel (*Ammodytes tobianus*, (Behrens & Steffensen, 2007)), Florida smoothhound shark (*Mustelus norrisi*, (Carlson & Parsons, 2001) and silver perch (*Bairdiella chrysoura*, (Hanke & Smith, 2011)). Similarly swimming speed has also been shown to decrease after an initial velocity peak in Atlantic cod (Herbert & Steffensen, 2005; Johansen *et al.*, 2006) and Atlantic herring (*Clupea harengus*, (Domenici *et al.*, 2000)). It is conceivable, however speculative, that inanga may have initially responded with a similar initial increase in swimming speed, yet focus was placed on the long-term effect of hypoxic exposure, hence initial responses were beyond the scope of this study.

While swimming speed increased in black mudfish in the moderate hypoxic group, it is not reflective of typical stress-induced locomotor activity and

may be significant due to docile behaviour of black mudfish in the beginning of the experiment. Furthermore, a similar increase was observed in the normoxic control group as well, which indicates that parameters beyond control of the experimental environment may have had an impact on the observed locomotor activity. Similar to the absence of locomotor depression or stress-induced burst-and-rest swimming style in banded kokopu and black mudfish, swimming speed has been shown to be unaffected by hypoxia in southern bluefin tuna (*Thunnus maccoyii*, (Fitzgibbon *et al.*, 2010)), and by moderate hypoxia in striped surfperch (*Embiotoca laterali*, (Cook *et al.*, 2014)).

The findings suggest an elevated hypoxia sensitivity in inanga, eliciting a decrease in swimming speed in moderate hypoxia, while locomotor activity in banded kokopu and black mudfish remains unaffected. Decreased swimming activity has been hypothesised to offset metabolic stress, by reducing energy demand (Fischer *et al.*, 1992; Nilsson *et al.*, 1993; Schurmann & Steffensen, 1994; Van Raaij *et al.*, 1996a) and thereby prolonging onset of anaerobic metabolism and minimisation of the concurrent accumulation of anaerobic metabolites (Vianen *et al.*, 2001). The adjustments in swimming speed demonstrated by inanga may reflect an adaptive strategy in the absence of normoxic refuge, to endure moderate hypoxic DO levels, while no such response was elicited in banded kokopu and black mudfish, thus characterising these species as more hypoxia-tolerant than inanga. These suggestions are in agreement with previous findings, where inanga avoided escapable hypoxia at levels below 5.9 mg L^{-1} DO concentration (Chapter 2) and displayed a critical oxygen concentration (C_{crit}) of 5.0 mg L^{-1} (Chapter 3), below which oxygen uptake decreases with declining ambient DO concentration (Pörtner & Grieshaber, 1993),

hence ceases to fuel aerobic metabolism (Herbert & Steffensen, 2005) and anaerobic metabolism is utilized (Chabot *et al.*, 2016). Banded kokopu on the contrary avoided hypoxia at levels below 2.3 mg L^{-1} and displayed a C_{crit} of 4.3 mg L^{-1}, while black mudfish did not demonstrate avoidance of hypoxia as severe as 1.2 mg L^{-1} and displayed no C_{crit} suggesting a distinct hypoxia tolerance.

These propositions appear to be in contrast to the elevated swimming speed that has been observed in inanga after 48 h of acclimation to severe hypoxia (Figure 4.5). However, this was caused by distinctly docile behaviour at 0 h and swimming speed at 48 h approximated data for inanga from moderate hypoxic acclimation. Furthermore, this difference may have been produced by a lower sample number acclimated to severe hypoxia.

Notable were distinct variability in, and group effect on, swimming speed, observed especially in inanga and banded kokopu during the course of the moderate hypoxia acclimation experiments (Figure 4.4), as well as the increase in swimming speed in both normoxic control and moderate hypoxic group, in black mudfish, indicating that while all experiments were conducted under distinctly identical conditions, factors unregistered and uncontrollable in the experimental environment may have had an effect on the fish behaviour and thus recorded swimming speed during the course of the experiment.

4.5.2 Gill morphology

Gill remodelling via cell apoptosis of interlamellar cell mass (ILCM) enlarges the respiratory surface area and thus affects maintenance of oxygen uptake during hypoxia (Sollid *et al.*, 2003). This strategy has been previously described in crucian carp (Sollid *et al.*, 2003) and goldfish (Mitrovic *et al.*, 2009), after one

day of exposure to 0.75 mg L^{-1} DO concentration, with a maximum gill morphometric alteration after seven days of exposure. Comparable modifications were observed in Lake Qinghai scale-less carp, initiated after 8 h exposure to 0.3 mg L^{-1} DO concentration (Matey *et al.*, 2008), and Atlantic stingray, when exposed to a 7 h hypoxia interval at approximately 2.0 mg L^{-1} within 20 days of experimental observations (Dabruzzi & Bennett, 2013). However, in all three species of the present study secondary lamellae were highly protruding with no ILCM in both normoxia and hypoxia acclimated groups (Figure 4.6). Therefore, increased oxygen capacity via gill remodelling is not a hypoxia response strategy observed in these galaxiids. A comparatively large respiratory surface area due to the absence of ILCM at normoxia, suggests an elevated oxygen uptake capacity at any DO level, potentially at the expense of elevated osmoregulatory costs (Sollid *et al.*, 2005; Nilsson, 2007). Similarly, respiratory surface area was not found to be increased in hypoxia acclimated groups via increased numbers or enlargement of gill filament or lamellae (Table 4.2). Chapman *et al.* (2000) previously studied phenotypic plasticity in the gill morphology of the African cichlid (*Pseudocrenilabrus multicolour victoriae*) from both steady hypoxic and steady normoxic habitats, as well as laboratory fish raised in normoxia and hypoxia. Their experiments demonstrated increased gill surface areas due to larger gill filament length and greater lamellar area in fish from the hypoxic habitat, as well as increased gill surface area due to more gill filaments of greater length in the experimental hypoxia-raised fish. Fish in the present study were not collected from hypoxic habitats or raised under stable hypoxic laboratory conditions. Therefore, the lack of gill morphometric adaptations may be due to a comparatively short 96 h acclimation period which was potentially insufficient to

induce changes in gill morphology. This clearly indicates that, in the presence of temporary inescapable moderate (and severe) hypoxia, the three species are not able to utilise morphometric adaptation of the gills for oxygen uptake maintenance. This is in agreement with Laurent and Perry (1991), who suggested that gill morphometric parameters depend to a greater extent on species-specific oxygen requirements than on changes in oxygen availability. In this context, banded kokopu filament length relative to total fish size was found to be longer than in inanga and black mudfish (Table 4.2). The comparatively elevated respiratory surface area may therefore be indicative of inter-species differences in oxygen uptake capacities and hence offer one explanation for the distinct hypoxia sensitivity dissimilarities between inanga and banded kokopu that have been observed in previous studies (Chapter 2 and Chapter 3). Similarly, black mudfish were found to have wider respiratory lamellae than inanga and banded kokopu (Table 4.2), while the number of lamellae per mm filament, lamellar height and interlamellar distances were not different between the three species. This is in contrast to findings from Davidson (1999), where black mudfish demonstrated lamellae not only wider but also shorter and more widely spaced than in inanga. While relative filament length in black mudfish was comparable between the two studies (14.6 versus 13.1 µm filament (mm fish size^{-1})), the number of lamellae per mm of filament were smaller in the present study, while interlamellar distances were greater, and lamellar height was smaller in black mudfish and inanga in the present study. Histological preparation of tissue may inherently cause artefacts and alteration, potentially affecting the morphometric results. However, it has also been shown that gill surface area is positively correlated to body size (Chapman & Chapman, 1998). Therefore, the distinct differences in

black mudfish gill morphometrics may be due to the distinctly greater body size of 88 and 128 mm, utilised by Davidson (1999) in comparison to the fish mean size of 61.5 ± 0.9 mm. In contrast, inanga body sizes were comparable (68.0 and 69.0 ± 1.6 mm). Similarly, differences in sampling size may have caused the distinct dissimilarities between the two studies, as six fish from both normoxic control and moderate hypoxic groups were utilised in the present study. In contrast, only one inanga and one black mudfish were analysed for comparative height of secondary lamellae and interlamellar distances, respectively, while another black mudfish was utilised for filament length and number of secondary lamellae per mm of filament by Davidson (1999). Corresponding to the present findings are observations on Canterbury mudfish (*Neochanna burrowsius*), where interlamellar distances and lamellar shape were found to be similar between mudfish and other aquatic fish species (Meredith, 1985). In contrast however, lamellar thickness was increased in black mudfish, suggesting an adaptation to aerial respiration in the context of emersion and aestivation commonly observed in this species (McDowall, 1990; McPhail, 1999), thereby potentially minimising gill collapse and functional loss (Meredith, 1985). Banded kokopu has been shown to sustain emersion for more than one week, while inanga survived emersion for less than one day (Meredith, 1985). However, these marked differences do not appear to be based upon distinct gill morphologies.

4.5.3 Neuroepithelial cells

In accordance with previous studies, where serotonergic neuroepithelial cells (NECs) have been detected in the gill epithelium of all fish examined, these putative oxygen sensing NECs (Jonz & Nurse, 2005) were demonstrated in the primary gill filament and secondary respiratory lamellae of inanga, banded

kokopu and black mudfish (Figure 4.7). Inanga and black mudfish exhibited comparable distribution patterns with NECs occurring especially along the longitudinal axis of the filament and in increased concentration at the distal filament tip, while fewer NECs were observed scattered within the lamellae, at times in close proximity to the lamellar edge. Similar distribution patterns have been observed previously in zebrafish (*Danio rerio*, (Jonz & Nurse, 2003)) and goldfish (Saltys *et al.*, 2006; Coolidge *et al.*, 2008). Banded kokopu on the other hand displayed a scattered distribution of NECs throughout the primary filament and respiratory lamellae, whereby lamellar NECs were occasionally observed near the external environment. Comparable species-specific differences in NEC distribution patterns have emerged from NEC studies on several species (Goniakowska-Witalinska *et al.*, 1995; Sundin *et al.*, 1998; Saltys *et al.*, 2006; Regan *et al.*, 2011) and have been suggested to be caused by distinct functional specifications of the NECs, potentially in coherence with specific hypoxia tolerances (Coolidge *et al.*, 2008). Present findings, however, indicate this not to be a general mechanism. In this context, inanga and black mudfish presented distinctly different hypoxia sensitivities, however, similar NEC distribution patterns were ascertained from both species.

Hypoxia acclimation did not induce any change in NEC density in inanga and banded kokopu (Figure 4.8 and Figure 4.9), which is in agreement with previous studies on mangrove rivulus (*Kryptolebias marmoratus*), where gill and skin NEC density upon hypoxia acclimation remained unchanged (Regan *et al.*, 2011). In this previous study, however, hypoxia did induce NEC hypertrophy, which has also previously been shown to occur in zebrafish, alongside an increase in NEC innervation (Jonz *et al.*, 2004). It therefore appears that, while hypoxic

stimulation of behavioural and physiological adaptations evidently originates from oxygen sensitive cells responding to internal and external changes in DO concentration (Burleson & Smatresk, 1990b), they are not accompanied by an increase in gill and skin NEC density.

4.5.4 Hypoxia-inducible factor 1 alpha

The hypoxia-regulated HIF-1 alpha pathway has been strongly indicated to mediate compensatory physiological responses through upregulated transcription of hypoxia-inducible genes by the stabilised and dimerised HIF-1 protein under hypoxic conditions (Semenza, 2001). In this context, increased HIF-1 alpha expression, presumably followed by HIF-1 alpha stabilisation, has been previously demonstrated in multiple fish species in response to aquatic hypoxia (Randall *et al.*, 2004; Rahman & Thomas, 2007; Terova *et al.*, 2008; Kodama *et al.*, 2012; Rimoldi *et al.*, 2012).

Findings from the present study, however, do not clearly reflect a comparable HIF-1 activation. Gill epithelial cells along the primary filament and respiratory lamellae exhibited HIF-1 alpha proteins in inanga, banded kokopu and black mudfish (Figure 4.10). However, the nuclei and total HIF-1 alpha density was markedly elevated in both, normoxic control and hypoxia acclimation, in comparison to the cytoplasm HIF-1 alpha density in all three species and at both hypoxia acclimation levels (Figure 4.11 and 4.12). Furthermore, a significant hypoxia-induced increase in HIF-1 alpha density was only observed in the cytoplasm of inanga and banded kokopu. However, due to the distinctly small scale of cytoplasm HIF-1 alpha density in comparison to that observed in nuclei at normoxia and hypoxia, it is debatable whether this detected increase reflects hypoxia-induced effects on gene transcription. These findings are highly

unexpected, as in mammals HIF-1 alpha has been shown to degrade under normoxic conditions and stabilise in the cytoplasm upon hypoxia, followed by transition into the nucleus, where dimerization with HIF-1 beta and upregulation of hypoxia-inducible genes occur (Lee & Percy, 2011). However, comparable results were demonstrated with similar immunohistological detection in gonad cells of rainbow trout and embryonic cells of chinook salmon, exposed in vitro to different levels of DO concentration (Soitamo *et al.*, 2001). Here, the HIF-1 alpha protein was stabilised and accumulated at cellular *in vitro* DO concentrations. These DO concentration levels are comparable to normal venous blood oxygen concentrations, and therefore are commonly observed in fish cells *in vivo* at normoxic environmental conditions. Hence it has been suggested by Soitamo *et al.* (2001) that oxygen-regulated gene expression may be of importance in the normoxic physiology of some fish. These results indicate that not only changes in HIF-1 alpha expression, but also stabilisation of HIF-1 alpha protein under distinct DO concentrations require investigation, possibly via immunohistological detection.

A distinct difference in HIF-1 alpha density between black mudfish and inanga as well as banded kokopu was observed. As black mudfish immunohistochemistry was conducted on different days than for inanga and banded kokopu, results may have been affected by laboratory environmental variations that remained unregistered and uncontrollable within the scope of this study. The absence of a significant hypoxia-induced increase in cytoplasm HIF-1 alpha density in black mudfish, however, may be reflective of increased hypoxia tolerance in this species. In this context, 96 h acclimation to a DO concentration

of 5 mg L^{-1} may not have been severe enough for black mudfish to initiate any cellular adjustments that entail stabilisation of HIF-1 alpha in the cytoplasm.

4.6 Conclusions

Distinct hypoxia sensitivity was demonstrated by inanga, reflected by decreased swimming speed in moderate inescapable hypoxia, while no comparable effect was elicited in banded kokopu and black mudfish, which characterises these species as comparatively more hypoxia tolerant. Gill morphological plasticity as a hypoxia-responsive strategy was not utilised in any of the three species. However, potential gill morphological adaptation to prolonged emersion and aestivation was observed in black mudfish. While oxygen sensing NECs were detected in distinct distribution patterns within the primary gill filament and respiratory lamellae, specific responses to hypoxia in the three species appear not to be mediated by an adaptation of NEC density. Similarly, the stabilisation and accumulation of HIF-1 alpha protein at normoxic conditions indicate differences between the piscine and mammalian HIF-1 alpha pathway and a distinct relevance of hypoxia-inducible genes in the normoxic physiology of fish, requiring further in-depth investigation of the piscine HIF-1 alpha protein stabilisation mechanisms and HIF-1 alpha pathway.

These findings offer further insight in the distinct differences of hypoxia sensitivities and responses between inanga, banded kokopu and black mudfish, which are reflected in their species specific habitat preferences. Hypoxic episodes have been shown to occur frequently in the natural habitat of all three species, and despite their distinct hypoxia sensitivities, all three species are able to withstand hypoxia of moderate extent, at least temporarily.

5 Concluding discussion

5.1 Research summary and implications

The overarching goal of this thesis was to increase comprehension of behavioural and physiological adaptive strategies enabling fish to sustain hypoxia and to elucidate whether related species, presenting distinct habitat requirements and preferences, display specific differences in hypoxia sensitivity and adaptive mechanisms. While aquatic hypoxia is of growing concern (Diaz, 2001) and potential effects on fish communities have been postulated (Wu, 2002), generalised conclusions towards the effect of hypoxia on fish may not be adequate in the context of species-specific habitat preferences and life strategies (Dwyer *et al.*, 2014). Behavioural and physiological adaptations to hypoxia were therefore investigated in the related species inanga, banded kokopu and black mudfish of the Galaxiidae family (Waters *et al.*, 2000), which inhabit distinctly different oxygen environments and dissimilar life strategies (McDowall, 1990), yet are not separated by large phylogenetic distances.

The specific aims of this study were to:

1) Determine whether the three species exhibit unique behavioural responses upon encountering a hypoxic environment;

2) Investigate whether distinct hypoxia sensitivities and behavioural responses towards hypoxia are based on different metabolic oxygen demands and oxygen consumption profiles; and

3) Examine whether locomotor activity, as a measure of metabolic oxygen demand, and gill morphology are affected by prolonged hypoxia, and

whether responses to hypoxia are mediated by oxygen sensing neuroepithelial cells (NECs) and by the transcription protein hypoxia inducible factor 1 (HIF-1).

To facilitate these objectives, a novel, comparative combination of behavioural, physiological, molecular and gill morphological studies was utilised. To enable specific aims (1) and (3), a circulatory water system with an embedded study device was constructed, which allowed an efficient and precise adjustment of the water DO concentration via nitrogen stripping in a deoxygenation tower (Chapter 1).

To achieve the first aim of this thesis (species-specific sensitivities and behavioural responses to hypoxia), the behaviour of isolated fish was studied in a normoxia–hypoxia choice chamber, providing progressive escapable hypoxia and access to the water surface (Chapter 2). Distinct different responses were found in the three species, with inanga exhibiting avoidance of mild hypoxia levels via horizontal migration, while banded kokopu primarily undertook ASR, prior to hypoxia avoidance via migration at a slightly lower DO level. Black mudfish, on the contrary, did not display any behavioural response at any level of hypoxia tested. These differences suggest distinct hypoxia sensitivities, characterising inanga as possibly a markedly hypoxia sensitive species, black mudfish on the other hand as distinctly hypoxia tolerant and banded kokopu as a species of moderate hypoxia tolerance, albeit they commonly inhabit environments not typically characterised by extensive DO level fluctuations (McDowall, 1990).

To achieve the second objective of this thesis (The effect of varying environmental DO concentrations on oxygen consumption), oxygen consumption

at normoxia and distinct hypoxic DO levels applied in a pseudo-randomised order were determined via intermittent-flow respirometry from isolated fish (Chapter 3). Concordant with findings from Chapter 2, oxygen consumption was distinctly different between the three species. Inanga displayed comparatively elevated oxygen consumption rates, thereby indicating an increased oxygen demand, characteristic for active, pelagic and shoaling species (Meredith, 1985). All three species were indicated as oxyregulators, with inanga displaying a shift to oxyconformation at moderate hypoxia, comparable with the level of hypoxia that previously elicited avoidance responses in Chapter 2. Banded kokopu shifted to oxyconforming at slightly more severe hypoxia levels while black mudfish demonstrated no shift at any DO concentration tested. The critical oxygen concentrations at which a shift occurred in inanga and banded kokopu are comparable, albeit higher than in the majority of previously described fish species that exhibit a range of life strategies (e.g. (McKenzie *et al.*, 2007; Cook *et al.*, 2013; Stoffels, 2015) and for a comprehensive review see Rogers *et al*., (2016)). This indicates that inanga and banded kokopu are hypoxia sensitive species, however, both presented C_{crit} values that were lower than some hypoxia sensitive species such as Dover sole (*Solea solea*) larvae (McKenzie *et al.*, 2008). In contrast, black mudfish did not exhibit a critical dissolved oxygen concentration within the range of DO levels tested (minimum values of between 2.5 – 2.1 mg L^{-1}). Therefore, black mudfish may be as tolerant to hypoxia as other highly tolerant species such as longjaw mudsucker (*Gillichthys mirabilis*) and shortjaw mudsucker (*Gillichthys seta*, (Gracey *et al.*, 2001), lesser sandeel (*Ammodytes tobianus*, (Behrens & Steffensen, 2007), and African mouth-brooding cichlid (*Pseudocrenilabrus multicolour*, (Reardon & Chapman, 2010), which exhibited

critical dissolved oxygen concentrations ranging from approximately 1.0 to 1.8 mg L^{-1}. These observations demonstrate the hypoxia tolerance of black mudfish and moderate-oxygen tolerance of banded kokopu, while increased oxygen demand and hypoxia responses at relatively high DO levels are reflected in the distinct hypoxia sensitivity of inanga.

The studies on critical oxygen concentration thresholds and hypoxia sensitivities eliciting clear behavioural and physiological responses revealed a distinct response-inducing hypoxia level of 5 mg L^{-1} in inanga. This distinct level DO concentration was therefore utilised to achieve the third goal of this thesis (The effect of prolonged hypoxia exposure on locomotor activity, gill remodelling capacities in response to hypoxia and mediation of hypoxia responses by adaptations in oxygen sensing NEC and HIF-1 alpha densities). Here, groups of fish were acclimated to moderate inescapable hypoxia of 5 mg L^{-1} without access to the water surface (Chapter 4). Prolonged hypoxia acclimation was found to decrease locomotor activity in inanga, but not in banded kokopu or black mudfish, which indicates energy conserving strategies in moderate hypoxia in inanga, but not in banded kokopu and black mudfish at this level of hypoxia. None of the three species demonstrated hypoxia-induced gill remodelling capacities to increase the respiratory surface. Concurrently, these scale-less species have been shown to maintain oxygen uptake in response to hypoxia via recruitment of aerial skin respiration upon emersion (Meredith, 1985; McPhail, 1999; Urbina *et al.*, 2011), albeit only for short periods in inanga. This strategy is not feasible in the commonly scale-covered teleosts that have been shown to employ gill remodelling in response to hypoxia (Sollid *et al.*, 2003; Matey *et al.*, 2008; Mitrovic *et al.*, 2009). Behavioural and physiological responses to hypoxia were

furthermore not mediated by adaptations in the density of gill epithelial NECs and HIF-1 alpha protein, while normoxic stabilisation of HIF-1 alpha protein indicated piscine specific utilisation of the HIF-1 alpha pathway at normoxia, not observed in the mammalian pathway (Semenza, 2001).

The findings of this thesis therefore permit the following main conclusions:

1) Inanga, banded kokopu and black mudfish exhibit distinct species-specific responses to hypoxia that are potentially a result of adaptive radiation to specific oxygen environments. These differences are, however, not mediated by adjustments in NEC or HIF-1 alpha cell density.

2) Inanga, banded kokopu and black mudfish demonstrate distinct species–specific hypoxia sensitivities; more specifically, inanga can be described as the most hypoxia sensitive of the three species, while black mudfish exhibited no responses to hypoxia and thus can be described as the most hypoxia tolerant species.

In this context, inanga, contrary to banded kokopu and black mudfish, is characterised not only as a markedly hypoxia sensitive species, but also by limited capacities of hypoxia response mechanisms. Comparable conclusions were previously drawn by Urbina (2013), who investigated tolerance and responses of inanga to changes in DO concentration and salinity. While all three species were shown to sustain inescapable moderate hypoxia for prolonged periods, in the presence of normoxic refuge, inanga and banded kokopu have demonstrated distinct avoidance of comparable decreased DO levels. Temporary DO

fluctuations and moderate hypoxia may therefore not impact established populations in any of the three species. However, these findings indicate potential effects on upstream recruitment of juvenile inanga and banded kokopu upon migration into adult freshwater habitats at the conclusion of their marine larval development. In this context, the avoidance of escapable hypoxia may potentially impede upstream migration through hypoxic zones, thereby leading to decreased abundance of these species upstream. These suggestions are of particular concern in the context of intensified anthropogenic impacts on the natural environment (Diaz & Breitburg, 2009) and the potential impeding effects of global warming on the oxygen solubility and availability in aquatic environments (Wu, 2002).

5.2 Recommendations for future work

While findings of this thesis demonstrate species-specific hypoxia sensitivities and response capacities in inanga, banded kokopu and black mudfish (Chapter 2 – 4), the exact molecular and physiological mechanisms mediating these distinct adaptations remain unclear. It is therefore advisable to investigate metabolic parameters typically associated with a hypoxia–induced stress response (Richards, 2009; Cook & Herbert, 2012b), as well as circulatory and ventilatory adaptations in escapable and inescapable hypoxia and upon emersion in all three species to understand the physiological foundation of the distinct hypoxia sensitivities in these species. It may prove useful to utilise a different camera set-up for choice chamber studies on progressive, escapable hypoxia that allows views of the water surface. By installing higher resolution cameras, not only above, but also in front of the study arena, parameters such as aquatic surface respiration and bubble respiration may be quantified much more precisely. Moreover, it may be valuable to repeat respirometry studies utilising smaller respirometry chambers and an automated DO concentration measuring system to facilitate an estimation of standard metabolic rate rather than routine metabolic rate. This approach may enable an evaluation of the effect of hypoxia-induced changes in swimming activity on the metabolic oxygen demand and oxygen consumption profile. Furthermore, a more in–depth continuation of studies on HIF-1 alpha protein stabilisation and accumulation as well as on the associated pathway regulating hypoxia-inducible gene transcription, and on NEC functionality in dependence on hypoxia may elucidate the molecular basis and control of hypoxia responses in fish. Finally, the distinct hypoxia tolerance of black mudfish may have impeded any observation of responses in this species that might be exhibited at much lower

DO levels that those tested. Therefore, it may prove valuable to subject black mudfish to more severe levels of hypoxia to determine a critical oxygen tension for this species.

6 References

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7 Appendices

Appendix 2.1: Documentation of object tracking program

A HERO3 action video camera (frame rate: 50 frames per second; GoPro, Inc.) was used to record the experiments on individual fish from above the test arena. A simple location tracking framework was constructed using Matlab®, to objectively analyse the fish position within the tank as well as the activity level of the fish. Full colour images from every 25 frames were read by the framework for analysis. Information and parameters used in this framework include: Location and size of the test arena, fish minimum body size, colour differences between fish and background, and maximum speed of fish movements. Surroundings of the test arena were excluded from the analysis and frame-to-frame image differences (with set interval) were analysed under the assumption, that any significant differences between the two frames were generated due to fish movements. These differences were quality-assured by matching them with the given fish size and with the maximum speed of movement, in order to avoid errors derived from light changes or water ripple reflections. Once a specific fish shape was generated by the frame differences, the centroid of this shape was detected and marked as the location of the fish. In addition, colour-contrast differences between fish and background noise were also used, to identify likely fish locations. Due to the video and test arena settings, the results from this method were not as evidential as the frame-to-frame movement detection method, therefore applied only when no or less movement was detected by the other methodology, which included when fish were slowly drifting between frames (K. Muraoka, 2015).

Appendix 2.2: Two-stage change-point model for residence time

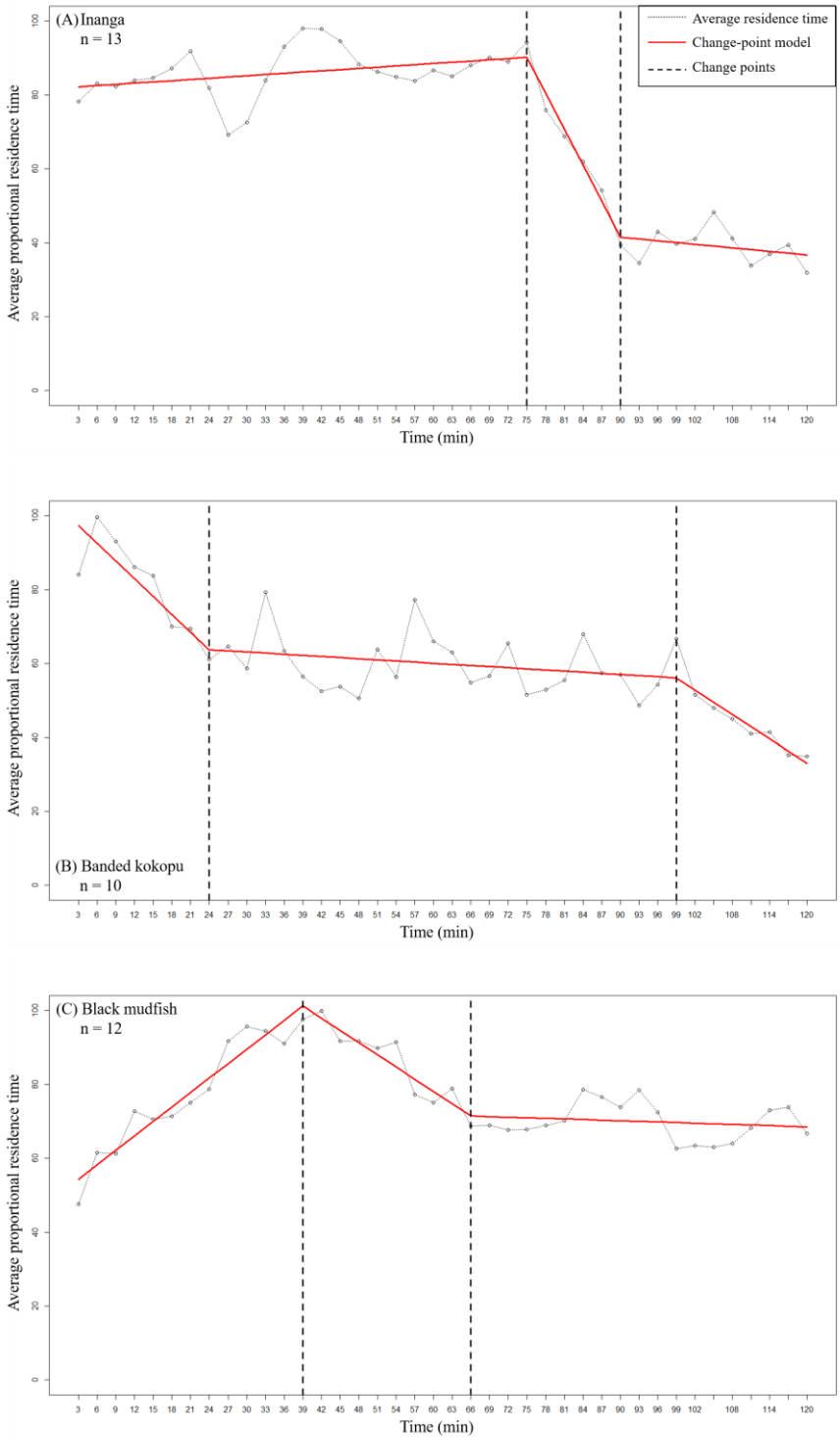

Figure 7.1: Change-point model graphs for average proportional residence time in inanga (A), banded kokopu (B) and black mudfish (C).

Appendix 2.3: Two-stage change-point model for side visits

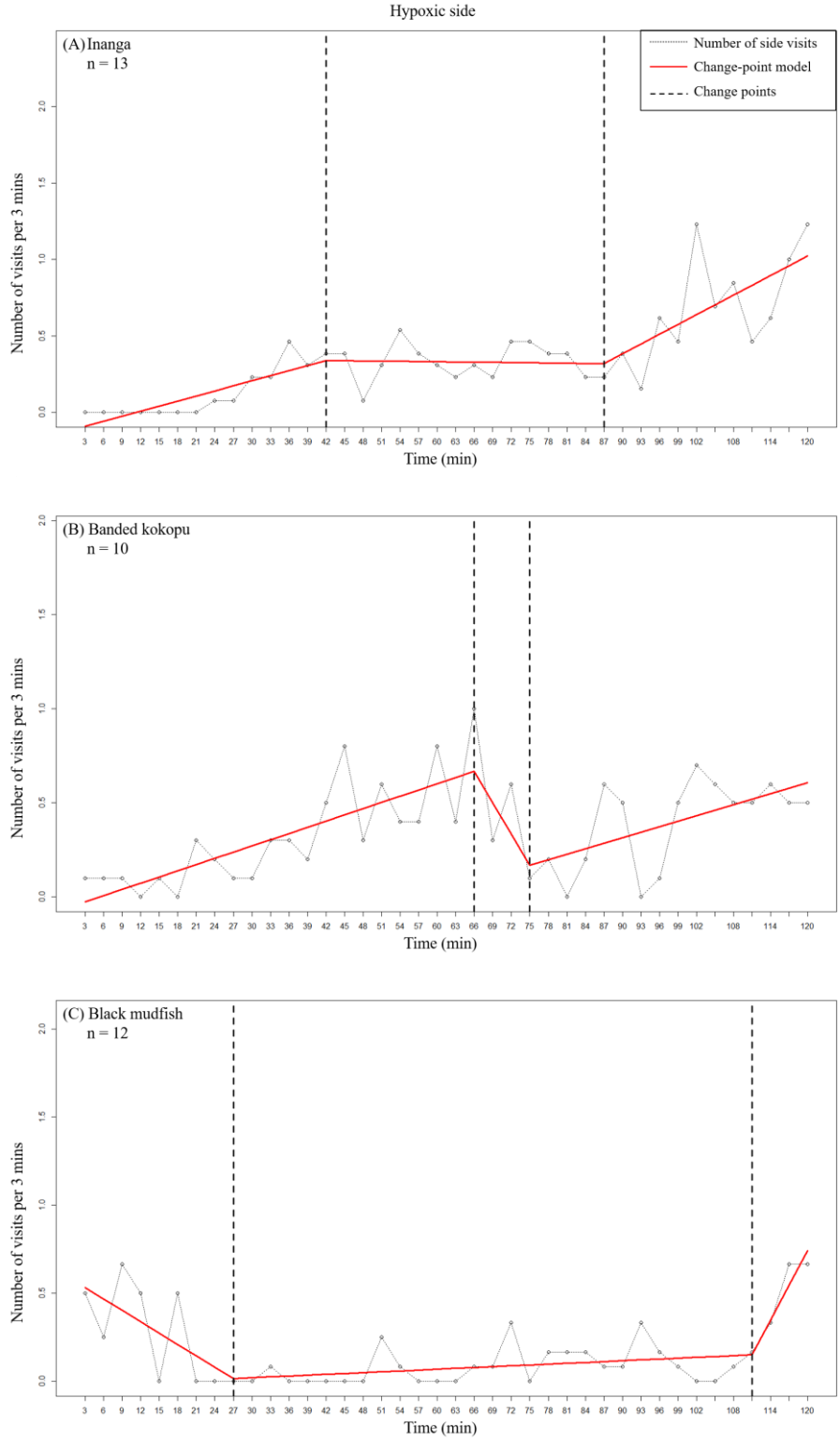

Figure 7.2: Change-point model graphs for average number of side visits into the hypoxic side in inanga (A), banded kokopu (B) and black mudfish (C).

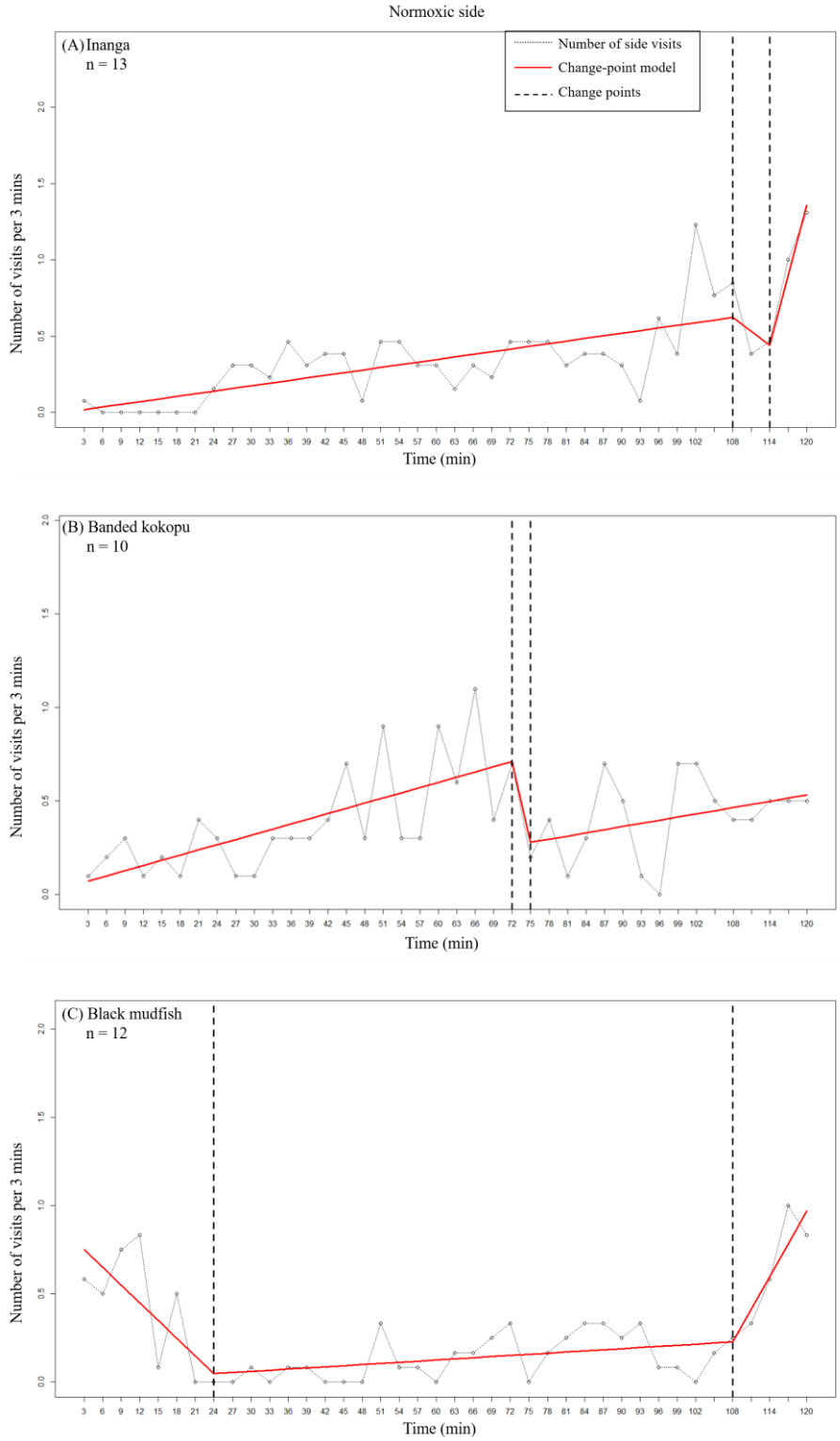

Figure 7.3: Change-point model graphs for average number of side visits into the normoxic side in inanga (A), banded kokopu (B) and black mudfish (C).

Appendix 2.4: Two-stage change-point model for aquatic surface respiration frequency

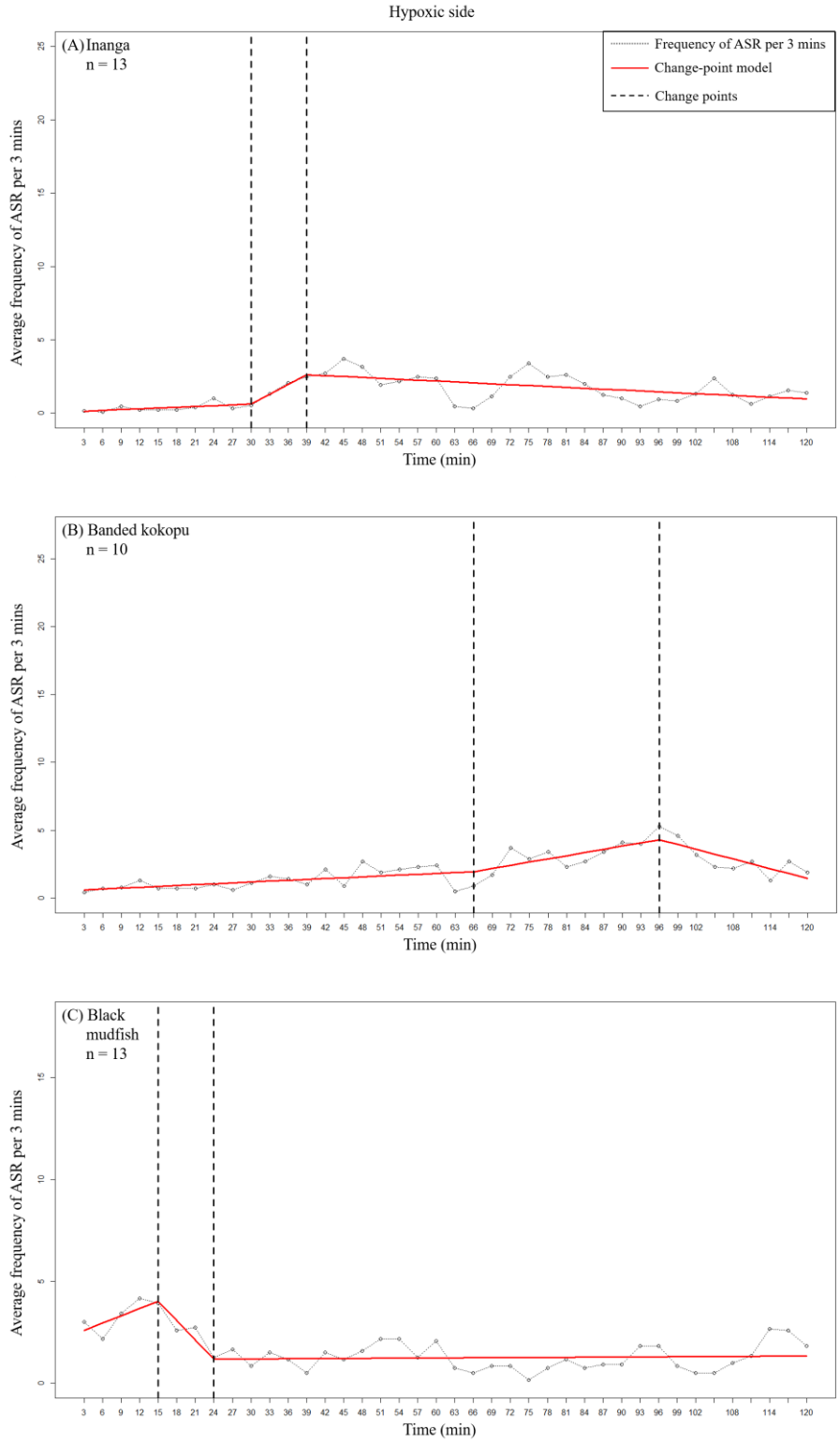

Figure 7.4: Change-point model graphs for average frequency of ASR per 3 mins in the hypoxic side in inanga (A), banded kokopu (B) and black mudfish (C).

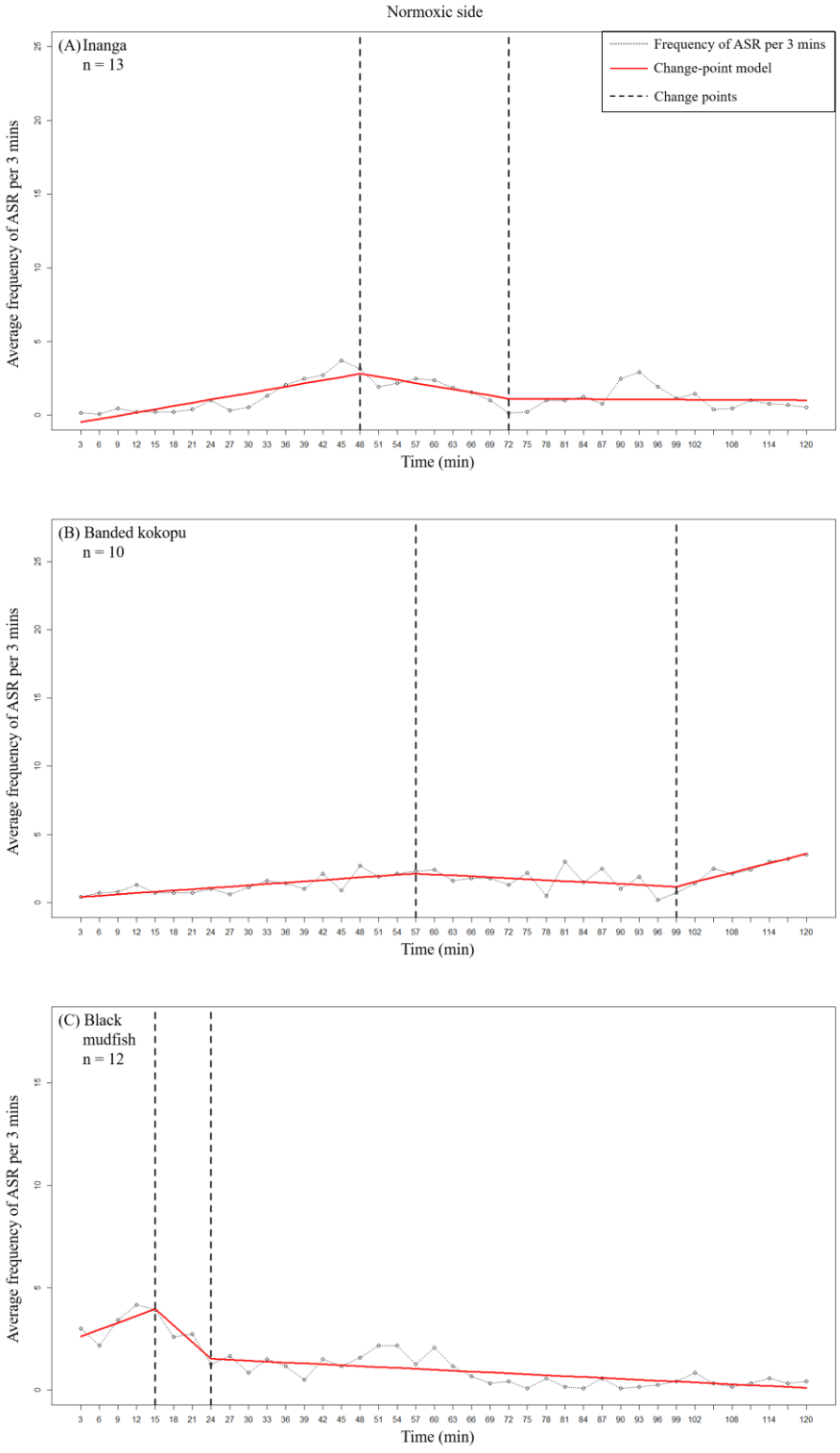

Figure 7.5: Change-point model graphs for average frequency of ASR per 3 mins in the normoxic side in inanga (A), banded kokopu (B) and black mudfish (C).

Appendix 2.5: Two-stage change-point model for aquatic surface respiration duration

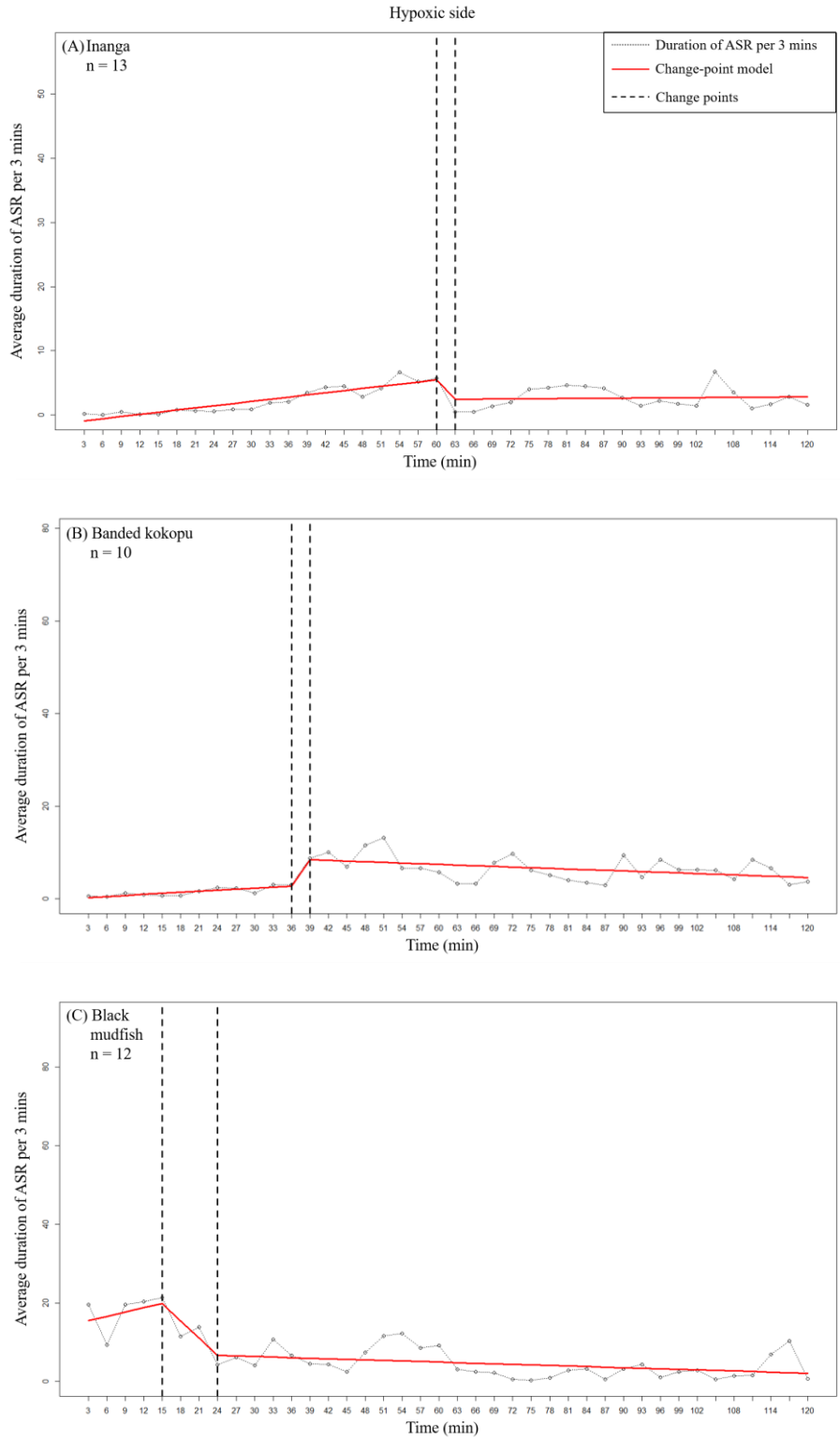

Figure 7.6: Change-point model graphs for average duration of ASR per 3 mins in the hypoxic side in inanga (A), banded kokopu (B) and black mudfish (C).

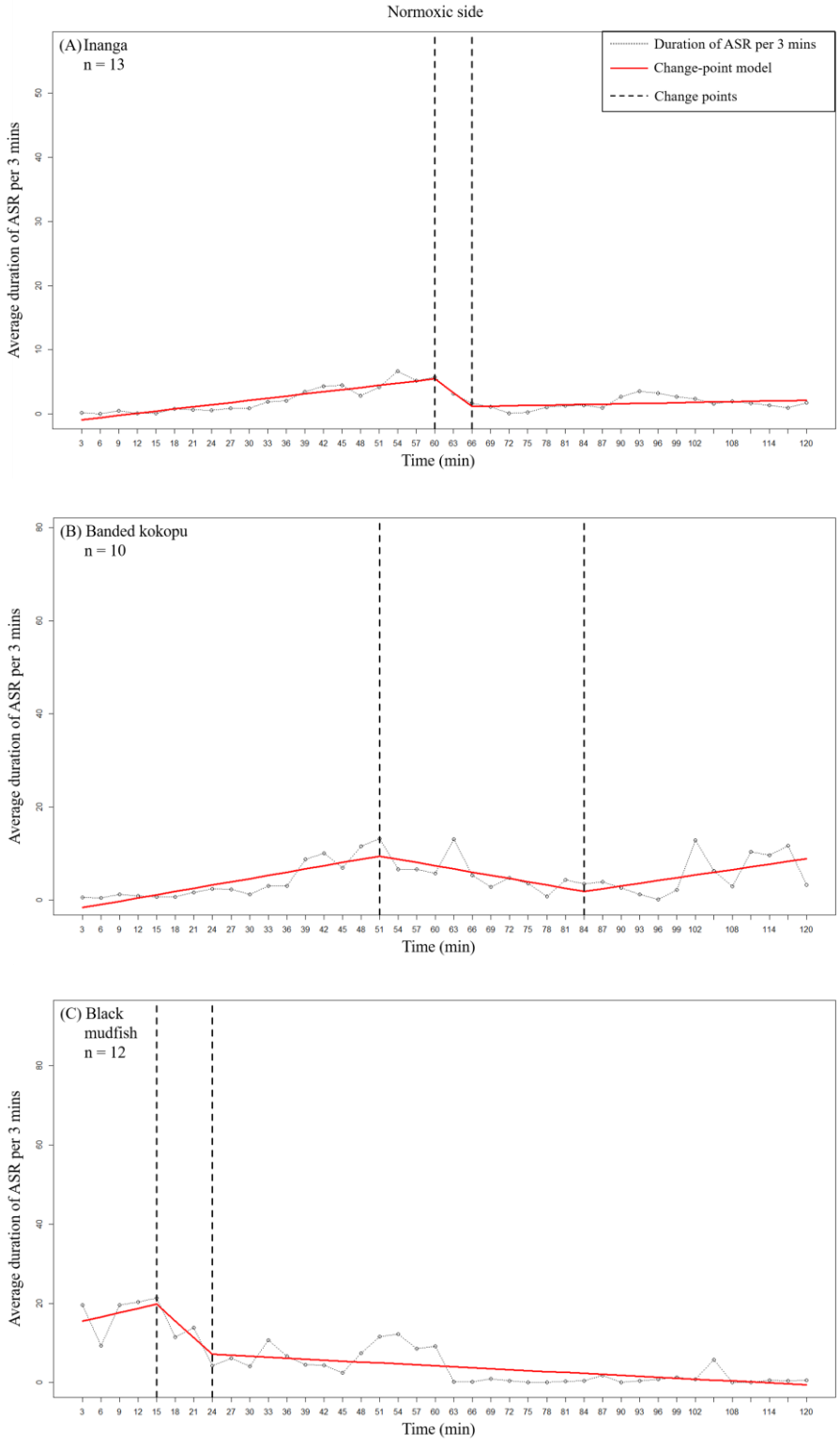

Figure 7.7: Change-point model graphs for average duration of ASR per 3 mins in the normoxic side in inanga (A), banded kokopu (B) and black mudfish (C).

Appendix 2.6: Two-stage change-point model for swimming speed

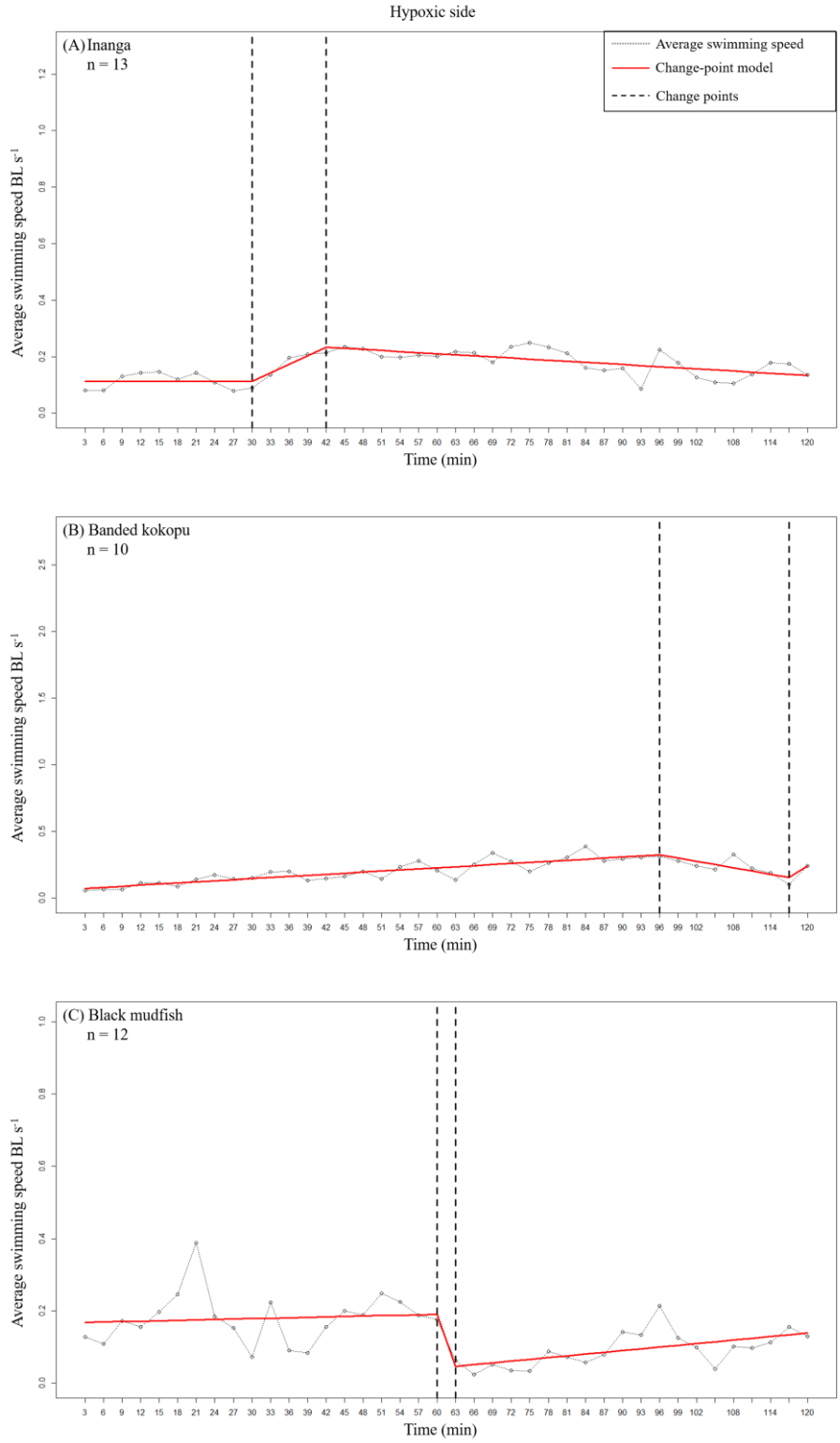

Figure 7.8: Change-point model graphs for swimming speed in the hypoxic side in inanga (A), banded kokopu (B) and black mudfish (C).

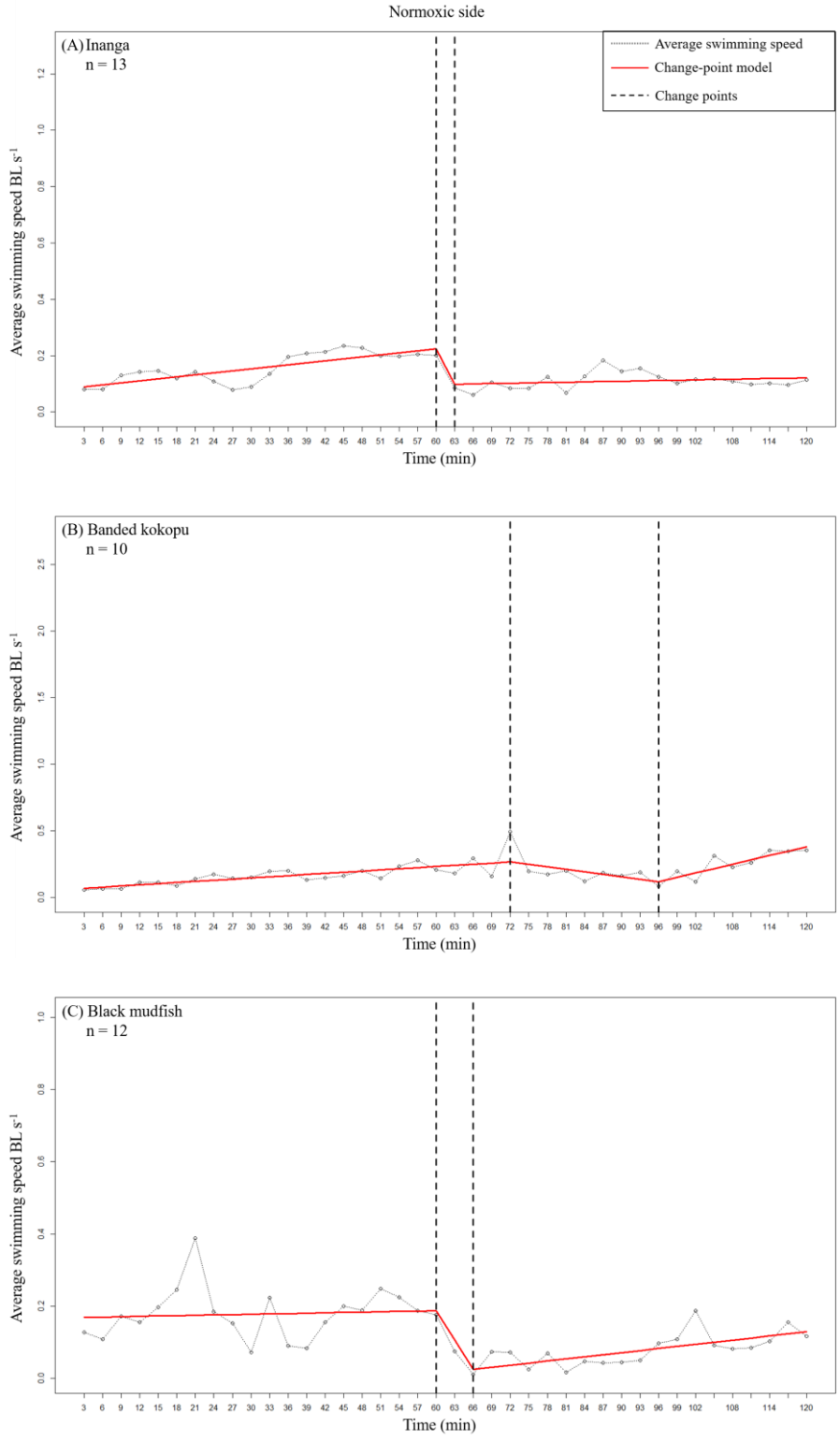

Figure 7.9: Change-point model graphs for swimming speed in the normoxic side in inanga (A), banded kokopu (B) and black mudfish (C).

Appendix 3.1: Respirometry chamber volume

Table 7.1: Measurements of empty and water-filled respirometry chambers required for respirometry chambers volume calculations.

	Weight (g)			
	Chamber #			
	1	2	3	4
Empty respirometer	808	850	828	800
Filled respirometer	1616	1646	1636	1616
Water	808	796	808	816

Appendix 3.2: Tukey's HSD inanga mass-specific oxygen consumption

Table 7.2: Post hoc Tukey's HSD for unequal n: *P*-values for inanga mass-specific oxygen consumption. $P < 0.05$ are marked by bold font.

	10.6 mg L^{-1}	7.2 mg L^{-1}	5.8 mg L^{-1}	4.8 mg L^{-1}	3.5 mg L^{-1}	2.5 mg L^{-1}
10.6 mg L^{-1}		0.9693	0.9926	0.1019	**0.0015**	**0.0002**
7.2 mg L^{-1}	0.9693		1	0.3747	**0.0131**	**0.001**
5.8 mg L^{-1}	0.9926	1		0.3838	**0.008**	**0.0007**
4.8 mg L^{-1}	0.1019	0.3747	0.3838		0.5456	0.0801
3.5 mg L^{-1}	**0.0015**	**0.0131**	**0.008**	0.5456		0.7967
2.5 mg L^{-1}	**0.0002**	**0.001**	**0.0007**	0.0801	0.7967	

Appendix 3.3: Tukey's HSD banded kokopu mass-specific oxygen consumption

Table 7.3: Post hoc Tukey's HSD for unequal n: *P*-values for banded kokopu mass-specific oxygen consumption. $P < 0.05$ are marked by bold font.

	10.6 mg L^{-1}	7.2 mg L^{-1}	5.8 mg L^{-1}	4.8 mg L^{-1}	3.5 mg L^{-1}	2.5 mg L^{-1}
10.6 mg L^{-1}		0.9905	0.3995	0.9997	0.2929	0.0975
7.2 mg L^{-1}	0.9905		0.1454	0.9996	0.095	**0.0287**
5.8 mg L^{-1}	0.3995	0.1454		0.2553	1	0.921
4.8 mg L^{-1}	0.9997	0.9996	0.2553		0.1764	0.0551
3.5 mg L^{-1}	0.2929	0.095	1	0.1764		0.9645
2.5 mg L^{-1}	0.0975	**0.0287**	0.921	0.0551	0.9645	

Appendix 3.4: Sample number of mean mass-specific oxygen consumption

Table 7.4: Particular n individual $\dot{M}O_2$, comprising mean mass-specific oxygen consumption at distinct DO levels in inanga, banded kokopu and black mudfish.

DO level (mg L^{-1})	Inanga	Banded kokopu	Black mudfish
10.6	10	10	9
7.2	10	10	8
5.8	10	9	9
4.8	10	10	9
3.5	10	9	9
2.5	6	7	8

Appendix 3.5: Tukey's HSD inter-species comparison of mass-specific oxygen consumption

Table 7.5: Post hoc Tukey's HSD *P*-values for inter-species comparison of mass-specific oxygen consumption. Applied was post hoc Tukey HSD for equal and unequal n respectively. $P < 0.05$ are marked by bold font. In: Inanga; Bk: Banded kokopu; Bm: Black mudfish.

	10.6 mg L^{-1}			7.2 mg L^{-1}			5.8 mg L^{-1}		
	In	Bk	Bm	In	Bk	Bm	In	Bk	Bm
In		**0.0392**	0.2149		**0.0481**	**0.0006**		**0.0002**	**0.0018**
Bk	**0.0392**		0.7429	**0.0481**		0.098	**0.0002**		0.428
Bm	0.2149	0.7429		**0.0006**	0.098		**0.0018**	0.428	

Appendix 3.6: Tukey's HSD inanga mass-independent oxygen consumption

Table 7.6: Post hoc Tukey's HSD for unequal n: *P*-values for inanga mass-independent oxygen consumption. $P < 0.05$ are marked by bold font.

	10.6 mg L^{-1}	7.2 mg L^{-1}	5.8 mg L^{-1}	4.8 mg L^{-1}	3.5 mg L^{-1}	2.5 mg L^{-1}
10.6 mg L^{-1}		0.9752	0.9396	0.9559	0.2586	**0.0347**
7.2 mg L^{-1}	0.9752		0.9999	0.5536	0.0527	**0.0062**
5.8 mg L^{-1}	0.9396	0.9999		0.5196	**0.0451**	**0.0035**
4.8 mg L^{-1}	0.9559	0.5537	0.5196		0.7613	0.1789
3.5 mg L^{-1}	0.2586	0.0527	**0.0451**	0.7613		0.8006
2.5 mg L^{-1}	**0.0347**	**0.0062**	**0.0035**	0.1789	0.8006	

Appendix 3.7: Tukey's HSD banded kokopu mass-independent oxygen consumption

Table 7.7: Post hoc Tukey's HSD for unequal n: *P*-values for banded kokopu mass-independent oxygen consumption. $P < 0.05$ are marked by bold font.

	10.6 mg L^{-1}	7.2 mg L^{-1}	5.8 mg L^{-1}	4.8 mg L^{-1}	3.5 mg L^{-1}	2.5 mg L^{-1}
10.6 mg L^{-1}		0.9886	0.3503	0.9976	0.2179	**0.0455**
7.2 mg L^{-1}	0.9886		0.1073	0.9011	0.0663	**0.0103**
5.8 mg L^{-1}	0.3503	0.1073		0.6904	0.9964	0.833
4.8 mg L^{-1}	0.9976	0.9011	0.6904		0.4343	0.1216
3.5 mg L^{-1}	0.2179	0.0663	0.9964	0.4343		0.9794
2.5 mg L^{-1}	**0.0455**	**0.0103**	0.833	0.1216	0.9794	

Appendix 3.8: Sample number of mean mass-independent oxygen consumption

Table 7.8: Particular n of individual $\dot{M}O_2$ comprising mean mass-independent oxygen consumption at distinct DO levels in inanga, banded kokopu and black mudfish.

DO level (mg L^{-1})	Inanga	Banded kokopu	Black mudfish
10.6	10	10	8
7.2	9	9	8
5.8	9	9	9
4.8	8	8	9
3.5	9	9	9
2.5	8	8	8

Appendix 3.9: Tukey's HSD inter-species comparison of mass-independent oxygen consumption

Table 7.9: Post-hoc Tukey HSD *P*-values for inter-species comparison of mass-independent oxygen consumption. Applied was post hoc Tukey HSD for equal and unequal n respectively. $P < 0.05$ are marked by bold font. In: Inanga; Bk: Banded kokopu; Bm: Black mudfish.

	10.6 mg L^{-1}			7.2 mg L^{-1}			5.8 mg L^{-1}		
	In	Bk	Bm	In	Bk	Bm	In	Bk	Bm
In		0.7239	0.2252		0.514	**0.0005**		**0.0004**	**0.0002**
Bk	0.7239		0.5776	0.514		**0.0046**	**0.0004**		0.9051
Bm	0.2252	0.5776		**0.0005**	**0.0046**		**0.0002**	0.9051	
	4.8 mg L^{-1}			3.5 mg L^{-1}			2.5 mg L^{-1}		
	In	Bk	Bm	In	Bk	Bm	In	Bk	Bm
In		0.7843	**0.0244**		0.1844	0.2737		0.3976	0.2967
Bk	0.7843		0.1005	0.1844		0.9132	0.3976		0.9732
Bm	**0.0244**	0.1005		0.2737	0.9132		0.2967	0.9732	

Appendix 4.1: Sample number of NEC density

Table 7.10: Distinct number of fish analysed for the effect of moderate hypoxia on NEC density.

	Inanga	Banded kokopu
Normoxic control	8	7
Moderate hypoxia	7	7

Appendix 4.2: Sample number of HIF-1 alpha density

Table 7.11: Distinct number of fish analysed for the effect of moderate hypoxia on HIF-1 alpha density.

	Inanga	Banded kokopu	Black mudfish
Normoxic control	8	9	9
Moderate hypoxia	7	8	8