EVOLUTION AND COMPARATIVE HAEMOGLOBIN OXYGEN BINDING IN NEW ZEALAND MUDFISHES

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Abstract

New Zealand's five endemic mudfish (*Neochanna* spp.) species have distributions that differ both geographically and by habitat type. Differences in habitat preferences between species have led to the proposal of an evolutionary series within the group. A morphological cline can be observed from the galaxiform Chatham Island and Canterbury species inhabiting lakes and streams, respectively, to the anguilliform Northland and brown mudfishes of ephemeral wetlands. Morphological specializations proposed for wetland dwelling include loss of pelvic fins, reduced eyes, enlarged nostrils, development of caudal flanges, and elongation of dorsal and anal fin bases to become almost confluent with the caudal fin. Another expectation of adaptation to wetland dwelling is specializations in respiratory physiology to obtain oxygen from highly hypoxic or acidic waters, and the ability to cope with seasonal exposure to air during the drought season. Expected respiratory specializations to wetland dwelling include high oxygen affinity haemoglobins, high levels of cooperative oxygen binding, the presence of multiple haemoglobins and the ability to aestivate and survive long periods of emersion.

The four mainland *Neochanna* species were examined to determine if differences in haemoglobin expression as well as differences in haemoglobin oxygen binding correlated with differing habitats and treatments. Whole blood oxygen affinity was determined at several pH levels (6.5, 7.0, 7.5 and 8.0) and temperatures (10ºC, 15ºC and 20ºC), as well as different treatments (aestivating, fasting and control) using a Hemox analyzer. The presence of multiple haemoglobins was determined by isoelectric focusing. All four species displayed high oxygen affinities (p50 = 6.5 to 9.5 mm Hg at pH 7.5 & 15ºC), moderate levels of cooperativity (Hill coefficients = 1.75 to 2.00 at pH 7.5 & 15ºC), pH sensitivity (Bohr coefficients = -0.62 to -0.94 between pH 7.5 and 7.0 at 15ºC), temperature sensitivity (ΔH = -2.20 to -15.78 k cal mol^{-1} between 10ºC and 15ºC) and the presence of multiple haemoglobins. Black, brown and Northland mudfish were able to survive aestivation for six weeks but there were no changes between air-breathing and water-breathing individuals with respect

to oxygen binding characteristics. Although there is evidence of habitat specialization in haemoglobin physiology between mudfish species, differences between species did not correlate with the evolutionary series proposed for specialization to dwelling in ephemeral wetlands and latitudinal distributions of mudfish species appear to strongly dictate oxygen binding properties of mudfish whole blood.

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Chapter One: General Introduction

1.1 The New Zealand mudfishes

The genus *Neochanna* was established by Günther (1867) and comprises highly distinctive members of a family of small, scaleless fishes (McDowall, 1997), the southern temperate Galaxiidae (Waters & McDowall, 2005). Although species of *Neochanna* have the typical galaxiid features such as being scaleless, having an elongated and tubular body with blunt heads and small eyes and a posterior dorsal fin, they are able to be distinguished from the genus *Galaxias* (McDowall, 1997). This is due to the reduced or absent pelvic fins, having flattened incisor-like jaw teeth, the presence of only a few or no endopterygoid teeth, long-based and low dorsal and anal fins that are nearly confluent with a distinctly rounded caudal fin, and elongated, tubular anterior nostrils that point forwards (McDowall, 1997).

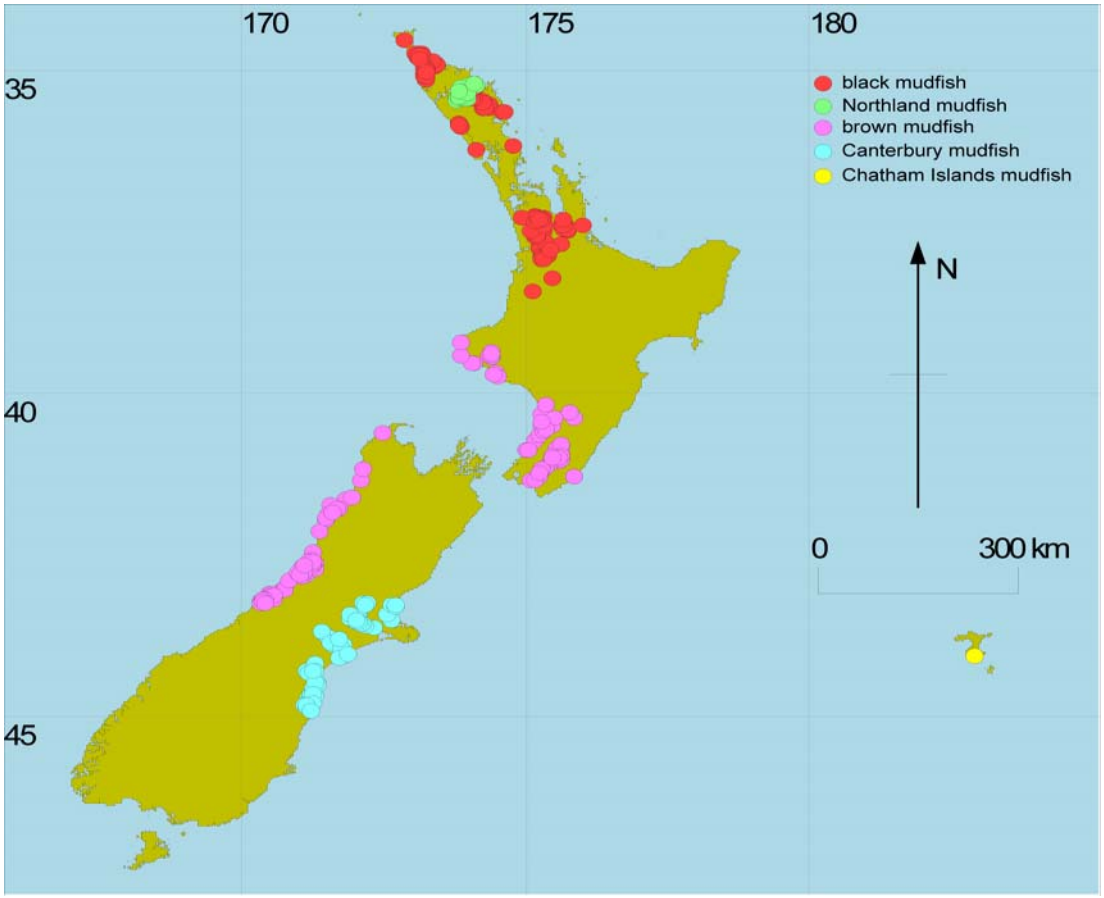

Figure 1: Distribution of mudfish species in New Zealand (FWFDB, NIWA).

There are five recognised species of *Neochanna* including brown mudfish (*N. apoda*), black mudfish (*N. diversus*), Northland mudfish (*N. heleios*), Canterbury mudfish (*N. burrowsius*) and Chatham Island mudfish (*N. rekohua*) that are endemic to New Zealand (Figure 1) and one species which is endemic to southern Australia, the Tasmanian mudfish (*N. cleaveri*). Of the six currently recognised *Neochanna* species, only the Tasmanian mudfish (Tasmania, Victoria) is diadromous (Fulton, 1986). These mudfish species show varying degrees of morphological and ecological specialization (Waters & McDowall, 2005). The five species of mudfish in New Zealand occupy different habitats which range from wetlands to streams and, in the case of the Chatham Island mudfish, lakes. These habitats vary widely throughout the country due to regional and local differences in soils, topography, climate, hydrology, water chemistry, vegetation and other factors including human disturbance (McDowall, 1990).

1.1.1 Brown mudfish (Neochanna apoda)

More than 100 years ago, Günther (1867) erected the genus *Neochanna* to contain a distinctive galaxiid which was found when drains were being dug in swamplands on the West Coast of the South Island of New Zealand. This small, cigar-shaped fish was named *Neochanna apoda* (Figure 2) and it was distinguished from previously described species of *Galaxias* by its lack of pelvic fins and girdle, teeth in the jaws being compressed and incisor-like, and the lack of endopterygoidal teeth (Günther, 1867).

Figure 2: A brown mudfish (*Neochanna apoda*)

Brown mudfish are found in a wide range of habitats, making it the most common and widespread of the five New Zealand mudfish species (Günther, 1867). Eldon (1968) found that they occupied habitats such as small, gravelly, spring-fed streams; over-grown weedy creeks; roadside drains; farm drains; open boggy swamps; forest swamps; pakihi bogs; coastal sand dune swamps; and water-filled depressions left after excavations. Eldon (1968), also found the brown mudfish to be most abundant in forest swamps and bogs, these habitats were once very widespread in the Wairarapa and are still common in Westland. Figure 1 shows that brown mudfish are widespread in the southern half of the North Island, from Opunake (Taranaki) southwards, west of the ranges and from Masterton (Wairarapa) southwards east of the ranges (McDowall, 1990). It is also common and widespread in the South Island on the West Coast, from north of Karamea to south of Okarito, mostly at low altitudes (Butler, 1999).

The brown mudfish is considered the most specialized of the mudfish species for wetland dwelling, as it has all the known mudfish morphological adaptations required for wetland dwelling. These adaptations are associated with living in a habitat filled with aquatic vegetation and debris and have resulted in changes to the body plan such as displaying "anguilliform" morphology, rounded fins, long nostrils and small eyes (Waters & McDowall, 2005). The brown mudfish exhibits modifications in fin morphology with the absence of pelvic fins and modifications to the dorsal, anal and caudal fins, making the three fins almost confluent and distinctly anguilliform (Waters & McDowall, 2005). Osteological adaptations such as an upfolded vomer, maxillary spur and palantine spur strengthen the anterior of the cranium and this has been proposed to be beneficial for pushing through aquatic vegetation and debris (McDowall, 1997). Mudfish are cryptic, essentially nocturnal, and thus have substantially higher forebrain-optic lobe ratios than many *Galaxias* species, which is correlated with reduced eye diameter (Cadwallader, 1975). The brown mudfish has a much bigger mouth, smaller eyes, and more rays in the dorsal and anal fins than other mudfish species in New Zealand. It has been recorded to grow up to 190 mm, however, few fish exceed 160 mm (N Ling, pers. com.). According to the

Department of Conservation New Zealand threats classification systems list in 2007, the brown mudfish are classified as a species in gradual decline.

1.1.2 Black mudfish (Neochanna diversus)

Stokell (1949) described a second species of mudfish from the northern part of the North Island, named *Neochanna diversus* (Figure 3) or the black mudfish. The black mudfish is very similar in many respects to the brown mudfish as it shares most of the same distinctive features of the brown mudfish, except that its jaw teeth are typical recurved fang-like teeth of other galaxiids, and it occasionally has endopterygoid teeth, even though when these are present they are few and small (McDowall, 1997). The black mudfish also lacks the bulbousness behind the eyes which occurs in the brown mudfish and it has a more rounded head, larger eyes, short-based dorsal and anal fins, and its caudal peduncle is neither as short nor as deep as the brown mudfish (McDowall, 1990).

Figure 3: A black mudfish (*Neochanna diversus*).

Black mudfish are distributed from the far north of New Zealand through to the lower Waikato region as far south as Piopio (Figure 1). They are typically found in swampy and overgrown habitats with either still or gently flowing water (McDowall, 1990). Black mudfish in the Waikato region are most commonly found in wetlands with an absence of water in summer, moderate depth of water in winter, limited modification of the vegetation and a low turbidity (Hicks & Barrier, 1996). Due to the draining of wetlands for agricultural development, their distribution has suffered greatly and they are mainly confined to large protected areas such as the Whangamarino wetland and the Kopouatai Peat Dome in the Waikato Region (Hicks

& Barrier, 1996), and the kauri-gum swamps around Kaitaia and Hikurangi in the Northland region (Ling, 2001).

In hypoxic water (<1.0 mg litre^{-1}), the black mudfish exhibits a primitive form of air-breathing which involves the fish travelling to the surface and ingesting air bubbles. The air bubble is held in the buccal cavity while the mudfish continues gill ventilation which suggests that the fish is passing water over the air bubble so that oxygen can diffuse from the air bubble to the water which can then be taken up by the gills (McPhail, 1999). This method of air-breathing has been described in goldfish (*Carassius auratus*), where a similar combination of air-gulping and gill ventilation improves blood oxygen transport under conditions of aquatic hypoxia (Burggren, 1982). The black mudfish has adaptations for prolonged respiration in air, these include a wider spacing of the secondary gill lamellae and a thinner epidermis its non air-breathing close relative, the inanga (*Galaxias maculatus*) (Dean, 1995). Drought resistance experiments showed that black mudfish can survive prolonged periods out of water where they remained quiescent but alert (McPhail, 1999). This quiescent behaviour and their reduced metabolic rate are mechanisms that conserve energy and reduce the production of nitrogenous wastes (Barrier et al. 1996). The black mudfish also produces a mucous layer on its skin during aestivation and a postulated function of this mucous is to inhibit desiccation (Graham, 1997). Thus, black mudfish appear to be well equipped behaviourally, morphologically, and physiologically to survive long periods without water. According to the Department of Conservation New Zealand threats classification systems list in 2007 the black mudfish is also classified as a species in gradual decline.

1.1.3 Canterbury mudfish (Neochanna burrowsius)

A third New Zealand species, the Canterbury mudfish was initially described as *Galaxias burrowsius* (Figure 4) by Phillipps (1926) and it closely resembles the other mudfish species in many characters. It was excluded from *Neochanna* by both Phillipps (1926) and Stokell (1949) as this species has pelvic fins, even though they are significantly smaller than those of other galaxiids (McDowall, 1997). It also has a

few, small endopterygoid teeth but like the black mudfish it has conical, fang-like jaw teeth. On closer observation of the bone structure of the snout it has been concluded that the Canterbury mudfish is very closely related to other mudfish species in New Zealand (McDowall, 1990). The key feature identifying this species of mudfish is that the pectoral fins (10-12 rays) are very small and fan-shaped, and the pelvic fins are very reduced: 4-5 rays compared to 6-7 rays found in *Galaxias* (McDowall, 1970). The very small eyes also distinguish it from most *Galaxias* species (McDowall, 1990). McDowall (1970) reclassified the Canterbury mudfish to be included within *Neochanna* because of the several distinctive similarities between the three species and considered the three species to form a radiation within the New Zealand Galaxiidae.

Figure 4: A Canterbury mudfish (*Neochanna burrowsius*)

The Canterbury mudfish is found on the alluvial Canterbury Plains on the east coast of the South Island (Figure 1), most often in weedy drains and irrigation races. It seems unlikely that the Canterbury mudfish naturally frequented creeks and drains, but that it, like the other mudfishes, occurred in wetlands (McDowall, 1990). Due to land development and agriculture these natural habitats have been drastically reduced, and populations of the Canterbury mudfish now persist mainly in creeks and streams (O'Brien, 2005).

This species is well documented for its ability to aestivate during droughts. Stokell (1949) found that these mudfish could survive drought by burying in mud if forced to do so by falling water levels. Cadwallader (1975) speculated that the fish may form small chambers in the mud into which they not only retreat for the duration of a

drought period but also for every-day protection against predators, leaving at night to forage. However, Eldon et al. (1978b) found that only 12 out of 71 fish were found aestivating in holes and burrows and thus concluded that the fish aestivated in whatever situation they occupied when the drought overtook them. Canterbury mudfish have since been found to aestivate in holes while others were in vegetative debris, root masses, car tyres and other similar microhabitats (O'Brien, 2005). According to the Department of Conservation New Zealand threats classification systems list in 2007 the Canterbury mudfish are classified as being nationally endangered.

1.1.4 Northland mudfish (Neochanna heleios)

Neochanna heleios (Figure 5) or the Northland mudfish was discovered in 1998 and is one of New Zealand's rarest native freshwater fishes (Ling & Gleeson, 2001). It was found during an investigation of the phylogenetic relationships and geographical structure between populations of black mudfish by Gleeson et al. (1999). Two mudfish populations previously described as black mudfish were found to be genetically and morphologically distinct, and were subsequently described as a new *Neochanna* species (Ling & Gleeson, 2001). They found that there were significant differences in both genetic diversity and divergence between populations of mudfish between Northland and Waikato (Ling & Gleeson, 2001). The similar appearance of the Northland species to the black mudfish, and the fact that it bisects the range of the black mudfish, has prevented its discovery until now (Ling & Gleeson, 2001).

Figure 5: A Northland mudfish (*Neochanna heleios*)

This species of mudfish combines several characters of the black and brown mudfishes. It resembles the black mudfish in the similarity of the tail and the

relatively slender caudal peduncle and moderately sized dorsal and anal fins (Ling & Gleeson, 2001). But it also resembles the brown mudfish, especially in the head region with a sloped forehead, small eyes, a dorsal bulbous swelling behind the eye and a mouth that extends far back to the posterior margin of the eye (Ling & Gleeson, 2001). The Northland mudfish differs from Canterbury mudfish by lacking pelvic fins and having smaller eyes (10-15% of head length). This species of mudfish is morphologically very specialized to wetland dwelling due to a number of morphological adaptations which results in more anguilliform characteristics such as the loss of pelvic fins, and the dorsal, anal, and caudal fins becoming more confluent (Ling & Gleeson, 2001). Associated with these changes to the body plan are elongated nostrils, small eyes and an increase in anterior cranial ossification (Waters & McDowall, 2005).

Northland mudfish are found only at sites on the Kerikeri volcanic plateau and coastal wetlands near Kerikeri (Figure 1). This new taxon bisects the range of black mudfish and is currently not known from any other region (Ling & Gleeson, 2001). The habitat requirements of the Northland mudfish are similar to those of the black mudfish, preferring non-turbid wetlands on acid soils (Ling, 2001). No surveys of wetland habitats in central Northland have recorded the presence of both black and Northland mudfish, which may indicate that these species do not co-occur. Through knowledge of Northland's geological history it has been suggested that Northland mudfish may be in fact an ancestral Northland mudfish species whereas the black mudfish may be a more recent immigrant (Gleeson et al., 1999). The Northland mudfish is a high priority for conservation action, as its distribution is extremely restricted and land development in the area poses a serious threat to its survival due to further destruction of its remnant habitat (Ling & Gleeson, 2001). According to the Department of Conservation New Zealand threats classification systems list in 2007 the Northland mudfish are classified as being nationally endangered.

1.1.5 Chatham Island mudfish (Neochanna rekohua)

Skrzynski (1967) speculated that the Chatham Islands should host mudfish populations due to the large areas of "mudfish-like" wetland habitat present on the islands. Mitchell (1995) responded to that speculation by stating that no mudfish had been found but that he had found a new species of *Galaxias* inhabiting a lake in a peat swamp which he named *Galaxias rekohua*. McDowall (2004) re-examined the species and concluded that the Chatham's endemic galaxiid is actually a mudfish, based on studying material such as the paratypes of specimens from Lake Tuku a Taupo provided by both Mitchell and Department of Conservation, and specimens from Lake Rakeinui. Adult Chatham Island mudfish (Figure 6) exhibited the general body form of other mudfishes, similar swimming style, air-breathing behaviour, small eyes, long tubular anterior nostrils and strongly developed caudal flanges (McDowall, 2004). With the exception of the distinctly forked caudal fins as juveniles, the lack of a deeply clefted, Y-shaped anterior ethmoid region with a vomer that turns dorsally up the anterior of the ethmoid region, and the absence of reduction in size and fin ray counts in the pelvic girdle, the Chatham Islands mudfish fits comfortably among the *Neochanna* species. The above characteristics appear to place Chatham Island mudfish as basal to the other mudfish species (McDowall, 2004).

Figure 6: A Chatham Island mudfish (*Neochanna rekohua*).

The Chatham Island mudfish are restricted to the Chatham Islands (Figure 1) and are known only in Lake Tuku a Taupo and Lake Rakeinui and not in associated wetlands (McDowall, 2004). Further lakes in the vicinity of Tuku a Taupo and Rakeinui have not yet been sampled, so the distribution of this species may be broader than presently known (McDowall, 2004). Chatham Island mudfish are unique among

New Zealand mudfish as it occupies lakes within peat swamps rather than the wetland habitats typically occupied by the mainland species. It has been hypothesised (Waters & McDowall, 2005) that as a result of this, the species has either retained or regained a number of morphological character states such as median ethmoid ossification and a truncate caudal fin which are absent in the mainland mudfish taxa. Thus, due to these differences in morphology, the Chatham Island mudfish is the least specialised at wetland dwelling out of all the New Zealand mudfish (McDowall, 2004). The aestivation abilities of the lake-dwelling Chatham Island mudfish are still unknown. Under the threat classification lists of the Department of Conservation, Chatham Island mudfish are classified as range restricted.

1.1.6 The proposed evolutionary series of Neochanna

Research carried out on the phylogenetics of the Australasian mudfishes by Waters & McDowall (2005) has suggested that an evolutionary transition in morphological characters has occurred. Differences in habitat preferences and geographical distributions between species have lead to the proposal of an evolutionary series within the endemic *Neochanna* species. Morphological specializations proposed for wetland dwelling include loss of pelvic fins, reduced eyes, enlarged nostrils, development of caudal flanges, and elongation of dorsal and anal fin bases to become almost confluent with the caudal fin. On the basis of these adaptations a morphological cline may be observed from the galaxiform Chatham Island and Canterbury species (least specialized to wetland dwelling) inhabiting lakes and streams, respectively, to the anguilliform Northland and brown mudfishes (most specialized to wetland dwelling) of ephemeral wetlands. Due to the fact that the wetlands seasonally dry out, mudfish have also had to adapt physiologically so that they are able to survive prolonged periods of time out of water. To be able to survive out of water mudfish must be able to avoid desiccation, respire in air, eliminate nitrogenous waste and be able to survive without feeding (McPhail, 1999). The ability to aestivate appears common to the group although this has not been investigated in the Chatham Island species. Until now there have been few

physiological studies carried out on the haemoglobin system of the New Zealand mudfishes.

1.2 The haemoglobin system

It has been found that the common mode of gas exchange in the Canterbury mudfish occurs through the skin with more than 50% of oxygen being taken up by the skin in either air or water (Wells et al, 1984). Once oxygen diffuses across the respiratory epithelium into the blood, it combines with a respiratory pigment that gives the characteristic colour to the blood. The respiratory pigment in mudfish blood, as in all other vertebrates, is haemoglobin and it is bright red when loaded with oxygen and a dark maroon-red when deoxygenated. Haemoglobin contains four iron-containing porphyrin prosthetic groups (haeme) which consist of two α- and β-polypeptide chains (Wells, 1999). Each haemoglobin molecule can combine with four oxygen molecules and the extent to which oxygen is bound to haemoglobin varies with the partial pressure of the gas (Randall et al, 2000). The amino acid sequence of the polypeptide chains determines the folding of each chain and it is the interaction between the subunits of the haemoglobin that determines the oxygen binding properties. Small changes in the tertiary structure of segments near the haems and a large shift in the quaternary structure from the tense state (deoxygenated state) to the relaxed state (oxygenated state) causes oxygen binding to the haemoglobin (Wells, 1999).

The essential functional feature of haemoglobin as a respiratory pigment is that it allows oxygen loading (at high pO_2) and unloading (at low pO_2) to occur over a relatively narrow range of oxygen pressures and so it can act as an oxygen carrier from the respiratory surface to the tissues (Randall et al., 2000). Haemoglobins found in mudfish have a relatively high oxygen affinity and are therefore saturated at low partial pressures. This facilitates the movement of oxygen into the blood from the environment because the oxygen is bound to haemoglobin at a low partial pressure but for the haemoglobin to release the oxygen the partial pressure must be even lower

(Wells et al., 1984). The oxygen affinity of whole blood is defined by the half-saturation oxygen tension (p50). This value is the partial pressure of oxygen required to saturate 50% of the haemoglobin, thus the lower the p50 value the higher the whole blood oxygen affinity. Hypoxia tolerant mudfish such as the Canterbury mudfish have a relatively high oxygen affinity, with p50 values of 11 mm Hg at pH 7.8 and 12ºC (Wells et al., 1984) which is comparable to the whole blood oxygen affinity of hypoxia tolerant common carp (*Cyprinus carpio*) with values of 7 mm Hg at pH 7.9 and 20ºC (Weber & Lykkeboe, 1978). However, these values are significantly lower than those recorded for hypoxia intolerant fishes such as the rainbow trout (*Oncorhynchus mykiss*) (22 mm Hg at pH 7.8 and 15 ºC; Weber et al., 1976), and the Antarctic dwelling *Pagothenia borchgrevinki* (31.1 mm Hg at pH 8.0 and -1.5 ºC; Wells et al., 1989). For haemoglobin with a high oxygen affinity it is significant that the oxygen affinity is affected by changes in chemical and physical factors that favour oxygen binding at the respiratory epithelium and oxygen release in the tissues (Randall et al., 2000).

1.2.1 The Bohr effect

The S-shaped oxygen equilibrium curve and the Bohr effect were first described by the Danish physiologist, Christian Bohr in 1904. Because of the Bohr effect, an increase in blood carbon dioxide level (Bohr et al., 1904) or a decrease in pH (Astrup & Severinghaus, 1985) causes haemoglobin to bind to oxygen with less affinity. The oxygen equilibrium curve shifts to the right when carbon dioxide concentration or hydrogen ion concentration is increased. Canterbury mudfish showed a rightward displacement of the oxygen equilibrium when pH changed from 7.8 to 7.4 with p50 values changing from 11 mm Hg to 18 mm Hg, respectively (Wells et al., 1984).

The Bohr effect significantly increases the release of oxygen from haemoglobin to the tissues under any given partial pressure of oxygen (pO_2) at the capillaries. It facilitates oxygen transport as the haemoglobin is close to fully saturated at the lungs or gills. As the blood is circulated through the tissues, oxygen is offloaded while passing the capillary beds, where pO_2 decreases (Jensen, 2004). When tissue oxygen

consumption increases, capillary pO_2 decreases further, and more oxygen is extracted from the blood. When a tissues metabolic rate increases, its carbon dioxide production increases and the carbon dioxide is quickly converted into bicarbonate molecules and acidic protons by the enzyme carbonic anhydrase (CA). This causes the pH of the tissues to decrease, and so increases the dissociation of oxygen from haemoglobin, allowing the tissue to obtain enough oxygen to meet its demands (Jensen, 2004). Carbonic anhydrase is essential for the Bohr effect, as it secures rapid carbon dioxide hydration and red blood cell acidification in tissue capillaries (and the reverse in lung/gill capillaries), which allows the Bohr shift to occur during capillary transit (Maren & Swenson, 1980). During exercise, when a critically low capillary pO_2 is reached, lactic acid in the muscles further increase oxygen dissociation and thus facilitates oxygen delivery (Stringer et al., 1994).

Research has shown that water-breathing fish have very low circulating carbon dioxide tensions compared with air-breathers, as most fishes have low rates of aerial carbon dioxide release (Graham, 1997). The water to air transition causes a rise in arterial pCO_2 and Rahn et al. (1971) predicted that this would cause a fall in erythrocyte pH which would cause a decrease in oxygen affinity. Wood et al. (1979) found that the acidosis caused by increased arterial pCO_2 is fully compensated by raised plasma bicarbonate and so erythrocyte pH is kept reasonably constant. Species of fish such as the reedfish (*Erpetoichthys calabaricus*; Beitinger et al., 1985) displayed a constant blood oxygen affinity during brief transitions to air but other species such as the Amazonian osteoglossids show a lower oxygen affinity when exposed to air (Powers et al., 1979). Thus it is not clear why differences in blood oxygen affinity between water-breathers and air-breathers occur.

1.2.2 Effects of organic phosphate compounds

The binding of organic phosphate compounds to haemoglobin reduces the oxygen affinity of most vertebrate haemoglobins (Randall et al., 2000). In most fishes, adenosine triphosphate (ATP) and/or guanosine triphosphate (GTP) are the phosphorylated compounds that modulate the oxygen affinity of haemoglobin

(Jensen, 2004). Organic phosphates bind preferentially to the T (deoxy) structure in the central cavity between the two β-chains of the haemoglobin and decrease oxygen affinity by further modulating the allosteric interaction between oxygen and hydrogen ion binding sites (Jensen, 2004). Phosphorylated compounds in the erythrocyte not only affect the oxygen affinity of haemoglobin but also increase the magnitude of the Bohr effect and may affect subunit interaction (Randall et al., 2000). An increase in erythrocyte organic phosphate concentrations results in a rightward displacement of the oxygen equilibrium (Wells, 1999). This phenomenon has been characterised in a number of different fishes such as species of Anguilliforms (Olianas et al., 2005), rainbow trout (Nikinmaa, 2001) and the southern blue fin tuna (*Thunnus maccoyii;* Yokoyama, 2004).

Blood oxygen-affinity is often increased as a short-term response to hypoxic challenge (Wood & Johansen, 1972). Wood and Johansen (1972) demonstrated that *Anguilla anguilla* showed an increase in blood oxygen affinity when subjected to hypoxic conditions. The principal mechanism for this is an increase in cell volume coupled with an increase in erythrocyte pH which is followed by a decrease in red cell organic triphosphates (RCOP) such as ATP and GTP (Powers, 1980), which are potent regulators of blood oxygen affinity (Nikinmaa, 2001). During exposure to hypoxia, the cellular nucleoside triphosphate (NTP) levels decrease markedly and within hours to days, there is a marked increase in haemoglobin oxygen affinity (Weber & Jensen, 1988).

Catecholamines are the proteins that mediate the increase in erythrocyte pH in response to hypoxic conditions (Tetens & Christensen, 1987). These proteins are responsible for activating the sodium/proton exchanger in the erythrocyte membrane which causes the extrusion of protons from the erythrocyte and increases erythrocyte pH as well as oxygen affinity (Nikinmaa, 2001). The adrenergically activated sodium/proton exchange is uniquely suited to increase erythrocyte pH in hypoxic conditions. This is because hypoxia is a powerful stimulus for the release of catecholamines, the number of ß-adrenergic receptors increase under hypoxic

conditions, in some species the concentration of cyclic adenosine monophosphate (cAMP) is higher under hypoxic conditions and also the turnover rate of the sodium/proton exchanger is greater during hypoxia (Nikinmaa, 2001). Due to adrenergic stimulation, erythrocyte volume also increases (accumulation of osmotically active sodium and chloride ions). This dilutes the intracellular medium and the dilution of haemoglobin and organic phosphates will tend to increase oxygen affinity by reducing the interaction between organic phosphates and haemoglobin (Nikinmaa, 1992).

The ATP/GTP ratio varies in different species of fish; *Labeo capensis* has high ATP concentrations in the blood (Frey et al., 1998), whereas in Canterbury mudfish, GTP appears to play a greater role in hypoxic adaptation (Wells, 1984). GTP appears to be more abundant in species which tolerate hypoxic conditions well than in species requiring well oxygenated waters. GTP generally has a greater effect on haemoglobin oxygen affinity than ATP at similar NTP/haemoglobin molar ratios. This is mainly because GTP can form an additional hydrogen bond with haemoglobin, compared to ATP, which leads to an increased stability of the low-affinity deoxy conformation of haemoglobin (Weber & Jensen, 1988).

1.2.3 Haemoglobin concentration and heterogeneity

An increase in blood haemoglobin concentration raises the oxygen capacitance coefficient and this is an energetically inexpensive means of adjusting blood oxygen transport to increased oxygen demand compared with increased ventilation or cardiac output (Weber & Jensen, 1988). The rate at which oxygen can bind to haemoglobin also depends on the concentration of haemoglobin in the blood. The more oxygen bound per unit time, the longer the persistence of a large diffusion gradient across the respiratory epithelium for oxygen and, therefore, the higher the rate of oxygen transfer (Randall et al., 2000). Changes in haemoglobin concentration during exercise may result from water movement from the extracellular to the intracellular lactate-loaded tissue compartments, increased diuresis, and/or a rapid release of stored erythrocytes via splenic contraction which can be observed in the Japanese

amberjack (*Seriola quinqueradiata*; Yamamoto et al., 1985). The stress of aquatic hypoxia evokes variable responses in fishes; haemoglobin concentration remains unchanged in plaice (*Pleuronectes platessa*; Woods et al., 1975), common carp (Weber & Lykkeboe, 1978) and tench (*Tinca tinca*; Jensen & Weber, 1985), but increases in European eel (*Anguilla anguilla*; Wood & Johansen, 1972), rainbow trout (Soivio et al., 1980) and Japanese amberjack(Yamamoto et al., 1985).

A common characteristic of teleost fish is the presence of multiple haemoglobins with differences in functional properties and intrinsic oxygen affinities (Weber & Jensen, 1988). These different types of haemoglobin increase the scope of oxygen, proton and heat transport in the blood of the organism (Weber & Jensen, 1988). As well as haemoglobin having anodic components with normal Bohr and Root effects, many teleosts have cathodic components, which display high oxygen affinities and small, often reversed, Bohr effects (Olianas et al., 2005). Under the conditions of internal hypoxia and acidosis, fish like rainbow trout and European eels have cathodal haemoglobin components which have a high, pH-insensitive oxygen affinity to transport oxygen to the tissues whereas under normoxic conditions the low-affinity, pH-sensitive anodal haemoglobin components unload oxygen to the tissues (Weber et al., 1975). Research carried out on ectothermic vertebrates has suggested that cathodic components of haemoglobin may safeguard oxygen uptake under hypoxic and acidic conditions (Weber, 1990).

Alterations in the relative abundance of the different haemoglobins provide organisms with a further mechanism for functional adaptation (Weber et al., 1976). Changes in teleost isohaemoglobins commonly accompany temperature acclimation and in rainbow trout the abundances of isohaemoglobins also depends on day length and acclimation to hypoxic conditions (Tun & Houston, 1986).

1.2.4 Effects of temperature

In fish, an acute temperature rise decreases oxygen affinity directly due to overall exothermy of haemoglobin oxygenation, and indirectly due to the associated pH

decrease (Randall et al., 2000). An increase in temperature usually causes a rightward displacement of the oxygen dissociation curve (Randall et al., 2000), although the haemoglobin of the endothermic southern bluefin tuna shows reversed temperature sensitivity (Bushnell & Jones, 1994).

Temperature acclimation influences erythrocytic NTP and p50 and thus temperature sensitivity, but the responses differ between species (Weber & Jensen, 1988). In the Australian blackfish (*Gadopsis marmoratus*), temperature acclimation increases blood NTP and p50 (Dobson & Baldwin, 1982). Acclimated blackfish exhibited greater p50 changes than acutely exposed blackfish, which suggests adaptive increases in unloading of haemoglobin as oxygen demand increases with temperature. However, in species such as common carp, the opposite response lowers the temperature effect in acclimated animals and thus may suggest that this is an adaptation to ensure oxygen loading at the respiratory surfaces at high temperatures (Albers et al., 1983).

1.2.5 Cooperative oxygen binding

Haemoglobins that bind ligands cooperatively are widely distributed in organisms from bacteria to mammals (Randall et al., 2000). Archibald Vivian Hill was the first to deduce the essential feature of cooperativity which was that the binding of the first ligand makes it easier to bind a second one (Hill, 1913). The Hill coefficient provides a means to quantify this effect. Cooperative binding is a special case of allostery and it requires that the macromolecule has more than one binding site as cooperativity results from the interactions between binding sites. If the binding of a ligand at one site increases the affinity for a ligand at another site then the macromolecule displays positive cooperativity and it has a Hill coefficient greater than 1. A negative cooperativity occurs when the Hill coefficient is less than 1 which means that when a ligand binds to a site it decreases the affinity for a ligand at another site. When the Hill coefficient equals 1 then the macromolecule is non-cooperative and the affinity of the enzyme to a ligand is not dependent on other ligands already bound (Riggs, 1998).

There are several theoretical models that try to explain cooperativity in haemoglobin but only two models have survived quantitative and qualitative testing (Eaton et al., 1999). These models are the MWC model (Monod et al., 1965) and the 'stereo-chemical mechanism' (Perutz, 1970). In the MWC model, cooperativity in the haemoglobin arises from an equilibrium between two structures having different arrangements of the subunits, the quaternary structure. The 'tense' or T quaternary structure has a low affinity for ligands such as oxygen while the 'relaxed' or R quaternary structure has a high affinity for ligands. Cooperativity arises from a shift in the population of T quarternary structures to R quaternary structures with increasing oxygen pressure (Monod et al., 1965). Perutz (1970) went further than just identifying the T and R structures by describing them in detail. He found that there was a set of salt bridges at the subunit interfaces that are present in the T structure but not in the R structure. Iron displacement associated with oxygen binding to the heme in the T quaternary structure could move a helix, break a salt bridge, release a proton, and destabilize the structure which would bias the quarternary equilibrium towards the R state. In the mechanism proposed by Perutz, the salt bridges had three roles: they stabilize the T structure relative to the R structure, lower the oxygen affinity in T because of the energy required to break them upon oxygen binding, and they release protons upon breakage which explains why the Bohr effect can operate (Eaton et al., 1999).

Cooperative oxygen binding is largely responsible for the sigmoidal nature of the haemoglobin-oxygen dissociation curve which is necessary for efficient oxygen transport in vertebrates as it allows large volumes of oxygen to be bound or released for relatively small changes in the partial pressure of oxygen in the blood (Weber & Jensen, 1988).

1.3 Air breathing and aestivation

There is a strong evolutionary selection pressure to breathe air over water. Water in equilibrium with air is 800-fold more dense and contains 30-fold lower oxygen

content (Wells, 1984). Air-breathing is one of many adaptive responses utilized by certain species of fish dwelling in habitats with low oxygen levels and signifies a marked departure from the "typically piscine" respiratory mode. An air-breathing fish may swim to the water surface, gulp air and then dive, or it may crawl onto land and either gulp air or passively exchange gases with the atmosphere across its respiratory surfaces. In vertebrates, the transition to air breathing involves two main strategies: a decrease in oxygen affinity and changes in other haematological parameters such as haematocrit (Morris & Bridges, 1994).

There are two types of air-breathing fishes; these are the facultative air breathers such as the armoured catfishes (Loricariidae; Graham & Baird, 1982) and the obligate air breathers such as the blue gourami (*Trichogaster trichopterus*; Burggren & Haswell, 1979). Facultative air breathers do not normally breathe air in normoxic water but need to adopt this mode when exposed to hypoxic conditions or in response to increased oxygen requirements. Species such as the bowfin (*Amia calva*; Johansen et al., 1970) are known facultative air breathers and they initially increase the rate of ventilation in response to decreasing oxygen levels. If deoxygenation increases further, then the onset of air breathing occurs. Obligate air breathers take air breaths at regular intervals, at all times, and under all aquatic conditions from hyperoxia to hypoxia (Graham, 1997).

McPhail (1999) found that black mudfish did not air-breathe until oxygen levels in the water column dropped below 2.5 mg l^{-1}. In hypoxic water, with oxygen levels below 1.0 mg mg l^{-1}, McPhail (1999) observed that all of the black mudfish displayed air-breathing behaviour. This involved rising up to the surface, gulping an air bubble and holding the bubble in the buccal cavity while the fish continued gill ventilation (McPhail, 1999). Mudfish are presumed to be nocturnal animals (Ling, 2001), so they are only active at night which is beneficial as they can avoid predators while feeding at the surface and carrying out other important activities. One problem that they may encounter if they are living in hypoxic waters (<1 mg l^{-1}) is that during the day when they are supposed to be "inactive" they will be required to go to the surface

to breathe air otherwise they will drown due to the fact that they are unable to meet their oxygen demands. Mudfish have been known to drown in minnow traps which are completely submerged in hypoxic water (Ling, 2001). This suggests that they must make frequent trips to the surface in order to survive. Research needs to be carried out on the diurnal activities of mudfish in hypoxic waters to see if they make air-gulping trips to the surface during daylight, thereby risking predation, or if they have some alternate means of meeting their metabolic demands.

As water levels drop, the black mudfish seeks moist sheltering areas and respires through its skin and gills. Mudfish populations are frequently found in areas that are subjected to seasonal drought which can extend for several months of the year (McDowall, 1990). During this time period the New Zealand mudfish have been known to survive by aestivating and breathing atmospheric air until the habitat becomes flooded again (Eldon, 1978a; Meredith, 1981). Storey and Storey (1990) suggested that "aestivation is typically defined as a dormancy that occurs in response to low water availability in the environment". The physiological features of aestivation typically include the paucity of body movements or entering a state of torpor (Smith, 1930), a decrease in metabolic activity (Lahiri et al., 1970), a shift in metabolic pathways, and the production and accumulation of urea and other nitrogenous metabolic end-products (McPhail, 1999). It has been found that both black and Canterbury mudfish do not go into torpor but rather enter a quiescent state where they remain alert during aestivation. This quiescent behaviour, along with their reduced metabolic rate are presumed to be mechanisms which conserve energy and reduce the production of nitrogenous waste (McPhail, 1999). Aestivation is an important adaptation to wetland dwelling, as these habitats dry out seasonally and therefore fish must be able to survive out of water. Also it is particularly important for NZ mudfish as it allows them to survive where other potential competitors are unable to. Hicks and Barrier (1994) indicated that black mudfish are particularly sensitive to competitive interactions with other fish so without seasonal droughts other species can colonise mudfish habitats and extirpate mudfish (McPhail, 1999).

1.4 Aims of this study

Differences in habitat preferences between species have lead to the proposal of an evolutionary series within the group based on morphological adaptations to wetland dwelling. The aim of the study was to examine whether the respiratory physiology of the *Neochanna* species follows the same pattern that we can observe in the morphological cline proposed by Waters & McDowall (2005) concerning adaptations to wetland dwelling.

The experimental aims of this study were therefore to:

- Examine the whole blood oxygen binding properties (p50, Hill coefficient, Bohr effect, heat of oxygenation) of all of the species of New Zealand mudfish (brown, Northland, black, Canterbury & Chatham Island) at a range of temperatures (10ºC, 15ºC and 20ºC) and pH (6.5, 7.0, 7.5 and 8.0).
- Examine the whole blood oxygen binding properties (p50, Hill Coefficient, Bohr effect) of all the species of New Zealand mudfish at a range of treatments (aestivating, fasting and control) at pH 7.5 and 8.0.
- Investigate the presence or absence of multiple haemoglobins in all of the New Zealand mudfish species.

Chapter Two: Haemoglobin oxygen affinities of NZ mudfish

2.1 Introduction

New Zealand's five endemic mudfish (*Neochanna*) species have distributions that differ both geographically and by habitat type. A morphological cline may be observed from the galaxiform Chatham Island and Canterbury species inhabiting lakes and streams, respectively, to the anguilliform Northland, black and brown mudfishes of ephemeral wetlands (McDowall, 1997).

The morphological specializations proposed for wetland dwelling show a trend towards anguilliform characteristics such as the loss of pelvic fins, reduced eyes, enlarged nostrils, development of caudal flanges, and elongation of dorsal and anal fin bases to become almost confluent with the caudal fin (Waters & McDowall, 2005).

An expectation of adaptation to wetland dwelling is specializations in respiratory physiology to obtain oxygen from highly hypoxic or acidic waters. Fish living in such conditions typically possess haemoglobins with tight oxygen binding. This allows haemoglobin to become fully oxygenated at low partial pressures of oxygen compared with the lower affinity haemoglobins of fish in normoxic habitats or air-breathing species where the haemoglobin becomes oxygenated at higher partial pressures of oxygen.

According to the proposed morphological cline concerning adaptation to wetlands we would expect respiratory physiology to follow the same trend. We would expect that the oxygen affinity of haemoglobin in the brown, black and Northland mudfish would be significantly higher than the haemoglobin found in Canterbury and Chatham Island mudfish. This is because mudfish that reside in ephemeral wetlands should be more adapted to extracting oxygen out of hypoxic conditions and thus have a higher oxygen affinity compared to the mudfish living in streams and lakes where oxygen levels are generally higher.

The aim of this study was to compare the haemoglobin oxygen binding characteristics of the endemic NZ mudfish species and the sensitivity of these characteristics to a range of temperatures and pH to determine whether physiological differences match the proposed morphological cline.

2.2 Methods

2.2.1 Capture and captive maintenance of NZ mudfish

Mudfish used in this study were captured throughout New Zealand. Canterbury mudfish were collected from Hororata (43º31'49.3"S, 171º57'17.4"E) and Christchurch (43º31'53.3"S, 172º36'29.5"E), black mudfish were collected from the Whangamarino wetland (37º17'47.8"S, 175º07'06.6"E) and in the Waiparera forest near Kaitaia (34º56'54.8"S, 173º09'46.5"E), Northland mudfish were collected from Lake Omapere wetlands near Kaikohe (35º21'19.6"S, 173º46'16.9"E), and brown mudfish were collected from Hokitika (42º44'34.7"S, 171º01'27.3"E) and Stratford, Taranaki (39º23'38.8"S, 174º21'54.2"E). Unfortunately, due to time and resource restrictions, we were unable to obtain specimens of Chatham Island mudfish. Mudfish from the South Island were sampled and analysed in Christchurch because of legal restrictions preventing the transfer of aquatic organisms between islands of New Zealand. All other experiments were conducted at the University of Waikato in Hamilton.

Mudfish were captured by setting 20 to 30 fine-mesh (3 mm) Gee minnow traps overnight in mudfish habitat. Fine-mesh Gee minnow traps are double-ended wire baskets that clip together as shown in Figure 7. The traps should be set with the openings of the entry cones just below the water surface (Ling 2001; McDonald, 2007). The reason for this is that it directs the nocturnal mudfish into the trap while they are feeding at the surface at night and also allows them an air space so that they do not drown (Ling, 2001). Fish were transported back to the laboratory in aerated wetland water and held in 60 l glass aquaria with 5-10 mudfish occupying a single aquarium depending on their size. Water was gradually exchanged for dechlorinated Hamilton City tap water and the aquaria had an average pH of 7.4 and temperature of 14˚C - 15˚C. Refuge was provided for the mudfish in the form of plastic pipes, rocks and terracotta pots. The mudfish were fed regularly on frozen blood worms.

Figure 7: Setting fine mesh Gee minnow traps in a wetland.

2.2.2 Collection of blood and analysis of oxygen binding

Mudfish were anaesthetized using MS222 (0.1g l^{-1}) and approximately 50 µl of whole blood was removed by caudal venepuncture using a heparinized 0.5 ml tuberculin syringe with a 28-gauge needle. Blood samples were kept on ice and mudfish were returned to oxygenated water to recover from anaesthesia.

The oxygen binding characteristics of the blood were analysed using a Hemox analyzer (TCS Scientific) (Figure 8) which is an automated system for recording the oxygen equilibrium curve using spectrophotometric measurement and the continuous monitoring of the oxygen partial pressure with a Clark oxygen electrode. Temperature was regulated by a Julabo F20 water bath connected to the Hemox analyzer water jacket via insulated hoses and the pH of the proprietary Hemox buffer was adjusted by the addition of 2 M hydrochloric acid (HCl) or 2 M sodium hydroxide). The Hemox analyzer was calibrated according to the manufacturer's instructions prior to analysis of samples. 20 µl of whole blood was diluted with 5 ml

of Hemox buffer solution, 20 µl of bovine serum albumin (BSA: 20% w/v) and 10 µl of antifoam solution. The sample was added to the Hemox analyzer cuvette and fully equlibrated with air (BOC Gases; dry air). The air saturated pO_2 value was determined as follows:

$pO_2 = (P_{atm} - P_{wv}) \times 0.209$ where P_{atm} is the ambient barometric pressure and P_{wv} is the water vapour pressure at the respective test temperature.

Once the Hemox analyzer was fully calibrated and the temperature had stabilized, the sample was deoxygenated by bubbling oxygen-free N_2 gas (BOC Gases; zero-grade N_2) through it. The disassociation curve was recorded from 100% to 0% saturation using a data acquisition system on a Dell Pentium III computer. Oxygen binding characteristics of the blood were examined at temperatures of 10˚C, 15˚C and 20˚C ($\pm$ 0.1˚C) and pH values of 6.5, 7.0, 7.5 and 8.0 ($\pm$ 0.1 pH unit). Where sample sizes allowed, 5 blood samples were analyzed for each combination of pH and temperature.

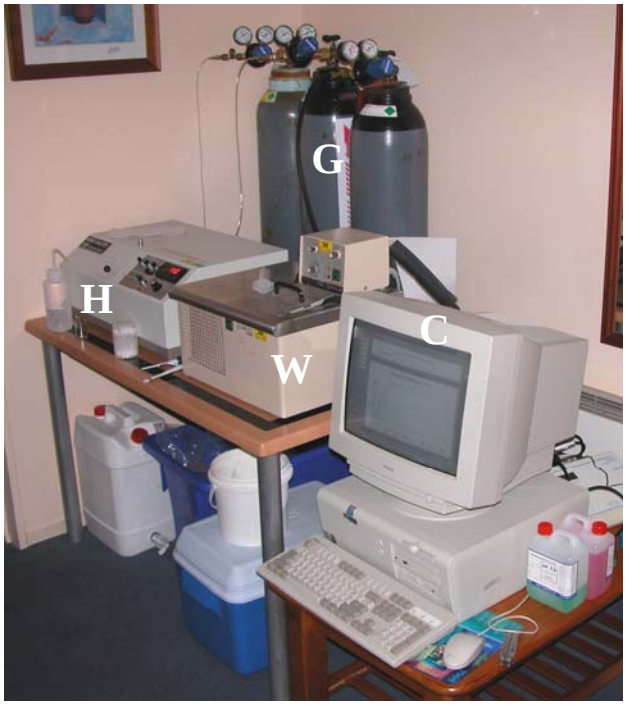

Figure 8: The hemox analyser.

H = Hemox analyzer, W = water bath, C = computer, G = gas cylinders.

2.2.3 Statistical analysis

Data were analysed using Statistica version 7.1 (Statsoft Inc., Tulsa. USA). Following tests for assumptions for normality and homogeneity of variance, factorial two-way analysis of variance and subsequent Tukey's post-hoc tests were performed on the log p50 and Hill coefficient data. All tests were considered significant at the 0.05 level.

2.3 Results

2.3.1 Oxygen equilibrium curves

Oxygen equilibrium curves obtained from mainland New Zealand mudfish species are presented in Figure 9. The curves appear hyperbolic, yet still display high levels of cooperativity as well as high oxygen affinities for all species. The p50 values ranged from 6 mm Hg to 12 mm Hg (20˚C), 7 mm Hg to 9 mm Hg (15˚C) and 4 mm Hg to 8 mm Hg (10˚C). At pH 7.5 there were no significant differences in mean p50 values between the four species at any of the temperatures. However, the ephemeral wetland dwelling black and Northland mudfish tended to have higher oxygen affinities over most of the temperatures and the stream dwelling Canterbury mudfish generally had the lowest oxygen affinity. All mudfish species displayed an increase in oxygen affinity as the temperature decreased.

2.3.2 pH dependence of the whole blood oxygen affinity

The pH-dependence (Bohr effect) of the whole blood oxygen affinity index, p50, of all the mainland mudfish species at different temperatures is depicted in Figure 10. As pH increases above 7.0, the oxygen affinity of the haemoglobins in all mudfish species increased, with the lowest p50 values occurring at pH 8. At lower temperatures and below pH 7.0, a reverse Bohr shift can be observed in the Northland mudfish, Whangamarino and Kaitaia black mudfish, and to some extent the Canterbury mudfish. However, the Taranaki and West Coast brown mudfish did not exhibit this trend, rather they displayed a pronounced Bohr effect right across the range of pH values at all temperatures. The largest Bohr effect was from pH 7.5 to 7.0 in all species at all temperatures.

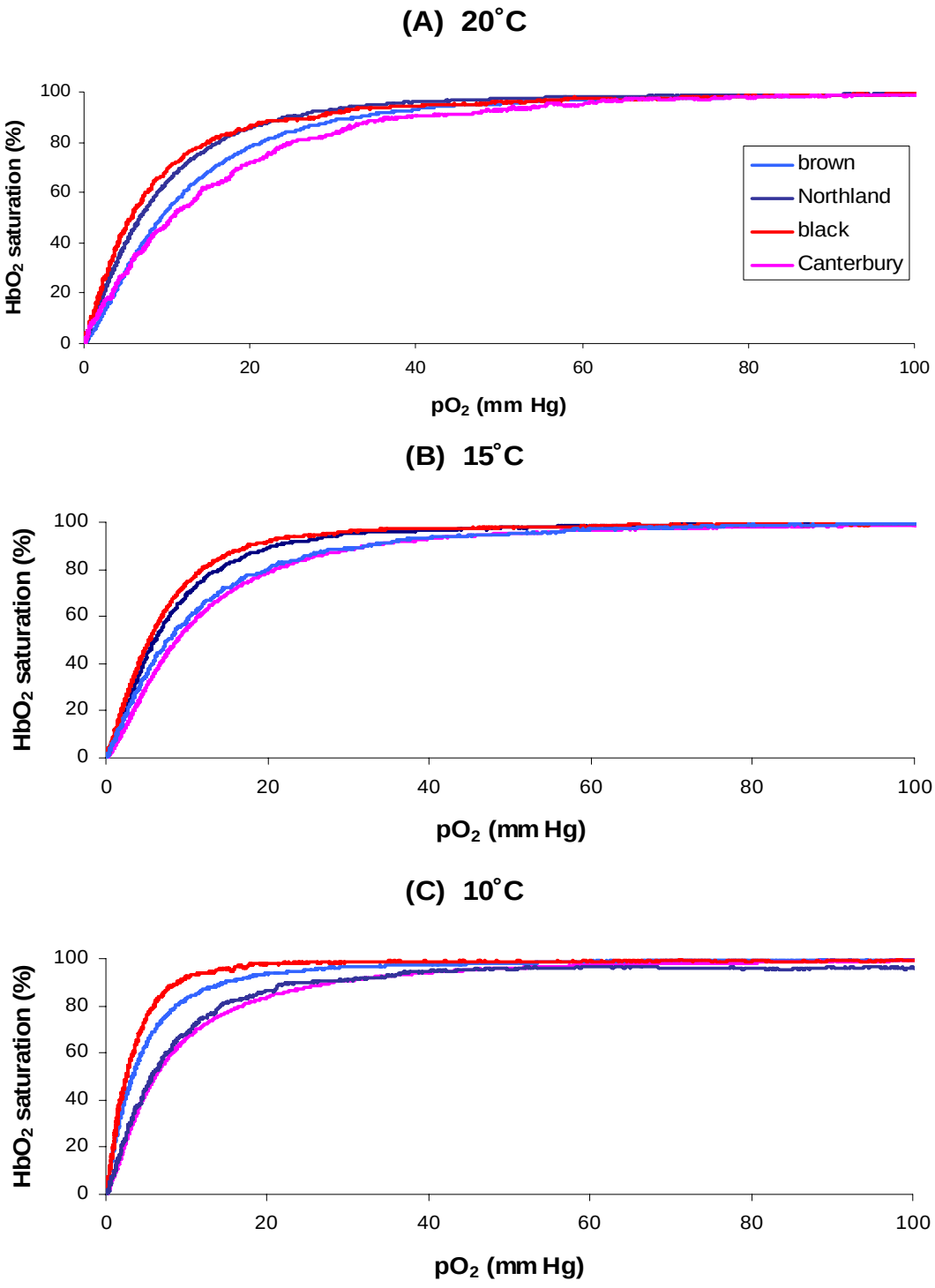

Figure 9: Representative oxygen equilibrium curves of the mainland NZ mudfish species at pH 7.5 and temperatures of 20˚C (A), 15˚C (B) and 10˚C (C).

*curves are from mudfish individuals with p50 values closest to the mean value for the species.

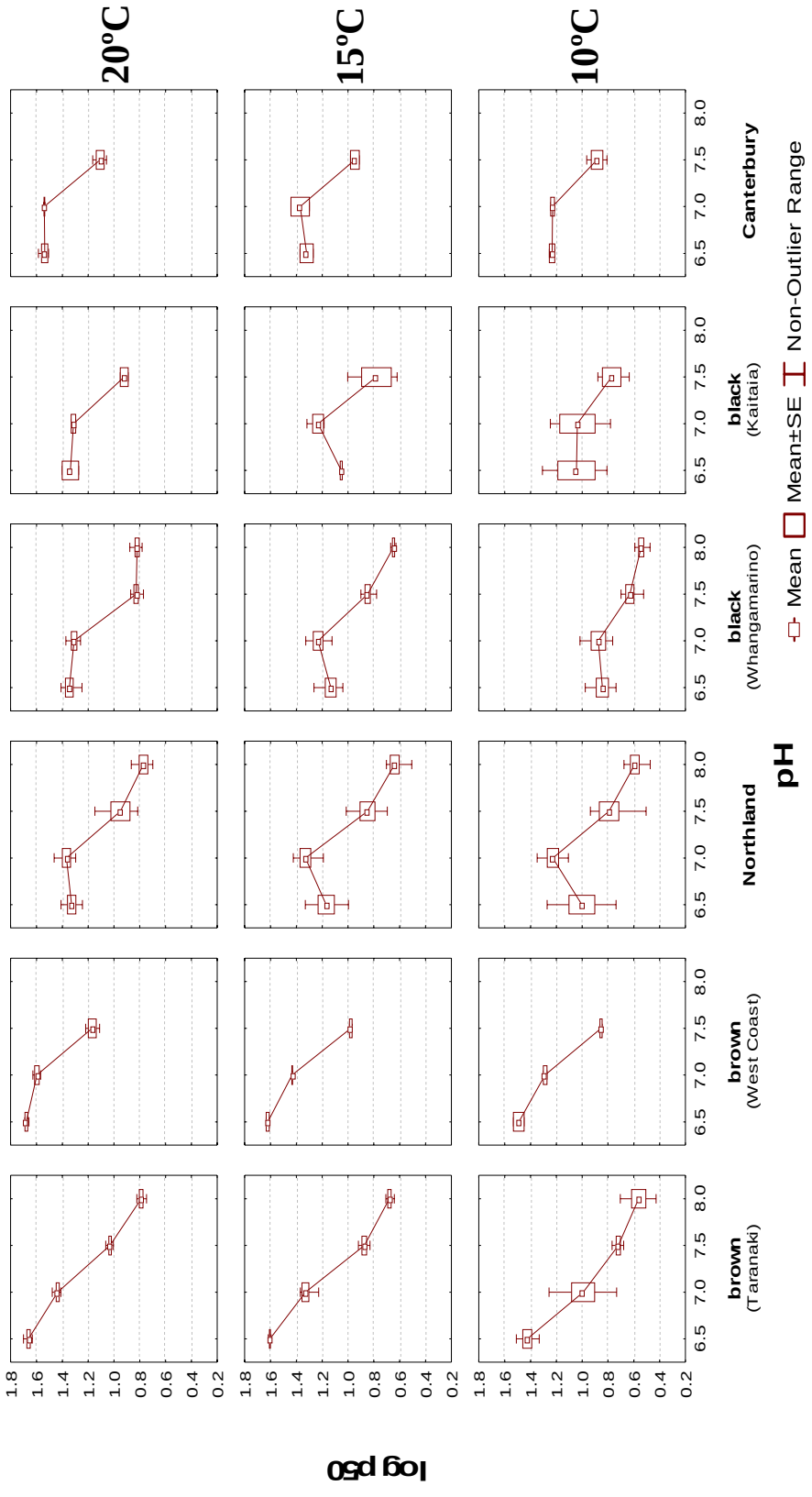

Figure 10: pH dependence of whole blood oxygen affinity for New Zealand mudfish species at a range of temperatures (10°C, 15°C and 20°C). n = 5 for all species except Kaitaia black, West Coast brown and Canterbury mudfish where n = 3. Log p50 values are not presented at pH 8.0 in the West Coast brown, Kaitaia black and Canterbury mudfish due to low sample numbers.

The whole blood of Whangamarino black mudfish exhibited the highest mean oxygen affinity at 10˚C throughout the entire pH range, while the Taranaki brown mudfish also displayed a high affinity but only at pH values 7.5 and 8.0. Whangamarino black mudfish had relatively low Bohr factors for all pH changes, with the largest Bohr factor of -0.38 occuring between pH 7.0 and 7.5. At the same temperature both the Taranaki and West Coast brown mudfish were the most pH sensitive with Bohr factors as large as -0.85 and -0.69 respectively. At 15˚C the Whangamarino black, Kaitaia black and Northland mudfish displayed very similar patterns of oxygen affinities throughout the range of pH values, which were significantly different to patterns produced by Taranaki and West Coast brown mudfish. The pH sensitivity of the Whangamarino black mudfish increased as temperature rose from 10˚C to 15˚C with increased Bohr factors of up to -0.62. At 20˚C all mudfish species, with the exception of the brown mudfish, showed similar patterns in oxygen affinity across the range of pH values with very small or non-existent Bohr effects (~ -0.06) between pH 6.5 and 7.0 and large Bohr effects (~ -0.6) between pH 7.0 and 7.5.

Mean log p50 values at different pH values of each mudfish species at 10˚C is presented in Figure 11. It shows that the oxygen affinity of whole blood of all species at pH 7.5 and 8.0 was higher than at other pH values and that at pH 6.5 and 7.0 there was an overlap of log p50 values for most species. Whole blood oxygen affinity was highest in the Whangamarino black mudfish at all pH values and lowest in the West Coast brown mudfish. A two-way ANOVA revealed significant differences in log p50 values at 10˚C between the different mudfish species ($p<0.001$, $F = 11.38$) and also at the different pH levels ($p<0.001$, $F = 46.05$). A subsequent Tukey's post-hoc test showed that there were no significant differences between mudfish species at pH 7.5 but significant differences were present at pH 6.5 and 7.0. At pH 7.0, there was a significant difference between the Whangamarino black mudfish and the Northland mudfish, and at pH 6.5 there were significant differences between the brown mudfish (lowest oxygen affinity), black mudfish (Whangamarino exhibited highest oxygen affinity) and Northland mudfish. There were also significant differences in log p50 values at the different pH values but only for the

Northland mudfish (between pH 7.0 & 7.5), Taranaki brown (between pH 6.5 & 7.0-7.5) and West Coast brown mudfish (between pH 6.5 & 7.5).

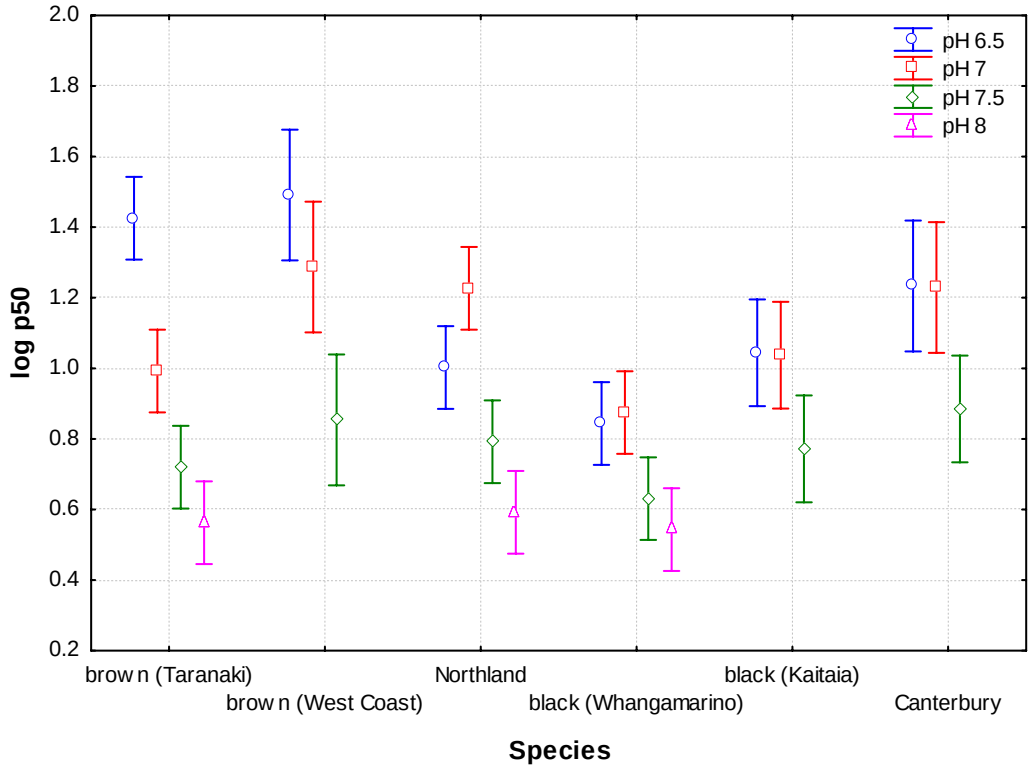

Figure 11: Mean log p50 values of mudfish species over a pH range at 10˚C.

Values are offset for clarity and some species are missing log p50 values at pH 8.0 due to low sample numbers. n = 5 for all species except West Coast brown, Kaitaia black and Canterbury mudfish where n = 3.

At 15˚C the oxygen affinity was highest at the highest pH values for all of the species as shown in Figure 12. For the Taranaki brown, Northland and Whangamarino black mudfish the highest oxygen affinities occurred at pH 8.0. For the West Coast brown, Kaitaia black and Canterbury mudfish the highest affinities were found at pH 7.5. The overlap of log p50 values at pH 6.5 and 7.0 is less pronounced compared to values displayed at 10˚C. Whole blood oxygen affinity at pH values of 7.0, 7.5 and 8.0 were similar for all species but at pH 6.5 the affinity was again highest in the Whangamarino black mudfish and lowest in the West Coast brown mudfish. A two-way ANOVA revealed significant differences in log p50 values between the different

mudfish species ($p<0.001$, $F = 17.1$) and also at the different pH levels ($p<0.001$, $F = 146.2$) at 15˚C. A subsequent Tukey's post-hoc test showed that there were no significant differences between mudfish species at pH 7.0 and 7.5. At pH 6.5 the Whangamarino black, Kaitaia black, Northland and Canterbury mudfish species all had significantly higher oxygen affinities than the brown mudfish. There were significant differences in log p50 values between the different pH values for all of the species with the oxygen affinity at pH 7.5 being significantly higher than the affinity at pH 6.5 and 7.0. The Taranaki brown mudfish had significant differences in log p50 values between pH values with the highest oxygen affinity occurring at pH 8.0 and the lowest affinity at pH 6.5.

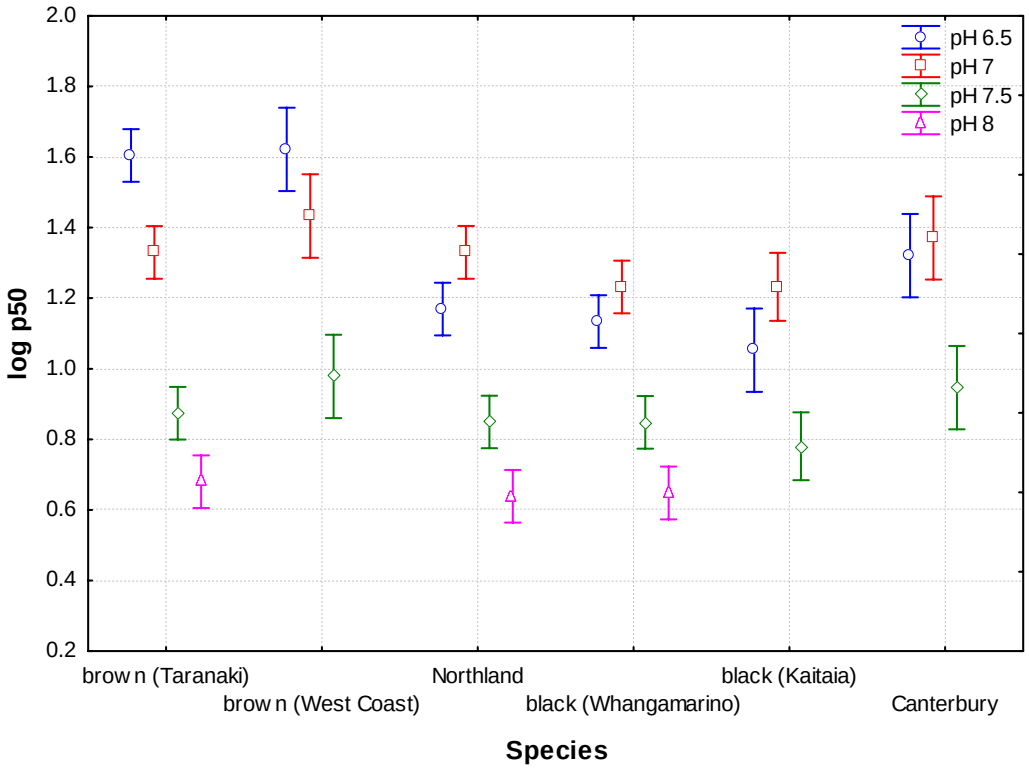

Figure 12: Mean log p50 values of mudfish species over a pH range at 15˚C.

Values are offset for clarity and some species are missing log p50 values at pH 8.0 due to low sample numbers. n = 5 for all species except West Coast brown, Kaitaia black and Canterbury mudfish where n = 3.

The effect of pH on haemoglobin oxygen affinity at 20˚C is presented in Figure 13. Higher oxygen affinities were displayed at pH 7.5 and 8.0 compared to pH 6.5 and 7.0 for all species. The overlap of log p50 values at pH values of 6.5 and 7.0 is pronounced in the black, Northland and Canterbury mudfish, however, in the Taranaki brown mudfish there was a significant difference in oxygen affinity at all pH values. Whole blood oxygen affinity was high in the black and Northland mudfish in comparison to the Canterbury and brown mudfish throughout the pH range. A two-way ANOVA revealed significant differences in log p50 values between the different mudfish species ($p<0.001$, $F = 42.8$) and also at the different pH levels ($p<0.001$, $F = 325.4$) at 20˚C.

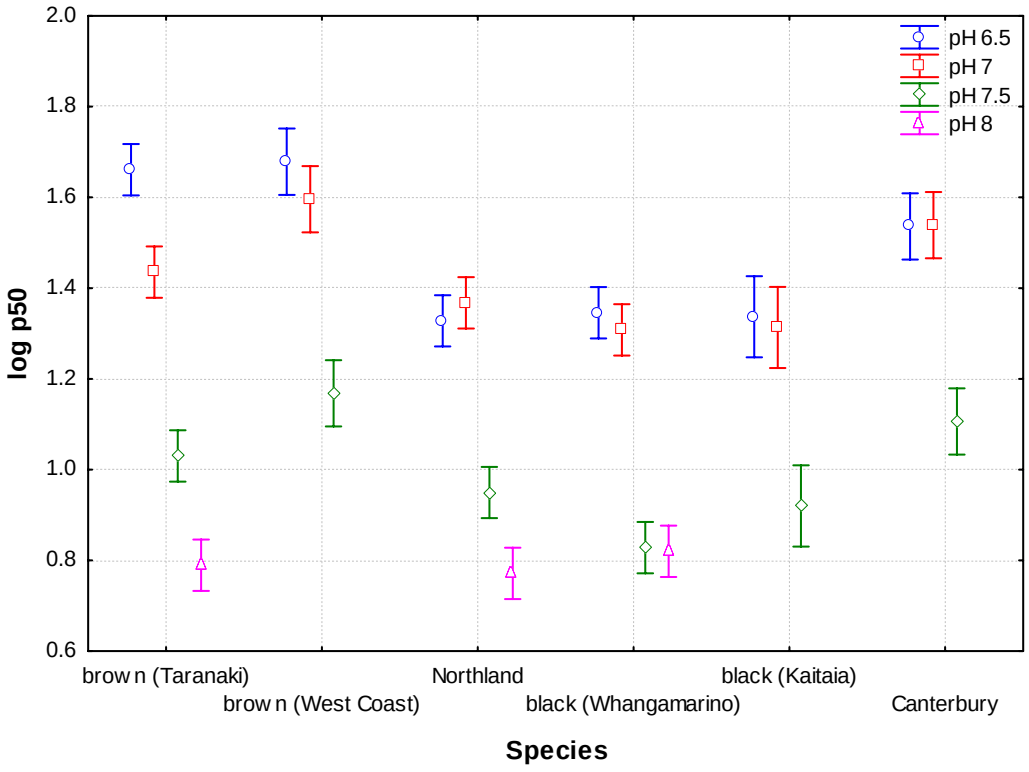

Figure 13: Mean log p50 values of mudfish species over a pH range at 20˚C.

Values are offset for clarity and some species are missing log p50 values at pH 8.0 due to low sample numbers. n = 5 for all species except West Coast brown, Kaitaia black and Canterbury mudfish where n = 3.

A subsequent Tukey's post-hoc test showed that there were significant differences between mudfish species at all pH values. At pH 6.5 the black and Northland mudfish had significantly higher oxygen affinities than the Canterbury and brown mudfish. At pH 7.0 the results were the same with the exception of the Taranaki brown mudfish no longer having a significantly lower oxygen affinity than the black and Northland mudfish. At pH 7.5 the Whangamarino black mudfish had the highest oxygen affinity and was significantly different to the brown and Canterbury mudfish. The Kaitaia black and Northland mudfish also had a significantly lower oxygen affinity than the West Coast brown mudfish which displayed the lowest oxygen affinity of all the species.

Table 1: Factorial two way ANOVA results for oxygen affinities of different species of mudfish with pH, temperature and the interaction between them.

Species	**pH**	**Temperature (˚C)**	**Interaction** pH*temp
brown (Taranaki)	F = 393.8 **p < 0.001***	F = 80.1 **p < 0.001***	F = 2.9 **p = 0.016***
brown (West Coast)	F = 605.2 **p < 0.001***	F = 124.8 **p < 0.001***	F = 4.1 **p = 0.026***
Northland	F = 80.26 **p < 0.001***	F = 12.87 **p < 0.001***	F = 0.68 p = 0.065
black (Whangamarino)	F = 161.6 **p < 0.001***	F = 125.1 **p < 0.001***	F = 6.7 **p < 0.001***
black (Kaitaia)	F = 11.66 **p < 0.001***	F = 3.96 **p = 0.043***	F = 0.46 p = 0.764
Canterbury	F = 119.9 **p < 0.001***	F = 45.8 **p < 0.001***	F = 0.7 p = 0.621

***** indicates a significant difference ($p < 0.05$)

Table 1 reveals a significant interaction between pH and temperature on the oxygen affinity of the brown and Whangamarino black mudfish. This means that the effect of pH on oxygen affinity changes if the temperature changes and vice versa. The two variables are not independent of each other in the brown and Whangamarino black mudfish, whilst in the Northland, Canterbury and Kaitaia black mudfish they are independent as there was no significant interaction.

2.3.3 Effects of temperature on haemoglobin oxygen affinity

All species displayed a decrease in oxygen affinity (increase in logp50 values) at all pH values as temperature increased (Figure 14). Oxygen affinity was highest at 10˚C and decreased as temperature increased to 15˚C and then decreased further once the temperature reached 20˚C. The effect of temperature on log p50 values decreased as pH increased for many of the species. For example, Whangamarino black mudfish had log p50 values ranging from 0.8 to 1.4 at pH 6.5 but at pH 8.0 the range was only from 0.6 to 0.8. This trend was also observed in the Kaitaia black, Northland and Taranaki brown mudfish. West Coast brown and Canterbury mudfish did not display this pattern.

The temperature dependence of oxygen binding equilibria was investigated in both the 10˚C - 15˚C range and the 15˚C - 20˚C range for all of the mainland New Zealand mudfish species at pH values ranging from 6.5 to 8.0 (Table 2). The overall oxygenation enthalpy change (ΔH) in kcal.mol^{-1} (1 kcal = 4.184 kJ), corrected for the heat of oxygenation solubilization (-3 kcal.mol^{-1}), was calculated by the integrated van't Hoff equation $\Delta H = -4.574[(T1xT2)/(T1-T2)]\ \Delta log_{p50}/1000$. Within the 10˚C - 15˚C range, ΔH reached a maximum value for all species with the exception of the Northland mudfish at pH 7.0 and tended to become less exothermic at higher or lower pH values. ΔH became endothermic in the Kaitaia black mudfish at pH 6.5, whereas in all the other species ΔH values were exothermic over the entire pH range (6.5 – 8.0). The Taranaki brown and Whangamarino black mudfish showed the highest absolute values of ΔH of all the species at all of the pH values investigated. The

West Coast brown mudfish maintained a rather constant absolute value across the pH range with only a slight exothermic increase in ΔH at pH 7.0. Within the 15˚C - 20˚C range the overall oxygenation enthalpy change for all species was very different when compared to the values observed at 10˚C - 15˚C. The ΔH in both the brown mudfish populations became increasingly more exothermic as pH increased from pH 6.5 to 7.5, but at pH 8.0 it became less exothermic. The absolute value of ΔH was lowest at pH 7.0 for the Northland and Kaitaia black mudfish and became increasingly exothermic at higher or lower pH. The Whangamarino black mudfish displayed an endothermic ΔH at pH 7.5, but at higher or lower pH the absolute value of ΔH increased significantly. An interesting point is that the most northern species or populations of New Zealand mudfish, the Northland and Kaitaia black mudfish, were the least temperature sensitive over both temperature ranges.

Table 2: Overall oxygenation enthalpy change, ΔH (kcal·mol^{-1}), of the whole blood of mainland mudfish species of New Zealand.

*Values were determined from the mean p50 values of the species at the specific conditions. Some species are missing values for pH 8 due to low sample numbers.

Species	Temp. range (˚C)	pH 6.5	pH 7	pH 7.5	pH 8
brown	10 - 15	-13.32	-21.61	-11.34	-7.93
(Taranaki)	15 - 20	-4.34	-7.97	-11.98	-8.43
brown	10 - 15	-9.63	-10.69	-8.91	-
(West Coast)	15 - 20	-4.28	-12.64	-15.18	-
Northland	10 - 15	-10.22	-7.39	-3.54	-3.49
	15 - 20	-11.05	-2.64	-8.82	-10.16
black	10 - 15	-21.15	-25.49	-15.78	-7.60
(Whangamarino)	15 - 20	-16.18	-5.74	1.72	-13.63
black	10 - 15	2.90	-11.65	-2.20	-
(Kaitaia)	15 - 20	-21.78	-5.74	-8.32	-
Canterbury	10 - 15	-6.74	-10.73	-4.29	-
	15 - 20	-16.43	-12.62	-12.15	-

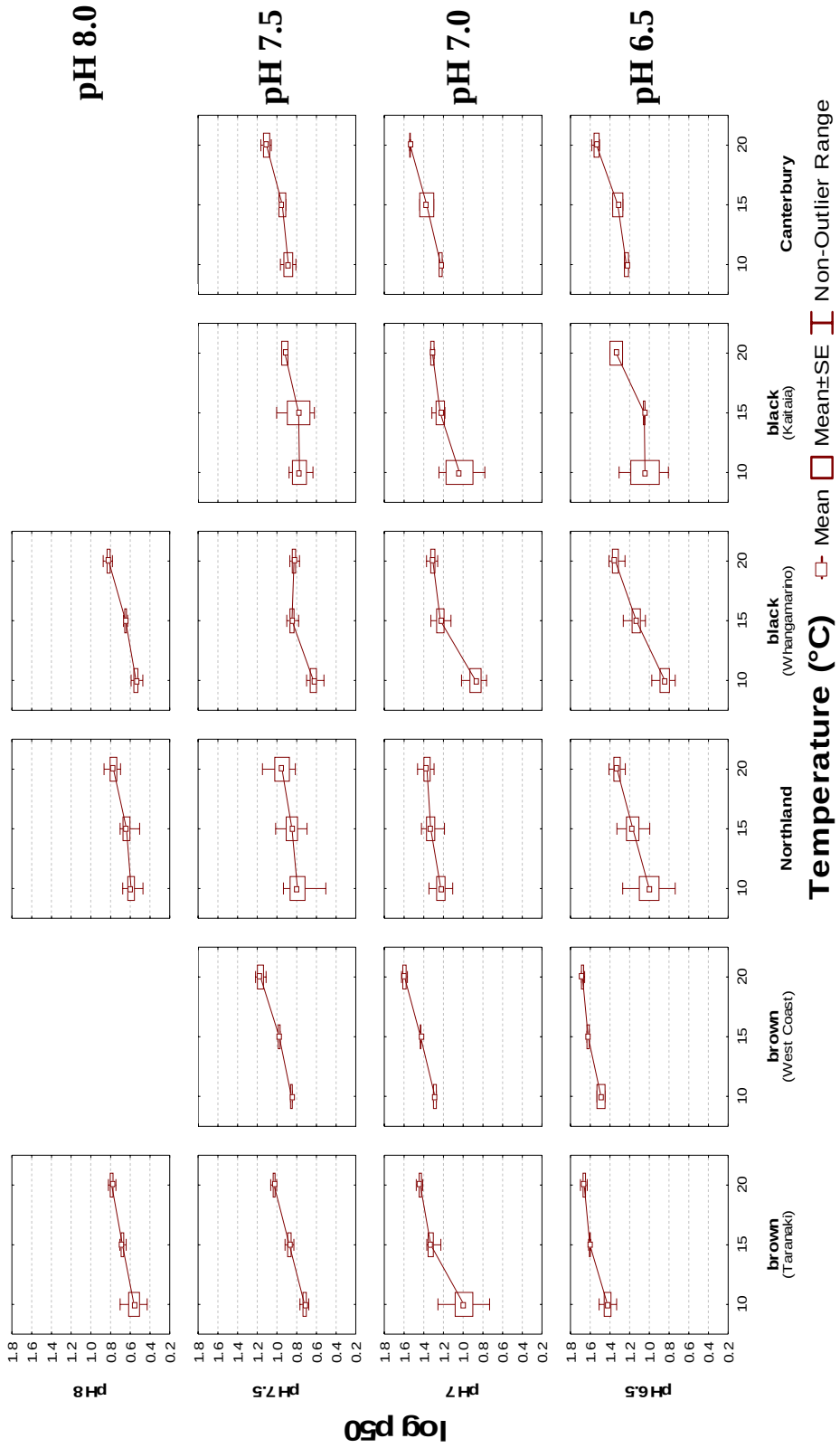

Figure 14: Temperature dependence of whole blood oxygen affinity for the New Zealand mudfish species at a range of pH's (6.5, 7.0, 7.5 and 8.0). n = 5 for all species except Kaitaia black, West Coast brown and Canterbury mudfish where n = 3. log p50 values are missing at pH 8.0 in the West Coast brown, Kaitaia black and Canterbury mudfish due to low sample numbers.

The decrease in oxygen affinity in response to an increase in temperature at pH 6.5 is presented in Figure 15. The brown mudfish exhibited the least changes in log p50 in response to increasing temperature with only a 0.3 increase in log p50 values for a 10˚C rise in temperature. The results of a two-way ANOVA found that there were significant differences in log p50 values between the different species ($p<0.001$, $F=44.23$) and between the different temperatures ($p<0.001$, $F=38.62$). Tukey's post-hoc tests revealed that, with the exception of the Whangamarino black and Northland mudfish, there were no significant differences in log p50 values as temperature increased. At pH 6.5 the post-hoc tests showed that at all temperatures the brown mudfish had significantly lower oxygen affinities than the Whangamarino black and Northland mudfish.

Figure 16 shows that at pH 7.0 there was little difference in log p50 values at 15˚C and 20˚C for all of the mudfish species. Both Whangamarino black and Taranaki brown mudfish had a higher oxygen affinity at 10˚C compared to the two higher temperatures. A two-way ANOVA revealed significant differences in log p50 values between different species ($p<0.001$, $F=11.65$) and at the different temperatures ($p<0.001$, $F=49.15$). A subsequent Tukey's post-hoc test revealed that the Whangamarino black and Taranaki brown mudfish displayed a significantly higher oxygen affinity at 10ºC compared to the higher temperatures. At 20˚C the only significant difference of log p50 values between species occurred between Whangamarino black which had a significantly higher oxygen affinity than the West Coast brown mudfish. At 15˚C there were no significant differences between any of the species and at 10˚C the Whangamarino black mudfish had a significantly higher oxygen affinity than the Northland, Canterbury and West Coast brown mudfish.

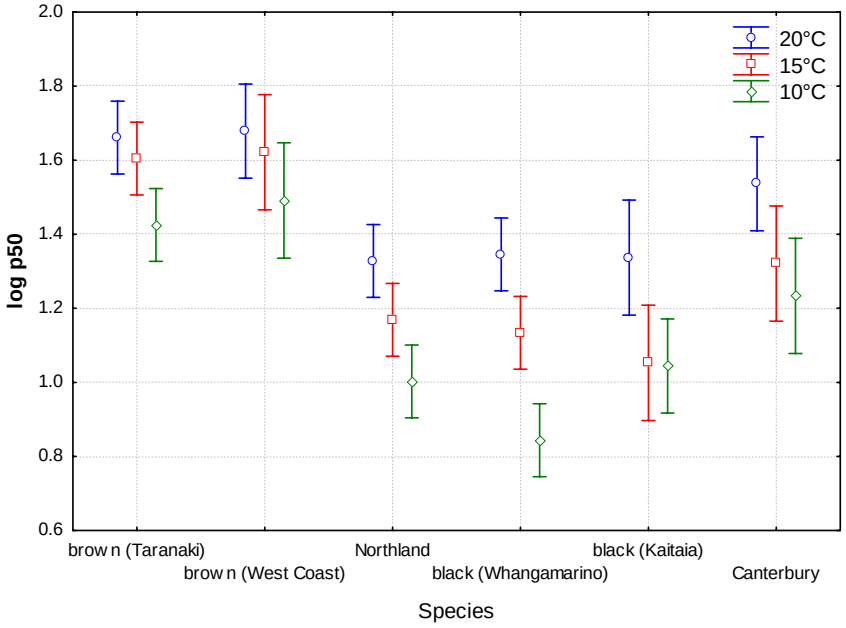

Figure 15: Mean log p50 values of mudfish species at 10ºC, 15˚C and 20ºC at pH 6.5.

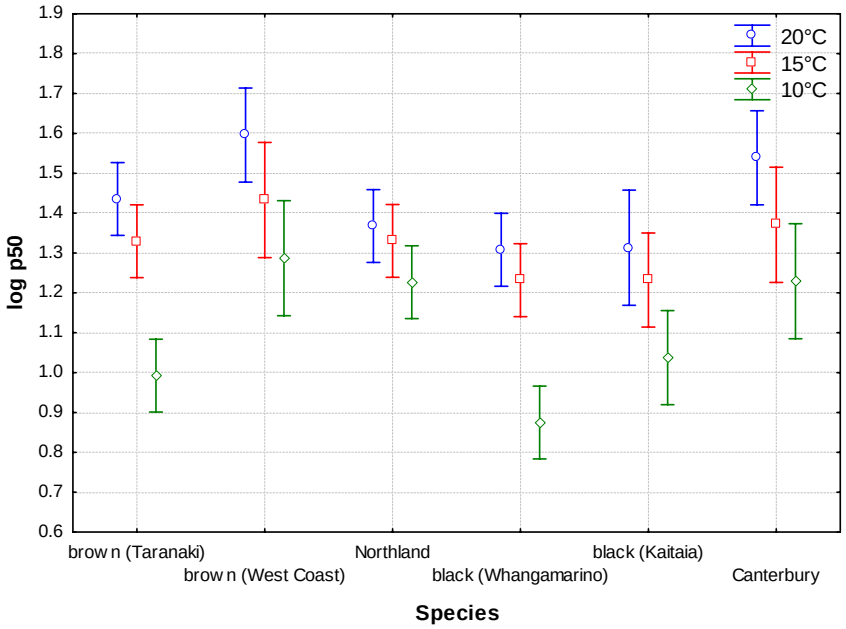

Figure 16: Mean log p50 values of mudfish species at 10ºC, 15˚C and 20ºC at pH 7.0.

Values are offset for clarity. n = 5 for all species except West Coast brown, Kaitaia black and Canterbury mudfish where n = 3.

Figure 17 shows that at pH 7.5 there were little differences in log p50 values at any of the temperatures for any of the mudfish species although the Whangamarino black mudfish seemed to have the highest oxygen affinity throughout the temperature range at pH 7.5. However, the results of a two-way ANOVA found that there were significant differences in log p50 values between the different species ($p<0.001$, $F=7.90$) and at the different temperatures ($p<0.001$, $F=27.07$). Tukey's post-hoc tests revealed that at pH 7.5 the increase in temperature had no significant effect on the oxygen affinity for all of the species, except the Taranaki brown mudfish where there was a significant difference between 10˚C and 20˚C. At 20˚C the only significant difference in log p50 values between species occurred between Whangamarino black (significantly higher oxygen affinity) and West Coast brown and Canterbury mudfish. At both 15˚C and 10˚C there were no significant differences between any of the mudfish species.

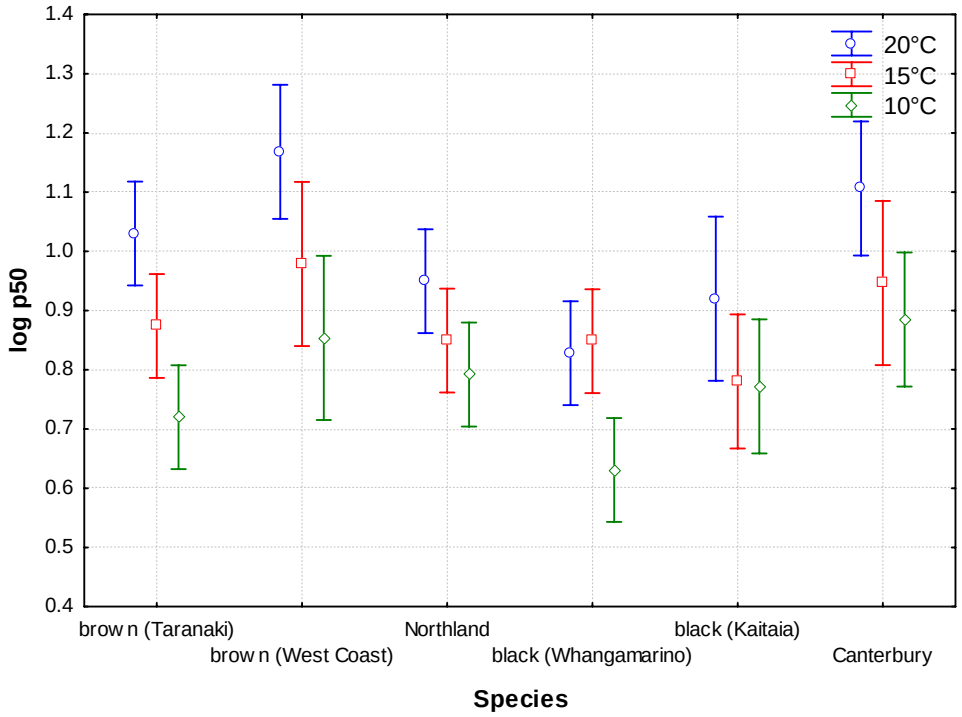

Figure 17: Mean log p50 values of mudfish species at 10ºC, 15˚C and 20ºC at pH 7.5.

Values are offset for clarity. n = 5 for all species except West Coast brown, Kaitaia black and Canterbury mudfish where n = 3.

Figure 18 shows the effect of an increase in temperature on oxygen affinity at pH 8.0. The oxygen affinity is lowest at 20˚C and increases as temperature decreases. At pH 8.0 there seemed to be no differences between the three species with all the species showing very similar oxygen affinities. The results of a two-way ANOVA revealed no significant differences in log p50 values between the different species ($p=0.915$, $F=0.09$) but there were differences in oxygen affinity within a species at the different temperatures ($p<0.001$, $F=46.26$). Tukey's post-hoc tests showed that Taranaki brown, Northland and Whangamarino black mudfish had significantly higher oxygen affinities at 10˚C than at 20˚C.

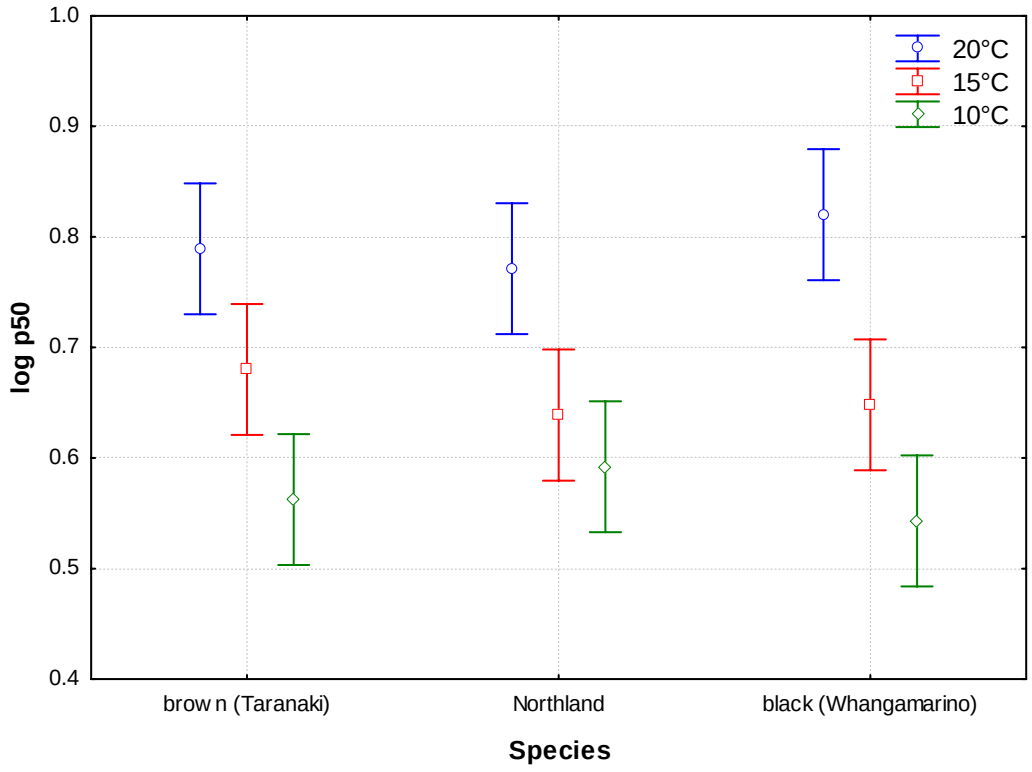

Figure 18: Mean log p50 values of mudfish species at 10ºC, 15˚C and 20ºC at pH 8.0.

Values are offset for clarity. West Coast brown, Kaitaia black and the Canterbury mudfish were not included due to low sample numbers.

2.3.4 Cooperative oxygen binding

The level of cooperative oxygen binding (Hill coefficient, n_{50}) for whole blood of all mudfish species at four different pH values and three different temperatures is displayed in Figure 19. The trend in all of the species was as pH increased so did the level of cooperative oxygen binding. In addition, at higher pH values (7.5 and 8.0), the cooperativity of the haemoglobin decreased as temperature increased. Also, the effects of pH on cooperative oxygen binding decreased as temperature increased for all species of mudfish. For example, at 10˚C the Taranaki brown mudfish had a large range of n_{50} values from 1.4 to 2.6 over the pH range while at 20˚C the range was significantly less with values from 1.6 to 2.0. All species of mudfish showed relatively high levels of cooperative oxygen binding with the Whangamarino black and the Taranaki brown mudfish exhibiting the highest n_{50} values.

At 10˚C an increase in pH caused significant differences ($p<0.05$) in the cooperativity of the haemoglobin of all mudfish species with the highest level of cooperative oxygen binding occurring at pH 7.5 and 8.0. Figure 20 displays the mean Hill coefficient values of the mudfish species at 10˚C. A two-way ANOVA revealed significant differences in mean Hill coefficients between the different mudfish species ($p<0.001$, $F = 52.1$) and also at the different pH levels ($p<0.001$, $F = 115.3$) at 10˚C. Tukey's post-hoc tests showed that there were significant differences between mudfish species at all pH values. At pH 6.5, the Whangamarino black and Northland mudfish had the lowest levels of cooperative oxygen binding, which were significantly lower than the Taranaki brown mudfish. As pH increased to 7.0 the Taranaki brown mudfish displayed significantly higher levels of cooperativity than the black and Northland mudfish with a mean of over 1.8. At pH 7.5 the levels of cooperativity significantly increased for the Whangamarino black mudfish and it was significantly higher than all of the species except the Taranaki brown mudfish. There were also significant differences within a species at the different pH values. For the Taranaki brown mudfish the Hill coefficients differed significantly at each pH level (6.5, 7.0 and 7.5) and increased as pH increased. In the black mudfish the level of cooperative oxygen binding was significantly higher at pH 7.5 than at the lower pH

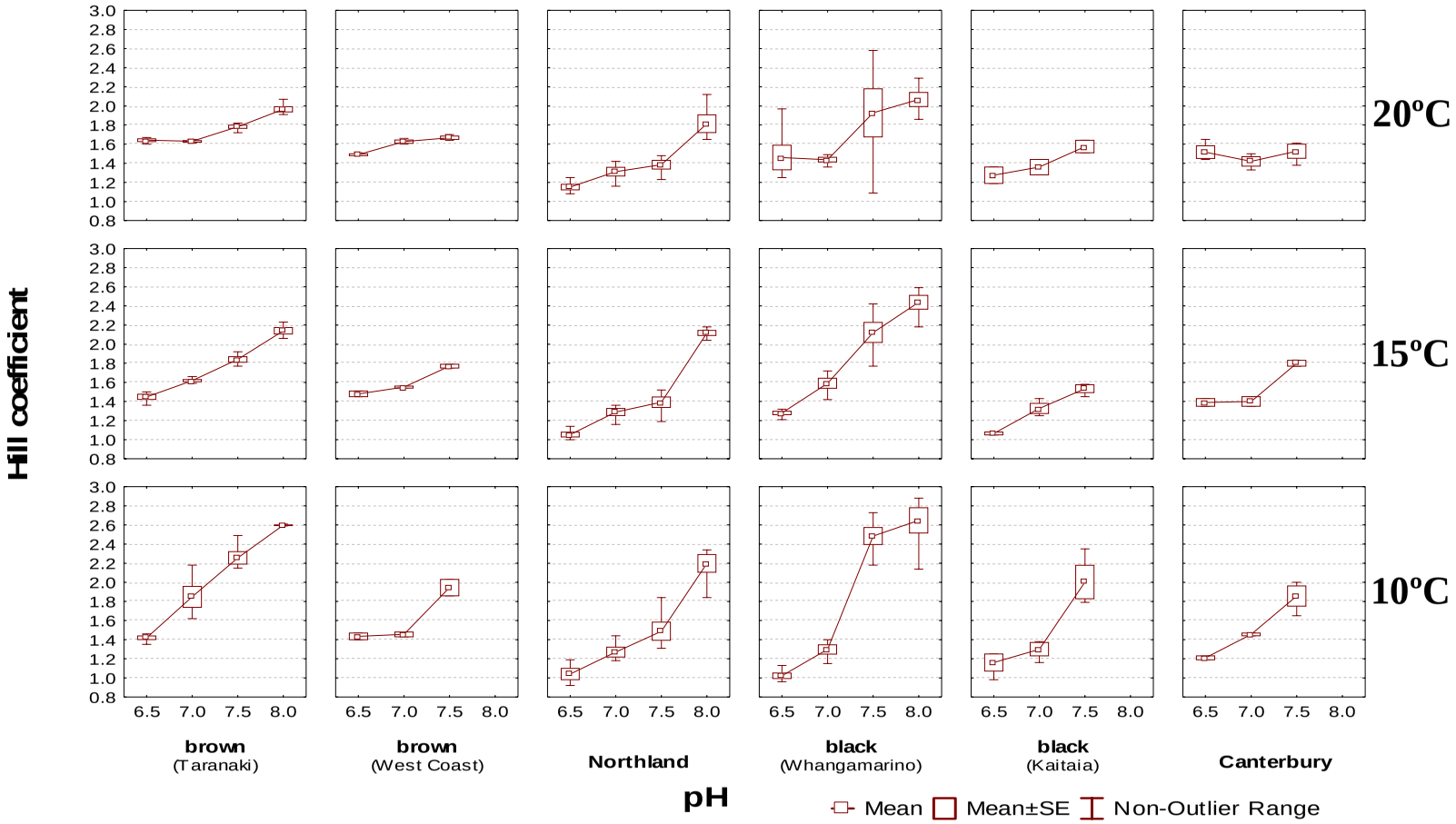

Figure 19: Levels of cooperative oxygen binding in the New Zealand mudfish species at a range of temperatures (10°C, 15°C and 20°C) and pH's (6.5, 7.0, 7.5 and 8.0). n = 5 for all species except Kaitaia black, West Coast brown and Canterbury mudfish where n = 3. log p50 values are missing at pH 8.0 in the West Coast brown, Kaitaia black and Canterbury mudfish due to low sample numbers.

values. Both the Canterbury and Northland mudfish also had significant differences in Hill coefficients between pH 7.5 and 6.5.

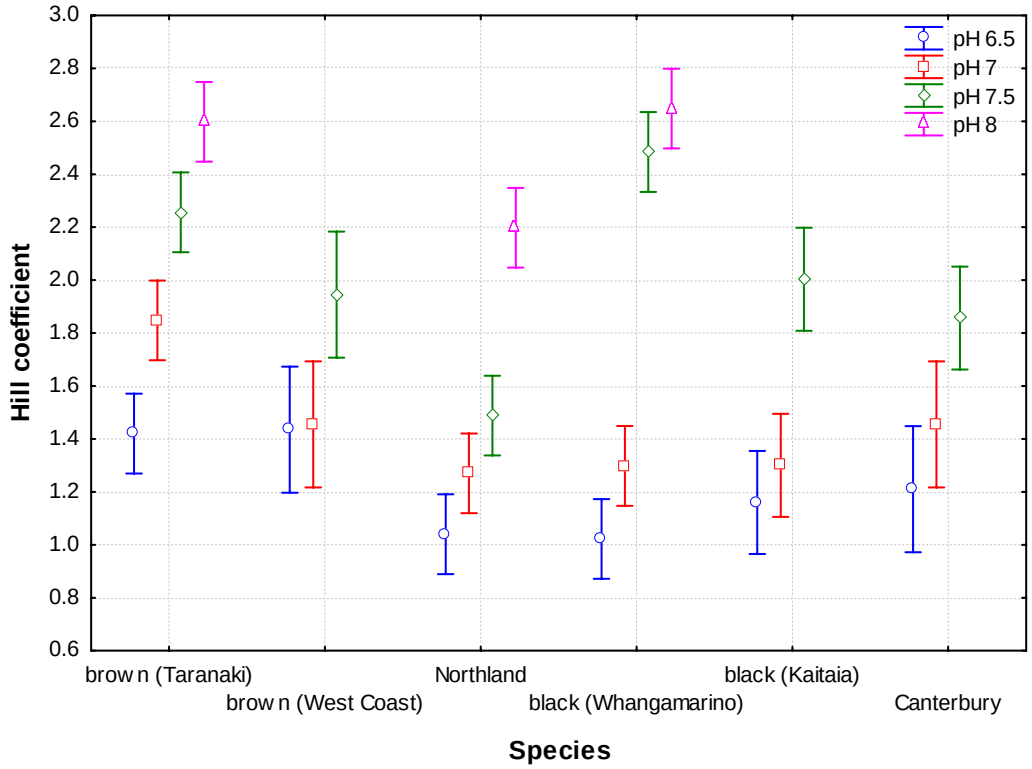

Figure 20: Cooperative oxygen binding in mainland New Zealand mudfishes over a range of pH values at 10˚C.

***** n = 5 for all species except West Coast brown, Kaitaia black and Canterbury mudfish where n = 3. Some species are missing values at pH 8.0 due to low sample numbers. The values are offset for clarity.

At 15˚C (Figure 21) the effect of pH on cooperativity decreased slightly and the Hill coefficients were lower than those found at 10˚C. A two-way ANOVA revealed significant differences in mean Hill coefficients between the different mudfish species ($p<0.001$, $F = 83.74$) and also at the different pH levels ($p<0.001$, $F = 94.38$) at 15˚C. A subsequent Tukey's post-hoc test showed that there were significant differences between mudfish species at all pH values. At pH 6.5 the Kaitaia black and Northland mudfish had the lowest levels of cooperative oxygen binding and they were significantly lower than the Canterbury and brown mudfish. At pH 7.0 the

levels of cooperativity were similar for all species with the only significant differences occuring between the lowest n_{50} values (Northland, Kaitaia) and the highest n_{50} values (Whangamarino, Taranaki). As pH increased to 7.5 the Hill coefficient for the Whangamarino black mudfish drastically increased and it was significantly different to all the other species. For all species except the West Coast brown mudfish the level of cooperativity was significantly higher at pH 7.5 than at pH 6.5. The Whangamarino black mudfish were the most pH sensitive at 15 ˚C with significant differences in the mean Hill coefficient at each pH.

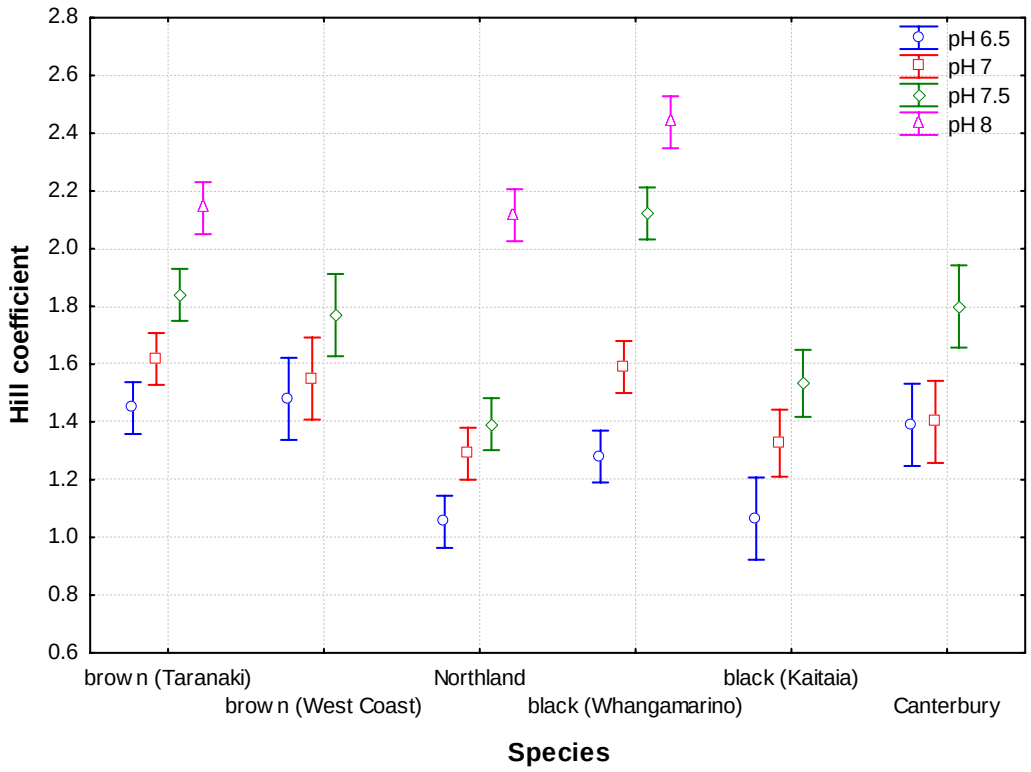

Figure 21: Cooperative oxygen binding in mainland New Zealand mudfishes over a pH range at 15˚C.

* n = 5 for all species except West Coast brown, Kaitaia black and Canterbury mudfish where n = 3. Some species are missing values at pH 8.0 due to low sample numbers. The values are offset for clarity.

The increase in temperature to 20˚C caused a significant decrease in the effect of pH on cooperativity. Figure 22 shows that generally all of the species were very similar ranging from just under 1.2 to just over 1.6 with the exception of the Whangamarino black and Taranaki brown mudfish. At 20˚C, a two-way ANOVA found significant differences in Hill coefficients between mudfish species ($p<0.001$, $F=20.62$) and at different pH levels ($p<0.001$, $F=8.31$) although these differences were minimal. Tukey's post-hoc test revealed significant differences between mudfish species at pH values of 6.5 and 7.0. At pH 6.5 the Taranaki brown mudfish had a slightly, yet significantly higher Hill coefficients than the Northland mudfish.

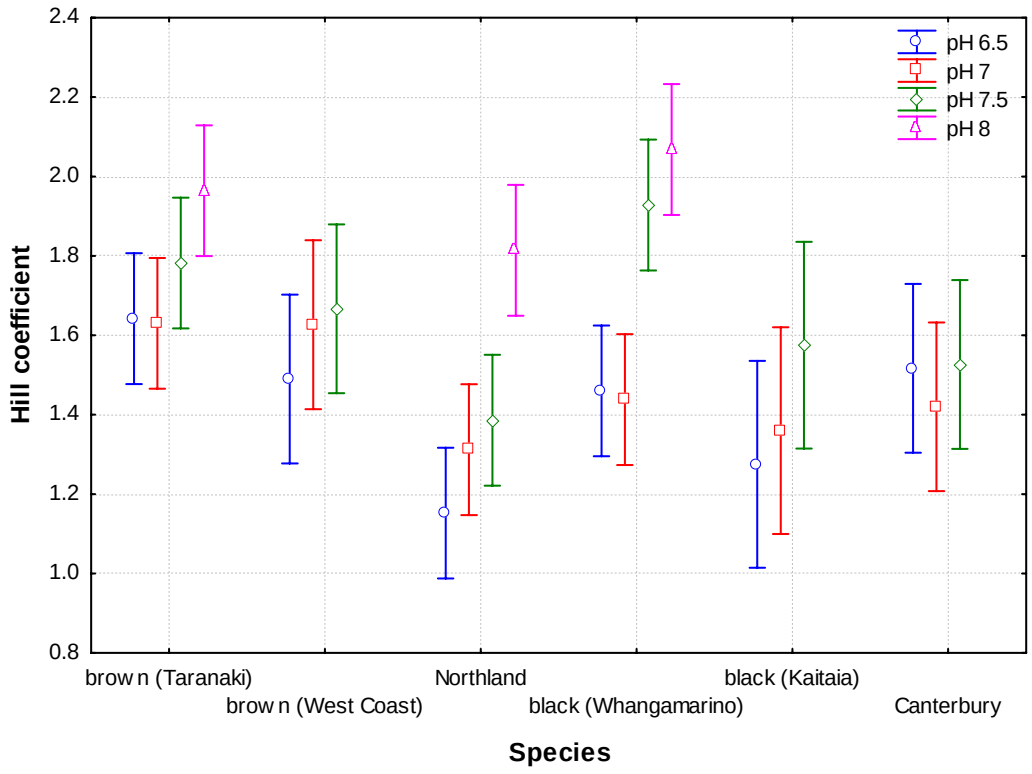

Figure 22: Cooperative oxygen binding in mainland New Zealand mudfishes over a range of pH's at 20˚C.

***** n = 5 for all species except West Coast brown, Kaitaia black and Canterbury mudfish where n = 3. Some species are missing values at pH 8.0 due to low sample numbers. The values are offset for clarity.

At pH 7.5 the haemoglobin of the Whangamarino mudfish displayed significantly higher levels of cooperativity than the Northland mudfish. The Whangamarino black mudfish was the only species to show a significant difference in the level of cooperative oxygen binding at different pH values.

Table 3 shows that there was a significant interaction between pH and temperature on the cooperativity of the haemoglobin of all the mudfish species. This means that the effect of pH on cooperative oxygen binding is modified by changes in the temperature and vice versa. The two variables are not independent of each other in any of the mudfish species.

Table 3: Factorial two way ANOVA results for cooperative oxygen binding in the different species of mudfish with pH, temperature and the interaction between them.

Species	**pH**	**Temperature (˚C)**	**Interaction** pH*temp
brown (Taranaki)	F = 174.6 **p < 0.001***	F = 57.2 **p < 0.001***	F = 20.5 **p < 0.001***
brown (West Coast)	F = 93.01 **p < 0.001***	F = 0.26 p = 0.779	F = 15.58 **p < 0.001***
Northland	F = 142.5 **p < 0.001***	F = 1.9 P = 0.157	F = 3.8 **p = 0.003***
black (Whangamarino)	F = 81.38 **p < 0.001***	F = 2.23 p = 0.118	F = 6.28 **p < 0.001***
black (Kaitaia)	F = 24.01 **p < 0.001***	F = 2.88 p = 0.09	F = 3.22 **p = 0.045***
Canterbury	F = 23.08 **p < 0.001***	F = 0.27 p = 0.764	F = 6.14 **p = 0.005***

*indicates a significant difference ($p < 0.05$)

2.4 Discussion

An evolutionary series within New Zealand's endemic mudfish has been proposed (Waters & McDowall, 2005) based on differences in morphology, distribution and habitat preference of the different *Neochanna* species. The resulting morphological cline places the galaxiform Chatham Island and Canterbury mudfish species as the least specialized to wetland dwelling and the anguilliform Northland and brown mudfishes as the most specialized to wetland dwelling. Wetlands can be very hypoxic and seasonally dry implicating the necessity for physiological as well as morphological adaptation.

The oxygen equilibrium curves obtained from the four mainland NZ mudfish species compared well in both shape and position with those reported for other fish species occupying low oxygen environments such as tench (*Tinca tinca*; Jensen & Weber, 1985) and common carp (*Cyprinus carpio*; Weber & Lykkeboe, 1978) at similar pH and temperature values. The curves for the mudfish were more left-shifted and steeper than curves reported for species occupying oxygen rich environments such as the rainbow trout (*Oncorynchus mykiss*; *Weber* et al., 1976). The shape and position of the curve is important as it represents a compromise between the need to obtain oxygen from an environment that, relative to air, is oxygen deficient, and the need to permit satisfactory unloading at oxygen tensions encountered in the tissues. Research has shown that species-specific differences in the oxygen equilibrium curves can be due to differences in environmental oxygen availability, with high oxygen affinities found in species that encounter hypoxic conditions, and lower oxygen affinities in species inhabiting well oxygenated waters (Powers et al., 1979; Riggs, 1979; Powers, 1980).

The oxygen affinities of the New Zealand mudfishes presented here were found to be consistently higher than a previous study of the blood oxygen affinity of the Canterbury mudfish (Wells et al., 1984) under the same conditions. These differences may not necessarily be significant, as they may be due to differences in

techniques used to determine blood oxygen affinity (thin layer versus diluted whole blood), differences in time spent in captivity (months versus days/weeks), and differences in location and season of capture. Small sample sizes in both studies and high individual variation within the species could also account for some of the differences observed.

Mudfish whole blood is very sensitive to changes in pH, with a decreasing pH leading to a reduction in oxygen affinity in all of the mudfish species. This has also been noted in a diverse range of fishes including rainbow trout (Weber et al., 1976), Australian blackfish (*Gadopsis marmoratus*; Dobson & Baldwin, 1982), tench (Eddy, 1973), common carp (Black, 1940) and many more. This is expected, as the functional significance of the alkaline Bohr effect lies in enhancing oxygen delivery to the tissues at times of greatest oxygen demand (Dobson and Baldwin, 1982). During exercise, acidic metabolites will be present in the blood in high concentrations which causes a reduction in pH and the subsequent uptake of hydrogen ions by the haemoglobin. This causes a decrease in oxygen affinity and concomitant release of more bound oxygen than would occur merely in response to the lowering of tissue pO_2 during activity (Stringer et al., 1994). In some mudfish species (Canterbury, Northland and black) there was some evidence of an apparent reverse Bohr shift below pH 7.0 but statistical comparisons found no significant difference in oxygen affinity between pH 6.5 and 7.0. It is possible that small sample numbers or large variations between individual's accounts for the above observations. It is also possible that haemoglobins of these species are pH insensitive within this range and significant oxidation may occur below pH 7.0, however, we can not validate this as haemoglobin oxidation was not measured in this study.

The whole blood oxygen affinity of the mudfish species examined was also temperature sensitive, with an increase in temperature causing an increase in p50 values and thus a decrease in oxygen affinity. The effect of temperature on oxygen affinity has been shown in a large range of teleosts and in most cases an increase in temperature causes a decrease in oxygen affinity. This has been observed in four

closely related species of *Trematomus* (Grigg, 1967), brook trout (*Salvelinus fontinalis*) and rainbow trout (Irving, 1941), Australian blackfish (Dobson & Baldwin, 1982), African lungfishes (*Protopterus aethiopicus*; Lenfant & Johansen, 1968) and many more. However, research carried out on the endothermic southern bluefin tuna (*Thunnus maccoyii*) has revealed a different pattern. Instead of the haemoglobin displaying a reduction in oxygen affinity as temperature increases it shows a reversed temperature sensitivity (Bushnell & Jones, 1994). The decrease in oxygen affinity in response to an acute temperature rise is directly due to overall exothermy of haemoglobin oxygenation as well as indirectly due to the associated pH decrease (Randall et al., 2000).

All of the mudfish species showed an increase in the cooperativity of the haemoglobin as pH increased. At low pH values of 6.5 and 7.0 the cooperativity of the haemoglobin was low and approaching unity in all of the mudfish species. The reduction in cooperativity at low pH values is due to the inhibition of the allosteric transition from the T to the R structure (Perutz, 1983). At the higher pH values the New Zealand mudfish species showed high oxygen affinity as well as a high degree of cooperativity. The high degree of cooperativity coupled with high oxygen affinities aids in the release of bound oxygen to the tissues under a relatively narrow range of oxygen tensions. The level of cooperative oxygen binding is important as it determines the shape of the oxygen equilibrium curve and thus determines the functional range of oxygen tensions over which oxygen can be unloaded from the blood (Dobson & Baldwin, 1982). The Hill coefficient (n_{50}) can also be used to determine how active the fish are. Fish utilizing sustained aerobic muscle work, like the salmonid species (Weber et al., 1976), generally display n_{50} values approaching 2.5-3.0 with distinctly sigmoidal curves, whereas, less active species like the Australian blackfish (Dobson & Baldwin, 1982) are characterized by a more hyperbolic-shaped curve with lower n_{50} values. The New Zealand mudfish species tend to display hyperbolic-shaped curves with approximate n_{50} values of 2, so this would place them among the less active fish species. A number of reviews (Powers, 1980; Brittain, 2005) discussing the role of haemoglobin in matching oxygen demand

with supply have suggested a general trend where active fish appear to have low-affinity and highly cooperative haemoglobins which favour oxygen unloading at the tissues. Less active species tend to have haemoglobins which have a high-affinity for oxygen and low levels of cooperativity, thus favouring oxygen uptake at the respiratory surfaces. However, a study done on four cold-temperate marine fishes (Herbert et al., 2006) found that although oxygen equilibrium curve characters such as p50, Bohr factors and n_{50} values provide clues to the efficiency of oxygen transport, they cannot themselves be used to make general trends on the physiology and ecology of phylogenetically different species.

A number of studies (Nikinmaa, 2001; Yokoyama, 2004; Olianas et al., 2005) have demonstrated the importance of organic phosphates in the regulation of haemoglobin oxygen affinity in fish blood. The binding of organic phosphate compounds to haemoglobin reduces the oxygen affinity of most vertebrate haemoglobins (Jensen, 2004). Phosphorylated compounds in the erythrocyte not only affect the oxygen affinity of haemoglobin but also increase the magnitude of the Bohr effect and may affect subunit interaction (Randall et al., 2000). In this study we collected fish from their natural habitats and took whole blood samples as quickly as possible, therefore, these samples should be representative of blood from wild mudfish. But due to some species spending more time in the lab than others and acclimating to the conditions in the lab, some of the effects we observed could be due to variation in organic phosphates as well as pH and temperature.

There is some evidence of habitat specialization in haemoglobin physiology between mudfish species but this did not match the proposed cline of Waters & McDowall (2005). As expected, Canterbury mudfish typically displayed the lowest oxygen affinity across the temperature range examined whilst the black mudfish, inhabiting ephemeral wetlands in the northern North Island, consistently displayed the highest affinity at all temperatures. Although the brown mudfish which were collected in Taranaki displayed low oxygen affinities at low pH values of 6.5 and 7.0, at higher pH values of 7.5 and 8.0 they displayed oxygen affinities almost as high as the black

mudfish. A low oxygen affinity, significant Bohr shift and a low degree of cooperativity was observed in the haemoglobin of the Canterbury and West Coast brown mudfish. These two species of mudfish happen to be the most southernly located where the mean annual temperature is around 7.5ºC - 10ºC and the water column is typically less hypoxic. The black and Northland mudfish had a high whole blood oxygen affinity at all temperatures, a significant Bohr shift (between pH 7.0 – 7.5) and a high degree of cooperativity. These mudfish species are located in the northern region of the North Island of New Zealand where mean annual temperatures are significantly higher than those experienced in the South Island. Also, there are higher seasonal as well as diurnal fluctuations in temperature in the northern regions of New Zealand. The Kaitaia black and Northland mudfish species were the least temperature sensitive with the lowest oxygenation enthalpy change, whereas, the southern species of mudfish had high oxygenation enthalpy changes. This pattern can be seen in the blood from the Australian (*Neoceratodus*) and African (*Protopterus)* lungfishes where *Neoceratodus*, which occupies waters that show large annual and diurnal changes in temperatures has blood that is notably insensitive to temperature changes. Conversely, the habitat that *Protopterus* abides in has slight temperature variations and thus the blood of *Protopterus* is much more sensitive to temperature changes. During summer, the habitats that the black and Northland mudfish abide in either dry out completely or become very shallow, warm and hypoxic. Thus, it is beneficial for the black and Northland mudfish to have a high oxygen affinity to safeguard oxygen loading when oxygen availability is reduced, as well as a reduced sensitivity to changes in pH to cope with the variable conditions often prevailing in ephemeral wetlands. This high oxygen affinity and reduced sensitivity to changes in pH is also a tactic used by the spotted robust triplefin (*Grahamina capito*) to cope with the variable conditions often prevailing in tidal pools and shallow estuarine areas (Brix et al., 1999).

The proposed cline with respect to morphological adaptations for wetland dwelling (Waters & McDowall, 2005) is as follows:

brown (most specialized) – Northland – black – Canterbury (least specialized)

Although there is evidence of habitat specialization in haemoglobin physiology between mudfish species, it did not match the proposed cline of Waters & McDowall (2005). With respect to haemoglobin physiology the cline was as follows:

black (most specialized) – Northland – brown – Canterbury (least specialized).

Chapter Three: Aestivation and oxygen affinities

3.1 Introduction

Slow-moving or temporary bodies of water can become periodically depleted of oxygen. This may cause the fish inhabiting these habitats to resort to breathing atmospheric air in order to survive. Air breathing is an ancient vertebrate specialization and fish from a great number of families are reliant on atmospheric air on occasion (Johansen, 1970). There is a strong evolutionary selection pressure to breathe air over water, as relative to air, the higher viscosity of water imposes a greater frictional resistance and its greater density requires more work from the fish to overcome inertia (Graham, 1997). Water also contains much less oxygen than air, and because its diffusion constants are much lower, gases diffuse more slowly through water than air (Graham, 1997). Air breathing can be accomplished in a few different ways such as the possession of an accessory air-breathing organ or on the reliance of gills and skin for aerial gas exchange (Berg & Steen, 1965).

Previous studies on closely related species of non-air-breathing and air-breathing osteoglossids and erythrinids (Johansen et al., 1978b) have shown that the blood of air-breathing fishes has a higher oxygen capacity, p50, Bohr shift and greater red cell organic phosphate concentrations than that of a water-breathing fish. However, comparisons among a diversity of air-breathing and non-air-breathing species fail to verify this as a "universal" evolutionary pattern (Graham, 1997). The pattern is not seen among the three lungfish genera (Lenfant et al., 1966) which have different air-breathing requirements.

Air exposure tests carried out on the marble swamp eel (*Synbranchus marmoratus*) by Johansen et al. (1978b) found that when the fish were exposed to air for up to 44 hours there were major effects on the respiratory properties of the blood. There was a dramatic increase in haemoglobin concentration, a decrease in haematocrit, a drop in plasma pH and a doubling of the red cell phosphate concentration. Air exposure also decreased haemoglobin oxygen affinity from a p50 value of 5 in water to 11 in air.

Research carried out by Barrier (1993) on black mudfish populations revealed that 87% of the sites where the mudfish were present became dry in summer, suggesting that these fish preferentially inhabit such areas. Aestivation is an adaptation that the New Zealand mudfish species have which allows them to survive periods of anoxia or drought in their ephemeral habitats (Eldon, 1978a; Meredith, 1981). They are able to aestivate for up to several months and during this time they respire atmospheric air through their gills and skin (Dean, 1995; Ling, 2001). During aestivation we would expect the closely related New Zealand mudfishes to show a decrease in haemoglobin oxygen affinity compared to when they are in water due to the increased availability of oxygen.

The aim of this study was to investigate the oxygen affinity and cooperative oxygen binding in the New Zealand mudfishes while they are aestivating, fasting and under normal conditions. To carry out this investigation we compared differences in p50 values and Hill coefficients between the treatments as well as between species.

3.2 Methods

3.2.1 Capture and maintenance of mudfish

Adult black, Northland and brown mudfish were obtained from the Whangamarino wetland, Lake Omapere wetlands and Stratford, respectively, as described in Section 2.2.1. Restrictions on the transfer of aquatic organisms between islands of New Zealand as well as the limited numbers of available specimens prevented the inclusion of Chatham Island and Canterbury mudfishes.

Fish were returned to the University of Waikato and allowed to acclimate to laboratory conditions (15 ºC, 12:12 L:D photoperiod) in 60 l aquaria (5 individuals per aquarium) for two weeks prior to the commencement of experiments. Aquaria contained short sections of PVC tubing and terracotta pots to provide refuge. Fish were fed to satiation daily on frozen blood worms and aquaria received weekly water changes of dechlorinated Hamilton City tapwater.

3.2.2 Experimental design

Following acclimation to laboratory conditions, five adult fish of each species were randomly assigned to one of three experimental conditions: aestivating, fasting, and control. Aestivating fish were placed into 20 cm x 15 cm x 5 cm ventilated plastic containers filled with damp sphagnum moss (one individual per container; Figure 23). Containers were checked for any fish deaths once a week but were otherwise left undisturbed for the duration of the experiment (6 weeks). Fasting fish remained in their aquarium without feeding but were otherwise treated identically to fish under normal conditions. Control fish were maintained identically to the period of acclimation and fed daily to satiation with frozen blood worms. All fish were anaesthetized (MS222, 0.1g l^{-1}), measured and weighed prior to assignment to each treatment.

Figure 23: Vivaria for aestivating mudfish. Vivaria consisted of ventilated plastic containers filled with damp sphagnum moss for the aestivating mudfish. The period of aestivation was confined to 6 weeks.

3.2.3 Collection of blood sample and processing

After 6 weeks duration in each experimental treatment, blood samples were obtained from anaesthetized fish by caudal venepuncture and whole blood oxygen dissociation curves obtained as described in Section 2.2.2 at 15˚C (± 0.1˚C) and at buffer pH values of 7.5 and 8.0 (± 0.1 pH unit). Five blood samples were analyzed for each combination of pH and treatment with the exception of fasting black mudfish which experienced high mortality. All fish were measured and weighed as before to assess growth and condition.

3.2.4 Statistical analysis

Data were analysed using Statistica version 7.1 (Statsoft Inc., Tulsa. USA). Following tests for assumptions for normality and homogeneity of variance, factorial two-way analysis of variance and subsequent Tukey's post-hoc tests were performed on the log p50 and Hill coefficient data. All tests were considered significant at the 0.05 level.

3.3 Results

3.3.1 Effect of treatment on mudfish condition

Table 4 displays the mean weight of the three mudfish species before and after the experiment for all three treatments. After 6 weeks, all of the mudfish lost weight in the aestivating and fasting treatments, but had gained weight in the control treatment. The brown mudfish had the smallest reduction in % body weight out of the three species in both the aestivating and fasting treatments, whereas the Northland mudfish had the greatest reduction in % body weight, losing almost 20% of original body weight over the experimental period of 6 weeks.

Table 4: Mean % body weight gain/loss (± standard error) of three mudfish species at different treatments over the 6 week experimental period.

n = 5 for all treatment groups with the exception of fasting black mudfish* where n = 1 due to a high mortality rate.

Species	Treatment	Original weight (g)	Weight after experiment (g)	% body weight gain or loss
brown	aestivating	13.08 ± 1.64	11.63 ± 1.36	-11.05 ± 0.90
	fasting	14.98 ± 1.43	13.98 ± 1.34	-6.64 ± 1.35
	control	17.1 ± 1.98	18.69 ± 2.12	9.32 ± 1.18
Northland	aestivating	5.58 ± 0.60	4.61 ± 0.51	-17.44 ± 2.03
	fasting	5.34 ± 1.35	4.32 ± 1.08	-19.14 ± 0.57
	control	5.02 ± 0.62	5.24 ± 1.03	20.31 ± 4.54
black	aestivating	7.76 ± 2.36	6.79 ± 2.02	-12.59 ± 0.72
	fasting*	7.88	6.72	-14.72
	control	6.77 ± 1.17	7.81 ± 0.90	15.36 ± 1.70

3.3.2 pH dependence of the whole blood oxygen affinity

As expected, all species showed an increase in oxygen affinity (decrease in log p50) as pH increased (Figure 24). There was no obvious trend for all three species except that the control treatment tended to have the lowest or nearly lowest oxygen affinity in all species. In the Northland and brown mudfish species, fasting fish displayed the highest oxygen affinities, while aestivating black mudfish displayed the highest

oxygen affinity at both pH values. However, this was not conclusive as the fasting data was not included due to a high mortality rate in that experiment.

Figure 25 shows that at pH 7.5, the brown mudfish displayed no differences in oxygen affinity between the three treatments and in the black and Northland mudfish the differences were very minor. The results of a two-way ANOVA found that there were no significant differences in log p50 values between the three different species ($p=0.079$, $F= 3.289$) for the three different treatment types (e.g. aestivating black = aestivating Northland= aestivating brown etc). Two-way ANOVA did reveal a significant difference ($p<0.05$, $F=8.444$) in log p50 values within the species across treatments. However, a subsequent Tukey's post-hoc test showed that at pH 7.5 the only significant difference between treatments within a species occurred in the Northland mudfish. It was found that fasting Northland mudfish had a significantly higher whole blood oxygen affinity than the Northland control treatment.

Figure 26 shows that at pH 8.0 there seem to be no significant differences in oxygen affinity between the species in any of the treatments. A two-way ANOVA found that there were no significant differences in log p50 values between the different species ($p=0.196$, $F=1.741$) and there were also no significant difference in oxygen affinity at the different treatments ($p=0.185$, $F=1.389$).

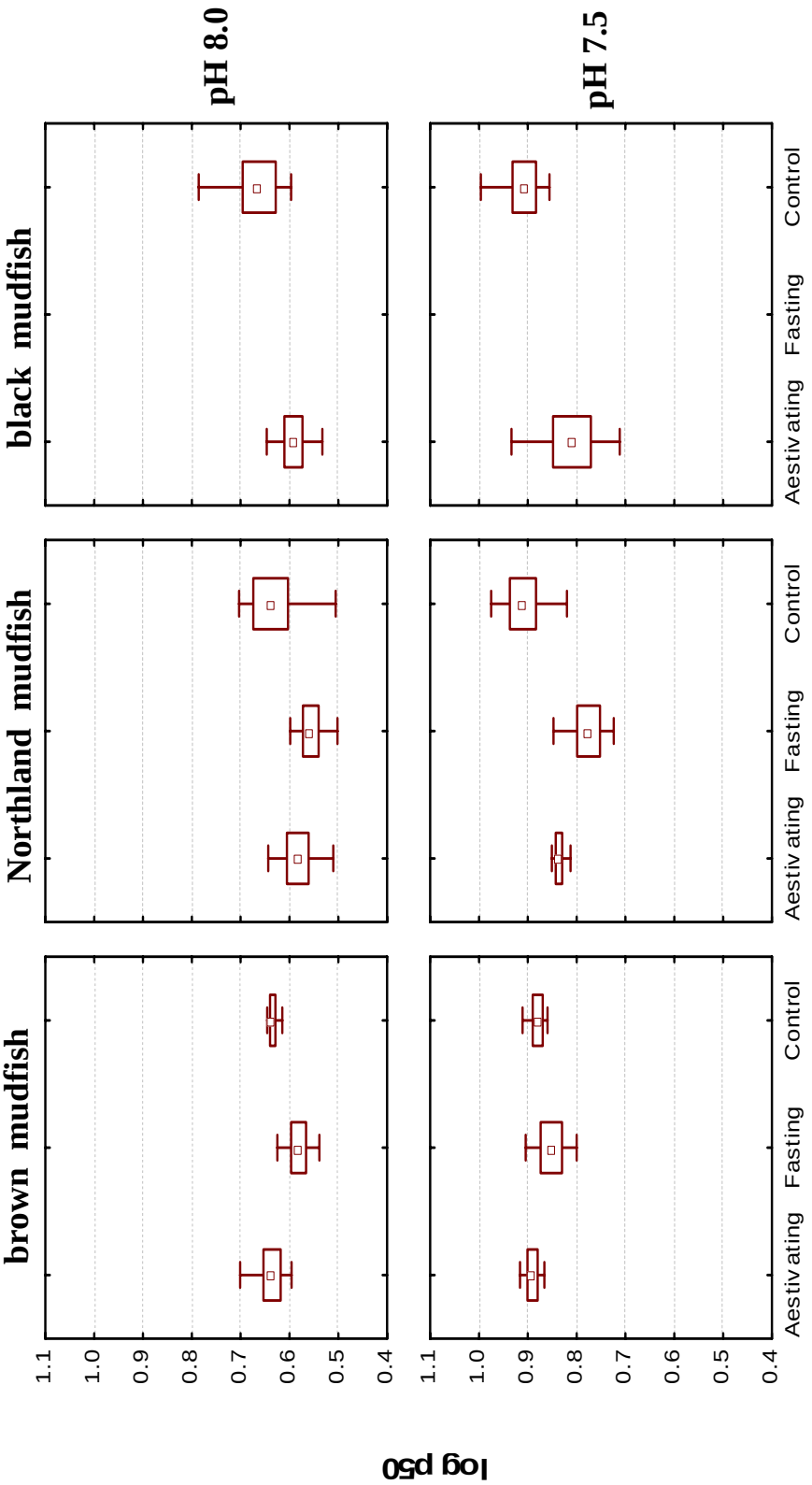

Figure 24: pH dependence of whole blood oxygen affinity at 15ºC for black, brown and Northland mudfish at different treatments (aestivating, fasting and control) at two pH's (7.5and 8.0). n = 5 for all species except the fasting black mudfish where n = 1 due to a high mortality rate in that group.

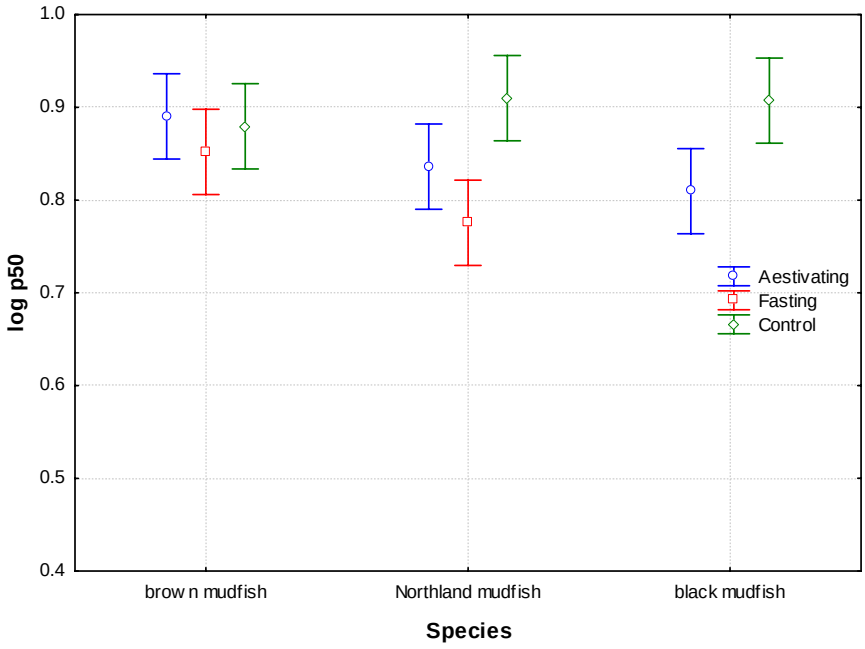

Figure 25: Mean log p50 values of black, brown and Northland mudfish under aestivating, fasting and normal conditions at pH 7.5 and 15ºC.

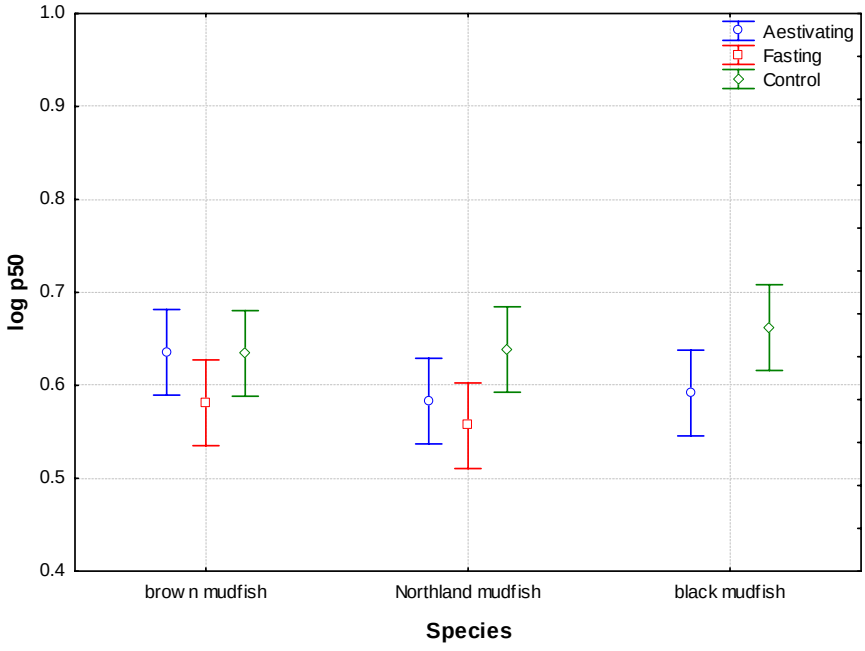

Figure 26: Mean log p50 values of black, brown and Northland mudfish under aestivating, fasting and normal conditions at pH 8.0 and 15ºC.

*Lines are offset for clarity and the fasting black mudfish data is missing due to a shortage of samples. n = 5 for all species.

Table 5 shows that there was a significant interaction between pH and treatment but only in the brown mudfish. This means that the effect of pH on oxygen affinity is modified by changes in the treatment and vice versa. In the Northland and black mudfish the two variables are independent of each other.

Table 5: Factorial two-way ANOVA results for oxygen affinities of different species of mudfish with pH, treatment and the interaction between them.

Species	**pH**	**Treatment**	**Interaction** pH*treatment
brown	F = 206.5 **p<0.001***	F = 63.3 **p<0.001***	F = 51.8 **p<0.001***
Northland	F = 165.9 **p<0.001***	F = 10.7 **p<0.001***	F = 0.6 p=0.539
black	F = 60.15 **p<0.001***	F = 7.92 **P=0.012***	F = 0.21 p=0.655

* indicates a significant difference ($p < 0.05$)

3.3.3 Cooperative oxygen binding

All species showed an increase in cooperative oxygen binding (increase in Hill coefficients) as pH increased (Figure 27). A trend that is displayed in the black and Northland species and at both pH values was that cooperativity was highest in the aestivating mudfish, then the fasting mudfish and lowest in the control treatments. For the brown mudfish this trend was the opposite as the cooperativity was highest in the control treatment, then fasting and the lowest in the aestivating treatment, although these differences were very small.

Figure 28 shows that at pH 7.5 there were some significant differences in cooperativity between the three species at the three different treatments. A two-way ANOVA found that there were significant differences in mean Hill coefficients between the different mudfish species ($p<0.001$, $F = 20.05$) and also between the different treatments ($p=0.004$, $F = 9.41$). A subsequent Tukey's post-hoc test

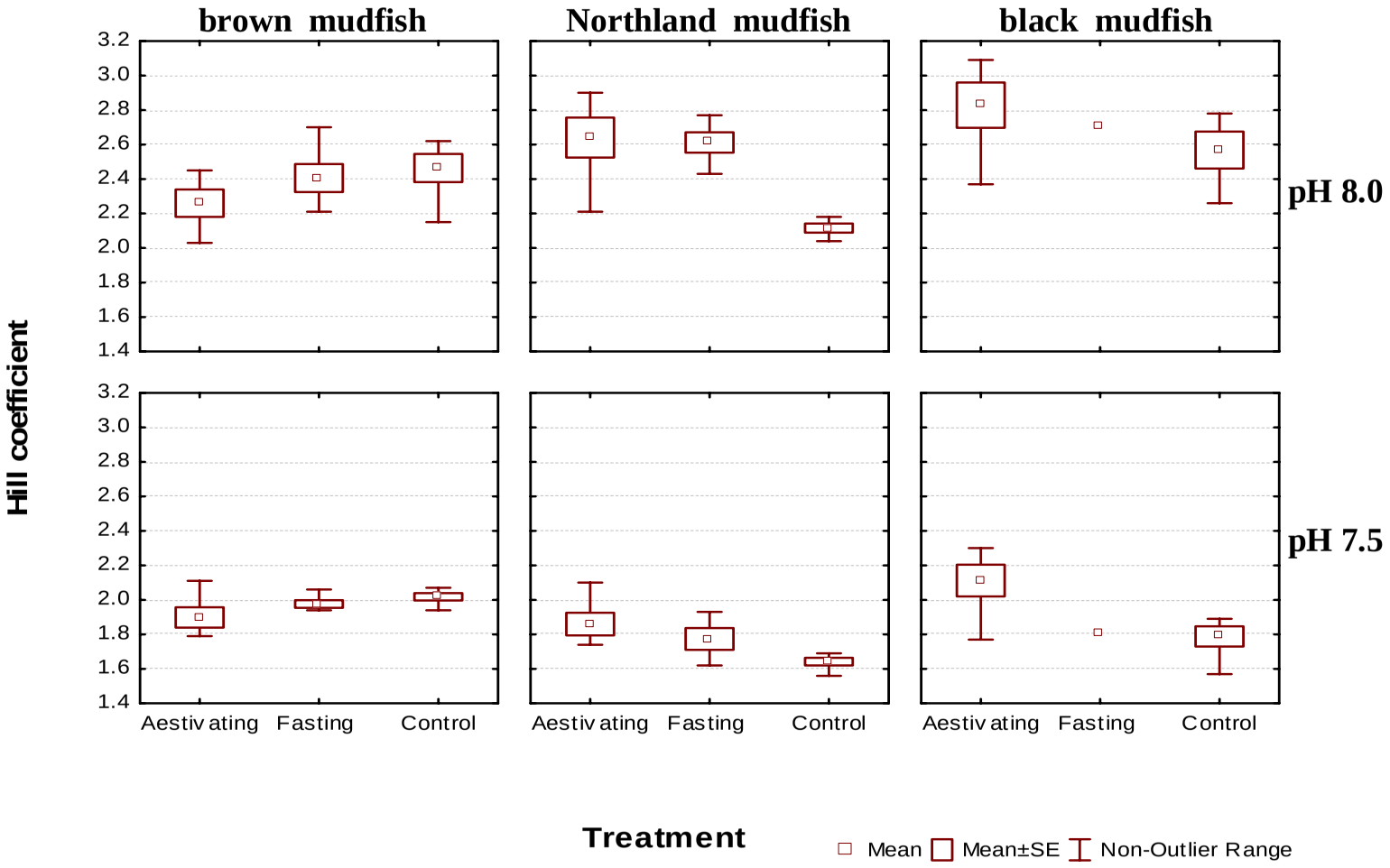

Figure 27: Levels of cooperative oxygen binding at 15ºC in black, brown and Northland mudfish at a range of treatments (aestivating, fasting and control) and pH's (7.5 and 8.0). n = 5 for all species except the fasting black mudfish where n = 1 due to a high mortality rate in that treatment.

showed that the only difference between species was that the control brown mudfish treatment showed a significantly higher level of cooperativity then the Northland control treatment. Significant differences between treatments within a species only occurred in the black mudfish where the aestivating fish had a significantly higher Hill coefficient than the fish in the control treatment.

At pH 8.0 (Figure 29), the black mudfish seemed to have the highest cooperativity for the aestivating and control treatments out of the three species. In the Northland mudfish there was a significant difference in cooperativity between the control treatment (lower cooperativity) and the fasting and aestivating treatments (higher cooperativity). Figure 28 does not indicate a significant difference between

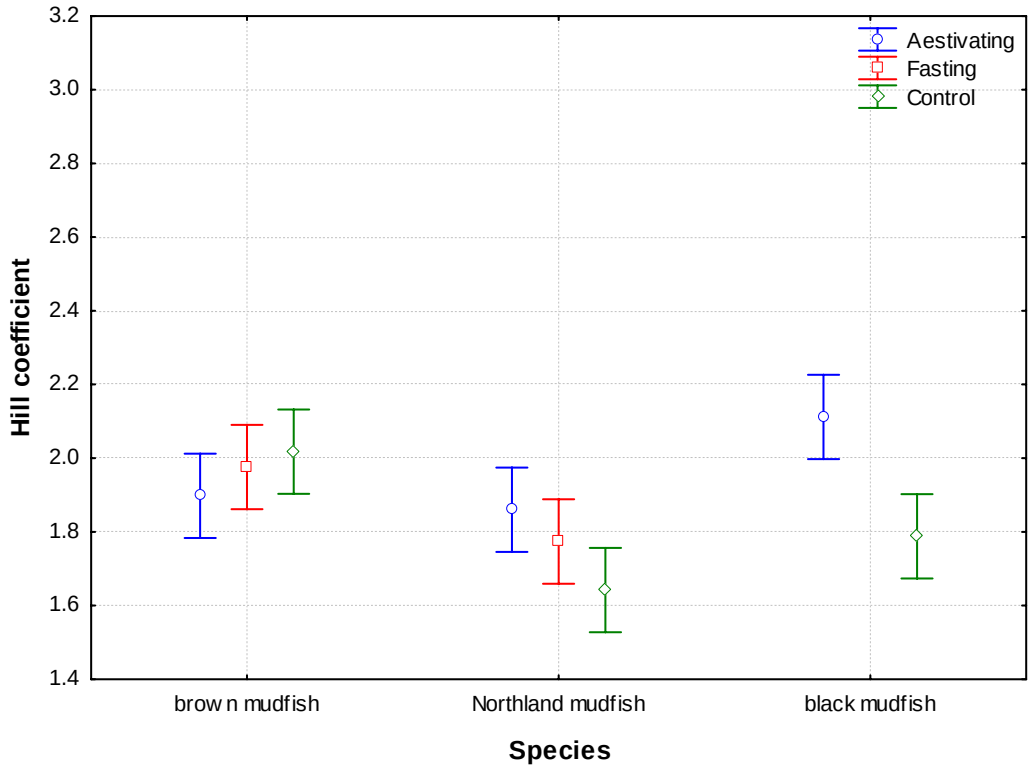

Figure 28: Cooperative oxygen binding in black, brown and Northland mudfish species under aestivating, fasting and normal conditions at pH 7.5 and 15˚C.

***Fasting black mudfish have been excluded due to low sample numbers. The lines are offset for the clarity. n = 5 for all species.

treatments for the brown mudfish. A two-way ANOVA found that there were no significant differences in mean Hill coefficients between the different mudfish species (p=0.293, F = 1.145) but there where significant differences between the different treatments (p=0.014, F = 6.798) at pH 8. A subsequent Tukey's post-hoc test showed that the mudfish in the control treatment for the Northland mudfish species had a significantly lower cooperativity compared to the aestivating and fasting fish.

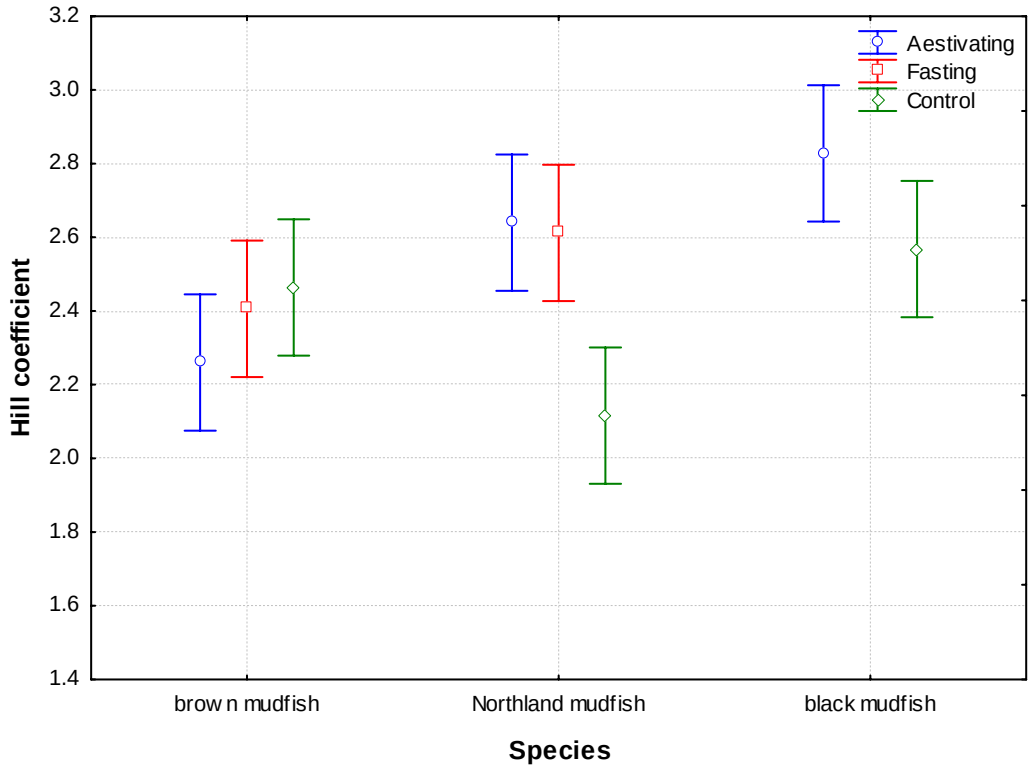

Figure 29: Cooperative oxygen binding in black, brown and Northland mudfish species under aestivating, fasting and normal conditions at pH 8.0 and 15˚C.

*****Fasting black mudfish have been excluded due to low sample numbers. The lines are offset for the clarity. n = 5 for all species.

There was a significant interaction between pH and treatment but only in the Northland mudfish (Table 6). This means that the effect of pH on oxygen affinity is modified by changes in the treatment and vice versa. In the brown and black mudfish the two variables were independent of each other.

Table 6: Factorial two way ANOVA results for the cooperativity of the haemoglobin of different species of mudfish with pH, treatment and the interaction between them.

Species	**pH**	**Treatment**	**Interaction** pH*treatment
brown	F = 63.87 **p<0.001***	F = 3.44 **P=0.049***	F = 0.25 P=0.782
Northland	F = 164.4 **p<0.001***	F = 18 **p<0.001***	F = 4.3 **p=0.025***
black	F = 55.13 **p<0.001***	F = 8.4 **P=0.010***	F = 0.10 p=0.755

* indicates a significant difference ($p < 0.05$)

3.4 Discussion

During aestivation and fasting, mudfish from all three species lost a significant proportion of weight while mudfish in the control treatment gained weight. The brown mudfish experienced the least reduction in % body weight whereas the Northland mudfish experienced the greatest proportional weight loss. However, the initial body weights of brown mudfish (15.05 ± 1.01g) were nearly three times that of the Northland mudfish (5.31 ± 0.57) and twice that of the black mudfish (7.80 ± 1.24). This inverse relationship between weight loss and initial body weight is to be expected because of the interdependence of mass-specific metabolic rate and body mass (Randall et al., 2000). Dean (1995) found that aestivating black mudfish lost, on average, 15.2 ± 2.19% of their initial body weight after 28 days but did not observe a relationship between weight loss and initial body mass despite her fish ranging in weight from 0.96 to 5.94 g. The results of my study of black and Northland mudfish are comparable with those of Dean (1995) and unpublished results of N. Ling (pers. com.) who found that fasted black mudfish in water lost 17.8 ± 1.39% of initial body weight compared to 13.2 ± 3.15% in aestivating fish after 42 days. The general pattern of increased weight loss in fasting versus aestivating fish presumably reflects a greater level of metabolic depression in the latter as a true physiological adaptation to survive prolonged periods of emersion. However, this relationship was not observed in the brown mudfish despite the well established aestivating abilities of this species.

Due to an increasing dependence on aerial gas exchange, McCutcheon & Hall (1937) suggested that the evolutionary transition from aquatic to aerial respiration would be generally characterised by an increase in metabolism and a decrease in haemoglobin oxygen affinity as it reflects an adjustment to the higher oxygen availability in air than water. This pattern has been observed in extant species such as the bullfrog (*Rana* sp.) and several other amphibians, where the metamorphosis from water-breathing tadpoles to air-breathing adults has been accompanied by a decline in haemoglobin oxygen affinity (Riggs, 1951; Lenfant & Johansen, 1967). This theme

of a right-shift in the oxygen equilibrium curves among air-breathing fishes has persisted for a considerable amount of time (Frey & van Aardt, 1995).

The three New Zealand mudfish species used for this study (black, brown and Northland mudfish) are phyletically very similar. They exhibited very similar p50 values at all of the treatments ranging from 6 to 8 mm Hg at pH 7.5 and 3 to 5 mm Hg at pH 8.0. Comparisons made in the haemoglobin oxygen binding properties of over 20 species of air breathing fishes (Graham, 1997) reveals p50 values ranging from 3 mm Hg to as high as 35 mm Hg at pH values ranging from 7.2 to 7.8. The New Zealand mudfish species display high p50 values compared to other air-breathing fishes, although a lot of the data on the other air-breathing fishes were determined at higher temperatures which would have an effect on the oxygen affinity (increased p50 values). The high variation in the oxygen affinity of air-breathers reflects specific differences in behaviour, physiology, habitat and air breathing as well as differences in the methods of previous studies with respect to techniques and conditions employed (Graham, 1997). After comparing the haemoglobin oxygen affinities of 42 different species of air-breathing and non air-breathing Amazonian fishes, Powers et al. (1979) revealed that the p50 values for air breathing fishes, which ranged from about 3 to 14 mm Hg, were completely encompassed by values for non-air breathers. This suggests no fundamental effect of air breathing on the haemoglobin oxygen affinity. However, a big problem with broad species surveys such as the one that Powers et al. (1979) carried out is that bias can be introduced by phyletic differences amongst the species and species-specific differences in activity, habitat and air-breathing efficacy (Graham, 1997).

To overcome this bias, studies should be carried out on phyletically similar groups of species. Research carried out on differences in air breathing in phyletically similar groups (lungfishes, osteoglossids, and erythrinids) have provided only minimal support for the hypothesis of an evolutionary trend towards a reduced haemoglobin oxygen affinity with air breathing (Graham, 1997). Studies carried out on closely related species of non-air-breathing and air-breathing osteoglossids (Johansen et al.,

1978a) have shown that the blood of the air-breathing arapaima (*Arapaima gigas*) has a higher oxygen capacity, p50, Bohr shift and greater red cell organic phosphate concentrations than that of the water-breathing silver arowana (*Osteoglossum bicirrhosum*). However, in the lungfishes (Lenfant et al., 1966) the data revealed that there were no significant differences between air-breathers and non-air breathers. The three species of New Zealand mudfish used in my research showed no significant differences ($p<0.05$) in p50 values between air-breathing individuals and water-breathing individuals for any of the species at both pH 7.5 and 8.0. Indeed, contrary to expectations, slight differences between treatments typically indicated higher affinity in air-breathing mudfish, although this was accompanied by concomitant increases in cooperativity. These inter and intraspecific differences in the haemoglobin oxygen affinity of air-breathing fishes could be due to differences in the primary structure of the haemoglobin molecule, expression of allosteric effectors, respiratory behaviours and also environmental differences which can affect blood gas tensions, plasma and red cell pH which determine the microenvironment of haemoglobin molecules (Graham, 1997).

During a summer drought period, water levels can drop and mudfish are required to aestivate. Summer air temperatures are typically warmer than water temperatures and thus during aestivation mudfish experience increased body temperatures. This increase in temperature would cause a decrease in oxygen affinity due to the overall exothermy of haemoglobin oxygenation, and indirectly due to the associated pH decrease (Randall et al., 2000). Aestivating or air-breathing fish also have higher circulating carbon dioxide tensions than water-breathing fish due to the low rates of aerial carbon dioxide release by fish (Graham, 1997). The water to air transition causes a rise in arterial pCO_2 which presumably could cause a fall in erythrocyte pH which further decreases oxygen affinity. In our experiment we have not considered the changes in abiotic conditions between the treatments as all treatments were run at the same temperature and the pH of the blood was altered by the pre-determined pH of the buffer. This could be a reason why we have not observed differences in oxygen affinity between the aestivating fish and the non-aestivating fish. Further

research needs to be carried out on the in vivo differences in blood temperature and pH between aestivating and non-aestivating mudfish. This would involve measuring the blood pH of the mudfish in the different treatments as well as measuring in situ temperature differences between water in mudfish habitats and air and then examining blood oxygen binding under those new conditions (i.e. lower pH and increased temperature in aestivating fish). Unfortunately, apparatus capable of accurately measuring the pH of microlitre blood samples was not available for this research.

Chapter Four: Presence of multiple haemoglobins

4.1 Introduction

Fish may adapt to changes in ambient temperature and oxygen levels through changes in organic phosphate concentrations or changes in the structure and relative concentration of individual haemoglobin components which may result in increased total haemoglobin content or changes in oxygenation characteristics (Frey et al., 1998). Multiple haemoglobins occur in a large number of temperate fish genera that experience large variations in oxygen availability and temperature.

Teleost fish show an extensive heterogeneity in adult haemoglobin structure and function. Class 1 species such as carp (Gillen & Riggs, 1972) have single or multiple haemoglobins all of which are sensitive to pH and temperature. Class II species such as the eel (Gillen & Riggs, 1973) have multiple haemoglobins that include anodic (similar to class 1) and cathodic haemoglobins (reduced pH and temperature effects). Class III comprises fish such as the tuna, whose haemoglobins are sensitive to pH but not to temperature (Yokohama et al., 2004)

An organism with different types of haemoglobin in its blood has an increased scope for the transport of oxygen, protons and heat (Weber & Jensen, 1988). Anodic components of haemoglobin have normal Bohr and Root effects while the cathodic components display high oxygen affinities and small, often reversed, Bohr effects (Olianas, 2005). Research on the effect of internal hypoxia and acidosis on fish haemoglobin has shown that during hypoxic conditions, fish like trout and eels (Weber et al., 1975) have cathodal haemoglobin components (high, pH-insensitive oxygen affinity) to transport oxygen to the tissues whereas under normoxic conditions the low-affinity, pH-sensitive anodal haemoglobin components unload oxygen to the tissues. Thus it is beneficial for fish such as trout to have these different haemoglobin components so that they are adapted to a range of conditions by altering the relative abundance of these components.

The aim of this study was to investigate the possibility of the New Zealand mudfish species possessing multiple haemoglobins and also to see if there were differences between the species in the abundance of these haemoglobins between species. A further aim was to investigate whether the relative abundance of mudfish isohaemoglobins changes during aestivation and fasting.

4.2 Methods

4.2.1 Preparing the haemoglobin for iso-electric focusing

The blood samples used for this chapter were taken from the blood samples obtained from fish in Chapters 2 and 3. 20µl of whole blood was reserved from each sample where surplus blood was available following determinations of oxygen binding. 5 samples were obtained from each of the brown, black and Northland mudfishes and 3 samples were taken from each of the experimental treatments for each of these three species (e.g., 3 aestivating black, 3 fasting black, 3 control black). Unfortunately, due to limited sample numbers and small blood volumes, no samples were obtained for Canterbury mudfish.

The red blood cells were pelleted by centrifugation (2500 x g, 5 min) and the plasma discarded. Red cells were then washed three times in 0.01 M Tris-HCl containing 0.7% NaCl with a pH of 7.8 (Frey et al., 1998). The cells were then lysed with 0.01 M Tris buffer at pH 8.0. 40 µl of the lysis buffer was added to the washed red cells, mixed thoroughly and then centrifuged at 5000 x g for 10 min to pellet cell debris. The haemolysate was stored on ice while an Amberlite MB3 mixed-bed ion exchange resin was being equilibrated. The ion-exchange columns were equilibrated by adding 200 µl of the lysis buffer to the top of the column and then centrifuging the column at 2000 x g for 2 min. This was repeated 3 times with new additions of buffer each time. Once the columns were equilibrated the haemolysate was added to the column and collected by centrifugation at 2000 x g for 2 min. The final haemolysate was rapidly frozen in a cryovial in liquid nitrogen and then stored at -80ºC until analysis by isoelectric focusing.

4.2.2 Protein solubilisation

The IPG rehydration solution was made fresh daily and contained 8M urea, 2% CHAPS, 50 mM DTT, and 0.2% 3-10 ampholytes (Bio-Rad). The urea was treated with mixed bed ion-exchange resin (Sigma) for 10 minutes to remove charged species

and vacuum filtered through #1 filter paper (Advantec) to remove the ion exchange beads. The aliquoted urea was stored frozen at -20ºC until it was required.

332.5 µl of IPG rehydration solution was added directly to 17.5 µl of purified haemolysate (from section 4.2.1) in a microcentrifuge tube. They were vortexed twice for 5 seconds each and left at 20ºC for 1 hour so that the protein could solubilise. The solution was then centrifuged at 14,000 x g at room temperature for 2 minutes to pellet unsolubilised material. The supernatants from each tube were transferred to individual tubes and stored at 20ºC until required.

4.2.3 Isoelectric focusing

0.2 µl of 1% bromophenol blue solution was added to each of the solutions made in Section 4.2.2 to track progress of the IEF. 300 µl of each solution was laid along the length of a 17 cm focusing tray (Bio-Rad) and a 17cm 3-10NL IPG strip (Bio-Rad) was laid on top of this. This was left for 1 hour and then overlaid with 1.4 ml of mineral oil. The focusing tray lid was replaced and the IPG was left to passively rehydrate for another 19 hours (20 hours in total) at 20ºC in a Protean® IEF cell (Bio-Rad).

Before running the IEF, the IPG strips were lifted at each end and a wet electrode wick (made wet with 8 µl of milliQ water) was placed between each electrode and the gel. The focusing tray was replaced in the IEF cell and run at 20ºC with a current limit of 50 µA per gel. The focusing steps were as follows: the gels were held at 250 V for 15 minutes, and then subjected to a rapid ramp over 3 hours to 10,000 V, then voltage was maintained at 10,000 V for 60,000 V/hours. After completion of the IEF the gels were held at 500 V until removed from the IEF cell, to limit diffusion of the proteins.

4.2.4 Staining and imaging

IPG strips were removed from the focusing tray and mineral oil remaining on strips was removed onto filter paper. The IPG strips were then laid gel side up in rehydration trays. 5 ml of fixative (40% methanol, 10% acetic acid) was added to the

strips and left for 30 minutes. Fixative was then removed and replaced with 5 ml of 0.1% Coomassie blue R-250 stain. The stain was left for 2 hours and then removed. The IPG strips were destained with fixative until the background of the strips became clear (approximately 48 hours).

IPG strips were laid gel-side up on a Kaiser Prolite 5000 light box and images were made with a Nikon Coolpix 5700 which was attached to a copy stand for clear and interpretable images.

4.2.5 Determination of dilution for IEF solution

To determine what ratio of purified haemoglobin to IEF rehydration solution was required to give clear and interpretable results a dilution experiment was run. Four dilutions of the haemolysate solution were made in IEF rehydration solution (refer to Table 7).

Table 7: Range of dilution ratios of purified haemoglobin to IEF rehydration solution.

Dilution ratio	µl of haemoglobin	µl of rehyd. sol.
1/5	70	280
1/10	35	315
1/20	17.5	332.5
1/50	7	343

IPG strips were rehydrated with the different dilution ratios, focused, stained and then an image was made as described in Section 4.2.3 and 4.2.4. The dilution experiment was repeated to ensure the correct dilution ratio was chosen. The dilution of haemolysate chosen was determined on the basis of whether intense bands (haemoglobin) were detected by Coomassie blue R-250 stain, whilst limiting the amount of 'smearing' caused by the detection of other cellular proteins which have not been resolved.

4.2.6 2-dimensional gel electrophoresis

2-dimensional gel electrophoresis was used to determine which bands on the IEF are haemoglobin so that comparisons of haemoglobin numbers between species and treatments (aestivating, fasting and control) could be carried out. The IPG strips were prepared in the same manner as in Sections 4.2.2 and 4.2.3 and then frozen at -70ºC after they have been iso-electrically focused. The IPG was prepared for the second dimension by equilibrating it in 6ml of SDS-PAGE equilibration buffer I (6 M urea, 0.375 M Tris-HCl, pH 8.8, 2% (w/v) SDS, 20% (v/v) glycerol, 2% (w/v) DTT) for 15 minutes to reduce disulphide bonds. This was decanted off and replaced with 6 ml of SDS-PAGE equilibration buffer II (6 M urea, 0.375 M Tris-HCl, pH 8.8, 2% (w/v) SDS, 20% (v/v) glycerol, 2.5% (w/v) iodoacetamide) for another 15 minutes to acetylate free thiol groups. The SDS-PAGE equilibration buffer was stored in 12 ml aliquots at -20°C until required and then supplemented with DTT or iodoacetamide on the day it would be used.

After removing excess liquid, the IPG was laid on top of a 18.5 cm by 20 cm, 1.5 mm thick, 18% SDS-PAGE gel consisting of 6.725 MQ H_2O, 12.5 ml 1.5 M Tris-HCl pH 8.8, 6.5 ml 10% SDS, 30 ml acrylamide-bis (30% acrylamide, 2.67% bis-acrylamide), 250 µl of 20% (w/v) APS prepared fresh daily, and 25 µl of TEMED. A 0.5% (w/v) agarose LE (Roche) gel plug, made in 1x electrode buffer, containing 10 kDa protein markers (GibcoBRL®) was placed next to the basic end of the IPG and both were covered in 2-D agar overlay, containing 0.003% (w/v) bromophenol blue to track progress of the electrophoresis.

The gel slab was loaded into a Protean II Xi gel apparatus with an IPG conversion kit (BioRad). The gels were maintained at 15°C by circulating water, chilled by a refrigerated waterbath (RB-12A/TE-8D, Techne), through the cooling chamber and run at 35 mA per gel (Power Supply 1000/500, BioRad) for 5.75 hours. At completion of the electrophoresis, the gel was removed and placed in fixative overnight. The following day the gel was stained in 0.1% Coomassie blue R-250 stain for 1 hour and destained in fixative until the background was transparent. Gels

were stained in the same manner as the IPG strips so that bands detected could be determined as haemoglobin components and not contaminating red blood cell proteins.

4.3 Results

4.3.1 Differences in haemoglobin multiplicity between species

2-dimensional electrophoresis of haemolysates from three species of New Zealand mudfish (brown, black and Northland) revealed that the majority of the bands visible on the IPG strips were haemoglobin subunits. Figure 30 shows 2-dimensional electrophoretograms of the three mudfish species, stained with Coomassie blue R-250 stain. The major proteins resolved were all at a molecular weight of approximately 17 kDa which is known to be the weight of most haemoglobin subunits (Randall et al., 2000).

All mudfish species produced haemolysates that resolved into multiple haemoglobin subunits during isoelectric focusing (Figure 31). All of the mudfish species displayed a major anodic haemoglobin subunit at the isoelectric point (pI) of approximately 9.5, as well as multiple cathodic haemoglobin subunits. The number and apparent concentration of cathodic haemoglobins was greater than that of anodic haemoglobins. Differences between species were most noticeable between the Taranaki brown mudfish (IPG strips 1-5, Figure 31) and the Whangamarino black mudfish (IPG strips 6-10, Figure 31). The haemoglobin subunits of the Taranaki brown mudfish mainly ranged from a pI value of 7.0 – 9.0 whereas the haemoglobin subunits of the Whangamarino black mudfish mainly ranged from 6.5 – 7.5. The Northland mudfish (IPG strips 12-16, Figure 31) and the Kaitaia black mudfish (IPG strip 11, Figure 31) were similar and had a range of haemoglobin components which encompassed the range of those displayed by both the Taranaki brown and Whangamarino black mudfish. The Northland, Kaitaia black and brown mudfish displayed a doublet of haemoglobin subunits with pI values of approximately 8.9 and 9, whereas these bands were apparently absent in the Whangamarino black mudfish.

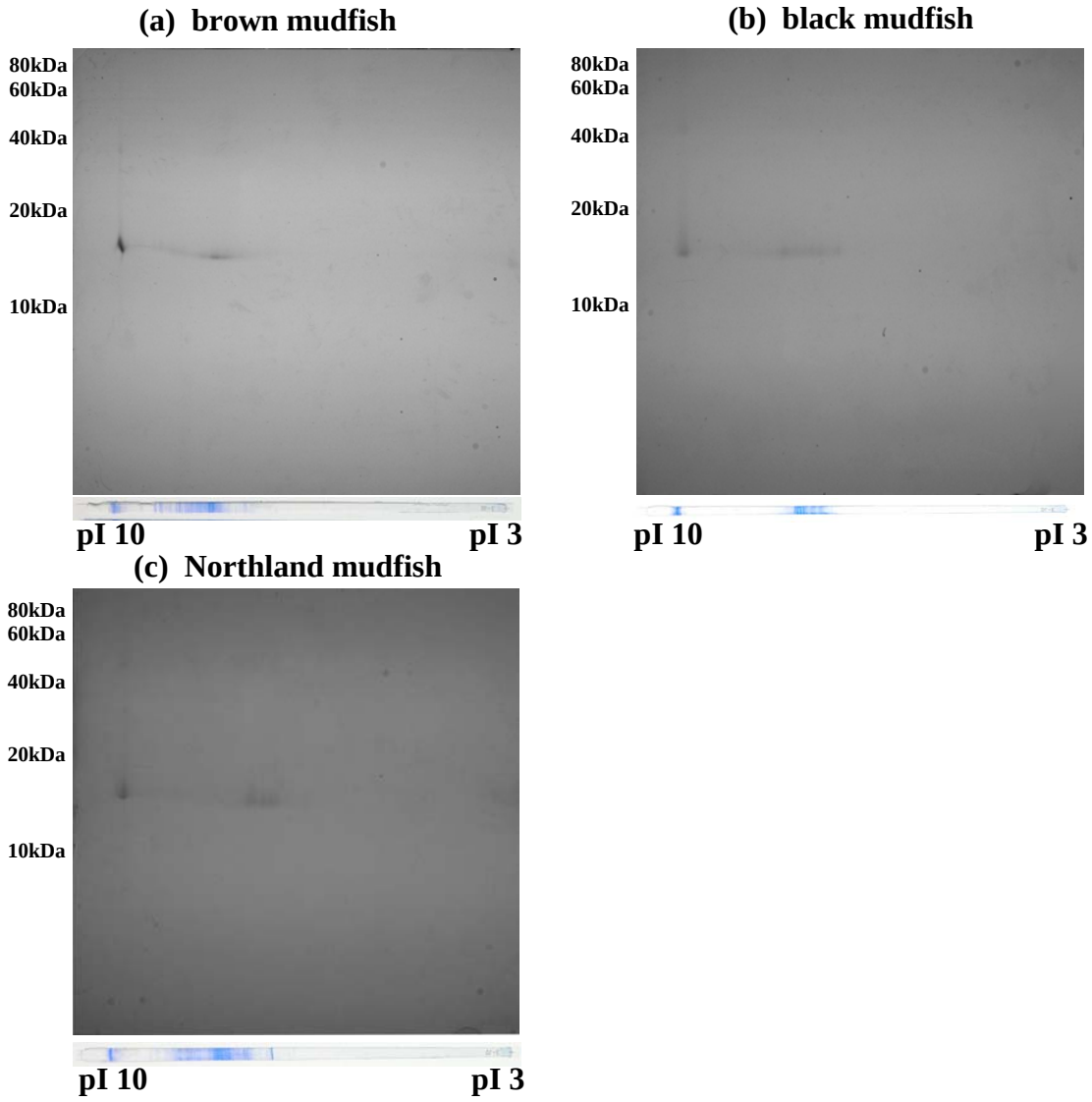

Figure 30: 2-dimensional gel electrophoresis of haemolysates from (a) brown, (b) black and (c) Northland mudfish.

*vertical axis on electrophoretograms displays molecular weights of proteins on the gel determined by kDa protein markers (GibcoBRL®) and horizontal axis displays pH gradient from IEF gels. Representative IEF gels of each species are overlaid on the electrophoretograms.

Mudfish species

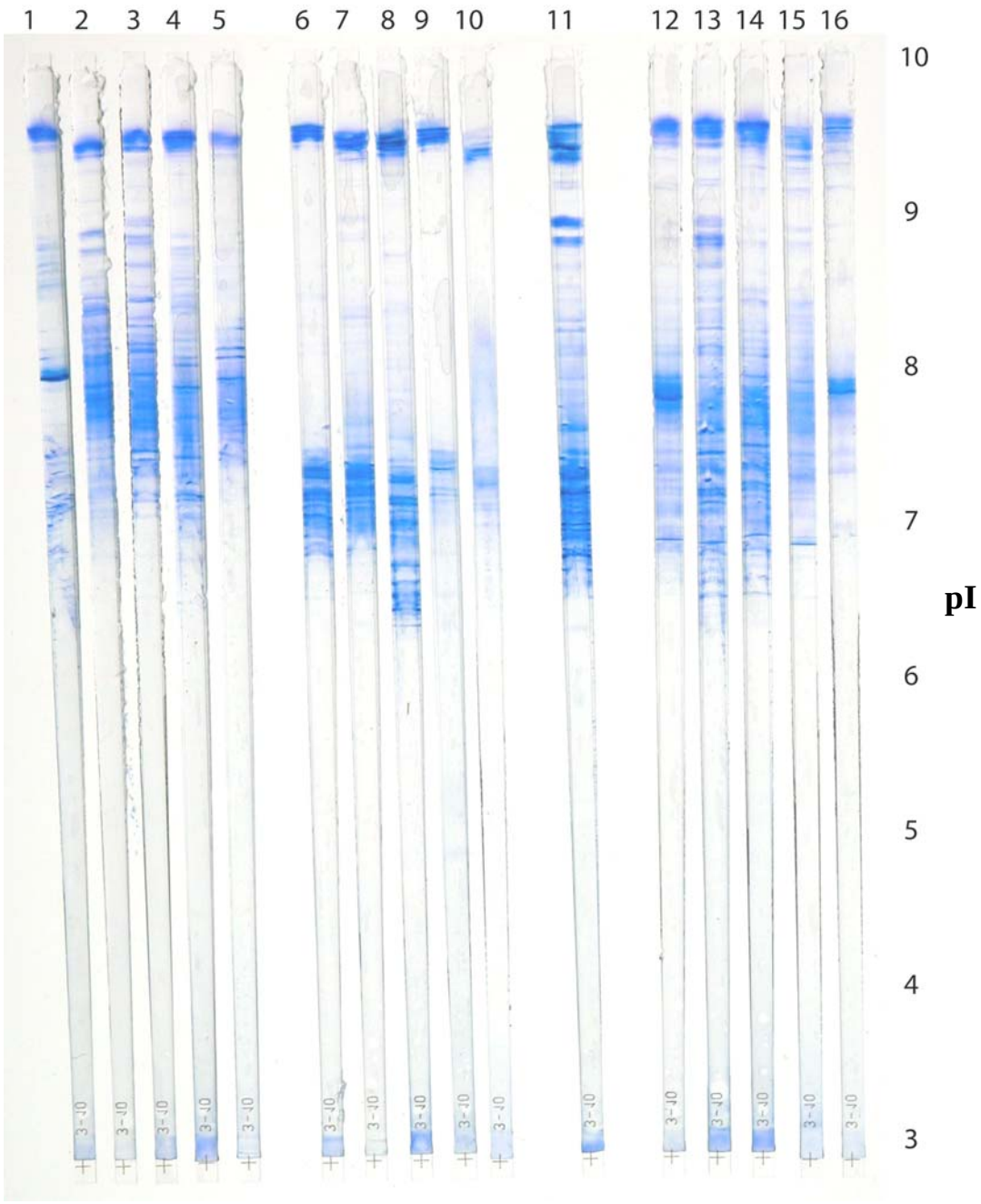

Figure 31: Isoelectric focusing (IEF) of haemolysates from Taranaki brown (1-5), Whangamarino black (6-10), Kaitaia black (11) and Northland mudfish (12-16).

*Each IPG strips represent an individual mudfish and n = 1 for the Kaitaia black mudfish due to limited sample numbers.

4.3.2 Differences in haemoglobin multiplicity between treatments

Figure 32 shows that expression of haemoglobin subunits did not seem to differ between the three different treatments for any of the mudfish species. In all of the treatments (aestivating, fasting and control), all of the mudfish species displayed a major haemoglobin subunit at a pI of approximately 9.5. As in Section 4.3.1 there were differences between species in the expression of haemoglobin subunits. The brown mudfish displayed a major band at a pI of 8, the black showed major bands between pI 7 to 7.5 and the Northland displayed distinct bands at pI values of 7, 7.5 and 8.

In the black mudfish there may be a slight difference between the aestivating treatment and the fasting and control treatment as there is a haemoglobin subunit at an approximate pI value of 8. However, this band can only be observed in one individual so it may not be truly representative. Also the aestivating brown mudfish seem to have wider bands at pI 9.5 compared to the fasting and control treatments which may suggest that more than one haemoglobin is being expressed.

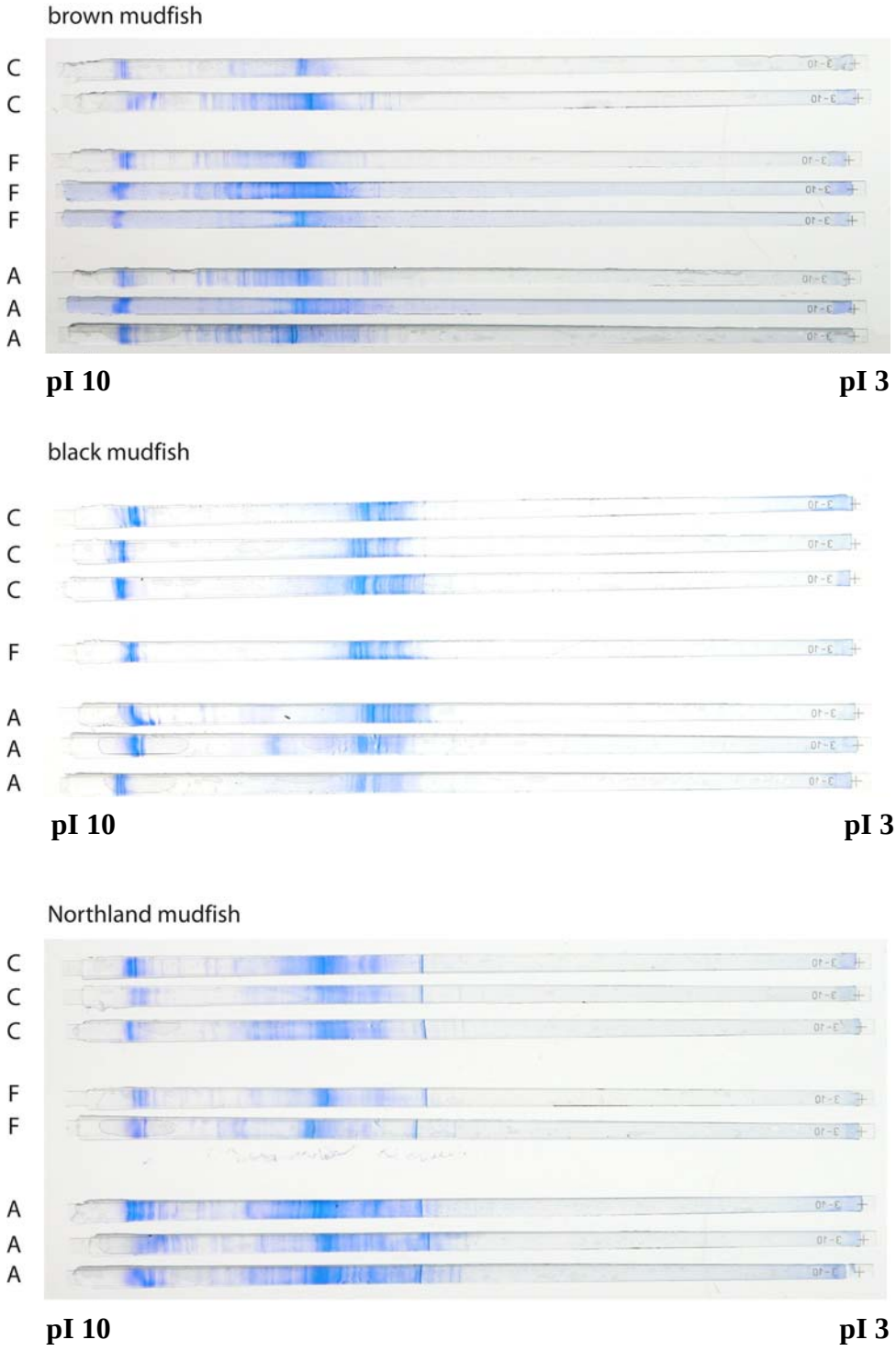

Figure 32: Isoelectric focussing (IEF) of haemolysates from brown, black, and Northland mudfish under contol (C), fasting (F) and aestivating conditions (A).

*Each IPG strip represents an individual.

4.4 Discussion

The presence of multiple haemoglobins is a common characteristic which occurs in a large number of fish genera that experience large variations in oxygen availability and temperature such as rainbow trout (*Oncorynchus mykiss*; Weber et al., 1976), goldfish (*Carassius auratus;* Houston & Cyr, 1974), European eel (*Anguilla anguilla*; Weber et al., 1976) and many more. Isoelectric focusing also revealed that brown, black and Northland mudfish all display multiple haemoglobins. The functional significance of multiple haemoglobins is that they increase the scope of oxygen, proton and heat transport in an organism due to differences in functional properties and intrinsic oxygen affinities (Weber & Jensen, 1988). The New Zealand mudfish species had a high number of cathodic (basic) haemoglobins and a low number of anodic (acidic) haemoglobins. Frey et al. (1998) showed that in *Labeo capensis*, the cathodic haemoglobins displayed higher oxygen affinities than the anodic haemoglobins at physiological pH values, and in the presence of phosphate the cathodic haemoglobins will favour oxygen loading at high temperatures and under hypoxia. Research on trout and eels subjected to internal hypoxia and acidosis by Weber et al. (1975) showed similar results with the expression of cathodal haemoglobins occurring under hypoxic conditions and anodal haemoglobins being expressed during normoxic conditions. This is because cathodal haemoglobins have a high, pH-insensitive oxygen affinity which can transport oxygen to tissues in low oxygen environments whereas the anodal haemoglobins are sensitive to pH and temperature and are characterized by strong Bohr and phosphate effects which aid oxygen unloading to tissues in high oxygen environments (Weber et al., 1975). New Zealand mudfish species are exposed to high temperatures and hypoxic conditions during summer due to a drop in the water levels of their respective habitats. The expression of a large number of haemoglobin components combined with a high oxygen affinity and variable sensitivity to changes in pH and temperature ensures that New Zealand mudfish species can cope with the variable conditions often prevailing in their respective habitats. Expression of numerous haemoglobin components with high affinities and low pH sensitivity also occurs in *Grahamina capito* as it has to

cope with living in very variable conditions often prevailing in tidal pools and shallow estuarine areas (Brix et al., 1999).

The functional properties of fish haemoglobins are strongly adapted to environmental conditions. Species differences in haemoglobin expression and oxygen binding characteristics have been found to occur in eels. Weber et al. (1976) suggested that haemoglobin multiplicity could be involved in the adaptation of eel haemoglobin to environmental conditions because the haemoglobin components in the Japanese eel (*Anguilla japonica*; Yamaguchi et al., 1962), American eel (*Anguilla rostrata*; Gillen & Riggs, 1973), and the European eel (*Anguilla anguilla*; Weber et al., 1976) have distinctly different oxygenation properties. The results of isoelectric focusing on the haemolysates of the New Zealand mudfish species revealed differences in haemoglobin expression between species as well as between different populations of the same species. There was a distinct difference in haemoglobin expression between the population of black mudfish located near Kaitaia compared to the population located in the Whangamarino wetland indicating that haemoglobin expression may reflect environmental rather than phylogenetic relationships. Further study needs to be carried out on the oxygenation properties of the individual haemoglobins in the different mudfish species to determine if there are functional differences between the haemoglobin components.

Alterations in the relative abundance of the different haemoglobins provide organisms with a further mechanism for functional adaptation (Weber et al., 1976). It is well known in species such as rainbow trout that changes in the expression of haemoglobin components commonly accompanies temperature acclimation, acclimation to hypoxia and changes in day length (Tun & Houston, 1986). However, not all species follow this pattern as species such as *Labeo capensis* (Frey et al., 1998) do not display changes in the relative abundance of haemoglobins in response to temperature acclimation. We are unable to predict whether New Zealand mudfishes display changes in the relative abundance of haemoglobins in response to temperature, hypoxia or day length as our experiment did not include these factors.

Expression of multiple, functionally different haemoglobins are characteristic of many Amazonian fishes and are presumed to be adaptive to differing water oxygen tensions (Val et al., 1990). However, research carried out on European eels by Weber et al. (1976) showed that there were no differences in multiplicity patterns of haemoglobins from hypoxic and normoxic eels, thus he suggested that haemoglobin itself does not contribute to the adaptation of blood oxygen affinity to ambient oxygen tensions. Instead he found that GTP was the more potent regulator of oxygen affinity, and that triphosphates may play the major role in the adaptation of eel blood to environmental oxygen levels rather than changes in the relative abundance of haemoglobin components. New Zealand mudfish species also did not display a change in expression of haemoglobin components in response to a change in ambient oxygen tensions as all three species showed very similar patterns of haemoglobin multiplicity between water-breathing and air-breathing individuals, although in this study it was an increase in ambient oxygen tension (air-breathing) rather than a decrease (hypoxia).

Recommendations for further study would be to examine the effect of acclimation of New Zealand mudfish species to different dissolved oxygen levels on the expression of haemoglobin components. Also it would be interesting to further examine differences in haemoglobin expression between different populations of the same species to determine the importance of environmental effects.

Chapter Five: Conclusions

The five recognized species of endemic New Zealand mudfish (brown, Northland, black, Canterbury and Chatham Island mudfish) are highly distinctive members of the southern temperate Galaxiidae. These mudfish species are generally small, elongated fishes which are distributed throughout New Zealand in a range of habitats which present different ecological challenges. Variations in abiotic and biotic factors of the different habitats have resulted in varying degrees of morphological and ecological specialization within the genus (McDowall, 1990; 1997). Molecular data from research by Waters & McDowall (2005) strongly supports the monophyly of the mudfish genus and indicates a single trajectory of mudfish evolution with changes in body plan being associated with specialization to wetland dwelling. These morphological adaptations consist of loss of pelvic fins, reduced eyes, enlarged nostrils, development of caudal flanges, and the elongation of dorsal and anal fin bases to become almost confluent with the caudal fin (McDowall, 1990; 1997). This has led to a proposal (Waters & McDowall, 2005) of an evolutionary transition from a plesiomorphic galaxiid morphotype (Chatham Island and Canterbury mudfish) to a more specialized "anguilliform" galaxiid morphotype (brown, Northland and black mudfish). Mudfish specialization to wetland dwelling is also expected to extend to adaptations in respiratory physiology so that oxygen can be extracted from highly hypoxic or acidic waters and transported to the tissues. Respiratory specializations to hypoxic environments include high oxygen affinity haemoglobins, high levels of cooperativity and the presence of multiple haemoglobins with differences in functional properties and intrinsic oxygen affinities (Weber et al., 1976; Dobson & Baldwin, 1982; Weber & Jensen, 1988; Brix et al., 1999; Nikinmaa, 2001). Another distinctive physiological adaptation to wetland dwelling is the ability to aestivate, as it presents clear advantages to fish living in ephemeral waters.

The oxygen binding characteristics of whole blood from four mainland New Zealand mudfish were investigated at a range of pH values (6.5, 7.0, 7.5 and 8.0),

temperatures (10ºC, 15ºC and 20ºC) and treatments (aestivating, fasting and normal) while the presence of multiple haemoglobins was determined by isoelectric focusing. However, Chatham Island mudfish were not included in any of the experiments and Canterbury mudfish were not included in the aestivating experiment due to restrictions on the transfer of aquatic organisms between islands of New Zealand.

Oxygen equilibrium curves obtained from the four mainland NZ mudfish species were similar in position and shape to those reported for other fish species occupying hypoxic environments such tench (*Tinca tinca*; Eddy, 1973) and common carp (Weber & Lykkeboe, 1978) at similar pH and temperature values. Oxygen affinities of all mudfish species were high at high pH values (7.5 and 8.0) and low temperatures (10ºC). Whole blood from all mudfish species was found to be sensitive to changes in pH (decreasing pH = reduction in oxygen affinity) which has also been noted in a diverse range of fishes including rainbow trout (*Oncorynchus mykiss*; Weber et al., 1976), Australian blackfish (*Gadopsis marmoratus*; Dobson & Baldwin, 1982), tench (Eddy, 1973), common carp (Black, 1940) and many more. As well as the whole blood of mudfish being pH sensitive it was also found to be temperature sensitive (increase in temperature = reduction in oxygen affinity). This phenomena has also been known to occur in a large range of teleosts such as species of *Trematomus* (Grigg, 1967), brook trout and rainbow trout (Irving, 1941), Australian blackfish (Dobson & Baldwin, 1982), marbled lungfish (*Protopterus aethiopicus*) (Lenfant & Johansen, 1968) but not in species such as the endothermic southern bluefin tuna (*Thunnus maccoyii*) which displays a reversed temperature sensitivity (Bushnell & Jones, 1994). As well as displaying high oxygen affinities, mudfish whole blood also displayed a high degree of cooperativity (at higher pH values of 7.5 and 8.0) which aids in the release of bound oxygen to tissues under a relatively narrow range of oxygen tensions.

During a summer drought period, water levels drop and mudfish are required to aestivate until water level rises again. The three species of New Zealand mudfish (black, brown and Northland) used in the aestivating experiment were all able to

survive emersion for a period of 6 weeks by aestivating, although all individual mudfish lost significant proportions of weight. The aestivating mudfish species did not provide support for the hypothesis of an evolutionary trend towards a reduced haemoglobin oxygen affinity with air breathing (Graham, 1997) as they displayed no significant differences in oxygen affinity between air-breathing individuals and water-breathing individuals. Results obtained from lungfishes by Lenfant et al. (1966) also revealed that there were no significant differences between air-breathers and non-air breathers.

The presence of multiple haemoglobins was observed in New Zealand mudfish species (brown, black and Northland mudfish) which is a common characteristic occuring in a large number of fish genera (Weber & Jensen, 1988). By increasing the scope of oxygen, proton and heat transport in an organism, multiple haemoglobins are functionally significant for organisms experiencing large variations in oxygen availability and temperature (Weber & Jensen, 1988). The three mudfish species displayed high numbers of cathodic (basic) haemoglobins and low numbers of anodic (acidic) haemoglobins. Cathodic haemoglobins display higher oxygen affinities at physiological pH values and in the presence of phosphate and will favour oxygen loading at high temperatures and under hypoxic conditions (Frey et al., 1998) which is beneficial for mudfish as they inhabit wetlands where these conditions prevail.

Research has shown that species-specific differences in oxygen binding characteristics can be due to differences in environmental oxygen availability, with high oxygen affinities found in species that encounter hypoxic conditions, and lower oxygen affinities in species inhabiting well oxygenated waters (Powers et al., 1979; Riggs, 1979; Powers, 1980). The black and Northland mudfish, inhabiting ephemeral wetlands in the northern North Island with large temperature fluctuations had the highest mean whole blood oxygen affinity, significant Bohr shifts (between pH 7.0 – 7.5), a high degree of cooperativity, ability to aestivate and possessed multiple haemoglobins. Canterbury mudfish, inhabiting less hypoxic waters with smaller temperature fluctuations, also displayed significant Bohr shifts and although they are

known to aestivate, they are different to the black and Northland mudfish as they display low oxygen affinities and a low degree of cooperativity. Species-specific differences in the expression of haemoglobins were found in New Zealand mudfish species but further study needs to be carried out on the oxygenation properties of the individual haemoglobins to determine if these changes in haemoglobin expression are directly correlated to habitat specialization.

Results of this study have shown that habitat specialization in haemoglobin physiology between mudfish species does occur but it does not follow the proposed cline of Waters & McDowall (2005). Thus with respect to haemoglobin physiology the black mudfish and Northland mudfish are most specialized, followed by the brown mudfish and finally the Canterbury mudfish which are the least specialized.

The implications of this research is that by studying the evolution of increasing specialization in oxygen uptake of species in the genus *Neochanna* we can get a better idea of the ability of each of the New Zealand mudfish species to tolerate physiological stresses in their respective habitats. By knowing the limits of dissolved oxygen levels and exposure that mudfish species can survive in, we will possibly be able to improve the management of remaining mudfish habitats as well as having knowledge which could be useful in the event of a translocation.

Potential future research includes the investigation of oxygen binding characteristics and aestivation abilities of the Chatham Island mudfish due to the habitat type and location where they reside. Isoelectric focusing needs to be carried out on both Chatham Island and Canterbury mudfish to determine the expression of haemoglobins and to examine whether or not expression varies from the other New Zealand mudfish species. An increase in sample size for all species would also be beneficial as it would prove to be more representative of the population by increasing the accuracy and precision of the results, although this is difficult due to the conservation status of all New Zealand mudfish species. Changes to the methodology which could possibly improve this study include acclimating the

mudfish species to a range of temperatures and pH values found in their natural habitat (rather than subjecting whole blood to changes in temperature and pH) as well as acclimating mudfish to varying levels of dissolved oxygen levels ranging from hypoxic waters to normoxic waters. This could be beneficial as it might display the effects of dissolved oxygen levels, temperatures and pH on oxygen binding characteristics, organic phosphate concentration and haemoglobin expression. Further research also needs to be carried out on the in vivo differences in blood temperature and pH between aestivating and non-aestivating mudfish. This would involve measuring the blood pH of the mudfish in the different treatments as well as measuring in situ temperature differences between water in mudfish habitats and air and then examining blood oxygen binding under those new conditions.

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