Temperature sensitivity of red blood cell physiology in Atlantic cod, *Gadus morhua*: Comparative, molecular, evolutionary and environmental aspects

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy by Samantha Leanne Barlow

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Abstract

The ability of fish to withstand increased temperatures has been directly linked with the capacity of their oxygen transport system and its ability to supply the tissues of the body with sufficient oxygen to meet demand. Therefore studies on the effects of temperature on the oxygen transport systems of aquatic organisms are important for understanding their capacity to cope with climate change. An early study in Atlantic cod (*Gadus morhua*) suggested haemoglobin I genotype affects temperature sensitivity of red blood cell oxygen binding, with one genotype found to be temperature insensitive. However this research had many limitations and subsequent research remains inconclusive as to the significance of the haemoglobin I polymorphism. In the most comprehensive study to date, results showed statistically indistinguishable red blood cell O_2 binding between any of the three haemoglobin I genotypes in wild-caught Atlantic cod. Red blood cells had an unusually low O_2 affinity, with reduced or even reversed thermal sensitivity, suggesting an endothermic nature to oxygen binding rarely seen in ectothermic teleosts.

Reduced thermal sensitivity of oxygen binding is often attributed to the presence of increased pH sensitivity or large Bohr effect, however this is usually only considered in heterothermic fish. However, our finding of temperature insensitive oxygen binding in Atlantic cod and the presence of one of the highest Bohr effects measured suggest this mechanism may be more wide spread than previously thought. To explore this we test the effect of pH sensitivity and temperature sensitivity of oxygen binding on in six diverse fish species. A lack of thermal sensitivity of red blood cell oxygen binding was observed in two tested species and reduced thermal sensitivity was found in a further two. A strong inverse correlation was observed between the temperature sensitivity and pH sensitivity of oxygen binding in red blood cells, with a regression coefficient of 0.88. This confirms that the occurrence of thermal insensitive oxygen binding is not exclusive to heterothermic fish and as previously suggested the mechanism behind this is linked to at least one allosteric modulator.

Haemoglobin polymerisation and subsequent red blood cell sickling has long been observed in fish, although little is still known about the phenomenon. Recent studies have

shown haemoglobin polymerisation in fish is the result of acidosis; we confirm this in Atlantic cod and observe a strong link between the occurrence of sickling and acid-induced haemoglobin deoxygenation or the Root effect. Further, we attempt to determine the effect of temperature on the sickling in cod, as despite the ectothermic nature of fish this information is lacking and we try to determine if this too may contribute to reduction in thermal sensitivity of oxygen binding. The occurrence of sickling decreased at increased temperatures, a new finding in fish and contrary to that found in mammals. The Root effect was similarly effected by temperature, a novel observation suggesting a strong association between the two phenomena. However, sickling appeared to be exothermic in nature and as such is likely to contribute to thermal insensitive oxygen binding.

Despite increasing interest in sickling in fish, the low number of observations make it difficult to determine a mechanism. Here we test a wide variety of fish to observe potential evolutionary pathways of sickling and attempt to find a genetic marker. Sickling was found to be prevalent throughout the Gadiformes, though no conclusive evidence was found in other tested species. Reconstruction of the evolution of fish RBC sickling on a composite phylogenetic tree of all studied species suggests two possible maximum parsimony evolutionary pathways and analysis of β globin chains gives two potential markers.

Finally, we determine a method to allow for DNA sequencing of the haemoglobin I polymorphism from fin clips, with the aim that this method can be used on samples taken from Atlantic cod fitted with thermal tracking tags. This will allow direct monitoring of the effect of the haemoglobin I polymorphism on temperature experience in the wild.

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Contents

Chapter 1. General Introduction

1.1 Atlantic cod- Big fish in a shrinking pond

Atlantic cod (*Gadus morhua*) has been utilised as a food source since the time of the Vikings and with almost £60 million worth netted by UK fisherman alone in 2013 (Radford, 2004), it is a huge commercial resource. Found in temperate climes ranging from the shallows near the shore to deeper waters near the continental shelf, their habitat stretches along both coasts of the North Atlantic Ocean; from the Bay of Biscay to the Barents Sea and from North Carolina to Greenland and off the coast of Iceland.

Once found in high abundance in the North Sea, intensive fishing practices and poor management plans have been partially attributed to severe decline in numbers and have driven some stocks close to collapse (Hutchings and Myers, 1994; Cook *et al*., 1997; O'Brien *et al*., 2000; Christensen *et al*., 2003; Hutchings, 2004; Rose, 2004; Worm *et al*., 2006; Pitcher *et al*., 2009). In conjunction with fishing pressures, warming seas as a result of climate change has also been ascribed a portion of the blame (Brander, 2005; Drinkwater, 2005; Perry *et al*., 2005; Deutsch *et al*., 2015).

Temperature is a prevailing factor influencing fish distribution (Drinkwater, 2005). Species specific thermal preferences can result in fluctuations in range which often correlate with long term temperature changes (Coutant, 1977). Shifts in distribution are most apparent at the north and south of the species limits, whereby increasing temperatures drive stocks towards the poles to cooler waters and temperature decrease causes shifts towards the equator (Rose, 2004).

The northeast Atlantic has experienced particularly acute warming, with sea surface temperatures raising 1.3°C over 30 years, four times faster than the global average (Smith *et al*., 2008). Here cod stocks near the south of their distribution limit in the Irish and Southern North Sea have indeed declined over the past decades. Projected future temperature changes have led to predictions of Atlantic cod stocks near their current upper thermal distribution

limit in the North East Atlantic to disappear entirely from the Celtic and Irish Seas by the end of this century (Drinkwater, 2005).

While debate continues as to which is to blame for the decline of cod, over fishing, climate change or a combination of both, a better understanding of thermal tolerance could aid conservation efforts and stock management as well as providing a more thorough understanding of physiological capabilities of withstanding elevated temperature.

1.2 Thermal tolerance in fish

The oxygen and capacity limited thermal tolerance (OCLTT) model suggests the thermal tolerance of marine ectotherms is limited by the failure of the cardiovascular system to supply sufficient oxygen to meet the increased demand at upper critical temperatures (Portner and Knust, 2007; Pörtner, 2010).

As ectotherms, the body temperature in most fish is dependent upon their environment. With increasing temperatures the basal metabolic rate (BMR or $\dot{M}O_2min$) also increases (Figure 1.1). The maximum capacity for oxygen consumption ($\dot{M}O_2max$) also varies with temperature, this tends to peak at an 'intermediate' temperature within thermal range of a species and reduces towards thermal extremes (Figure 1.1). Aerobic scope is defined as the difference between $\dot{M}O_2min$ and $\dot{M}O_2max$ and has become a commonly used proxy for whole-animal performance and fitness (Pörtner, 2010). The temperature at which aerobic scope is at a maximum is known as the optimal temperature (T_{opt}). Above (and below) T_{opt}, the aerobic scope will decrease (at T_{pejus}) and less energy can be devoted to processes like feeding and reproduction, thereby reducing the fitness of the animal. The critical (lethal) temperature (T_{crit}) is reached when the maximum capacity for oxygen uptake is needed just to supply basal oxygen needs resulting in falling blood oxygen levels and a transition to anaerobic metabolism, survival under these conditions is often short. Indeed, whole animal studies on the acute thermal tolerance of Atlantic cod have shown cardiac function suggested to become compromised close to the T_{crit} (Sartoris *et al*., 2003; Lannig *et al.*, 2004; Gollock *et al*., 2006).

This implies the circulatory system as a primary limiting factor in the O_2 supply cascade from the environment to the tissues.

Aerobic capacity can be increased to meet the oxygen demand by increasing (1) cardiac output, $\dot{Q}$ or (2) the arterial-venous O_2 difference, C_aO_2 - C_vO_2. The contribution of changes in C_aO_2 - C_vO_2 in the assessment of maximal O_2 supply capacities during warming of marine ectotherms is largely unknown, although it has long been recognised that in humans, the increase in C_aO_2 - C_vO_2 may surpass the increase in $\dot{Q}$ in its contribution to meeting elevated $\dot{M}O_2$ during heavy exercise (Ekelund and Holmgren, 1964; Dejours, 1975). In light of this, to understand how climate change will effect fish we must first characterise the oxygen transport system.

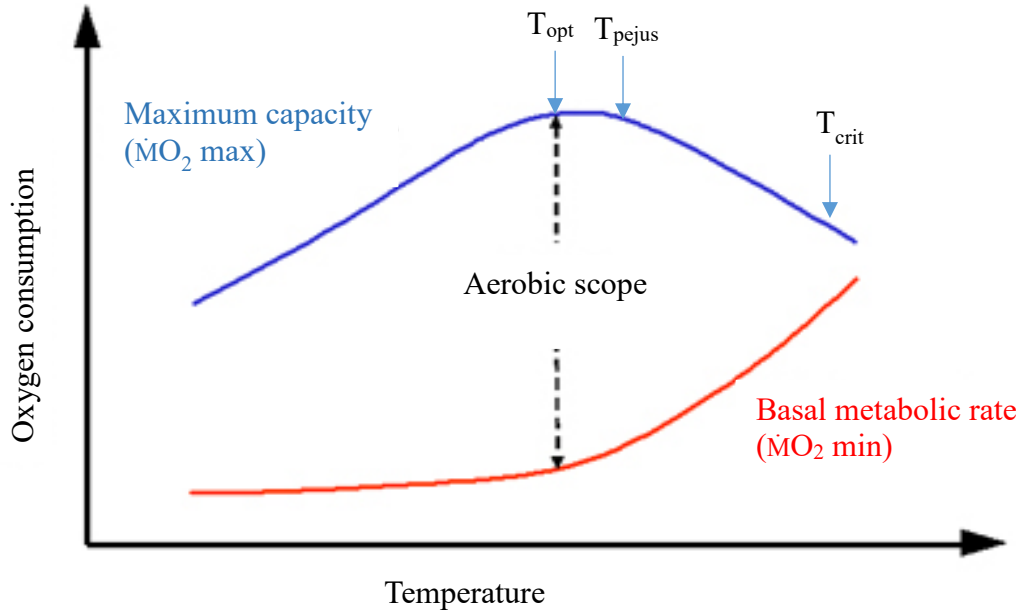

Figure 1.1: *Thermal dependency of basal metabolic rate (BMR) and maximum capacity ($\dot{M}O_2$ max), as well as the difference between both, the aerobic scope. Aerobic scope; is maximized at "intermediate" temperatures (T_{opt}), declines at T_{pejus} and can no longer be sustained beyond T_{crit}.*

1.3 Haemoglobin- structure and function

Haemoglobin (Hb) is the primary respiratory pigment found in vertebrate blood. At normal human body temperature and air pressure, Hb presence can increase the amount of oxygen dissolved in blood from 4.5 mL of O_2 per litre of blood to about 200 mL O_2 L^{-1}. The tetrameric Hb molecule consists of four polypeptide chains: two alpha (α) and two beta (β) subunits (Figure 1.2A). Despite variations in amino acid number between species, Hbs all exhibit a characteristic tertiary structure; helical segments (labelled A-H) interspersed with no helical regions (labelled to reflect the adjacent helices, eg AB) (Jensen *et al*., 1998; Keates, 2004). Within a hydrophobic pocket between two helices of each subunit, an oxygen binding haem (iron protoporphyrin XI) is covalently bound to the proximal histidine (Figure 1.2A).

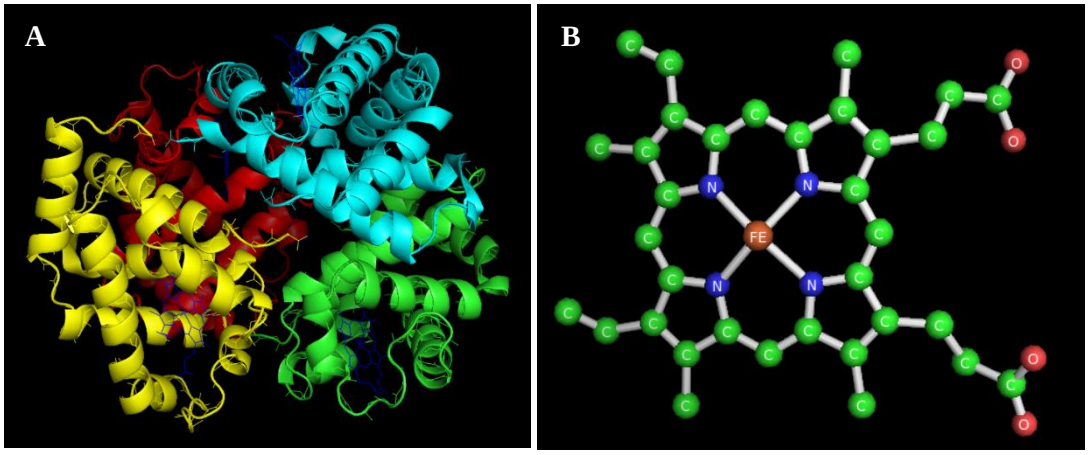

Figure 1.2: *(A) Haemoglobin molecule with four globin chains, two alpha (red and green) and two beta (yellow and cyan) chains each bearing a haem group (dark blue). (B) Haem group with element labels. Figures were generated by PyMol (DeLano Scientific LLC, CA, USA) using pdb file 1OUU and 1OUT, respectively, from trout haemoglobin, Oncorhynchus mykiss.*

The mechanism of oxygen binding is not oxidation of the ferrous ion (Fe^{2+}) at the centre of the haem (Figure 1.2B), but instead formation of a Fe^{2+}-O_2 complex. This ensures reversible oxygen binding unlike the former, without the presence of the globin haem cannot prevent oxidation. Similarly, the presence of a number of key amino acids within the globin are highly conserved and help maintain integrity of Fe^{2+}-O_2 binding, for example, the distal

histidine which discriminates against binding of other ligands such as carbon monoxide and stabilises bound oxygen (Springer *et al*., 1989; Perutz, 1990). However, mutations at other positions within the primary structure can alter globin structure and adjust the oxygen affinity between and within species.

The close interaction between the four Hb subunits results in cooperative binding of oxygen. Binding of oxygen to one of the four subunits increases affinity of the remaining three deoxygenated subunits. It is this process which results in the characteristic sigmoidal shape of the oxygen binding curve (Figure 1.3). The Monod, Wyman and Changeux or MWC model (Monod *et al*., 1965) proposes this allosteric interaction is due to Hbs existing in equilibria in two conformational states. The low affinity (T)ense state shifts to the higher affinity (R)elaxed. Deoxygenated Hb primarily exists in the T-state but alterations in the quaternary structure after the binding of the first O_2 molecule facilitates subsequent binding (Monod *et al*., 1965).

The oxygen binding properties of Hb can be visualised as oxygen equilibrium curves (OEC), where Hb-O_2 saturation is shown as a function of partial pressure of oxygen PO_2 (Figure 1.3). Oxygen affinity can be quantified by partial pressure of oxygen required to saturate 50% of the oxygen binding sites and is termed 'P_{50}'. A reduction in affinity will increase P_{50} and this is translated as a rightwards shift of the entire curve.

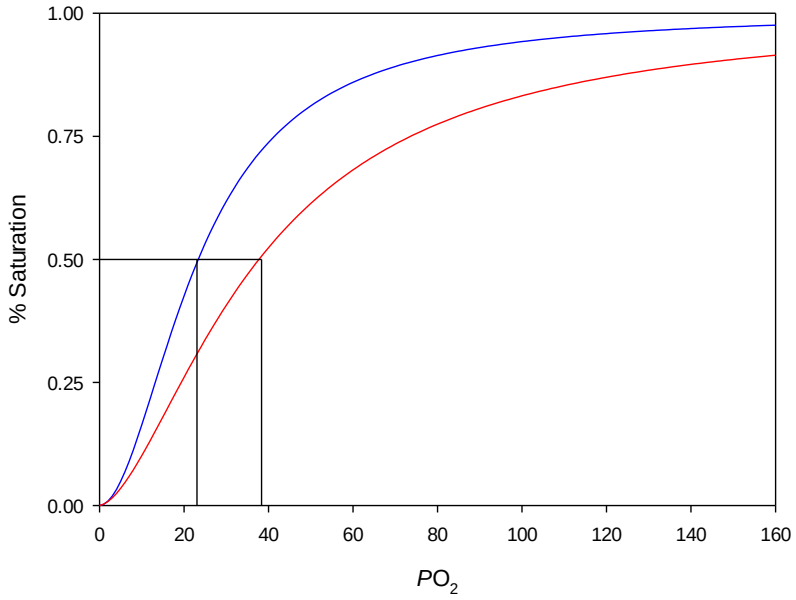

Figure 1.3: *Example oxygen equilibration curves, oxygen saturation as a function of PO_2. A right shift, blue to red, is the result of increased temperature or decreased pH indiating in a reduction of oxygen affinity. Drop lines indicate P_{50} values.*

The steepness of the central portion of the sigmoidal curve is indicative of cooperativity of oxygen binding between the Hb subunits. A shift in steepness is representative of a change in cooperativity. The Hill coefficient (n_H) is a measure of the apparent cooperativity, values greater than 1 denotes positive cooperativity, equal to one indicates a lack of cooperativity and less than one represents negative cooperativity.

Oxygen binding characteristics are determined by; (1) Hb morphology and its intrinsic affinity for oxygen and binding of heterotropic ligands (hydrogen ions, H^+; carbon dioxide, CO_2; nucleotide triphosphates, NTP; and chloride ions, Cl^-); and (2) the micro-environment it is exposed to including presence of oxygen, heterotropic ligands and temperature. Here we are specifically interested in the effect of the heterotropic ligand, H^+.

1.3 Effect of pH on oxygen binding

A reduction in pH, increases the number of H^+, these protons preferentially bind to T-state Hb and thus stabilises this conformation (Perutz, 1990). This reduces Hb propensity to bind

oxygen, lowering affinity and shifting the OEC to the right (increasing P_{50}). The decrease in oxygen binding affinity due to H^+ is known as the Bohr effect (Bohr *et al*., 1904), it's magnitude, known as the Bohr coefficient (Φ) can be calculated as $\Phi = \Delta \log P_{50} / \Delta pH$.

In fish Hbs, in conjunction with decreasing oxygen affinity, a reduction in pH can also decrease cooperativity in oxygen binding between the subunits. A decline in cooperativity between the four oxygen binding sites of the Hb tetramers at low pH, together with a large decrease in Hb O_2 affinity, mean Hbs may not become fully oxygenated even at PO_2 as high as 150 mmHg (Scholander and Van Dam, 1954; Pelster, 2001). This mechanism, known as the Root effect, is believed to facilitate oxygen secretion in the choroid and swim bladder *retia mirabilia* (singular: *rete mirabile*; Wittenberg and Wittenberg, 1962; Waser and Heisler, 2005; Berenbrink *et al*., 2005; Berenbrink 2007).

Both the Bohr and Root effects have been hypothesised to be part of the mechanism that provides more oxygen to muscles under acidotic stress (Rummer *et al*., 2013). In fish Hbs displaying the Bohr effect, the His at the C- terminal of the beta chains (His HC3β) is conserved. Its carboxy group binds with a Lys side chain (C5α) while the imidazole ring forms a 2nd salt bridge to Glu (FG1β), when transitioning from T to R state, these salt bridges break and protons are released.

1.4 Effect of temperature on oxygen binding

It is generally accepted that increasing temperature facilitates the unbinding of oxygen, shifting the OEC curve to the right and increasing $P_{50.}$ This is due to the exothermic nature of oxygenation of the haem groups (Wyman, 1948). This decrease of affinity can be advantageous or disadvantageous. Providing arterial oxygen saturation can be maintained, decreased affinity can increase oxygen unloading at the tissue (Barcroft & King 1909). However, if O_2 loading in the gills is significantly reduced by the decrease in O_2 affinity, O_2 supply to the tissues will be inhibited.

Thermal sensitivities of OECs can be expressed as apparent heat of oxygenation (ΔH'). These were calculated using the van't Hoff equation $\Delta H' = 2.303\ R\ ((\Delta \log P_{50})/(\Delta 1/T))$, where R = universal gas constant (0.008314 kJ K^{-1} mol^{-1}), and T = temperature in K.

The overall heat of oxygenation describes the net energy release associated with the binding reaction between oxygen and haemoglobin. However, the temperature sensitivity of Hb-O_2 equilibria is not solely dependent upon oxygen binding to Hb. Heterotropic ligands, such as protons (H^+) and organic phosphates (ATP/GTP) bind to the Hb T-state, this stabilises this Hb conformation. During oxygenation the heterotropic ligands are released from their binding sites, this process is endothermic and balances the exothermic nature of oxygenation. Thus the effect of temperature on Hb-O_2 binding is reduced in the presence of heterotropic ligands. This is best known for heterothermic tuna, billfishes, and lamnid sharks, where exothermic Hb O_2 binding may cause problems in heat-conserving vascular counter-current exchangers (Weber and Campbell, 2011), although it has recently been observed in the ectotherm Pacific mackerel (Clark *et al*., 2010).

This thesis focuses on the interactions between Hb, pH and temperature in the Hb-O_2 equilibria, particularly focusing on Atlantic cod, wherein mutations in the primary structure of the β subunit of the main Hb component are believed to affect individual sensitivity to temperature.

1.5 HbI polymorphisms in cod

Over 50 years ago Sick (1961) demonstrated the main Hb component in Atlantic cod blood had two electrophoretic different isoforms. This was explained by Atlantic cod having two polymorphic Hb alelles, HbI 1 and HbI 2, resulting in three HbI genotypes HbI 1/1, HbI 1/2 and HbI 2/2 (Sick, 1961). In 2009, two independent studies found the molecular basis of the HbI polymorphisms. Two non-synonymous substitutions in the β1 Hb gene were congruent with the HbI 1 and HbI 2 alleles determined by protein gel electrophoresis (Andersen *et al*., 2009, Borza *et al*., 2009). At position 55 in the β chain HbI 1 has a Met amino acid which

changes to a smaller Val in HbI 2 alleles. Similarly at position 62 HbI 1 contains a polar Lys while HbI 2 has a neutral Ala (Andersen *et al*., 2009, Borza *et al*., 2009).

Despite five decades of study, no conclusive decision has been reached as to the biochemical significance of the HbI genotypes (for review, Andersen, 2012; Ross *et al*, 2013). The frequencies of these alleles vary along a latitudinal cline in the North East Atlantic, from the Barents Sea, which is dominated by the HbI 2 allele with HbI 1 frequency as low as 0-0.1, to the Southern North Sea where HbI 1 frequency rises as high as 0.6-0.7 (Sick, 1965; Jamieson and Birley, 1989; Andersen *et al*., 2009; Ross *et al.* 2013). A similar, though less extreme cline of HbI gene frequencies has been observed along the East Coast of North America, where with HbI 1 frequency varies from 0.1 in the North to 0.3 in the South (Sick, 1965).

These clines have been attributed to divergent temperature sensitivities of cod with the different HbI genotypes, regarding growth, physiology and behaviour (reviewed by Andersen, 2012; Ross *et al*, 2013). Indeed, two independent temperature preference studies on Atlantic cod from the Øresund, Denmark, also infer a link between haemoglobin genotype and temperature (Petersen and Steffensen, 2003; Behrens *et al*., 2012). Both these studies found that cod with HbI 1/1 show preference for higher water temperature than HbI 2/2 cod in laboratory temperature choice experiments.

Karpov and Novikov (1980) were the first to suggest functional differences in RBC-O_2 affinity between the HbI genotypes. Looking at RBC OECs in White Sea Atlantic cod (67° North) and assessing changes in P_{50} values at physiologically low pH (7.5), they showed that HbI 2/2 RBCs had a greater O_2 affinity than HbI 1/1 RBCs at temperatures ranging from 0-12°C. This trend was reversed between 12°C and 20°C where HbI 1/1 RBCs attained the higher O_2 affinity (Figure 1.4, Karpov and Novikov, 1980). Close inspection of their data suggests that HbI 2/2 RBCs showed typical exothermic O_2 binding, whereby affinity decreased with increased temperature, whereas, interestingly, HbI 1/1 RBCs showed O_2 affinity which lacked any sensitivity to temperature. Similar, although less pronounced results have been

observed in haemolysates of Atlantic cod (Brix *et al*, 1998 and 2004; Pörtner *et al*., 2001). In contrast to these findings, Colosimo *et al*. (2003), also working on haemolysates, found the highest Hb-O_2 affinity at 12°C, independent of Hb genotype. Based on acclimation studies, they further suggested that individual physiological adaptation in Atlantic cod had a greater influence on thermal sensitivity of Hb-O_2 binding than Hb genotype (Colosimo *et al*., 2003).

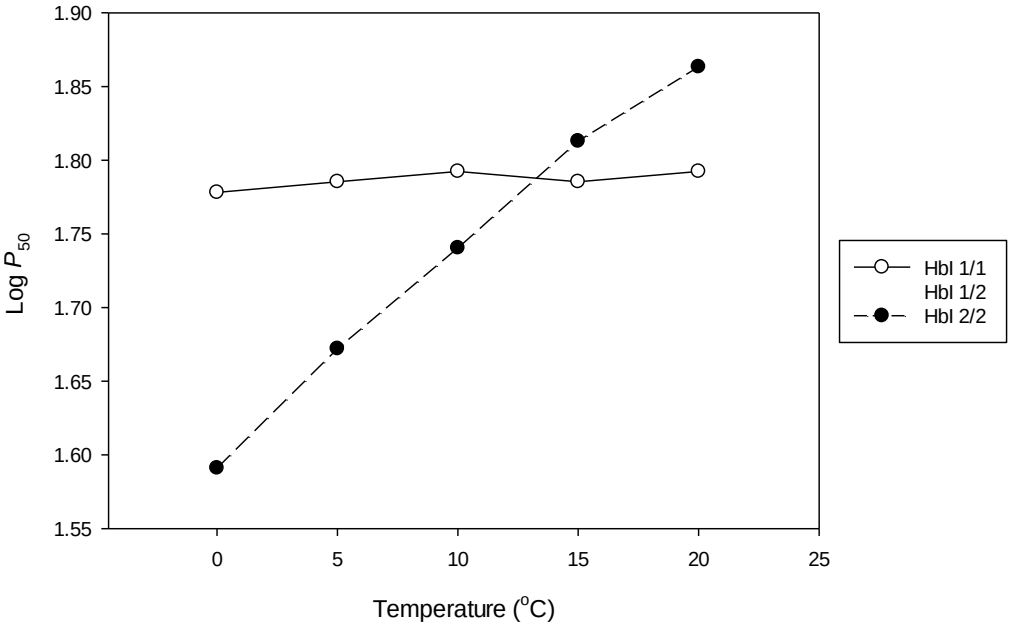

Figure 1.4: *Replica of findings by Karpov and Novikov, change in log P_{50} with temperature of red blood cells in White Sea Atlantic cod for the two homozygous HbI genotypes (Karpov and Novikov, 1980).*

Given the breadth of the research into RBC and Hb O_2 affinity, most has been performed primarily on northern Atlantic cod populations or farmed cod, whereby environmental temperatures tend to be cooler or constrained, respectively. However, little has been done to assess the thermal sensitivity of RBC O_2 binding of Atlantic cod near the southern, warmer limit of their distribution in the North East Atlantic, where the effects of climate change will act first.

1.6 Red blood cell sickling

In fish, as in humans and other mammalian vertebrates, sickle cells form due to Hb polymers forming inside the cell and distorting its shape. In most species where sickling occurs the polymers consist of deoxyHb (Bookchin *et al*., 1976; Harosi *et al*., 1998). It is possible that sickling formation is another mechanism used to stabilise the T-state Hb.

Sickling is widespread throughout vertebrate groups, including deer (Undritz et al., 1960), goats (Holman and Dew, 1964), mongoose (Hawkey and Jordan, 1967) multiple species of sheep (Pritchard *et al*., 1963; Whitten, 1967; Jain and Kono, 1977; Butcher and Hawkey, 1979), along with various fish species (Harosi *et al*., 1998). As with humans, in many of these species sickling is restricted to certain Hb genotypes and is also the result of Hb forming polymers (Taylor and Easley, 1974; Butcher and Hawkey, 1977).

In humans, sickling is primarily stimulated by low oxygen partial pressure or deoxygenation (Hargens *et al*., 1980); however, in whiting (*Merlangius merlangus*) the partial pressure of oxygen alone does not solely determine sickling or a lack thereof (Koldkjaer and Berenbrink, 2007). In these fish it is pH which plays the crucial role prompting the production of Hb aggregates and provoking sickling of RBCs. In humans pH takes the secondary role, having only partial effect on sickling (Lange *et al*., 1951; Ueda and Bookchin, 1984). However, in both species it is low, more acidic pH levels which incite Hb polymer formation. However, this effect of pH in whiting was established *in vitro* and many teleost fishes have a mechanism to combat severe acidosis *in vivo*, known as βNHE (beta- adrenergic Na/H Exchanger) (Berenbrink *et al*., 2005). This causes an increase in intracellular pH and as such reduces the chance of Hb polymerisation *in vivo*. Nevertheless, the study by Koldkjaer and Berenbrink (2007) demonstrated that *in vivo* sickling can occur under extreme exercise stress, showing that whiting RBCs do reach pH values low enough to result in sickling. In the whiting studied by Koldkjaer and Berenbrink (2007) it was found that high PO_2 provides some protection to the effects of pH.

The effects of temperature RBC sickling are generally unknown for non-human vertebrates and is difficult to determine as most studies into sickling occurs at room temperature and to date there have been no incidents of *in vitro* sickling occurring at temperatures within possible physiological regions. However, the *in vivo* sickling in whiting suggests it can occur (Koldkjaer and Berenbrink, 2007).

The observation that Hb polymerisation in fish occurs only under low pH conditions suggest a further link to the Hb-O_2 equilibration.

1.8 Aims and Hypotheses

The primary aim of this thesis is to explore influence of temperature on RBC oxygen binding in the primary study species, Atlantic cod. Focusing particularly upon the effects of HbI genotype, temperature, pH, and Hb polymerisation or sickling.

Chapter 2: Life on the edge: Temperature and Hb genotype in-sensitive oxygen binding in Atlantic cod (Gadus morhua) red blood cells near their upper thermal distribution limit

Our first approach will be to characterise the effect of the enigmatic HbI polymorphism. With much controversy still surrounding the relevance of this genetic variation, we begin by repeating and improving the initial experiment to suggest the polymorphism influences the temperature dependence of oxygen binding affinity. In this chapter we aim to observe the effect of HbI genotype on the thermal sensitivity of RBC O_2 binding, under a wide range of physiologically relevant conditions in Atlantic cod near their upper thermal distribution limit in the North East Atlantic. We hypothesise HbI genotype will have little effect upon the thermal sensitivity of RBC oxygen binding.

Chapter 3: Comparative study of the effect of pH sensitivity on thermal sensitivity of red blood cell oxygen binding

Evidence of low thermal sensitivity of oxygen binding in chub mackerel and now in Atlantic cod has been linked to large Bohr effects in these species. Correlations between the two have been observed in heterothermic fish such as tuna, but no study has been performed on non-

heterothermic fish. Here we test for a correlation between pH and temperature sensitivity in red blood cell oxygen binding in six fish species. We predict that as pH sensitivity (Bohr effect) increases the thermal sensitivity of RBC oxygen binding will decrease.

Chapter 4: Acidification-induced red blood cell sickling and loss of oxygen capacity in Atlantic cod, Gadus morhua: Effects of temperature and haemoglobin genotype.

RBC sickling and the Root effect have both been shown to have a high dependence on pH, Atlantic cod have been shown to exhibit both. In this *in vitro* study we explore the occurrence of both in RBC suspensions of Atlantic cod. Further we observe the effect of temperature on both RBC sickling and acid induced deoxygenation, as despite the ectothermic nature of fish this information is lacking. We propose low pH will incite RBC sickling and HbI genotype will alter the occurrence of sickled cells. Additionally, we anticipate RBC sickling to be endothermic as in other vertebrates, whereby an increase in temperature will facilitate sickling.

Chapter 5: Red blood cell sickling in fish: evolution and potential genetic marker

Limitations in number of species studied for sickling, particularly under physiologically relevant conditions, prevent understanding of the causes and consequences. As part of a larger collaborative study we explore the occurrence of acid induced sickling in a range of species from a number of orders and attempt reconstruction of the evolution of sickling and identification of a sickling marker. We hypothesise, sickling will be prevalent in the Gadidae and the mechanism is linked to the HbI polymorphism in Atlantic cod with similar genetic variations occurring in all sickling species.

Chapter 6: DNA extraction from fin tissue and sequencing of HbI β1 polymorphism in Atlantic cod

With the influence of the HbI polymorphism and its effect of Atlantic cod thermal tolerance still contested, linking direct observations of thermal experience of individual wild animals with HbI polymorphism would be invaluable. Such tracking data is now available due to large scale tagging programs, along with fin clips for genomic data. In this chapter we outline a method for reliable, cost effective genomic DNA (gDNA) extraction which yields high

concentration and quality from small quantities of fin tissue. The isolated gDNA was used to test a simple method of polymerase chain reaction (PCR) amplification to determine HbI genotype. This method was then applied to archival fin tissues.

Chapter 2. Life on the edge: Temperature and Hb genotype in-sensitive oxygen binding in Atlantic cod (*Gadus morhua*) red blood cells near their upper thermal distribution limit

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2.1 Abstract

Atlantic cod are a commercially important species believed to be threatened by warming seas near their southern, equatorward upper thermal edge of distribution. Previous studies have suggested separately that limitations to circulatory O_2 transport, in particular cardiac output, or the geographic distribution of their functionally different haemoglobin genotypes may play a role in setting thermal tolerance in this species. The present study assessed the thermal sensitivity of O_2 binding in Atlantic cod red blood cells with different Hb genotypes near their upper thermal distribution limit and modelled its consequences for the arterial-venous O_2 saturation difference, $S_{a\text{-}v}$, another major determinant of circulatory O_2 supply rate. Results showed statistically indistinguishable red blood cell O_2 binding between any of the three HbI genotypes in wild-caught Atlantic cod from the Irish Sea (53°North). Red blood cells had an unusually low O_2 affinity, with reduced or even reversed thermal sensitivity between pH 7.4 and 7.9 and 5.0 and 20.0°C. This was paired with large Bohr and Root effects as shown by strongly pH-dependent affinity and cooperativity of red blood cell O_2 binding. Modelling of $S_{a\text{-}v}$ at physiological pH, temperature and arterial and venous O_2 partial pressures revealed a substantial capacity for increases in $S_{a\text{-}v}$ to meet rising tissue O_2 demands at 5.0 and 12.5°C, but not at 20°C. There was further no evidence for an increase of maximal $S_{a\text{-}v}$ with temperature. It is suggested that Atlantic cod at such high temperatures may solely depend on

increases in cardiac output and blood O_2 capacity, or thermal acclimatisation of metabolic rate, for matching circulatory O_2 supply to tissue demand.

2.2 Introduction

The 5th assessment report of the Intergovernmental Panel on Climate Change documents an increase in average global sea surface temperatures over the last century and predicts their continued rise (Field *et al*., 2014). The body temperature of marine ectothermic organisms is directly affected by warming seas, which makes an understanding of their physiological capabilities to withstand elevated temperatures vital for predicting future redistributions of species and influencing management regimes (e.g., Deutsch *et al*., 2015).

Atlantic cod (*Gadus morhua*) are widely distributed in coastal and shelf seas throughout the North Atlantic, but stocks near the southern, equatorward upper thermal margin of their historic distribution limit in the Irish and Southern North Sea have declined over the past decades, which has in part been ascribed to warming seas (Brander, 2005; Drinkwater, 2005; Perry *et al*., 2005; Beggs *et al*., 2014; Deutsch *et al*., 2015). The high commercial importance and resulting fishing pressures, has led to extensive research into thermal effects on Atlantic cod life history traits, physiology, behaviour, abundance and distribution (Mork *et al*., 1984; Petersen and Steffensen, 2003; Gamperl *et al*., 2009; Righton *et al.*, 2010; Behrens *et al*., 2012; Engelhard *et al*., 2014; Kreiss *et al*., 2015; Rutterford *et al*., 2015). Based on the thermal sensitivity of life history traits and projected future temperature changes, Atlantic cod stocks near their current upper thermal distribution limit in the North East Atlantic have been predicted to disappear entirely from the Celtic and Irish Seas by the end of this century (Drinkwater, 2005). Likewise, alternative mechanistic models based on a metabolic index of the O_2 supply to demand ratio and projected future temperature and O_2 partial pressure (PO_2) changes predict reductions in the current habitat volume (occupied area x depth range) by 12-32% at the equatorward upper thermal margin of Atlantic cod by the end of the present century (Deutsch *et al*., 2015).

The oxygen- and capacity-limited thermal tolerance (OCLTT) hypothesis attempts to provide a general mechanistic explanation for the thermal distribution limits of aquatic organisms, suggesting that the capacity of O_2 supply mechanisms in aquatic ectotherms, such as the circulatory and ventilatory systems, becomes insufficient to meet rising O_2 demands at thermal extremes, thus affecting their ability to maintain an adequate aerobic scope for activities such as feeding, digestion, growth, migration, reproduction and predator evasion (Pörtner and Knust, 2007; Pörtner, 2010).

Studies on the acute thermal tolerance of Atlantic cod have identified the circulatory system as a primary limiting factor in the O_2 supply cascade from the environment to the tissues, with cardiac function suggested to become compromised close to the critical thermal maximum (Sartoris *et al*., 2003; Lannig *et al*., 2004; Gollock *et al*., 2006). According to the Fick equation, cardiac output, $\dot{Q}$ (the product of heart rate, f_H, and stroke volume, V_S) and the arterial-venous O_2 difference, C_aO_2 - C_vO_2, together determine the rate of circulatory O_2 delivery ($\dot{M}O_2$) between respiratory organs and tissues (Fick, 1870):

$$\dot{M}O_2 = \dot{Q}\ (C_aO_2 - C_vO_2) \qquad \text{Equation 2.1}$$

The contribution of changes in C_aO_2 - C_vO_2 in the assessment of maximal O_2 supply capacities during warming of marine ectotherms is largely unknown, although it has long been recognised that in humans, the increase in C_aO_2 - C_vO_2 may surpass the increase in $\dot{Q}$ in its contribution to meeting elevated $\dot{M}O_2$ during heavy exercise (factorial increases of 3.45 and 2.51, respectively; Ekelund and Holmgren, 1964; Dejours, 1975). C_aO_2 - C_vO_2 essentially equals the maximal blood O_2 binding capacity multiplied by the arterio-venous O_2 saturation difference, $S_{a\text{-}v}$ (ignoring the relative small contribution of physically dissolved O_2 in blood with average haemoglobin (Hb) concentration). $S_{a\text{-}v}$ is in turn determined by the arterial and mixed venous PO_2 values (P_aO_2 and $P\bar{v}O_2$, respectively) and the shape and properties of the blood O_2 equilibrium curve (OEC; e.g. Weber and Campbell, 2011). In fact, right-shifts of the OEC with increasing temperature or decreasing pH have classically been linked to improved rates of tissue O_2 supply (Bohr *et al*., 1904; Barcroft and King, 1909). Yet the contribution of

such OEC changes to meeting increased O_2 demands in marine ectotherms at elevated temperatures is poorly known.

Atlantic cod are of particular interest in this context because the different Hb genotypes of their polymorphic major HbI component (Sick, 1961) have been associated with differences in the thermal sensitivity of O_2 binding in their red blood cells (RBCs) (Karpov and Novikov, 1980; Andersen *et al*., 2009). The frequencies of the two co-dominant alleles underpinning the HbI polymorphism vary inversely along a latitudinal cline in the North East Atlantic, from the Barents Sea with frequencies of the HbI 1 allele as low as 0-0.1, to the Southern North Sea, where HbI 1 frequency rises as high as 0.6-0.7 (Sick, 1965; Jamieson and Birley, 1989; Andersen *et al*., 2009; Ross *et al.* 2013). These clines have been attributed to natural selection acting on divergent temperature sensitivities of Atlantic cod harbouring the different HbI genotypes regarding growth, physiology and behaviour (reviewed by Andersen, 2012; Ross *et al*., 2013). However, the brief but influential report by Karpov and Novikov (1980) that first suggested functional differences in RBC-O_2 affinity between the HbI genotypes, was based on RBC OECs of White Sea Atlantic cod near their northern, lower thermal distribution limit (67° North) and measured at a single, physiologically rather low pH value (7.5; Karpov and Novikov, 1980), and its findings and extrapolations for the efficiency of RBC O_2 transport in Atlantic cod HbI genotypes near their southern, upper thermal limit of distribution have to our knowledge never been experimentally verified.

The present study was undertaken to assess the thermal sensitivity of RBC O_2 binding, and its consequences for $S_{a\text{-}v}$ under *in vivo*-relevant conditions in Atlantic cod HbI genotypes near their upper thermal distribution limit in the North East Atlantic. The results showed statistically indistinguishable RBC O_2 affinities and their pH and temperature sensitivities between all three HbI genotypes in wild-caught Atlantic cod from the Irish Sea (53°North). All animals showed an unusually low RBC O_2 affinity, with no -or even reversed- thermal sensitivity over much of the physiological pH and temperature range. This was paired with strongly pH-dependent affinity and cooperativity of RBC O_2 binding. Modelling of $S_{a\text{-}v}$ at

physiological values for pH, temperature and PO_2 revealed a substantial capacity for increases in this factor to meet rising tissue O_2 demands at 5.0 and 12.5°C, but not at 20°C, where further increases in the maximal rate of O_2 delivery by the circulatory system are predicted to solely rely on increases in cardiac output and O_2 capacity.

2.3 Materials and Methods

Wild Atlantic cod with a total length of 46.4 ± 0.45 cm (here and elsewhere: mean ± S.E.M; N = 106 animals) were caught by hook and line on board of commercial fishing boats in the Mersey Estuary adjoining the Irish Sea near Liverpool, U.K., (53°25' North, 3.02°1' East) between mid-January and end of February 2015 at sea surface temperatures between 6.8 and 7.9°C. Animals were killed by a British Home Office approved Schedule 1 method, involving concussion and destruction of the brain. Blood was removed from caudal vessels using heparinized 1 ml syringes, whose dead space had been filled with 9.0 units ml^{-1} sodium heparin solution (from porcine intestinal mucosa, Sigma-Aldrich). Up to 8 animals were bled on the day before each experiment and samples were kept on ice for maximally 10 h before landing and genotyping. Immediately after, blood of a single individual was selected for experiments the next day in accordance with a pre-determined random selection of genotype order.

Genotype determination

RBCs were isolated from plasma and buffy coat by centrifugation (3000 rcf, 4°C, 4 mins) and 20 µl of RBC pellet were lysed by adding 64 µl cold distilled water. Hbs in the haemolysate were separated by horizontal agarose gel electrophoresis, modified from Sick (1961). A 1% agar gel was prepared in diluted (1:1, with water) Smithies buffer (45 mM Tris, 25 mM boric acid and 1 mM EDTA, adjusted to pH 8.8 at room temperature). Undiluted Smithies buffer was used as an electrode buffer and samples were run towards the positive pole at 120 volts for 40 minutes at 4°C in a cold room, whereupon Hb bands were viewed immediately without staining.

Preparation of RBC suspensions

Remaining RBC pellets of selected samples were resuspended in physiological saline composed of (in mmol l^{-1}) NaCl (125.5), KCl (3), $MgCl_2$ (1.5), $CaCl_2$ (1.5), D-glucose (5) and Hepes (20), adjusted to pH 7.97 at 15°C (Koldkjaer and Berenbrink, 2007). The above washing procedure of centrifugation and resuspension in fresh saline was repeated twice and during the last step RBCs were re-suspended at an approximate haematocrit (Hct) of 5-10 % and stored overnight at 4°C in a 15 ml falcon tube with a large air reservoir, placed on the side to maximise exchange surface area between saline and sedimented cells. Following the overnight rest and immediately prior to establishing RBC O_2 equilibrium curves (OECs), RBCs were washed again, re-suspended in fresh saline at 8-13% Hct, and the concentrations of tetrameric Hb [Hb_4], mean corpuscular Hb (MCHC), ATP, and GTP were determined.

Analytical procedures

[Hb_4] was determined by the cyan-methaemoglobin method using modified Drabkin's solution (11.9 mmol l^{-1} $NaHCO_3$, 0.61 mmol l^{-1} $K_3[Fe(CN)_6]$ and 0.77 mmol l^{-1} KCN) and a haem-based extinction coefficient of 11.0 l $mmol^{-1}$ cm^{-1} at a wavelength of 540 nm, as described earlier (Völkel and Berenbrink, 2000). Hct was measured in micro-haematocrit tubes using a SpinCrit Micro-Hematocrit centrifuge and MCHC was calculated as [Hb_4]/(Hct/100). For ATP and GTP determinations, equal volumes of washed RBC suspension and 0.6 M perchloric acid (PCA) were mixed before freezing at -80°C for later analysis. Samples were defrosted and centrifuged at 4°C and 13,000 rcf. The PCA extract was neutralised to an approximate pH of 7 by the addition of concentrated potassium carbonate to the supernatant and the resultant precipitate was removed by centrifugation. ATP and GTP concentrations in the supernatant were then determined enzymatically via the two step process outlined by Albers *et al*. (1983), with the following modifications: The enzymes hexokinase with glucose-6 phosphate dehydrogenase (H8629, Sigma-Aldrich) and Nucleoside 5′-diphosphate kinase (N0379, Sigma-Aldrich) were used at concentrations of 13 units/mL and 5000 units/mL, respectively. The accuracy of the test and potential losses of nucleotide triphosphates (NTPs) during PCA extractions were examined using ATP and GTP standard

solutions (Sigma-Aldrich, A2383 and G8877). Recovery was 96.4 ± 0.9% and 80.4 ± 0.64% (n = 18) for ATP and GTP respectively, and all measurements were corrected accordingly. Concentrations were converted to mmol l^{-1} RBCs using equation presented by Albers *et al*., (1983), then standardised using MCHC and presented as ATP/Hb_4 and GTP/Hb_4 molar ratios.

Oxygen equilibrium curve determinations

After the above measurements were taken, RBC suspensions were further diluted 10-fold in pH 7.97 saline and then pH was varied by final 10-fold dilutions in salines of pH 7.45, 7.70 and 7.97 (all adjusted at 15°C). Thermally-induced saline pH changes were assessed in air equilibrated RBC suspensions using a Lazar Model FTPH-2S pH electrode with a Jenco 6230N meter (Jenco Collaborative, California, USA). Given the buffering properties of the saline (20 mM Hepes) and small quantity of cells (0.08 – 0.13% Hct) oxygenation-linked changes in pH of RBC suspensions during OEC measurements were deemed negligible. For each individual, 1.2 ml aliquots of final RBC suspension were incubated, at the three pH values in parallel, in 50 ml capacity Eschweiler glass tonometers (Eschweiler GmbH, Engelsdorf, Germany) with custom attached 1 cm path length optical glass cuvettes (following a design by Brix *et al*., 1998). This was performed at temperatures of 5.0, 12.5 and 20.0°C and a minimum of five PO_2 values covering the range between of 20 to 80% RBC O_2 saturation. PO_2 was varied by mixing air and N_2 in pre-determined ratios using a Wösthoff gas mixing pump (Wösthoff GmbH, Bochum, Germany) and the final gas mixture was fully humidified at the experimental temperature. RBC suspensions were equilibrated for at least 20 minutes with each gas mixture. Solutions remained sealed within the tonometer to ensure PO_2 remained constant while an optical spectrum was taken between 500-700 nm (Unicam UV 500 spectrophotometer, Thermo Electron Corporation, Ohio, USA; with Vision 32 Software) and O_2 saturation of RBC suspensions was determined by spectral deconvolution (Völkel and Berenbrink, 2000).

Data analysis and statistics

Spectral deconvolution of the optical spectra (see Völkel and Berenbrink, 2000) was used to determine the concentrations of haemoglobin derivatives within RBC suspensions

(oxyhaemoglobin, HbO_2; deoxyhaemoglobin, deoxyHb; and the two forms of methaemoglobin, acid Hb^+ and alkaline Hb^+) at each temperature, pH and PO_2 value using SigmaPlot 12.5 software (Jandel Scientific, San Rafael, CA, USA). The unknown concentrations (mmol l^{-1}) of the different tetrameric Hb derivatives, were calculated using

$$f = au + bv + cw + dx \qquad \text{Equation 2. 2}$$

where a, b, c, and d, represent [HbO_2], [deoxyHb], [acid Hb^+], and [alkaline Hb^+], respectively and were restricted to values greater than or equal to zero. f is the predicted dependent variable to be fitted to the measured absorption data for each nm step between 500 and 700 nm. u, y, w and x represent the respective experimentally determined absorption coefficients for each Hb derivative at each wavelength between 500 and 700 nm, respectively. Absorption coefficients for HbO_2 and deoxyHb were created with RBC suspensions in pH 8.05 saline at 5.0°C, exposed to 100% oxygen or 100% nitrogen. Acid Hb^+ and alkaline Hb^+ absorption coefficients were constructed using Hb suspensions oxidised with tri-potassium hexacyanoferrat at pH 6.5 and 8.05 respectively, although the analysis showed that no methaemoglobin formation had occurred in any of our samples. In all cases, the predicted values by the curve fitting procedure were plotted for each wavelength between 500 and 700 nm together with the measured spectra for visual inspection of the accuracy of the prediction.

The level of RBC O_2 saturation (S) was calculated as [HbO_2]/ ([HbO_2] + [deoxyHb]). Hill plots on data between 20 and 80% saturation were created using log (S/(1-S)) versus log PO_2. Log P_{50} was calculated by linear regression as the log PO_2 when log (S/(1-S)) equalled 0. The slope of the regression line indicated the apparent cooperativity of RBC O_2 binding or Hill number (n_H). The Bohr coefficient was calculated by $\Phi = \Delta\log P_{50}/ \Delta\text{pH}$ for each pH interval. Because of nonlinearity, at each temperature, log P_{50} and n_H were plotted against measured saline pH and 2nd order polynomials were used to standardise them to pH 7.40, 7.65 and 7.9, removing the effect of temperature-induced pH shifts on these variables. Once standardised to fixed pH, thermal sensitivities of OECs were expressed as apparent heat of oxygenation, ΔH'. These were calculated using the van't Hoff equation $\Delta H' = 2.303\ R\ ((\Delta\log$

P_{50})/($\Delta 1/T$)), where R = universal gas constant (0.008314 kJ K^{-1} mol^{-1}), and T = temperature in K.

OECs for a series of fixed pH values were produced using values for n_H and P_{50} predicted at a given pH for each individual from the same 2nd order polynomial equations used above for standardising log P_{50} and n_H. RBC O_2 saturation S was then calculated as a function of PO2 using:

$$S = PO_2 \exp(n_H) \, [PO_2 \exp(n_H) + P_{50} \exp(n_H)]^{-1} \qquad \text{Equation 2. 3}$$

$S_{a\text{-}v}$ during acute temperature and/or pH changes was modelled as the differences between S_aO_2 and S_vO_2 at physiologically relevant pH and arterial and venous PO_2 values read from RBC OECs. An arterial pH of 7.86 and average values of 85 and 30 mmHg for P_aO_2 and $P\bar{v}O_2$ were assumed for resting normoxic Atlantic cod at 12.5°, based on literature values for this species close to this temperature (Kinkead *et al*., 1991; Perry *et al*., 1991; Claireaux and Dutil, 1992; Nelson *et al*., 1996; Larson *et al*., 1997; Karlsson *et al*., 2011; Petersen and Gamperl, 2011). P_aO_2 was assumed constant during acute thermal change (Sartoris *et al*., 2003), whereas values for $P\bar{v}O_2$ at 5.0 and 20.0°C of 60 and 15 mmHg, respectively, were based on the percentage changes observed by Lannig *et al*. (2004). Changes in arterial pH were assumed to follow the relationship with temperature established for marine teleosts and elasmobranchs by Ultsch and Jackson (1996). Owing to the generally larger deoxygenation-linked proton uptake in teleosts Hbs compared to other vertebrates (Berenbrink *et al*., 2005), venous pH was assumed to be similar to arterial pH, as previously recorded in normoxic Atlantic cod (Perry *et al*., 1991).

Maximal $S_{a\text{-}v}$ at each temperature was taken as the maximally observed $S_{a\text{-}v}$ at any pH and P_aO_2 and $P\bar{v}O_2$ equalling 85 and 15 mmHg, the lowest average $P\bar{v}O_2$ reported for Atlantic cod in the literature under any condition.

All values are reported as means ± standard error of the mean (SEM). Sigmaplot 12.5 software was used for all statistical analysis and significance was accepted at $p < 0.05$. Differences between mean values were generally assessed by one-way ANOVA, followed by

a post-hoc Tukey test, if relevant. Other test statistics (two-way, three way ANOVA, $\chi 2$ and one-sample *t*-tests) were used as indicated directly in the text.

2.4 Results

In 106 Atlantic cod caught between mid-January and end of February 2015 in the River Mersey Estuary near Liverpool, the HbI 1/1 genotype dominated with 45% of individuals, followed by 41% HbI 1/2 heterozygotes and just 14% HbI 2/2 homozygotes (Table 2.1). These genotype frequencies did not significantly deviate from the expectations according to the Hardy-Weinberg equilibrium, ($\chi^2 = 1.09$, d.f. = 2, $p > 0.5$) or from the average value recorded for the Irish Sea between 1971 and 1977 ($\chi^2 = 5.73$, d.f. = 2, $p > 0.05$; Jamieson and Birley, 1989). HbI 1 allele frequency was 0.66 and thus among the higher values recorded for Atlanitc cod stocks across their geographical range (Ross *et al*., 2013), and similar to values reported in recent years for the Southern North Sea (0.66, Pörtner *et al*., 2001; 0.64, Andersen *et al*. 2009). There was no difference in total length between HbI genotypes in 84 animals that were available for length measurement, nor in the subset of 16 animals selected for OECSs (p = 0.073 and 0.226, respectively; Table 2.1). In the latter group there were also no significant HbI genotype related differences in haematocrit (p = 0.834), haemoglobin concentration (p = 0.697), MCHC (p = 0.371) and ATP/Hb_4 (p = 0.284) or GTP/Hb_4 (p = 0.620) ratios of washed RBC suspensions immediately prior to experiments (Table 2.1). The ATP/Hb_4 and GTP/Hb_4 ratios further were similar to values previously reported on whole blood (Leray, 1982).

OECs of Atlantic cod RBCs at all three temperatures and for all three HbI genotypes revealed strong Bohr and Root effects, as shown by strong pH-induced reductions in RBC O_2 affinity and O_2 saturation at atmospheric PO_2, respectively (Figure 2.1A-C). At each nominal saline pH, increasing temperatures appeared to reduce O_2 affinity, shifting OECs to the right and increasing P_{50} (Figure 2.1A-C). However, this effect will have been partially due to the temperature-induced shifts in the pH of the HEPES buffer. Thus, for example, the actual pH values experienced by RBCs suspended in saline with a nominal pH of 7.90 were 7.99, 7.89 and 7.81 at 5.0, 12.5 and 20.0°C, respectively, with SEM values for pH below 0.005.

Table 2.1: *Summary of captured and experimental animals. Number of Atlantic cod of each genotype (HbI 1/1, HbI 1/2 and HbI 2/2) captured and selected for further experiments, total lengths, and values for haematocrit (Hct), haemoglobin concentration ([Hb_4]), mean cellular haemoglobin concentration (MCHC), and RBC ATP/Hb_4 and GTP/Hb_4 molar ratios in washed red blood cells (RBCs) immediately before experiments (mean values ± SEM., differing numbers of experimental individuals are indicated in brackets).*

	HbI 1/1	**HbI 1/2**	**HbI 2/2**
Number of captured individuals	48	43	15
Total Length (cm)	46.1 ± 0.7 (38)	47.5 ± 0.8 (36)	43.9 ± 1.2 (10)
Number of experimental individuals	6	5	5
Total Length (cm)	43.8 ± 1.7	49.5 ± 3.3	46.0 ± 2.6 (4)
Properties of washed RBC cell suspensions			
Hct (%)	11.2 ± 0.63	10.8 ± 0.48	10.9 ± 0.64
[Hb_4] (mmol per litre RBC suspension)	0.27 ± 0.01	0.27 ± 0.02	0.28 ± 0.01
MCHC (mmol per litre RBC)	2.43 ± 0.11	2.48 ± 0.08	2.62 ± 0.09
ATP/Hb_4 (mol/mol)	1.39 ± 0.11	1.57 ± 0.18	1.27 ± 0.09
GTP/Hb_4 (mol/mol)	0.80 ± 0.10	0.81 ± 0.08	0.76 ± 0.06

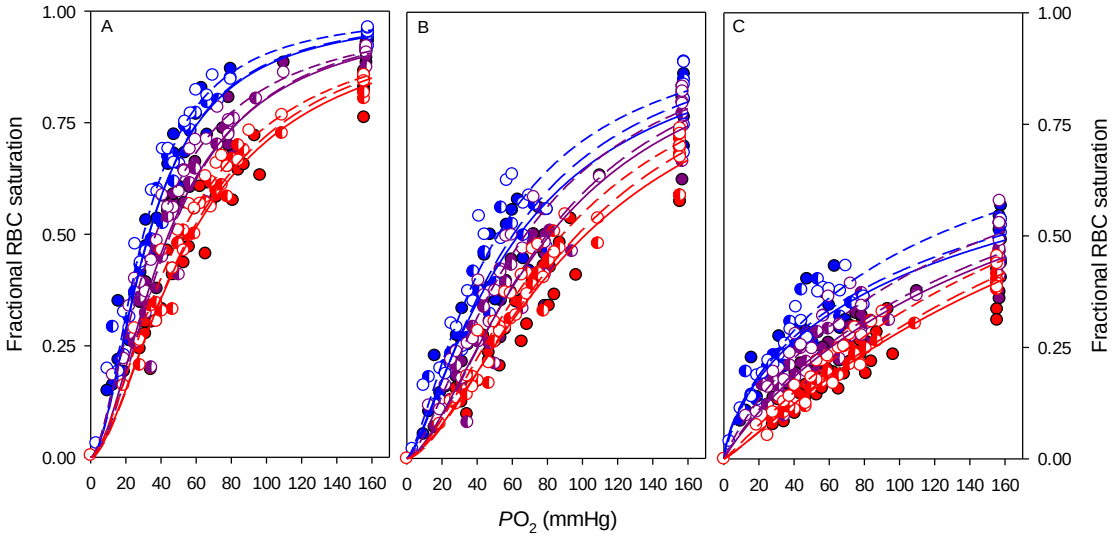

Figure 2.1*: Oxygen equilibrium curves of Atlantic cod RBCs with different HbI genotypes at 5.0, 12.5 and 20.0°C (blue, purple, red symbols and lines, respectively) and at nominal saline pH values of A) 7.90, B) 7.65 and C) 7.40. Circles indicate measured values while lines are based on sigmoidal curve fits for each temperature and HbI genotype (HbI 1/1, solid lines,*

filled symbols; HbI 1/2, long-dashed lines, half-filled symbols; HbI 2/2, short-dashed lines, open symbols). N = 5 - 6 animals for each genotype at each pH and temperature.

In the Bohr plot (Figure 2.2) the stepwise reductions of pH from nominal pH 7.90 to 7.65 and then 7.40 resulted in significant increases in log P_{50} within all genotypes and all temperatures ($p < 0.001$). Thus, the southern HbI 1/1 genotype at 5.0°C and pH 7.99 had a log P_{50} of 1.52 ± 0.02 (corresponding to a P_{50} of 33 mmHg). As pH decreased, O_2 affinity showed a corresponding decrease, with a log P_{50} of 1.79 ± 0.03 (P_{50} 62 mmHg) at pH 7.75 and a further decrease at pH 7.51 to 2.20 ± 0.06 (158 mmHg). Similar effects of pH were also observed at 12.5 and 20.0°C, although increasing temperatures caused a general shift of curves towards higher log P_{50} values and lower pH values (Figure 2.2).

Surprisingly, log P_{50} values were not affected by HbI genotype at any tested pH or temperature ($p = 0.161 – 0.421$), although there was a tendency of values in the northern HbI 2/2 type to be consistently lower than the other two genotypes.

The relationship between log P_{50} and pH appeared distinctly curvilinear and a three-way ANOVA with pH-range, temperature and genotype as factors, revealed that the Bohr coefficient, $\Delta \log P_{50} (\Delta \text{pH})^{-1}$, significantly increased in magnitude from around -1.08 in the higher pH range, to about -1.65 in the lower pH range ($p < 0.001$). This increased pH dependence of RBC O_2 affinity at lower pH is likely due to the more pronounced Root effect at the lowest pH values. Both genotype and temperature had no significant effect on the Bohr coefficient ($p = 0.183$ and 0.840, respectively).

Hill's cooperativity constant n_H did not vary significantly between the upper two saline pH values at any temperature, attaining values between 1.5 and 2.0. At the lowest saline pH however, n_H was significantly reduced down to values between 1.0 and 0.7 compared to the highest saline pH ($p < 0.001$) indicating the onset of the Root effect. Similar to log P_{50} above, n_H also remained unaffected by HbI genotype at all pH values and temperatures ($p = 0.161 – 0.421$). Given the lack of significant Hb genotype differences in all analyses above, data for all animals were pooled for the following analyses.

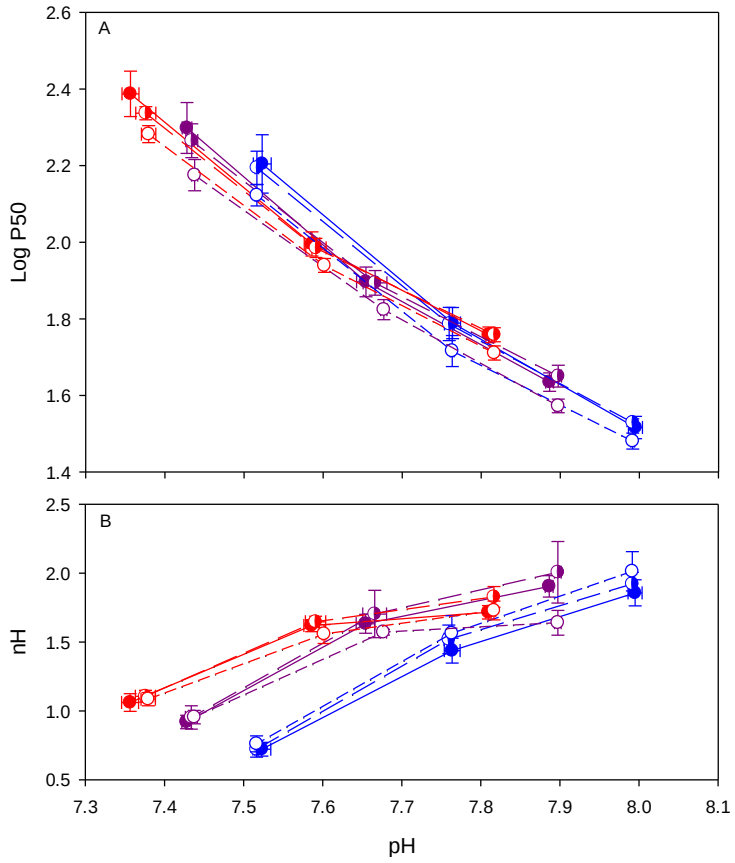

***Figure 2.2**: Effect of pH, HbI genotype, and temperature on the affinity and cooperativity of O_2 binding in Atlantic cod red blood cells. A) log P_{50} versus pH for HbI 1/1 (closed symbols, solid lines N = 6), HbI 1/2 (semi-open symbols, long-dashed lines, N = 5) and HbI 2/2 (open symbols, short-dashed lines, N = 5), at 5.0, 12.5 and 20.0°C (blue, purple and red symbols and lines, respectively). B) n_H, Hill's cooperativity coefficient at 50% RBC O_2 saturation, for the same data as in A.*

***Table 2.2:** Parameters (mean ± SE) for 2nd order polynomial fits of log P_{50} or n_H (y) as a function of pH (x), according to $y = ax^2 + bx + c$, with all genotypes pooled together (N = 16)*

	Log [P_{50} (mmHg)]			**n_H**		
***t* (°C)**	*a*	*b*	*c*	*a*	*b*	*c*
5.0	1.2 ± 0.2	-20.2 ± 3.5	85.4 ± 13.4	-2. 8 ± 0.8	45.6 ± 12.7	-185.1 ± 49.3
12.5	1.1 ± 0.2	-18.6 ± 3.4	78.2 ± 9.2	-4.4 ± 0.7	69.8 ± 10.5	-273. 5 ± 40.2
20.0	1.3 ± 0.1	-21.5 ± 1.7	88.7 ± 6.6	-3.8 ± 0.6	59.6 ± 8.8	-230.4 ± 33.4

Table 2.3: *Oxygen equilibrium curve properties, corrected for pH change with temperature, of Atlantic cod red blood cells, with all haemoglobin genotypes combined, when exposed to a range of temperatures and pH values; log P_{50}, with P_{50} in mmHg and n_H (co-operativity at 50 % saturation); mean ± SE, N = 16. For each parameter different subscript letters within a row indicate significant differences (One-way ANOVA for log P_{50} and One-way ANOVA on ranks for n_H).*

	Log [P_{50} (mmHg)]			**n_H**		
pH	5.0°C	12.5°C	20.0°C	5.0°C	12.5°C	20.0°C
7.90	1.60 ± 0.01^a	1.61 ± 0.02^a	1.69 ± 0.01^b	1.79 ± 0.06^a	1.86 ± 0.09^a	1.71 ± 0.05^a
7.65	1.93 ± 0.02^a	1.89 ± 0.02^a	1.90 ± 0.01^a	1.19 ± 0.04^a	1.61 ± 0.05^b	1.68 ± 0.03^b
7.40	2.42 ± 0.04^a	2.31 ± 0.03^b	2.28 ± 0.02^b	0.25 ± 0.08^a	0.80 ± 0.04^b	1.18 ± 0.03^c

After standardising log P_{50} values of the combined HbI genotypes to constant pH values (Table 2.2), log P_{50} at pH 7.65 was completely independent of temperature over the entire range from 5.0 to 20.0^{0}C (Table 2.3). At pH 7.90, log P_{50} was also statistically indistinguishable between 5.0°C and 12.5°C, and only increased significantly at 20.0°C compared to these values ($p = 0.002$ and $p < 0.001$, respectively; Table 2.3). At pH 7.40, log P_{50} was unaffected by temperature between 20.0 and 12.5^{0}C, and only significantly increased at 5^{0}C compared to these values ($p < 0.001$), revealing a reversed temperature sensitivity at the lower temperature range.

The pH-adjusted cooperativity coefficient n_H (Table 2.2) was unaffected by temperature at pH 7.9 (($p = 0.412$; Table 2.3), but at pH 7.65 it was significantly reduced at 5.0°C when compared to 12.5 and 20.0°C ($p < 0.001$), although values12.5 and 20°C did not differ significantly. At pH 7.4, n_H significantly increased with temperature over the whole range ($p < 0.001$; Table 2.3).

ΔH' for the oxygenation reaction of Atlantic cod RBCs was significantly affected by both pH ($p < 0.001$) and temperature range ($p < 0.001$), with no significant interaction ($p =$

0.574) between factors (two-way ANOVA, with temperature range and pH as factors; Figure 2.3). Between 12.5 and 20.0°C and at pH 7.90, Atlantic cod RBCs showed a typical exothermic oxygenation reaction, with a negative Δ*H*' value of -15.7 ± 2.9 kJ mol^{-1}. However, in the same thermal range, thermal sensitivity was significantly reduced at pH 7.65 and 7.40, where Δ*H*' values amounted to -2.5 ± 1.9 kJ mol^{-1} and +5.8 ± 3.9 kJ mol^{-1}, respectively. These values were not significantly different from each other and One-sample *t*-tests showed that they also did not significantly differ from zero (p = 0.208 and 0.158, respectively; Figure 2.3). At all pH values, the magnitude of Δ*H*' was significantly higher between 5.0-12.5°C than between 12.5-20.0°C. In the lower temperature range at pH 7.9 this resulted in a Δ*H*' value of -3.8 ± 2.3 kJ mol^{-1}, which was not significantly different from zero (One-sample *t*-test, p = 0.119). Stepwise, significantly more endothermic RBC oxygenation was observed at pH 7.65 (+8.9 ± 2.4 kJ mol^{-1}) and then pH 7.40 (+23.2 ± 4.4 kJ mol^{-1}).

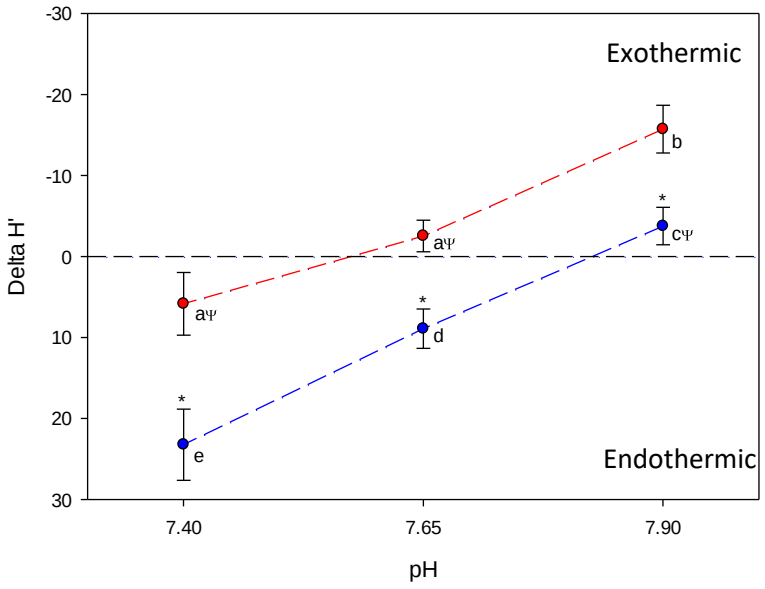

Figure 2.3: *Apparent heat of oxygenation, ΔH', for Atlantic cod red blood cells between 5.0 and 12.5°C (blue line and symbols) and 12.5 and 20.0°C (red line and symbols) at each reference pH (mean ± SEM, N = 16). Note reversal of y axis, with negative values at top. Differing letters within a temperature range or at constant pH indicate significantly different ΔH' values (two-way ANOVA), Ψ indicates values not significantly different from zero (one-sample t-test).*

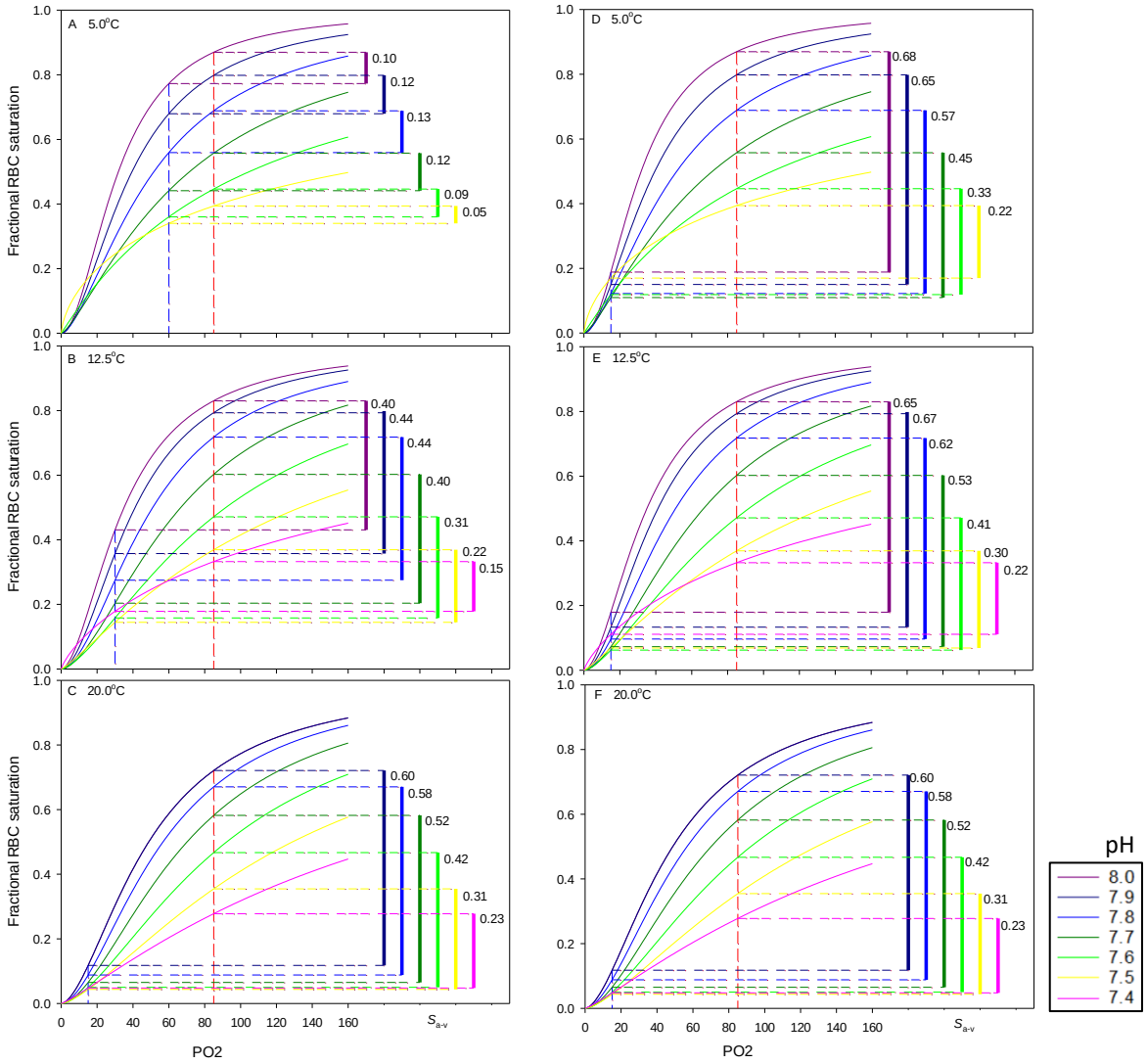

Figure 2.4: *Modelled oxygen equilibrium curves of Atlantic cod RBCs at a series of standardised pH values (see inset) and 5.0 (A, D), 12.5 (B, E), and 20.0°C (C, F). Red dashed vertical line indicates resting arterial PO_2 values, PaO_2. Blue dashed vertical line indicates either resting mixed venous PO_2 values ($P\bar{v}O_2$, A-C), or minimally observed mixed venous PO_2 values ($P\bar{v}O_2$ min, D-F), respectively (see Materials and Methods). Corresponding arterial and venous O_2 saturations, S_aO_2 and S_vO_2, and their difference, S_{a-v}, are indicated for each pH by numbers and colour matched horizontal dashed lines and vertical bars, respectively. Due to the pH shifts with temperature in the underlying data sets (Figure 2.2) oxygen equilibrium curves have not been modelled for pH 8.0 at 20.0° and pH 7.4 at 5.0°C.*

Using 2nd order polynomials (Table 2.2), log P_{50} and nH values from Figure 2.2 were standardized for a series of fixed pH values and the corresponding OECs shown for three temperatures (Figure 2.4). At each temperature, literature values for *in vivo* P_aO_2 and $P\bar{v}O_2$ and the resulting $S_{a\text{-}v}$ are indicated for each pH. The curves suggest *in vivo* arterial O_2 saturations, across temperature, of no more than 80% at resting arterial pH (7.91 to 7.81 between 5.0°C and 20.0°C, respectively) and constant arterial PO_2 (85 mmHg; Figure 2.4A-C). Increasing temperatures are associated with greater use of the venous reserve, as shown by decreases in $P\bar{v}O_2$ and consequent increases in $S_{a\text{-}v}$ from 0.11 to 0.44 and 0.58 at 5.0, 12.5 and 20.0°C, respectively. Further, at each temperature and fixed P_aO_2 and $P\bar{v}O_2$ values, acidification-induced decreases in S_vO_2 were accompanied by similar, or even greater decreases in S_aO_2 (Figure 2.4A-C). This suggests that in Atlantic cod RBCs under general acidosis and a given $P\bar{v}O_2$, the benefits of the Bohr effect in facilitating O_2 offloading to tissues are minimised by parallel or even greater decreases in arterial O_2 loading.

Estimates of maximal $S_{a\text{-}v}$ values at the three temperatures (Figure 2.4 D-F) show a substantial potential for increasing $S_{a\text{-}v}$ above routine values at 5.0 and 12.5°C, where $S_{a\text{-}v}$ rises, by factors of 4-5 and 1.5-2.0, respectively, when $P\bar{v}O_2$ is allowed to drop to the minimally observed value of 15 mmHg (Figure 2.4E, F vs. A, B). However, there was no additional capacity for $S_{a\text{-}v}$ increases above routine values at 20.0°C (Figure 2.4C, F). Similarly, across pH values, maximal $S_{a\text{-}v}$ values tended to decrease, rather than increase with temperature, such that even taking into account a temperature-associated decrease in *in vivo* arterial pH from 7.91 at 5.0°C to 7.81 at 20.0°C did not increase $S_{a\text{-}v}$ (Figure 2.4D-F).

2.5 Discussion

The results of the present study suggest that the O_2 binding properties of Atlantic cod RBCs near their southern, upper thermal distribution limit in North East Atlantic are contrary to common expectation; being (1) independent of HbI genotype, (2) characterized by an unusually low O_2 affinity that is strongly affected by pH and (3) remarkably temperature insensitive over much of the physiological pH range. These factors combine to create a blood

O_2 transport system in which maximal $S_{a\text{-}v}$ under *in vivo* conditions does not increase with temperature or general blood acidosis, which universally accompanies elevated temperature across ectothermic vertebrates (Ultsch and Jackson, 1996). This is surprising in light of the fact that increased temperature and general blood acidification, are the classic, textbook examples of how the rate of O_2 supply to tissues can be increased by right-shifts of the OEC and increased $S_{a\text{-}v}$ (Barcroft and King, 1909; Bohr *et al.* 1904; Dejours, 1975; Berenbrink 2006, 2011a). Similarly, temperature-dependent differences in O_2 affinity between the HbI genotypes of Atlantic cod have been held crucial in the adaptation of this species to environmental temperature for more than 35 years (Karpov and Novikov, 1980; Andersen, 2012; Ross *et al*., 2013). The clear lack of both a temperature and HbI genotype effect on RBC O_2 affinity demonstrated in the present study, together with results from carefully controlled whole animal temperature preference studies (Gamperl *et al*., 2009), points to an emerging paradigm shift in our understanding of thermal adaptation of O_2 supply mechanism and the roles of HbI genotypes differences in Atlantic cod. In the following the results are critically evaluated and the underlying mechanisms and consequences for maximal circulatory O_2 supply rates of Atlantic cod at elevated temperatures are discussed.

Low O_2 binding affinity of Atlantic cod RBCs

The average P_{50} of Atlantic cod RBCs across the three genotypes was 40 mmHg (calculated from log P_{50} values between 5.0 and 12.5°C at pH 7.90 in Table 2.3). This value is among the lowest O_2-affinities that have been reported for blood or RBCs of any fish under the standardised conditions given above (e.g. Herbert *et al*., 2006). Such a low P_{50} results in arterial blood in gills lying on the edge of the steep part of the OEC, with modelled RBC O_2 saturations of no more than 80% at typical PO_2 and pH values and at any temperature between 5.0 and 20.0°C (Figure 2.4). This guarantees that across all temperatures, small decreases in venous PO_2 enable large increases in O_2 unloading in the tissues at a relatively high venous PO_2, which will safeguard a sufficiently large diffusion gradient from the blood plasma to tissue mitochondria. Blood O_2 tissue extraction [$S_{a\text{-}v}$ x $S_aO_2^{-1}$] was accordingly as high as 53%

for normoxic resting animals at pH 7.90 and 12.5°C (calculated from Figure 2.4A), which compares well with estimates in Atlantic cod *in vivo* under similar conditions (57%, Perry *et al*., 1991; 51%, Petersen and Gamperl, 2011). The high venous unloading PO_2 may be particularly important for cardiac O_2 supply in species like Atlantic cod, where the ventricle lacks a coronary blood supply and consists entirely of spongey myocardium that relies exclusively on the O_2 remaining in luminal blood that is returned from the other tissues (Santer and Walker, 1980; Farrell *et al*., 2012). However, too low a blood O_2 affinity comes at the cost of potentially reducing the efficiency of a further right-shift of the OEC for increasing S_{a-v} under, e.g., warming or general acidosis.

(In)efficiency of the Bohr effect in enhancing O_2 supply under general acidosis

The low O_2 affinity of Atlantic cod RBCs was paired with one of the largest Bohr effects reported for blood or RBCs under controlled standard conditions ($\Delta \log P_{50} (\Delta pH)^{-1} = -1.08 \pm 0.05$, pH 7.9 to 7.65 and 5.0 to 20.0°C). Low O_2 affinity and a strong Bohr effect were both previously reported for Atlantic cod whole blood (Herbert *et al*., 2006) and haemolysates in the presence of saturating ATP concentrations (Pörtner *et al*., 2001; Brix *et al*., 2004; Verde *et al*., 2006). Bohr *et al*. (1904) first emphasized the biological importance of elevated blood carbon dioxide partial pressures (PCO_2s) and thereby blood acidification for enhancing blood O_2 utilisation in the tissues, without affecting O_2 uptake at the higher PO_2 values in the respiratory organ. The present study surprisingly suggests that these generally accepted benefits of the Bohr effect are partially cancelled in Atlantic cod due to their low blood O_2 affinity, whereby any decrease in S_vO_2 during general acidosis is accompanied by a similar, or even larger decrease in S_aO_2, such that S_{a-v} remains the same or even decreases upon acidification (Figure 2.4). Thus, the unusually large effect of elevated CO_2 or low pH on Atlantic cod RBC O_2 binding affinity and capacity (Krogh & Leitch, 1919; Herbert *et al*., 2006; Berenbrink *et al*., 2011) will be mainly useful during localized tissue acidification, such as at the tissue poles of the vascular counter-current exchangers (*retia mirabilia*) in the eye and swim bladder of cod, where they are crucial for generating super-atmospheric PO_2 values

that support the high metabolic demands of the poorly vascularized retina, and for swim bladder gas filling against increasing hydrostatic pressures at depth (Bohr, 1894; Wittenberg and Wittenberg, 1962; Berenbrink *et al.*, 2005; Berenbrink, 2007).

These considerations do not negate the benefits of the Bohr effect in increasing S_{a-v} due to arterio-venous pH and PCO_2 differences. Instead they emphasize that parallel pH shifts in arterial and venous blood, such as during exercise induced lactacidosis or environmental warming, are unlikely to increase S_{a-v} in Atlantic cod at physiological P_aO_2 and minimal $P\bar{v}O_2$. Any increases in circulatory blood O_2 supply under these conditions must come from increases in cardiac output ($\dot{Q}$, Fick, 1870), blood O_2 capacity, or alternative mechanism that may increase S_{a-v}.

Reduced and reversed thermal sensitivity of O_2 binding in Atlantic cod RBCs

Whole body or local increases in temperature, such as in working muscle, are classically thought to increase blood O_2 transport by increasing S_{a-v} (Barcroft and King, 1909). In many animals the intrinsically exothermic nature of haem O_2 binding determines the overall heat of Hb oxygenation, resulting in a lowered Hb O_2 affinity at elevated temperature (Weber and Campbell, 2011). However, binding of allosteric effectors such as protons and ATP or GTP preferentially to deoxy Hb requires their endothermic release during oxygenation and this can compensate for the heat released by exothermic haem oxygenation, leading to a reduced or even reversed temperature sensitivity of Hb O_2 affinity. This is best known for heterothermic tuna, billfishes, and lamnid sharks, where exothermic Hb O_2 binding may cause problems in heat-conserving vascular counter-current exchangers (Weber & Campbell, 2011). The finding of largely thermally insensitive RBC O_2 affinity in Atlantic cod in this study, together with the study by Clark *et al.* (2010) on Pacific mackerel, suggests that low thermal sensitivity of RBC O_2 affinity may be more widespread among ectotherm fishes than previously thought.

Normally, with an overall exothermic reaction of Hb O_2 binding, increased temperatures decrease Hb O_2 affinity and cause a right-shift of the OEC. This will generally

allow an increase $S_{a\text{-}v}$ in any organism with S_aO_2 and P_aO_2 in the flat upper part of the OEC because a decrease in S_vO_2 allows of a greater exploitation of the venous reserve. However, for a species with a RBC O_2 affinity as low as reported for Atlantic cod in the present study, any gain in O_2 offloading by a decrease in S_vO_2 will be obliterated by a parallel decrease in S_aO_2 at typical P_aO_2. This may be the ultimate, evolutionary driving cause behind the reduced thermal sensitivity of O_2 binding in Atlantic cod RBCs.

The proximate, mechanistic explanation for the phenomenon may involve at least two not necessarily exclusive factors. First, the large Bohr effect suggests an above average increase in the number of proton binding sites in deoxyHb compared to oxyHb (for review see Berenbrink, 2006, 2011a). The release of these protons during oxygenation may compensate for exothermic haem O_2 binding. This is supported by the strong effect of pH on the overall enthalpy of RBC oxygenation over the whole temperature range (Figure 2.3). Second, the increase in cooperativity of RBC O_2 binding with temperature at low pH (Table 3) suggests that the over-stabilisation of deoxy Hb by the Root-effect (with $n_H \leq 1$; see Berenbrink, 2011b) is weakened at higher temperatures, where increasing values of n_H indicate an endothermic transition to the oxy conformation of Hb. This is consistent with previous work demonstrating the large endothermic nature of the deoxy to oxy Hb conformational transition in teleosts (Saffran and Gibson, 1979). In addition, the endothermic release of the organic phosphate modulators ATP and GTP from deoxy Hb upon oxygenation may contribute to the overall heat of oxygenation of Atlantic cod RBCs, a mechanism that has previously been shown to contribute to the reduced and reversed oxygenation enthalpy of several species of billfish (Weber *et al*., 2010). However, elucidation of the detailed molecular mechanism(s) behind reduced or even reversed thermal sensitivity of Atlantic cod RBC O_2 affinity awaits detailed studies on purified Hbs under tightly controlled conditions of allosteric modifiers.

Lack of HbI genotype effects on O_2 binding in Atlantic cod RBCs

The increased frequency of the HbI 1 allele towards the Southern range of Atlantic cod has been widely related to a parallel cline in environmental temperature and to a presumed

advantage of HbI 1/1 cod in having a higher RBC O_2 affinity at temperatures above 15°C compared to HbI 2/2 cod where this is higher below 15°C (e.g., Karpov & Novikov, 1980; Andersen *et al*., 2009; reviewed by Andersen, 2012, and Ross *et al*., 2013). The current study establishes the absence of any statistically supported differences in the RBC O_2 binding characteristics between Atlantic cod of all three HbI genotypes near their southern upper thermal distribution limit. This result has been consistently obtained over a range of pH values at each of three physiologically relevant temperatures and is considered robust, because factors well known to modify the genetically determined, intrinsic O_2 binding affinity of Hb inside RBCs have carefully been controlled. To ensure environmental relevance but at the same time minimise differences in prior thermal or hypoxic acclimatization of individuals, RBCs were obtained immediately after capture from wild Atlantic cod at a single location and over a 6 week period in winter where long term annual water temperature changes were minimal and stratification was absent (Neat *et al*. 2014; O'Boyle & Nolan, 2010). In contrast to earlier studies (Karpov and Novikov, 1980; Gollock *et al*., 2006; Petersen and Gamperl, 2011) RBCs were washed in glucose-containing physiological saline and incubated overnight before experimentation. This removes any catecholamine hormones, which are known to be released into plasma during blood sampling stress and modify the concentration of intracellular allosteric modifiers of Hb O_2 binding, and allows, any catecholamine-initiated effects to wear off during pre-incubation in standardised physiological saline (Berenbrink and Bridges, 1994a, b). This ensures equilibration of extra and intracellular ion concentrations and well defined RBC extracellular and intracellular pH values (Berenbrink and Bridges, 1994a, b) and resulted in comparable RBC intracellular Hb and nucleotide triphosphate concentrations HbI genotypes that were similar to values in fresh whole blood (Table 2.1; Leray, 1982). Extreme dilution of RBCs (Hct 0.08-0.13%) in buffered physiological saline ensured full control of RBC extracellular pH and ion composition during the actual OEC measurements and avoided the need for correction of points on the OECs to constant pH, which may otherwise vary by more than 0.1 pH units with oxygenation status in Atlantic cod whole blood *in vitro* (Herbert *et al*., 2006). Extreme dilution also avoided potential problems with RBC O_2 consumption that

may have been behind O_2 contents of zero at PO_2 values of 15 mmHg in OECs obtained at high Hct with a gasometric method (Gollock *et al*., 2006; Petersen and Gamperl, 2011). Full spectrophotometric assessment of RBC O_2 saturation between 500 and 700 nm in the present study also avoided having to assume full RBC O_2 saturation at some arbitrary high PO_2 which may have led to a systematic overestimation of O_2 saturation and affinity in some previous studies (Karpov and Novikov, 1980; Gollock *et al*., 2006; Herbert *et al*., 2006; Petersen and Gamperl, 2011). Finally, 5-6 specimens per HbI genotype were used to reduce outlier effects in the interpretation of results. Together this makes the present study the most comprehensive test yet for HbI genotype differences in RBC O_2 binding properties. The negative finding in this study raises the question what other characteristic(s), if any, of the different HbI alleles is behind the documented differences in geographical distribution, growth rates, hypoxia tolerance, and preference temperature (reviewed by Andersen, 2012; Ross *et al*., 2013)?

Possible reasons for the variability of HbI genotype effects

In theory, genetic differences in the intrinsic O_2 binding characteristic between the Hb genotypes, or genetic differences in their interactions with allosteric modulators, could have been masked by the large phenotypic plasticity in Hb O_2 binding properties of ectotherms (Weber and Jensen, 1988). However, the difficulties in fully reproducing in purified haemolysates the genotype effects found in RBCs by Karpov and Novikov (1980) by others (Brix *et al*. 1998; Colosimo *et al*., 2003; Brix *et al.*, 2004) suggest that the adaptive value of different Hb genotypes on O_2 supply rates in different environments may have been overemphasized, as discussed by Gamperl *et al.* (2009). Alternatively, natural selection may act on a different life history stage than the juveniles or adults that are most commonly studied. For example, unfertilised eggs of Atlantic cod have been shown to contain transcripts of all four major adult expressed globins, including the β1 globin responsible for the HbI polymorphism (Wetten *et al*., 2010). The functional relevance of these gene products, by necessity of maternal origin, is unclear and transcripts disappear upon fertilisation in the embryonic stages until expression is switched on again later in juveniles and adults (Wetten

et al., 2010). However, if the maternal HbI genotype in eggs affects their fertilisation success, than this may explain the significantly skewed HbI genotype ratios in offspring of heterozygote parents that was observed by Gamperl *et al*. (2009) and was later in life balanced by significantly higher growth rates of the underrepresented genotype. Thus, differing costs and benefits during different life history stages and/or in different micro environments may lead to balanced HbI polymorphisms that differ in HbI 1 frequencies across the distributional range.

In addition, the HbI polymorphism may be genetically linked to other traits that are under selection, such as the regulatory polymorphism of the HbI promoter in Atlantic cod (Star *et al*., 2011; Andersen, 2012) that may be responsible for HbI genotype-associated differences in Hct and Hb concentration observed in some studies (Mork and Sundnes, 1984). Clearly our understanding of molecular mechanisms enabling adaptation of marine ectotherms to environmental temperature change is just at the beginning and more studies linking the genetics, physiology, ecology and evolution of these organisms are required.

Concluding remarks on physiological consequence of Atlantic cod RBC O_2 binding characteristics

Atlantic cod are regularly exposed to acute temperature shifts in their natural environments, similar to those employed in the present study, e.g. during upwelling and turbulent mixing events of water bodies with different temperatures (Freitas *et al.* 2015), or when crossing the thermocline (Righton *et al.*, 2010). The latter is particularly relevant for Irish Sea cod that continue actively changing depth during the warmer summer months, compared to North Sea cod that remain confined in bottom waters throughout June to September (Righton *et al*., 2001; Righton and Metcalfe, 2002). Our modelling approach suggests that during acute warming the O_2 binding characteristics of Atlantic cod RBC will enable uncompromised gill O_2 loading at *in vivo* arterial PO_2 values and at the same time permit increased O_2 offloading at falling venous PO_2. However, the theoretical maximal $S_{a\text{-}v}$ at physiological pH and arterial and venous PO_2 does not increase with temperature (Figure 2.4 D-F), and is already reached under

conditions of acute gradual warming to 20^0C (Figure 2.4 C, F). Under these conditions Atlantic cod can only further increase the capacity of their circulatory O_2 transport system by increasing blood O_2 capacity and/or cardiac output. However, in a complex network of feedback systems an increase in cardiac output may itself be limited, firstly by low PO_2 of cardiac luminal blood returning from systemic tissues, secondly by an increased cardiac workload and thus O_2 demand imposed by the higher viscosity of blood with an increased RBC number, and lastly by O_2-supply-independent physiological and anatomical limits to cardiac performance such as maximal heart rate and ventricle size, respectively. Ultimately, when all these avenues to increased blood O_2 transport rate are exhausted, long term preservations of aerobic scope for activity at elevated temperature may rely on the extent to which standard metabolic rate can be reduced by thermal acclimatisation.

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Chapter 3. Comparative study of the effect of pH sensitivity on thermal sensitivity of red blood cell oxygen binding

3.1 Abstract

Until recently, heterothermic fish were believed to be unique among teleosts due to the development of a markedly reduced, or even reversed, temperature dependence of blood–oxygen (blood–O_2) binding. The molecular basis of this phenomenon can likely be attributed to the endothermic dissociation of allosteric effectors upon oxygenation, counteracting the heat released upon the binding of oxygen to haem. Recent evidence in chub mackerel and Atlantic cod suggest these unusual binding properties to reduce or reverse thermal sensitivity of oxygen binding may be more widespread than in heterothermic fish. Accordingly, this study examined oxygenation of red blood cells (RBCs) in six fish species at a range of pH and temperatures and investigate the influence of one allosteric effector, H^+. A lack of thermal sensitivity of RBC oxygen binding was observed in two tested species and reduced thermal sensitivity was found in a further two. A strong inverse correlation was observed between the temperature sensitivity and pH sensitivity of oxygen binding in RBCs, with a regression coefficient of 0.88. This confirms that the occurrence of thermal insensitive oxygen binding is not exclusive to heterothermic fish and as previously suggested the mechanism behind this is linked to at least one allosteric modulator.

3.2 Introduction

The provision of oxygen is vital to ensure survival in most organisms. Haemoglobin (Hb) is the principle molecule responsible for transporting oxygen from the respiratory surfaces to the respiring tissues in vertebrates. The ability of Hb to uptake oxygen or its oxygen affinity, is modulated by a number of factors, including but not limited to; oxygen availability, interactions with allosteric effectors and temperature. The latter is particularly important in ectothermic animals.

The oxygenation of the haem is exothermic nature, this results in a reduction in oxygen affinity with increasing temperatures and is characterised by a negative oxygenation

enthalpy, ΔH^{O2} (Weber and Campbell, 2011). This is generally considered to be advantageous as in warm tissues, such as working muscles, O_2 unloading from the blood is enhanced and so can compensate for increased requirements (Barcroft and King, 1909). However, in regional heterothermic or endothermic fish, the decrease in O_2 affinity with increased temperature may be problematic, differences in temperatures between tissues and respiratory surface may result in disparity in O_2 delivery and demand. A number of tuna, sharks and billfish have warm swimming muscles and/or warm eyes and brains (Carey *et al*., 1971; Carey and Gibson, 1977; Block and Carey, 1985; Block, 1986; Larsen *et al*., 2003) supported by the countercurrent heat-exchangers systems (Larsen *et al*., 2003). Typical decrease in Hb-O_2 affinity with increasing temperatures may cause abrupt O_2 unloading from blood perfusing the warm, red swimming muscles while unloading may be inhibited in cold organs such as the liver and gut (Clark *et al*., 2008; Weber and Campbell, 2011). Interestingly, previous studies have demonstrated haemoglobin–oxygen (Hb–O_2) binding properties of tuna respond differently to temperature. In Atlantic bluefin tuna (*Thunnus thynnus*) Hb–O_2 affinity was essentially independent of temperature in purified haemolysates (Rossi-Fanelli and Antonini, 1960). This temperature insensitivity was also documented in other tuna species in whole blood and a reversed temperature dependence (where an increase in temperature increases Hb–O_2 affinity) has also been discovered (Cech *et al*. 1984; Jones *et al*. 1986; Brill and Bushnell 1991; Lowe *et al*. 2000; Brill and Bushnell 2006; Clark *et al*. 2008). The molecular basis for the reduced and reversed temperature dependence of Hb–O_2 binding is likely attributed, at least in part, to the counteractive effects of the endothermic dissociation of organic phosphates (NTPs), chloride ions (Cl^-) and protons (H^+) (Wood, 1980; Weber and Jensen, 1988; Larsen *et al*., 2003; Weber and Fago, 2008; Rasmussen *et al*., 2009). These molecules preferentially bind to deoxy Hb, the input of heat required during the bond breaking process can compensate for the heat released by exothermic haem oxygenation. Thus the overall oxygenation enthalpy in blood ($\Delta H'$) includes these endothermic contributions and so is close to zero or positive in these species.

The finding of largely thermally insensitive O_2 binding in red blood cells (RBCs) of Atlantic cod (*Gadus morhua*) in a previous study (Chapter 2), together with the study by Clark *et al.* (2010) in the blood of chub mackerel (*Scomber japonicus*), suggests that low temperature sensitivity of O_2 affinity is not uniquely associated with heterothermy. This study assesses the thermal sensitivity of RBC in six non- heterothermic fish species; Atlantic cod, burbot (*Lota lota*), lesser spotted dogfish (*Scyliorhinus canicula*), Nile tilapia (*Oreochromis niloticus*), North African catfish (*Clarias gariepinus*) and rainbow trout (*Oncorhynchus mykiss*). These species were selected to represent a phylogenetically diverse group, as well as varying habitats and energetic demands.

As explained, allosteric effectors are believed to be at least partially responsible for the unusual binding properties in heterothermic fish. In tuna species, H^+ appears to be particularly influential and a strong inverse correlation has been shown between temperature sensitivity and pH sensitivity, or the Bohr effect (Cech *et al*., 1984; Brill and Bushnell 1991; Lowe *et al*., 2000). Extremely high Bohr effects are exhibited in both cod and mackerel, and suggest H^+ ions are also potentially responsible for reduced temperature sensitivity of oxygen affinity in these species. As such this study will explore the influence of pH in conjunction with temperature on RBC oxygen binding, to our knowledge this has never been performed in non-heterothermic teleosts.

3.3 Materials and Methods

Animals and blood sampling

Atlantic cod were caught by hook and line method from the Mersey estuary, Merseyside, U.K, between December 2010 and February 2011 (mean standard length 28.7 ± 0.8 cm (S.D.)) and mean mass 245.6 ± 26.6 g, $N = 8$). These were kept at the University of Liverpool for several weeks in a 1000 litre tank equipped with a biological filter and aerated, recirculated artificial seawater kept at a temperature range of 11-13°C. Rainbow trout were similarly housed in a fresh water system. Lesser spotted dogfish (mean ± stdev total length, cm; weight, g; and number of individuals; 56 ± 3; 727 ± 125; 6) were kept at the University of Liverpool for

several weeks in a 1000 litre tank equipped with a biological filter and aerated, recirculated artificial seawater kept at a temperature range of 11-13°C. Nile tilapia (23 ± 3; 219 ± 64; 5), and North African catfish (35 ± 5; 277 ± 88; 5) were kept individually in 80-200 litre tanks at approximately 28°C. Burbot (size unknown, but all classified as adults, $N = 12$) were captured by hook and line under ice near Oslo, Norway during January 2014.

The fish were netted with minimal stress and killed by a British Home Office Schedule 1 method, concussion and destruction of brain. Blood was removed by puncturing of the caudal vessels with a 1 ml syringe, whose dead space was filled with 50 mg ml^{-1} of heparin solution (180 units/mg, sodium salt from porcine intestinal mucosa, Sigma-Aldrich). Samples were kept on ice and RBCs were isolated by centrifugation (3000 rcf, 4°C, 4 mins) and washed in an isotonic saline solution within an hour of extraction.

Saline recipe varied between species to account for different plasma osmolality and blood components. For all teleosts, saline recipe contained the same basic components (all concentrations given in mmol l^{-1}) NaCl (125.5 or 145), KCl (3), $MgCl_2$ (1.5), $CaCl_2$ (1.5), D-glucose (5) and Hepes (20) (as used by Koldkjaer and Berenbrink, 2007). NaCl concentration was varied to adjust saline osmolality depending on plasma osmolality in each species, this was determined using a sample of supernatant from first centrifugation using an Advanced instruments 3MO Plus micro osmometer. Dogfish saline recipe: NaCl (253.6), KCl (4), $MgCl_2$ (1), $CaCl_2$ (4.6) Urea (400), TMAO (80), Hepes (20) and D-glusoces (5).

All salines were adjusted to pH 7.97 at 15°C. RBC washing was repeated a further two times before the cells were re-suspended at an approximate haematocrit of 5-10 % and stored overnight at 4°C in a 15 ml falcon tube with a large air reservoir, placed on the side to maximise exchange surface area between saline and sedimented cells. Prior to experiments the following day, the wash step was repeated and cells resuspended in fresh 7.97 saline to approximately 10% haematocrit.

Oxygen affinity determination

The RBC suspension was then diluted 10 fold in 7.97 saline and a further 10 fold in saline of pH 7.45, 7.70 or 7.97 (all adjusted at 15°C). For each individual, RBCs were incubated, at the three pHs in parallel (two pHs for burbot and rainbow trout), in Eschweiler tonometers (Eschweiler GmbH, Engelsdorf, Germany) with custom attached quartz cuvettes, this was repeated at three temperatures (two for burbot) and a minimum of five oxygen tensions. Experimental temperatures used were determined by acclimation temperature ± 7.5°C, thus for cod and dogfish RBCs were exposed to 5.0, 12.5 and 20.0°C and for tilapia and African catfish 20.0, 27.5 and 35.0°C. Exceptions to this were burbot, where only 5.0 and 12.5°C were used, and rainbow trout, where 5.0, 15.0 and 20.0°C were used. Air and N_2 were mixed in pre-determined ratios using a Wosthoff gas mixing pump (Wosthoff GmbH, Bochum, Germany) and the final gas mixture fully humidified at the experimental temperature. RBC suspensions were equilibrated for at least 20 minutes with each gas mixture. Solutions remained sealed within the tonometer to ensure PO_2 remained constant while a spectrum was taken from 500-700 nm in 1 nm steps using a Unicam UV 500 spectrometer (Thermo Electron Corporation, Ohio, USA) and using Vision 32 Software. Oxygen saturation of RBC suspensions was determined by spectral deconvolution (Völkel and Berenbrink, 2000).

pH of the saline solutions without RBCs was measured using a Lazar Model FTPH-2S Micro Flow Through pH Measurement Electrode and Jenco 6230N combined pH/mV/temperature portable meter (Jenco Collaborative, California, USA) at each temperature under air, this was used to account for thermal induced pH change of the salines. Given the buffering properties of the saline and small quantity of cells it was assumed differences in oxygen saturation would have limited effect upon saline pH.

Data analysis and statistics

Spectral deconvolution of the optical spectra (see Volkel and Berenbrink, 2000) was used to determine the composition of haemoglobin derivatives within RBC suspensions (oxyhaemoglobin, HbO_2; deoxyhaemoglobin, deoxyHb; and the two forms of

methaemoglobin, acid Hb^+ and alkaline Hb^+) at each temperature, pH and PO_2 value using SigmaPlot 12.5 software (Jandel Scientific, San Rafael, CA, USA). The unknown concentrations (mmol l^{-1}) of the different Hb derivatives were calculated using $f = au + bv + cw + dx$, they were represented by letters *a-d* and were restricted to values greater than zero. *f* is the predicted dependent variable to be fitted to the measured absorption data for each nm step between 500 and 700 nm and *u*, *y*, *w* and *x* represent the experimentally determined absorption coefficients for each species. For each species absorption coefficients for the HbO_2 and deoxyHb were created with Hb suspensions in pH 8.05 saline at 5.0°C, exposed to 100% oxygen or 100% nitrogen. Acid Hb^+ and alkaline Hb^+ templates were constructed using Hb suspensions at pH 6.5 and 8.05 respectively, although neither (Hb^+) was seen in any of our samples. The predicted values were plotted with the measured spectra to confirm the accuracy of the prediction.

The level of RBC O_2 saturation (*S*) was calculated as $[HbO_2]/ ([HbO_2] + [deoxyHb])$. Hill plots on data between 20 and 80% saturation were created using log (*S*/(1-*S*)) versus log PO_2. Log P_{50} was calculated by linear regression as the log PO_2 when log (*S*/(1-*S*)) equalled 0. The slope of the regression line indicated the apparent cooperativity of RBC O_2 binding or Hill number (n_H). The Bohr coefficient was calculated by $\Phi = \Delta\log P_{50}/ \Delta pH$ for each pH interval. Because of nonlinearity, at each temperature, log P_{50} and n_H were plotted against measured saline pH and 2nd order polynomials were used to standardise them to pH 7.40, 7.65 and 7.9, removing the effect of temperature-induced pH shifts on these variables. Once standardised to fixed pH, thermal sensitivities of OECs were expressed as apparent heat of oxygenation, ΔH'. These were calculated using the van't Hoff equation $\Delta H' = 2.303\ R\ ((\Delta\log P_{50})/(\Delta 1/T))$, where *R* = universal gas constant (0.008314 kJ K^{-1} mol^{-1}), and *T* = temperature in K.

Comparisons between data were conducted using two-way ANOVA, with Tukey post-hoc test (unless stated otherwise). Statistics were performed on log P_{50}, *n*H, Bohr coefficient and ΔH' for each species independently, accounting for pH and temperature.

Comparisons on these parameters between species were performed only at the relevant acclimation temperature. All results are given as means ± standard error of the mean (SEM), unless otherwise stated.

3.4 Results

Oxygen equilibrium curves (OECs) in Figure 3.1 show marked differences between the six studied species and reflect differences in P_{50}, pH and temperature sensitivity. While the magnitude of response to pH and temperature indeed differ, two general trends emerge. 1) Decreasing pH appeared to reduce O_2 affinity, shifting OECs to the right and increasing P_{50}, and 2) increasing temperatures had a similar effect. However, the temperature effects will have been partially due to the temperature-induced shifts in the pH of the HEPES buffer (Figure 3.1), this must be corrected for prior to additional analysis.

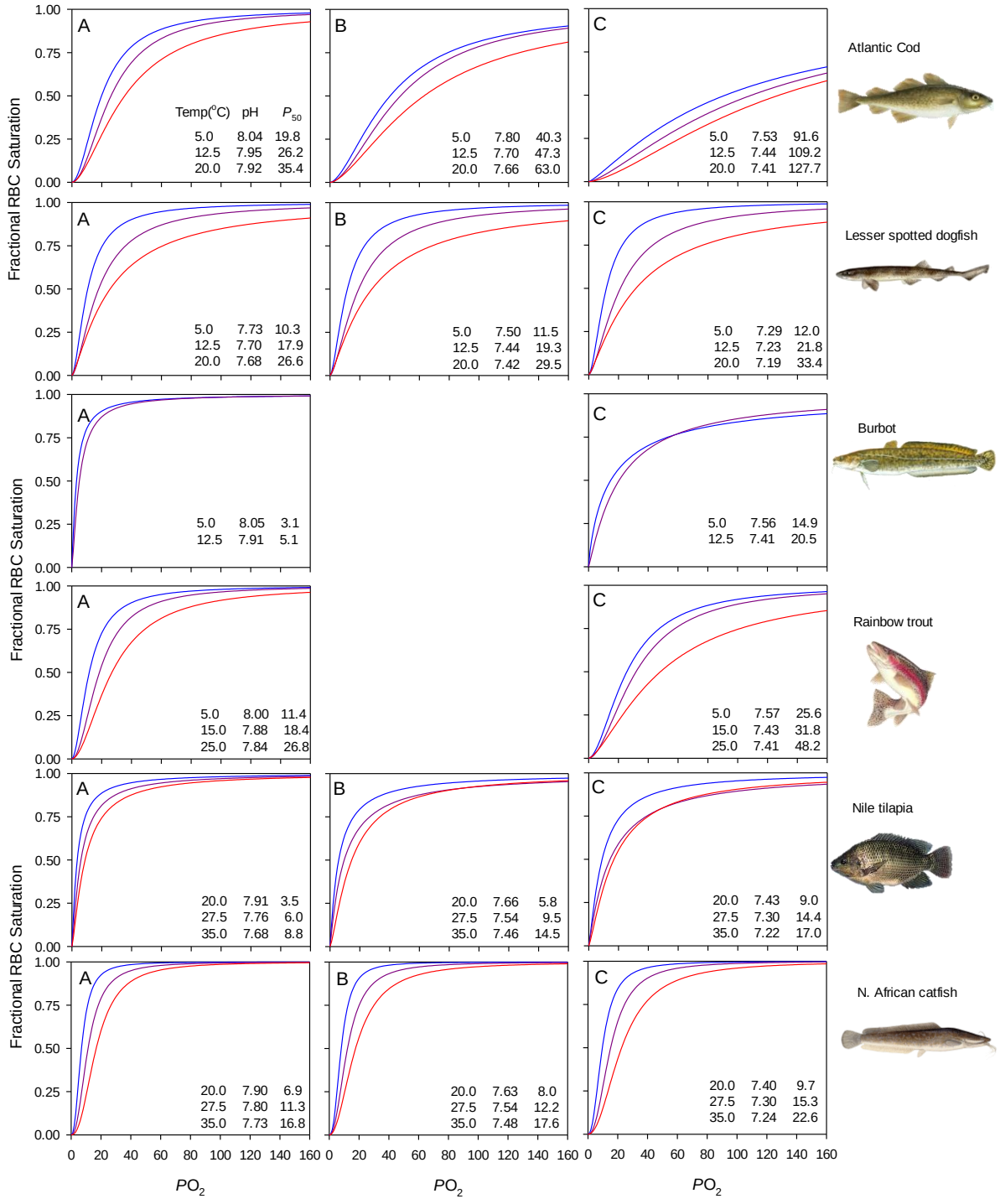


Figure 3.1: *Effects of temperature and pH on oxygen equilibration curves for six species: Atlantic cod (N = 8), lesser spotted dogfish (N = 6), Nile tilapia (N = 5), rainbow trout (N = 7), burbot (N = 6) and North African catfish (N = 5). Mean fractional saturation as determined from measured values at a minimum of five PO_2 (mmHg) values. Each rows of graphs represents one species (named and pictured to the right), each panel within the row shows data from nominal pH; A) 7.9, B) 7.65 and C) 7.4, and within each panel each line*

represents OECs at experimental temperatures (coldest- blue line, mid temperature- purple, high temperature- red). Mean measured pH values at each temperature are shown in inset along with mean P_{50} (mmHg).

At standardised pH and individual acclimation temperatures there was no significant difference in terms of log P_{50} and hence Hb-O_2 affinity across the species, with the exception of Atlantic cod. Cod RBCs had significantly higher log P_{50} (1.46 ± 0.04; at pH 7.90 and acclimation temperature, P_{50} = 29 mmHg) than all other species but Rainbow trout (1.25 ± 0.02; 18 mmHg) (Figure 3.2, Table 3.1).

At all temperatures for all species decreasing pH significantly decreases oxygen affinity (increasing log P_{50}) (Table 3.1). The Bohr plots of Atlantic cod and burbot (Figure 3.2) show a very strong pH effect on log P_{50}, a less pronounced effect was observed in Nile tilapia and Rainbow trout. In the lesser spotted dogfish and North African catfish pH had a much reduced effect on Hb-O_2 affinity. In dogfish this effect was only apparent at the lowest temperature, whereby log P_{50} at pH 7.90 (0.96 ± 0.02) was significantly lower than log P_{50} at 7.65 and 7.40 (1.03 ± 0.02 and 1.07 ± 0.01, respectively). Catfish log P_{50} also varied significantly with pH in the typical manner at 20.0 and 27.5°C, with significant differences at all pHs at the lowest temperature and log P_{50} at pH 7.40 significantly higher than at pH 7.65 and 7.90 at 27.5°C (Table 3.1).

These differences in pH sensitivity across the species are reflected in differences in the Bohr coefficient. Interestingly, there was no significant difference in Bohr coefficients between cod (-1.29; mean across all pH at acclimation temperature) and burbot (-1.47); trout (-0.54) and tilapia (-0.89); and catfish (-0.16) and dogfish (-0.17). However, beyond these pairings Bohr coefficients varied significantly across all species (data not shown).

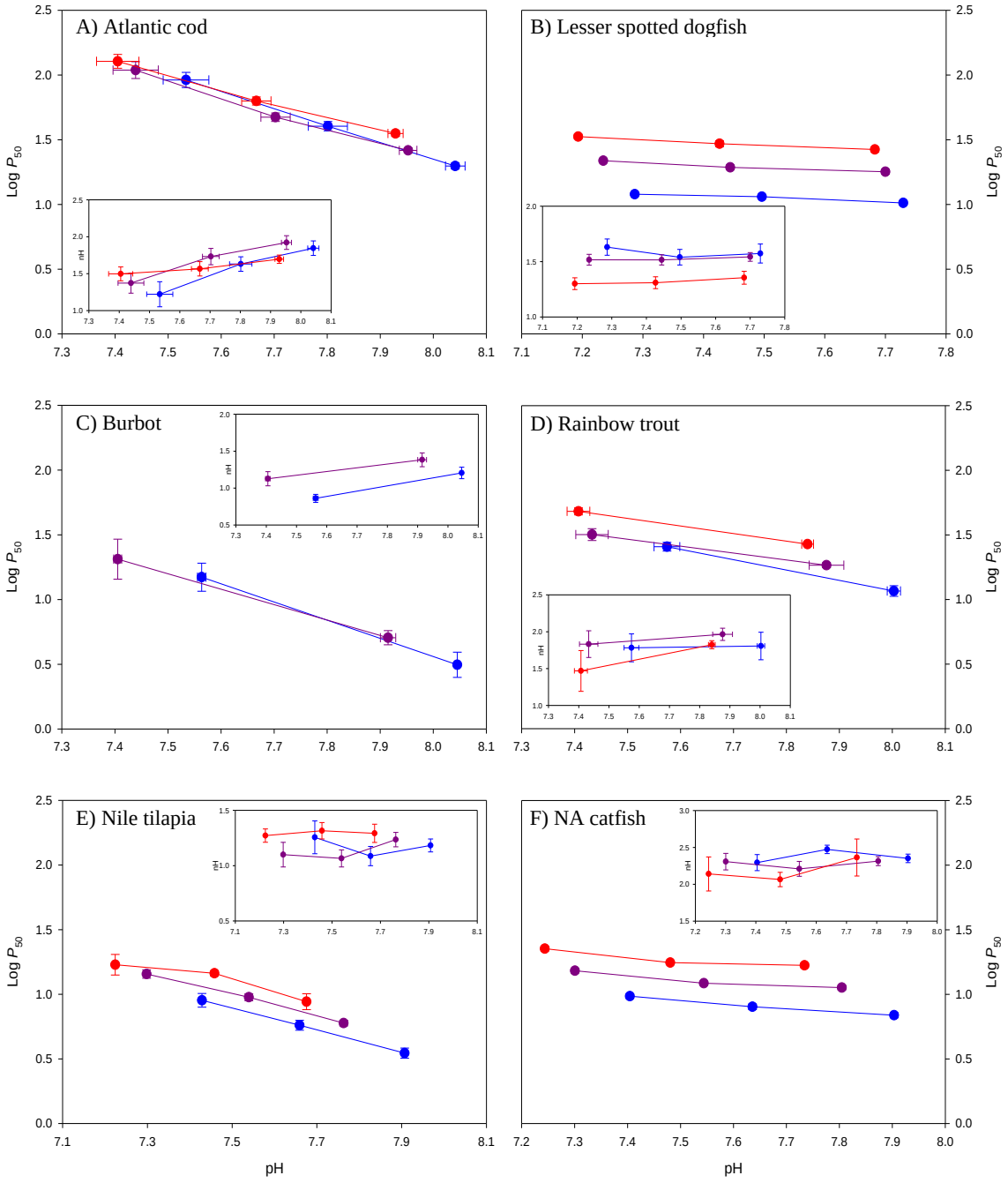


Figure 3.2: *Effect of pH and temperature on the affinity and cooperativity of O_2 binding in red blood cells of six fish species. Log P_{50} (mean ± SE) versus pH for A) Atlantic cod (N = 8), B) lesser spotted dogfish (6) at 5.0, 12.5 and 20.0°C; C) burbot (6) at 5.0 and 12.5°C; D) rainbow trout (7) at 5.0, 15.0 and 25.0°C; E) Nile tilapia (5) and F) North African catfish (5) and 20.0, 27.5 and 35.0°C (blue, purple and red symbols and lines represent cold, middle and warmest experimental temperatures respectively). Insets display n_H, Hill's cooperativity coefficient at 50% RBC O_2 saturation (mean ± SE), for the same species and data.*

Table 3.1: *Log P_{50} (mmHg), Bohr coefficient ($\Delta \log P_{50} / \Delta pH$) and n_H (co-operativity at 50 % saturation) corrected for pH change with temperature, of red blood cells of six fish species, when exposed to a range of temperatures (°C) and pH values (mean ± SE). For log P_{50} and n_H different superscript letters indicate significant differences between pHs and different subscript numbers indicate significant differences in temperature, for each species. For Bohr coefficient different superscript letters indicate significant differences between both temperatures and pH range in each species (Two-way ANOVA with temperature and pH/ 'pH range' as factors, followed by a post-hoc Tukey test). No letters/ numbers indicate no significant difference was found in that species for the relevant parameters.*

Species	*N*	Temp (°C)	Log P_{50}			Bohr coefficient		n_H		
			7.90	**7.65**	**7.40**	**7.90-7.65**	**7.65-7.40**	**7.90**	**7.65**	**7.40**
		5.0	$1.46 \pm 0.04^{a}{}_{1}$	$1.78 \pm 0.04^{b}{}_{1}$	$2.15 \pm 0.05^{c}{}_{1}$	-1.30 ± 0.03^{a}	-1.47 ± 0.14^{b}	$1.77 \pm 0.07^{a}{}_{1}$	$1.45 \pm 0.10^{ab}{}_{1}$	$1.01 \pm 0.25^{b}{}_{1}$
Atlantic cod	8	12.5	$1.46 \pm 0.03^{a}{}_{1}$	$1.74 \pm 0.03^{b}{}_{1}$	$2.11 \pm 0.05^{c}{}_{1}$	-1.11 ± 0.03^{a}	-1.47 ± 0.09^{b}	$1.88 \pm 0.06^{a}{}_{1}$	$1.71 \pm 0.10^{a}{}_{1}$	$1.41 \pm 0.19^{a}{}_{12}$
		20.0	$1.57 \pm 0.03^{a}{}_{1}$	$1.81 \pm 0.02^{b}{}_{1}$	$2.11 \pm 0.03^{c}{}_{1}$	-0.97 ± 0.03^{a}	-1.18 ± 0.05^{a}	$1.66 \pm 0.08^{a}{}_{1}$	$1.60 \pm 0.08^{a}{}_{1}$	$1.63 \pm 0.16^{a}{}_{2}$
		5.0	$0.96 \pm 0.02^{a}{}_{1}$	$1.03 \pm 0.02^{b}{}_{1}$	$1.07 \pm 0.01^{b}{}_{1}$	-0.26 ± 0.04	-0.18 ± 0.02	1.69 ± 0.16	1.55 ± 0.07	1.57 ± 0.07
Lesser spotted dogfish	6	12.5	$1.25 \pm 0.02^{a}{}_{2}$	$1.26 \pm 0.02^{a}{}_{2}$	$1.30 \pm 0.02^{a}{}_{2}$	-0.13 ± 0.06	-0.20 ± 0.03	1.59 ± 0.09	1.53 ± 0.04	1.51 ± 0.05
		20.0	$1.40 \pm 0.01^{a}{}_{3}$	$1.43 \pm 0.02^{a}{}_{3}$	$1.47 \pm 0.02^{a}{}_{3}$	-0.17 ± 0.05	-0.21 ± 0.02	1.42 ± 0.08	1.35 ± 0.06	1.31 ± 0.05
Burbot	6	5.0	$0.70 \pm 0.09^{a}{}_{1}$	$1.05 \pm 0.11^{b}{}_{1}$	$1.40 \pm 0.13^{c}{}_{1}$	-1.41 ± 0.18*		$1.10 \pm 0.06^{a}{}_{1}$	$0.92 \pm 0.05^{ab}{}_{1}$	$0.75 \pm 0.07^{b}{}_{1}$
		12.5	$0.73 \pm 0.07^{a}{}_{1}$	$1.03 \pm 0.11^{b}{}_{1}$	$1.32 \pm 0.16^{c}{}_{1}$	-1.20 ± 0.21*		$1.38 \pm 0.10^{a}{}_{2}$	$1.25 \pm 0.09^{a}{}_{2}$	$1.12 \pm 0.10^{a}{}_{2}$
		5.0	$1.15 \pm 0.03^{a}{}_{1}$	$1.35 \pm 0.03^{b}{}_{1}$	$1.55 \pm 0.05^{c}{}_{1}$	$-0.81 \pm 0.08^{*a}$		1.82 ± 0.13	1.75 ± 0.14	1.69 ± 0.28
Rainbow trout	7	15.0	$1.25 \pm 0.02^{a}{}_{2}$	$1.38 \pm 0.03^{b}{}_{1}$	$1.52 \pm 0.05^{c}{}_{1}$	$-0.54 \pm 0.09^{*b}$		1.98 ± 0.09	1.89 ± 0.12	1.81 ± 0.19
		25.0	$1.39 \pm 0.02^{a}{}_{3}$	$1.54 \pm 0.02^{b}{}_{2}$	$1.69 \pm 0.03^{c}{}_{2}$	$-0.60 \pm 0.07^{*ab}$		1.90 ± 0.07	1.67 ± 0.12	1.45 ± 0.26
		20.0	$0.55 \pm 0.04^{a}{}_{1}$	$0.77 \pm 0.04^{b}{}_{1}$	$0.98 \pm 0.06^{c}{}_{1}$	-0.87 ± 0.07	-0.84 ± 0.14	1.18 ± 0.06	1.09 ± 0.09	1.29 ± 0.16
Nile tilapia	5	27.5	$0.64 \pm 0.04^{a}{}_{12}$	$0.88 \pm 0.02^{b}{}_{12}$	$1.09 \pm 0.03^{c}{}_{1}$	-0.97 ± 0.18	-0.81 ± 0.08	1.44 ± 0.09	1.13 ± 0.07	1.06 ± 0.09
		35.0	$0.77 \pm 0.05^{a}{}_{2}$	$0.96 \pm 0.06^{b}{}_{2}$	$1.13 \pm 0.07^{c}{}_{1}$	-0.78 ± 0.11	-0.67 ± 0.09	1.20 ± 0.16	1.30 ± 0.08	1.31 ± 0.07
		20.0	$0.84 \pm 0.02^{a}{}_{1}$	$0.90 \pm 0.02^{b}{}_{1}$	$0.99 \pm 0.02^{c}{}_{1}$	-0.24 ± 0.02^{a}	-0.35 ± 0.03^{b}	2.36 ± 0.06	2.48 ± 0.06	2.29 ± 0.11
North African catfish	5	27.5	$1.06 \pm 0.02^{a}{}_{2}$	$1.06 \pm 0.01^{a}{}_{2}$	$1.14 \pm 0.02^{b}{}_{2}$	-0.02 ± 0.04^{a}	-0.29 ± 0.01^{b}	2.41 ± 0.10	2.23 ± 0.09	2.24 ± 0.10
		35.0	$1.26 \pm 0.03^{a}{}_{3}$	$1.22 \pm 0.01^{a}{}_{3}$	$1.27 \pm 0.01^{a}{}_{3}$	0.17 ± 0.11^{a}	-0.21 ± 0.03^{b}	2.77 ± 0.59	2.22 ± 0.15	2.05 ± 0.11

*Only two saline pHs used nominally '7.9' and '7.4', Bohr coefficient taken over whole range.

Table 3.2: *The effect of temperature and pH on the apparent heat of oxygenation (ΔH') for red blood cells of six fish species. Underline values indicate endothermic oxygen binding. P-values indicate ΔH' significance from zero (Students T-test) with non-significant values (no temperature sensitivity) underlined.*

	Temperature range (°C)	**ΔH (kJ mol^{-1})**			***P*- values**		
		7.90	**7.65**	**7.40**	**7.9**	**7.65**	**7.4**
	5.0 - 12.5	1.7 ± 3.9	6.0 ± 3.8	10.8 ± 4.0	0.776	0.136	0.025
Atlantic cod	12.5 - 20.0	-18.6 ± 4.9	-14.7 ± 2.0	-7.1 ± 3.1	0.001	0.001	0.114
	5.0 - 20.0	-8.2 ± 3.7	-4.1 ± 2.4	2.1 ± 2.0	0.008	0.122	0.349
	5.0 - 12.5	-48.9 ± 3.6	-47.9 ± 2.2	-49.5 ± 2.6	0.001	0.001	0.001
Lesser spotted dogfish	12.5 - 20.0	-38.4 ± 3.6	-40.1 ± 3.3	-40.4 ± 3.3	0.001	0.001	0.001
	5.0 - 20.0	-43.8 ± 2.6	-44.1 ± 2.0	-45.1 ± 1.6	0.001	0.001	0.001
Burbot	5.0 - 12.5	-5.2 ± 7.6	5.3 ± 18.3	15.6 ± 33.9	0.521	0.785	0.665
	5.0 - 15.0	-15.0 ± 4.6	-4.8 ± 2.7	5.4 ± 2.1	0.017	0.130	0.043
Rainbow trout	15.0 - 25.0	-23.5 ± 2.9	-25.9 ± 2.8	-28.3 ± 4.0	0.001	0.001	0.001
	5.0 - 25.0	-19.1 ± 2.9	-15.0 ± 1.7	-10.9 ± 1.4	0.001	0.001	0.001
	20.0 - 27.5	-26.9 ± 8.2	-24.7 ± 7.2	-22.4 ± 6.4	0.030	0.027	0.025
Nile Tilapia	27.5 - 35.0	-45.3 ± 6.3	-34.9 ± 6.0	-24.6 ± 5.8	0.007	0.014	0.032
	20.0 - 35.0	-37.4 ± 7.5	-30.6 ± 7.0	-23.9 ± 6.5	0.021	0.007	0.046
	20.0 - 27.5	-40.2 ± 1.5	-38.3 ± 1.2	-36.4 ± 1.0	0.001	0.001	0.001
N. African catfish	27.5 - 35.0	-38.3 ± 4.6	-35.6 ± 1.6	-32.5 ± 2.0	0.001	0.001	0.001
	20.0 - 35.0	-39.3 ± 2.6	-37.0 ± 1.2	-34.5 ± 1.4	0.001	0.001	0.001

Generally, as temperature increased log P_{50} also increased, indicating a reduction in oxygen affinity. However, this trend was only significant across all tested conditions for lesser spotted dogfish and North African catfish, both of which had highly temperature sensitive oxygen binding (Table 3.1). RBC oxygen binding in rainbow trout and Nile tilapia exhibited reduced thermal dependence under some conditions, with no significant variation of log P_{50} with temperature, particularly at low pH (Table 3.1). While log P_{50} in both Atlantic cod and burbot RBCs was unaffected by temperature at all pHs ($P < 0.001$, Table 3.1).

Indeed, thermally insensitive oxygen binding was observed in Atlantic cod, burbot and rainbow trout. This was determined when ΔH' did not vary significantly from zero ($P > 0.05$; Table 3.2). Furthermore, reversed thermal sensitivity was found in cod at pH 7.4 and in the low temperature range ($\Delta H' = 10.8 \pm 4.0$) this was determined to be significant from zero ($P = 0.025$) and thus was classified as endothermic oxygen binding (Table 3.2).

Correlation and regression analyses were conducted to examine the relationship between Bohr coefficient (pH sensitivity) and $\Delta H'$ (temperature sensitivity). These factors show an inverse relationship, whereby an increase in the magnitude of the Bohr coefficient results in a decrease in thermal sensitivity of oxygen binding (Figure 3.5) ($R^2 = 0.884$, $F = 25.34$, $P = 0.007$).

Under conditions of progressively low pH and temperature, n_H was significantly reduced in cod and burbot, but remained unchanged in all other species (Table 3.1). Burbot, exhibited very low cooperativity, with n_H around or occasionally below 1, this is reflected in the hyperbolic OECs (Figure 3.1). North African catfish had the highest level of cooperativity between subunits, with n_H values as high as 2.77 ± 0.59 (Table 3.1).

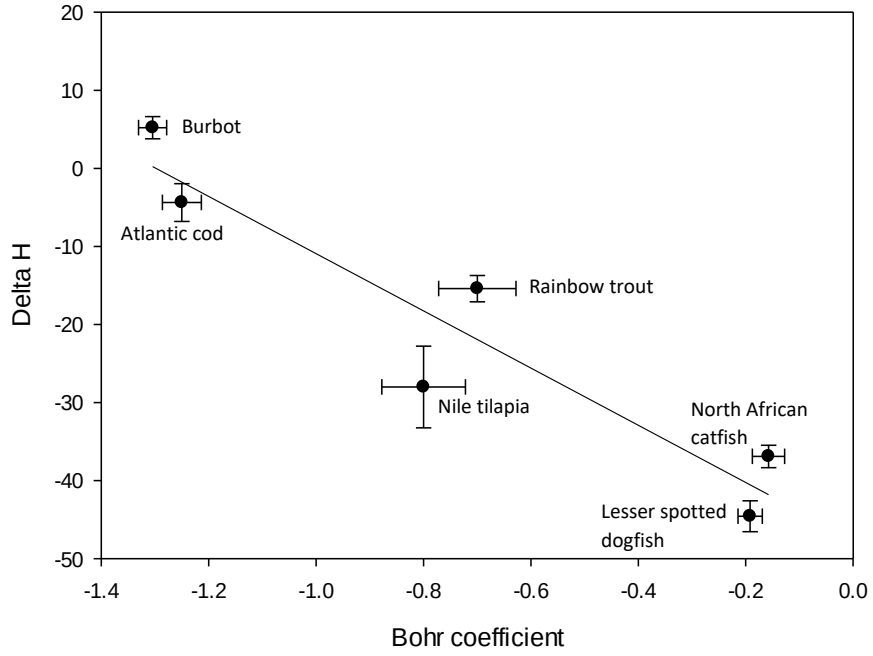


Figure 3.5: *Inverse relationship between pH (Bohr coefficient) and temperature (Delta H') sensitivity of red blood cell oxygen binding in six species of fish. Linear regression (R^2 = 0.884).*

3.5 Discussion

This study confirms reduced and reversed thermal effects on O_2 binding are not limited to only heterothermic fish. Building on the findings by Clark *et al*. (2008) in mackerel and Barlow *et al*. (2016; chapter 2) in Atlantic cod, the oxygen binding properties which were once considered 'a-typical' appear to be much more widespread. The strong correlation (r^2= 0.88) between pH sensitivity (Bohr coefficient) and temperature sensitivity (ΔH'), is similar to that found in tuna (Cech *et al*., 1984; Brill and Bushnell 1991; Lowe *et al*., 2000). This suggests that, as in heterotherms, H^+ may be partially responsible for this unusual oxygen binding phenomenon (Wood, 1980; Weber and Jensen, 1988; Larsen *et al*., 2003; Weber and Fago, 2008; Rasmussen *et al*., 2009).

Oxygen affinity and Cooperativity

Oxygen affinity and cooperativity varied considerably between species (Figures 3.1 and 3.2; Table 3.1). Notably, log P_{50} of Atlantic cod was significantly higher than all other species in this study under almost all conditions, signalling very low RBC oxygen affinity. With an average P_{50} of 29 mmHg (calculated from log P_{50} values between 5.0 and 12.5°C at pH 7.90 in Table 3.1), Atlantic cod has an O_2 affinity among the lowest that have been reported for blood or RBCs of any fish under the standardised conditions given above (e.g. Herbert *et al*., 2006). Low O_2 affinity has been previously reported for Atlantic cod in; whole blood (Herbert *et al*., 2006), RBCs (Chapter 2) and haemolysates in the presence of saturating ATP concentrations (Pörtner *et al*., 2001; Brix *et al*., 2004; Verde *et al*., 2006). Such a low P_{50} results in arterial blood PO_2 lying on the edge of the steep part of the OEC and may present problems with oxygen loading at the gills under low oxygen tensions. Indeed, in Chapter 2 modelled RBC O_2 saturations resulted in RBC oxygen binding of no more than 80% at typical PO_2 and pH values and at any temperature between 5.0 and 20.0°C. However, this guaranteed that across all temperatures, small decreases in venous PO_2 facilitated large increases in O_2 unloading in the tissues (Chapter 2).

The high affinity (P_{50} = 7 mmHg) in North African catfish is believed to be a-typical of air breathing fish. Compared to water breathers, air breathers are suggested to have higher oxygen carrying capacities and lower blood oxygen affinities (Johansen *et al*., 1978; Johansen *et al*., 1978; Morris and Bridges, 1994; Wells, 1999). However, a close (also air breathing) relative of the *Clarias gariepinus*, the catfish *Clarias batrachus* also has a surprisingly high blood–oxygen affinity (Singh and Hughes, 1995a and b). Such high affinity may have evolved to reduce the rate of transfer of O_2 across the blood/water pathway at the gills. The high n_H (2.36 ± 0.06) in the RBCs of North African catfish indicates significant haem–haem interactions in the haemoglobin of the water breather. The highly sigmoidal OEC favours oxygen unloading and is generally more typical of aerobically active fish (Wells 1999; Weber

2000), however such high co-operativty has also been observed in the low activity catfish, *Arius leptaspis* (Wells *et al*., 2005).

Atlantic cod, burbot and Nile tilapia exhibit low co-opertivity, under some circumstances. It is postulated that hyperbolic OECs may benefit fish living in more variable environments this reduces the chance of becoming compromised at low PO_2. The apparent pH dependence of the n_H value in cod and burbot is a manifestation of the Root effect (Brittain 2005; Table 3.1 and Figure 3.1) (Herbert *et al*., 2004). This results in lower cooperative binding at low pH. This is less apparent in the other four species with small or absent Root effects.

The trends and values of P_{50} and n_H in all species are comparable to previous work in these or closely related species, under similar conditions (Eddy, 1971; Weber *et al*., 1976; Wells and Weber, 1986; Singh and Hughes, 1995a and b; Narahara *et al*., 1996; Brix *et al*., 2004; Herbert *et al*., 2006).

Temperature and pH sensitivity

This study has shown RBC oxygen binding was essentially independent of temperature in burbot between 5.0 and 12.5°C and pH 7.40-7.90 (Table 3.2). Interestingly both Atlantic cod and rainbow trout also exhibit reduced thermal dependence and additionally reversed temperature dependence of RBC oxygen binding. At pH 7.4 and between the low temperature ranges (5.0-12.5 and 5.0-15.0°C, respectively) oxygen binding is endothermic (and varies significantly from zero, Table 3.2) and, as such, an increase in temperature will facilitate oxygen uptake. While in Nile tilapia temperature insensitivity was not achieved, reducing pH significantly reduced ΔH', particularly in the higher temperature range. The low pH and low temperature conditions required to incite reduced or reversed thermal sensitivity are similar to those observed in ectothermic scombrid, mackerel (Clark *et al*., 2008).

These results indicate that the OEC *in vivo* may be less influenced by temperature in arterial blood (where pH is highest) than in venous blood (where pH is lower). It is possible that maintaining temperature-independent O_2 binding in arterial blood at the gills ensures a

consistent supply of oxygen from the ambient water over the thermal range experienced (RossiFanelli and Antonini, 1960), whereas there may be a requirement for O_2 affinity to be differentially affected by temperature in the venous system where blood pH levels can be substantially reduced during high levels of sustained exercise (Brauner *et al*. 2000). The functional significance of these findings is not yet understood.

The reduced thermal dependence of oxygen binding at low pH can likely be attributed to increased H^+ concentration, these allosteric effectors preferentially bind to deoxy Hb, during oxygenation their endothermic dissociation can compensate for the heat released by exothermic haem oxygenation. This is the same mechanism observed in heterothermic fish, including tuna, billfishes, and lamnid sharks (Larsen *et al*., 2003; Clark *et al*., 2008; Weber and Campbell, 2011). Differences between species may be due the propensity to bind H^+ and the number of available proton binding sites caused by structural variations of haemoglobin (Berenbrink, 2006).

Other allosteric effectors, such as ATP/GTP and Cl^-, are also used in fish as a means of rapidly adapting Hb function to tissue O_2 demand. Indeed, it is believed organic phosphates are the most important heterotropic effectors in ectothermic vertebrates (Weber and Jensen, 1988). The interpretation of data from intact RBCs is complicated by presence of these compounds. When present, ATP and GTP depress the oxygen affinity of haemoglobin directly by allosteric interaction and indirectly by decreasing intracellular pH through modification of H^+ across the red cell membranes (Wood and Johansen, 1972; Weber *et al*., 1976; Johansen *et al*., 1976; Greaney and Powers, 1977; Qvist *et al*., 1977; Weber *et al*., 1977; Weber and Lykkeboe, 1978). Temperature acclimation influences erythrocytic NTP concentration and thus temperature sensitivity of oxygen affinity, but the responses between species vary widely. Thus, the effects we have observed cannot be solely attributed to H^+. Unfortunately accurate alteration of ATP/GTP levels is difficult in RBC solutions. Further investigation into the oxygen-binding properties of purified haemoglobin components is necessary to elucidate interactions of ATP and GTP. However, previous experiments in Atlantic cod suggest NTP

levels, after the washing and storage process used in this study, remain comparable to *in vivo* concentrations (Chapter 2) we assume this is true for all species.

A further complication is the presence of catecholamines, these compounds modulate intracellular pH, this may also alter intracellular allosteric effector concentrations, affecting P_{50}, n_H and the Bohr coefficient (Nikinmaa, 1997 and 2001). Washing in standardised physiological saline and overnight incubation of RBCs removes any catecholamine hormones, and allows any catecholamine-initiated effects to wear off (Berenbrink and Bridges, 1994a, b). This ensures equilibration of extra and intracellular ion concentrations and well defined RBC extracellular and intracellular pH values (Berenbrink and Bridges, 1994a, b) and allows us to assume consistent levels of NTPs throughout the experiments.

The finding of thermally independent and reversed thermal dependence of blood–O_2 binding existing in the ectothermic mackerel, suggests that reduced or reversed thermal effects on blood–O_2 binding may have evolved prior to regional heterothermy, not in response to it (Clark *et al*., 2008). However, this earlier evolution of reduced or reverse temperature dependence was still only predicted within the Scombroidei lineage. The widespread findings discovered in this study may have implications of the evolution of mechanisms to successfully overcome thermal influence on oxygen binding occurring much earlier in the teleost lineage. This may have allowed enhanced gill oxygen uptake over a broad range of ambient temperatures and potentially facilitated thermal niche expansion. The presence of such strong evidence for reduced or reversed temperature sensitivities of RBC oxygen binding across a wide range of physiological conditions in both gadiforms (Atlantic cod and burbot) may indicate more advance thermal tolerance in this lineage. Further phylogenetic analysis is required to better understand this potential.

In conclusion, our RBC O_2 binding results reinforce the arguments of the presence of reduced and reversed thermal dependence in a number of non- heterothermic fish within physiological ranges of pH, temperature and PO_2 conditions. This thermal independence is strongly linked to the sensitivity of RBC oxygen binding to pH. The evidence of this

phenomenon in a number of lineages suggests potential for evolution of mechanisms to reduce temperature dependence of oxygen binding earlier in the teleost lineage than previously proposed.

Chapter 4. Acidification-induced red blood cell sickling and loss of oxygen capacity in Atlantic cod, *Gadus morhua*: Effects of temperature and haemoglobin genotype.

4.1 Abstract

In vivo sickling has recently been observed in the whiting (*Merlangius merlangus*, Gadidae) and has been observed *in vitro* in several other species at low pH. In many teleost fishes the maximum oxygen saturation of Hb is also greatly reduced by acidification, this is known as the Root effect. Given the strong dependence of sickling and the Root effect on pH, in this *in vitro* study we explore the effect of acidification to incite these phenomena in red blood cell (RBC) suspensions of another Gadidae, Atlantic cod (*Gadus morhua*). RBCs were exposed to a range of physiological pH salines and temperatures. Acidification caused a reduction in RBC oxygen saturation of over 60 ± 1% (mean ± SE) and induced sickling in 100% of RBCs when air-equilibrated at 5°C, confirming a strong pH dependence found in previous studies. The occurrence of sickling decreased at increased temperatures, a new finding in fish and contrary to that found in mammals. The Root effect was similarly effected by temperature, a novel observation suggesting a strong association between the two phenomena. Given the similar influence of pH, we propose that the mechanisms behind both phenomena rely on a common haemoglobin protonation site for activation,. Interestingly, unlike in mammalian vertebrates, haemoglobin genotype within Atlantic cod had minimal effect on RBC sickling. Occurrence of sickling in close relatives suggest the underlying mutation may have evolved prior to the speciation of Atlantic cod.

4.2 Introduction

Haemoglobin polymerisation and subsequent red blood cell (RBC) sickling has been found to occur in a range of vertebrates (Butcher and Hawkey, 1979; Simpson *et al*., 1982; Taylor, 1983), including widespread evidence of *in vitro* sickling in a number of fish species (Harosi *et al*., 1998; Koldkjær and Berenbrink, 2007; Koldjkaer *et al*., 2013). This phenomenon is particularly prevalent in members of the fish family Gadidae, where an early *in vitro* observation of distorted RBCs in the spleen of several species (Yoffey, 1929; Dawson, 1932;

Thomas, 1971) has more recently been related to sickle cells and followed by descriptions of sickling in a number of other Gadidae (Harosi *et al*., 1998; Koldkjær and Berenbrink, 2007). However, many of these observations in fish were made *in vitro* under extremes of pH and anoxia (Harosi *et al.,* 1998). Nevertheless, recent evidence suggests sickling may occur *in vivo* after capture stress or exercise in the whiting (*Merlangius merlangus*) and Gulf toadfish (*Opsanus beta*) (Koldjkaer and Berenbrink, 2007; Koldjkaer *et al*., 2013).

Harosi *et al*. (1998) correlated erythrocyte morphology and haemoglobin oxygen state and found that in those fish species that they investigated, only deoxygenated haemoglobin polymerised, as is found in humans (Bookchin *et al*., 1976). However, unlike in human sickle cells, in whiting deoxygenation alone did not induce sickling (Koldjkaer and Berenbrink, 2007). In the latter study, sickled cells began to appear only as pH was decreased. Oxygen did have an ameliorating effect, with a larger pH decrease required to observe sickling under 100% oxygen when compared to air or 100% nitrogen. However, this study, along with that by Koldjkaer and colleagues (2013) demonstrated that a reduction in extracellular pH is fundamental for sickling in both whiting and Gulf toadfish.

Interestingly, in most species of fish, decreasing pH also results in a reduction in maximum Hb oxygen binding, known as the Root effect (Root, 1931; Bridges *et al*., 1983, Berenbrink *et al.,* 2005; Berenbrink *et al*., 2011). A decline in cooperativity between the four oxygen binding sites of the Hb tetramers at low pH, together with a large decrease in Hb O_2 affinity, mean Hbs may not become fully oxygenated even at PO_2 as high as 150 mmHg (Pelster, 2001, Barlow *et al*., submitted). This mechanism is believed to facilitate oxygen secretion in the choroid and swim bladder *retia mirabilia* (singular: *rete mirabile*; Wittenberg and Wittenberg, 1962; Berenbrink *et al*., 2005) and has been hypothesised to be part of a mechanism that provides more oxygen to muscles under acidotic stress (Rummer *et al*., 2013). Given the strong dependence both sickling and the Root effect have on pH, and given that both are associated with Hb deoxygenation, it is possible that both processes may influence each other. To investigate if a link may be present we selected a species in which sickling is

known to occur across multiple age ranges, from larval to juvenile and adult fishes, and which has one of the largest known Root effects, the Atlantic cod (*Gadus morhua*, Harosi *et al*., 1998; Krogh and Leitch, 1919, as discussed in Berenbrink *et al*., 2011).

In mammalian vertebrates Hb polymerisation is endothermic in nature, an increase in temperature increases sickling (Bookchin *et al*., 1976; Jain and Kono, 1977; Butcher, 1979; Butcher and Hawkey, 1979; Bunn, 1997; for summary see Koldkjaer *et al*., 2013), however knowledge of the effect of temperature on sickling in fish is lacking. This is an important gap in knowledge as blood temperature cannot be regulated by internal means and so is environmentally dependent in ectothermic animals. If endothermic in nature, sickling in cod could provide a mechanism to oppose the greatly reduced temperature sensitivity of oxygen binding that was observed by Barlow *et al*. (submitted). Similarly, there has been no systematic study that has assessed the effect of temperature on the Root effect in fish RBCs.

The molecular mechanisms behind both the Root effect and haemoglobin polymerisation in fish remain unknown. However, a number of theories have been suggested, including attempts at molecular mechanisms. While the general consensus about the mechanism for the Root effect appears to be that a single mutation is unlikely to be the cause and that it is the result of multiple factors (Brittain, 1987; Berenbrink, 2007), for sickling there is an established trend which may continue in fish. In humans and several species of sheep and deer, the occurrence of sickled cells is strongly linked to the occurrence of specific haemoglobin types (Butcher, 1979; Butcher and Hawkey, 1979). In humans, sickling is the result of a single amino acid substitution from a polar glutamic acid to a non-polar valine in the β chains of the Hb tetramer, which results in a reduction of solubility of deoxygenated Hb and thus haemoglobin polymerisation (Ingram, 1957; Perutz and Mitchison, 1950). Atlantic cod haemoglobin is known to have two alleles coding for its Hb β1 chain, which differ in two amino acids, namely at positions 55 and 62 of the β1 chain. These give rise to the so called HbI 1 and HbI 2 polymorphism (Sick, 1961; Andersen *et al*., 2009). While all studies into Atlantic cod have observed sickling in all individuals at the activating conditions (Thomas,

1971; Harosi *et al*., 1998), thus far none have established if they were all of either the HbI 1/1 or HbI 2/2 homozygote, or even the HbI 1/2 heterozygote genotype. It is also possible that the polymorphic HbI may simply affect the degree of sickling or merely its response to temperature. For the substitution at position 55, from a Met (HbI 1) to a smaller Val (HbI 2) amino acid side chain, Andersen *et al*. (2009) propose it occurs at a crucial point of interaction between α and β subunits and may result in destabilisation of the low oxygen affinity T(ense)-state of haemoglobin. Thus it may be possible that animals with the HbI 2 variant haemoglobin may employ sickling as a mechanism to stabilise haemoglobin when deoxygenated, however this mechanism may only be able to be activated after occupation of a specific proton binding site. For the Root effect, the preferred theory suggests that multiple substitutions result in the stabilisation of the low oxygen affinity T(ense)- state of haemoglobin at low pH or destabilisation of the R(elaxed)-state (Berenbrink, 2007).

The aim of the present study was to establish the effect of physiologically relevant pH and temperature changes on simultaneously measured RBC sickling and the Root effect in Atlantic cod of specified HbI genotype. Based on the strong similarities in pH and temperature profiles of RBC sickling and deoxygenation at atmospheric PO_2, it is proposed that the mechanisms for both processes likely are closely related.

4.3 Materials and Methods

Animals

Atlantic cod were caught by hook and line method from the Mersey Estuary, Merseyside, U.K, between December 2010 and February 2011 (mean standard length 28.7 ± 0.8 cm (S.D.)) and mean mass 245.6 ± 26.6 g, $N = 8$). These were kept at the University of Liverpool for several weeks in a 1000 litre tank equipped with a biological filter and aerated, recirculated artificial seawater kept at a temperature range of 11-13°C. The fish were netted with minimal stress and killed by a British Home Office Schedule 1 method, concussion and destruction of brain.

To increase likelihood of obtaining HbI 2/2 genotype, young juvenile cod (< 10 cm) were obtained from Millport Marine Biological Station, Scotland in June. These were killed immediately upon capture by the same method as above.

Blood sampling

For all animals, immediately following death, blood was removed by puncturing of the caudal vessels with a 1 ml syringe, whose dead space was filled with 50 mg ml^{-1} of heparin solution (180 units/mg, sodium salt from porcine intestinal mucosa, Sigma-Aldrich). Samples were kept on ice and RBCs were isolated by centrifugation (3000 rcf, 4°C, 4 mins) and washed in an isotonic saline solution no more than 10 hours post extraction: (all concentrations given in mmol l^{-1}) NaCl (125.5), KCl (3), $MgCl_2$ (1.5), $CaCl_2$ (1.5), D-glucose (5) and Hepes (20) (as used by Koldkjaer and Berenbrink, 2007), adjusted to pH 7.97 at 15°C (using a Lazar Model FTPH-2S Micro Flow Through pH Measurement Electrode and Jenco 6230N combined pH/mV/temperature portable meter (Jenco Collaborative, California, USA). Washing was repeated a further two times before the cells were re-suspended at an approximate haematocrit (Hct) of 5-10 % and stored overnight at 4°C in a 15 ml falcon tube with a large air reservoir.

Root effect and sickling in Mersey cod

Prior to experiments the following day cells were washed again and resuspended at a Hct between 8-13%. This RBC suspension was then diluted 10 fold in 7.97 saline and a further 10 fold in saline of pH ranging from 7.09-8.05 (all adjusted at 15°C). These mixed solutions were incubated at temperatures 5.0, 12.5 and 20.0°C under air. pH of the saline solution mixtures without RBCs were measured at each temperature under air. RBC suspensions were equilibrated for at least 20 minutes before an aliquot was taken for fixation (see below) and a spectrum was taken from 500-700 nm using a Unicam UV 500 spectrometer (Thermo Electron Corporation, Ohio, USA) and using Vision 32 Software. Oxygen saturation of RBC suspensions was determined by spectral deconvolution (Völkel and Berenbrink, 2000).

Sickling in Scotland cod

RBCs were treated as above for Mersey cod, however mixed solutions were exposed to 5, 10, 15 and 20°C and only an aliquot for fixation was taken.

Cell fixation and light microscopy

A 2% gluteraldehyde fixation solution was made from a 25% stock (Grade I; Sigma-Aldrich Company Ltd, Gillingham, UK) using distilled water and saline to reach an osmolality of 300 mOsm. Equal quantities of sample and fixation solution, to reach a final 1% glutaraldehyde concentration and stored at 4°C until analysis on the microscope. The samples were counted on an Axiovert 135 TV microscope (Carl Zeiss Microlmaging GmbH, Göttingen, Germany; using a 100x oil immersion lens and direct interference contrast) and viewed with Scion Image software (Scion Corporation, Frederick, MD, USA).

RNA Extraction and Reverse Transcription

25-50 mg of spleen tissue was homogenised in 1 ml TRIzol® Reagent (Invitrogen, Life Technologies, Paisley, UK). RNA was extracted from the homogenate as described in the Invitrogen TRIzol® Reagent instructions for animal tissues (Invitrogen, Life Technologies, Paisley, UK). The RNA was diluted 1:10 in sterile distilled water and quantified using a NanoDrop ND-1000 spectrophotometer (LabTech International, UK) and stored at -80 °C until reverse transcription.

RNA was reverse transcribed following a standard first strand cDNA synthesis protocol (Invitrogen, Life Technologies, Paisley, UK). Briefly, RNA (>1 µg) was reverse transcribed in a 20 µl reaction containing 50 ng/µl random hexamers, 10 mM dNTP mix (10 mM each of dATP, dGTP, dCTP and dTTP at neutral pH), 5X First strand buffer, 0.1 M DTT and 200 U SuperScript™ III Reverse Transcriptase (Invitrogen, Life Technologies, Paisley, UK). Cycling parameters were 65 °C for 5 mins, 50 °C for 60 mins and 70 °C for 15 mins in a Techne TC-5000 Thermal Cycler. The cDNA was stored at -20 °C.

PCR amplification, purification and DNA sequencing

1uL of DNA was PCR-amplified in a 10 µl reaction volume containing 5uL 2x BioMix Red (Bioline, UK) and 10 pM (each) of the forward (5'-GGACCACTCGCGTTGCAGAG-3') and reverse (5'-CGGAAACCACCGCGCACAGG-3') primers (Eurofins MWG Operon, Germany). Cycling parameters were 95 °C for 10 mins, 30 three step cycles (30 s at 95 °C, 30 s at 63 °C, 30 s at 72 °C) and 72 °C for 10 mins.

Unconsumed primers and nucleotides were removed from PCR products using an ExoSAP-IT digest (USB, UK). 10 uL reaction mix, containing 5 uL PCR product, 1 uL ExoSAP-IT and 4 uL nuclease free water was incubated at 37ºC then 80ºC each for 15 minutes. The cycle sequencing reaction was set up using the BigDye® Terminator v3.1 Cycle Sequencing kit. One µl of ExoSAP-IT product was added to a 9 µl reaction mix containing 1.5 uL 5X sequencing buffer, 1.0 µl BigDye 3.1 (Applied Biosystems, Life Technologies, Paisley, UK) and 1.6 pM of forward or reverse primer. Cycling parameters were 30 three step cycles (96 °C for 10 sec, 50 °C for 5 sec, 60°C for 4 mins). The sequencing product was then precipitated using 3 M sodium acetate prior to resuspension in HiDi™ formamide (Applied Biosystems, Life Technologies, Paisley, UK), and then run on an ABI3130xl. Genotype was determined by viewing spectrograms of DNA sequences on BioEdit (Ibis Biosciences, Carlsbud, CA) and visually identifying polymorphic sites.

Spectral deconvolution for Root effect, data analysis and statistics

Spectral deconvolution of the optical spectra (see Völkel and Berenbrink, 2000) was used to determine the concentrations of haemoglobin derivatives within RBC suspensions (oxyhaemoglobin, HbO_2; deoxyhaemoglobin, deoxyHb; and the two forms of methaemoglobin, acid Hb^+ and alkaline Hb^+) at each temperature, pH and PO_2 value using SigmaPlot 12.5 software (Jandel Scientific, San Rafael, CA, USA). The unknown concentrations (mmol l^{-1}) of the different tetrameric Hb derivatives, were calculated using $f = au + bv + cw + dx$, where a, b, c, and d, represent [HbO_2], [deoxyHb], [acid Hb^+], and alkaline

Hb^+], respectively and were restricted to values greater than or equal to zero. *f* is the predicted dependent variable to be fitted to the measured absorption data for each nm step between 500 and 700 nm and *u*, *y*, *w* and *x* represent the respective experimentally determined absorption coefficients for each Hb derivative, respectively. Absorption coefficients for the HbO_2 and deoxyHb were created with RBC suspensions in pH 8.05 saline at 5.0°C, exposed to 100% oxygen or 100% nitrogen. Acid Hb^+ and alkaline Hb^+ templates were constructed using Hb suspensions oxidised with tri-potassium hexacyanoferrat at pH 6.5 and 8.05 respectively, although no methaemoglobin formation was seen in any of our samples. The predicted values by the curve fitting procedure were plotted for each wavelength between 500 and 700 nm with the measured spectra to confirm the accuracy of the prediction.

Fractional oxygen saturation and sickle cells were plotted against measured pH values at each temperature. Mean apparent cooperativity constant for sickling-linked proton binding (n_HS) and an apparent extracellular pH at half maximal sickling (pK_aS) were determined using

$$f=-1*x^n/(x^n+(10^{-a})^n) \qquad \text{Equation 4.1}$$

where *n* is the slope of the curve and equates to n_HS, *a* is pH when 50% of cells are sickled and *f* is the predicted dependent variable.

Mean apparent cooperativity constant for oxygen-linked proton binding (n_HR) and an apparent extracellular pH at half maximal oxygenation (pK_aR) were determined using

$$f=c-b*x^n/(x^n+(10^{-a})^n) \qquad \text{Equation 4.2}$$

where *a* and *n* are as above, only for oxygenation. However, minimum (*c*) and amplitude (*b*) of the curves were not restrained.

Statistics comparing pK_a and n_H in temperature and oxygenation or sickling were performed using a two-way ANOVA, all others comparisons used a one-way ANOVA with Holm-Sidak pairwise comparison where necessary. All values are reported as mean ± SE, unless stated otherwise.

4.4 Results

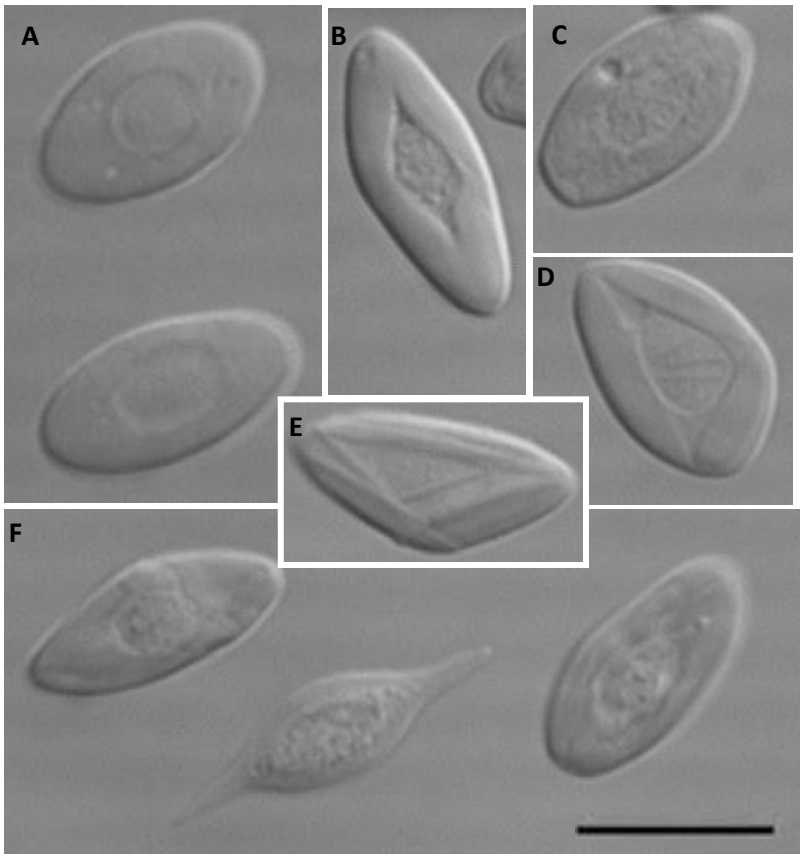


Figure 4.1: *Light microscopy images using differential interference contrast of representative normal (A) and sickled Atlantic cod RBCs (B-F), near pH 8.0 and 7.1-7.2, respectively. Heterozygote, HbI 1/2, animals. The scale bar applies to all panels and corresponds to 10 µm.*

Light microscopy of air-equilibrated Irish Sea HbI 1/2 heterozygote Atlantic cod RBCS showed an increased presence of irregularly shaped RBCs with angular or granular appearance when pH was lowered from about 8.0 to between 7.1 to 7.2 (Figure 4.1). Normal cells at high pH were elliptical in shape and smooth in appearance with homogenous cytoplasms and nucleoplasms (Figure 4.1A), while sickled cells had a distorted shape, often angular and with visible striations or granular texture in the nucleus and/or cytoplasm (Figure 4.1B-E). Cells with spindle like protrusions (Figure 4.1F, centre) were also observed on rare occasions.

At 5°C and pH greater than 7.8, all Irish sea HbI 1/2 Atlantic cod RBCs had a normal appearance, but occasional sickled cells began to occur at a mean pH as high as 7.75 (Figure

4.2A). As saline pH was further decreased the presence of sickled cells increased rapidly and a complete change from normal to sickled cells was observed within ~0.5 pH units, with a mean apparent cooperativity constant for sickling-linked proton binding (n_HS) of 11.8 ± 2.44 and an apparent extracellular pH at half maximal sickling (pK_aS) of pH 7.54 ± 0.03 (Table 4.1). Oxygen binding of these air-equilibrated RBCs was also strongly affected by pH via the Root effect, resulting in sigmoidal curves (Figure 4.2). At 5°C and pH > 7.8, oxygen binding reached a fractional saturation of 0.98 ± 0.01, but when saline pH was reduced, oxygen binding in air equilibrated cells dropped to an asymptotic minimum of 0.38 ± 0.01 and an n_HR of 4.81 ± 0.27 (Figure 4.2A). Interestingly, at a pH 7.49 ± 0.03 pK_aR (extracellular pH at half maximal saturation) was not significantly different from pK_aS, ($p < 0.001$).

As temperature was increased from 5 to 12.5 and 20°C, the transition from a zero number of sickled RBCs at high pH to 100% sickled RBCs at lower pHs continued to occur. This consistently took place within a relatively small pH range, which was reflected by the high n_H S at all temperatures (Table 4.1).

In contrast, temperature had a significant effect on maximum oxygen binding of air equilibrated RBCs at high pH, where RBCs only reached an asymptotic maximum of 0.90 ± 0.01 fractional saturation at 20°C, this is significantly different from maximum saturation at 5 (0.98 ± 0.01) and 12.5°C (0.96 ± 0.01) ($p < 0.001$) (Table 4.1). Similarly, minimum oxygen saturation at low pH also significantly decreased as temperature was increased. Between 5 and 20°C a significant decrease in the n_HR was seen ($p < 0.02$).

***Table 4.1**: Temperature dependence of pK_a values, n_H of red blood cell sickling and oxygenation, and minimum, maximum and amplitude of fractional RBC saturation associated with the Root effect in Atlantic cod from the Irish Sea determined using Equations 4.1 and 4.2 (see Materials and Methods). Mean ± SE, N = 5. Different letters indicate significant differences between temperatures, and between sickling and Root effect parameters (pK_a and cooperativity only).*

	pK_aR	pK_aS	n_H R	n_H S	Minimum R	Maximum R	Amplitude R
5	7.49±0.03^{a}	7.54±0.03^{a}	4.81±0.27^{d}	11.8±2.44^{f}	0.38±0.01^{g}	0.98±0.01^{j}	0.60±0.01^{l}
12.5	7.37±0.03^{b}	7.41±0.02^{b}	4.22±0.35^{de}	13.0±2.44^{f}	0.32±0.01^{h}	0.96±0.01^{j}	0.64±0.01^{l}
20	7.31±0.03^{c}	7.37±0.04^{c}	3.30±0.17^{e}	16.5±6.74^{f}	0.23±0.02^{i}	0.90±0.01^{k}	0.68±0.03^{l}

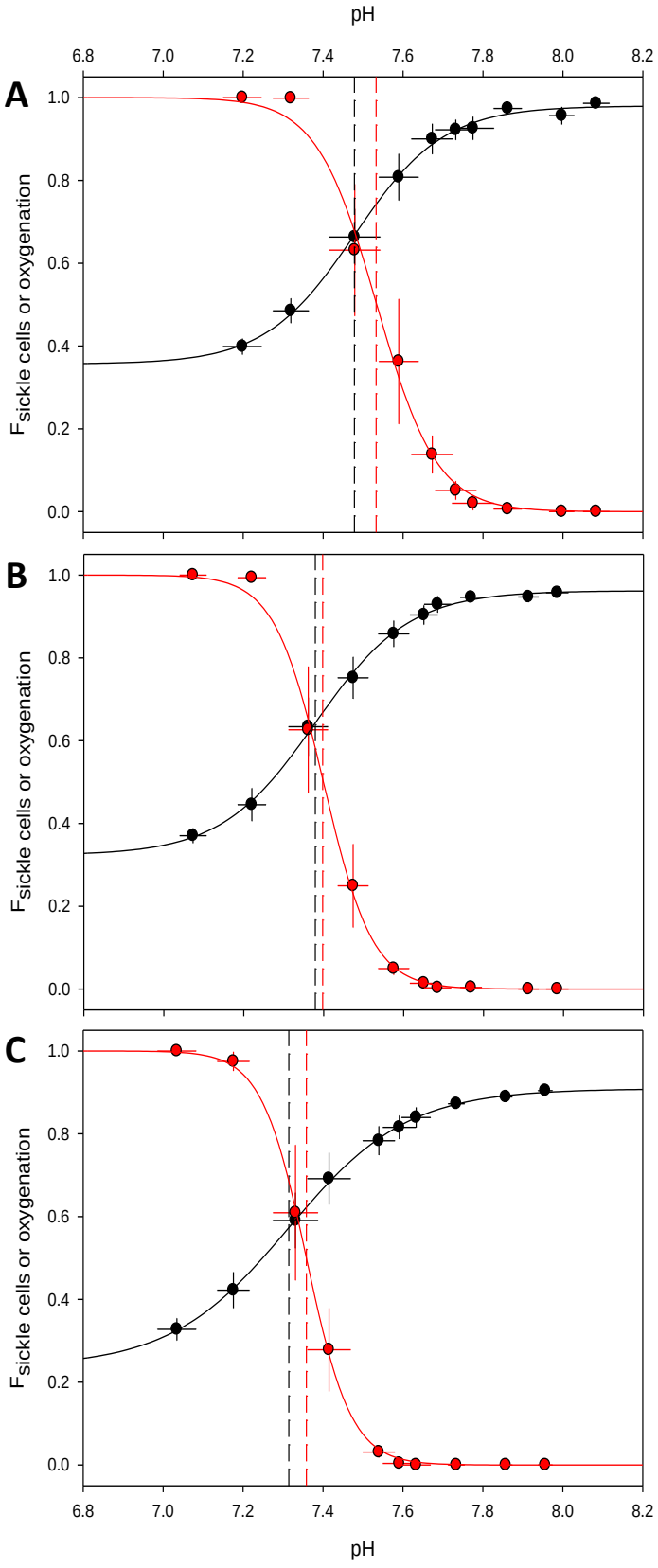


***Figure 4.2**: Fraction of sickle cells (red) and fractional oxygen saturation (black) in red blood cell suspensions of heterozygote HbI 1/2 Mersey Atlantic cod as a function of saline pH at 5 (A), 12.5 (B) and 20°C (C). Symbols represent mean values ± SE (N = 5). Curves have been*

calculated using the mean apparent pK_a values (dashed lines) and mean n_H constants obtained from 5 individual sigmoidal curve fits (see Materials and Methods).

Two-way Repeated Measures ANOVAs with Holm-Sidak pairwise comparisons were performed to compare the pK_a and proton-binding cooperativity of RBC sickling and oxygenation at different temperatures, with the respective observed RBC process as one factor and temperature as the second.

Comparatively, apparent cooperativity of deoxygenation-linked proton binding was greatly reduced in comparison to sickling-linked proton binding at the three measured temperatures ($p = 0.016$).

Notably, the apparent pK_aS and pK_aR were statistically indistinguishable from one another across all temperatures ($p = 0.129$) (Figure 4.2A). As temperature was increased from 5 over 12.5 to 20^{0}C there was a successive shift in both the sickling and Root effect curve to lower pH values, indicating that the induction of both sickling and deoxygenation required a stronger degree of acidification at elevated temperatures (Figure 4.2A-C). This was also seen by the significant reduction in pK_aS and pK_aR between all three temperatures ($p < 0.001$). There was no significant difference between pK_a of sickling and the Root effect at any of the three temperatures.

It is known that as body temperature increases, the pH regulated in the arterial blood of all vertebrates, including fishes will decrease, as reviewed by e.g. Ultsch and Jackson (1996). Taking this experimentally determined relationship between *in vivo* arterial pH and temperature in marine fish into account, Figure 4.3 demonstrates how the shift in the apparent pK_a of sickling and the Root effect with temperature is not parallel to the predicted shift in arterial *in vivo* pH, such that at 5^{o}C a significantly smaller reduction of *in vivo* arterial pH is required to induce half-maximal sickling and half maximal Root effect than at 12.5 and 20^{o}C ($p < 0.001$).

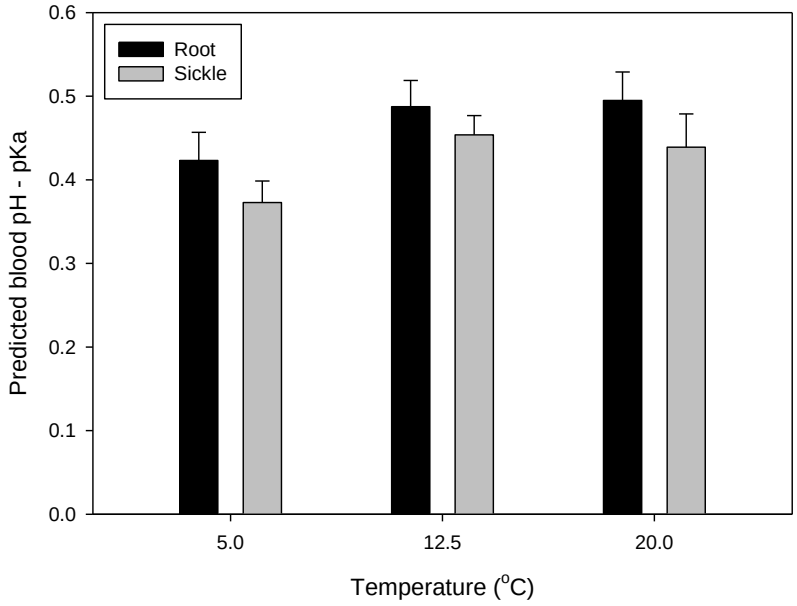


Figure 4.3: *Difference between arterial pH [as determined for teleost fishes (Ultsch and Jackson, 1996)] and pH of half-maximal Root effect and sickling as a function of temperature in red blood cells of HbI-1/2 Atlantic cod from the Irish Sea.*

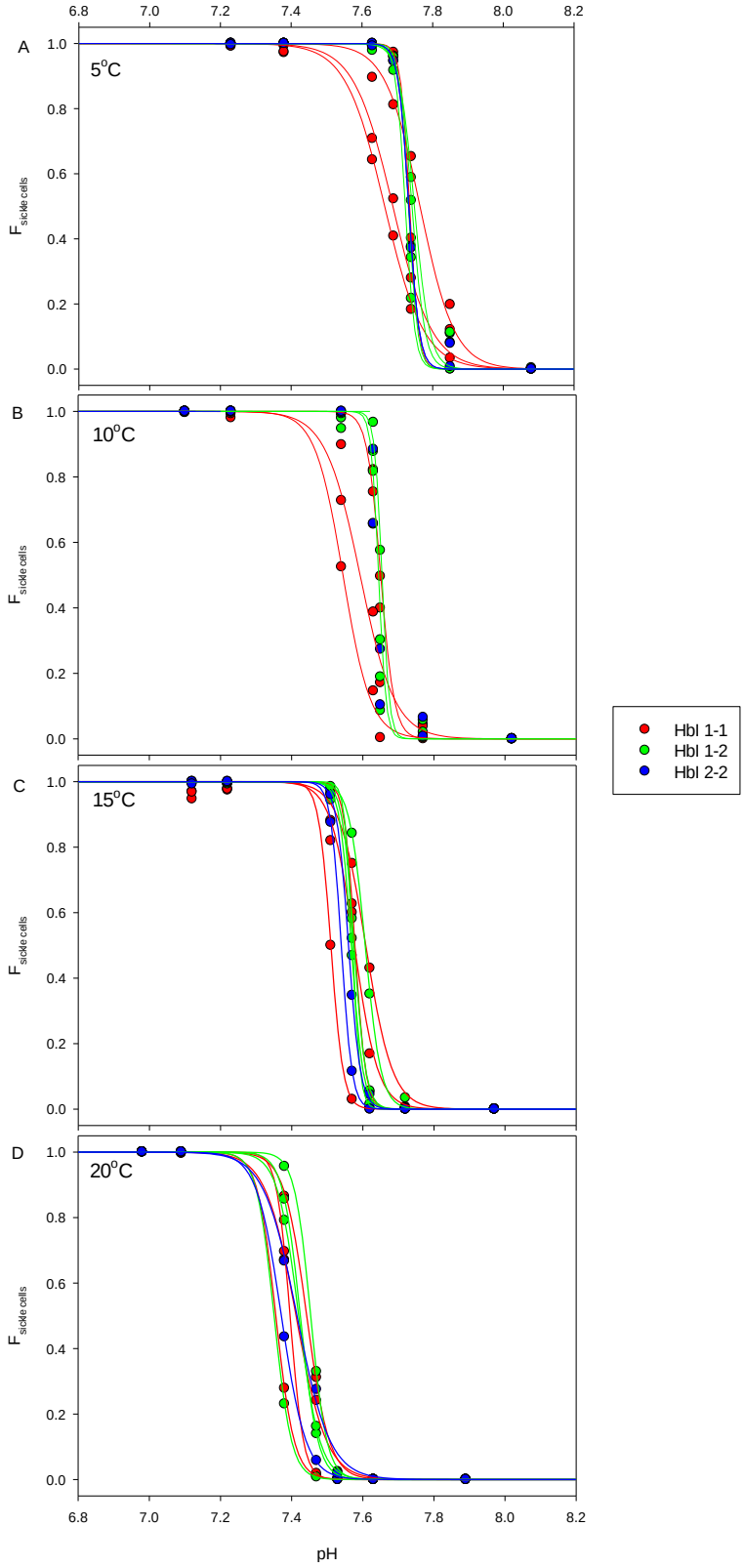


Figure 4.5: *Fraction of sickle cells for 3 genotypes against saline pH for NW Scotland Atlantic cod red blood cell suspensions equilibrated with air and at four different temperatures 5 (A), 10 (B), 15 (C) and 20°C (D). Symbols represent individual values and*

curves are fitted using the pK_aS and n_H S for each individual (see Materials and Methods). HbI 1/1; red (N = 4), HbI 1/2; pink (N = 4) and HbI 2/2; blue (N = 2).

Table 4.2: *Hb genotype and temperature dependence of pKa values and apparent cooperativities of proton binding of red blood cell sickling in Atlantic cod from NW Scotland determined using Equation 4.1 (see Materials and Methods). Mean values ± SE or range (for N = 4, 4, and 2 for HbI 1/1, 1/2, and 2/2 RBCs, respectively). Different superscript letters indicate significant difference between temperatures and genotypes for each parameter (Two-way Repeated Measures ANOVA, with Holm-Sidak pairwise comparison).*

	pK_aS			n_H S		
	HbI 1/1	HbI 1/2	HbI 2/2	HbI 1/1	HbI 1/2	HbI 2/2
5	7.71±0.02^{a}	7.75±0.01^{a}	7.73±0.00^{a}	14.66±6.43^{e}	27.28±3.16^{fg}	29.27±0.02^{fg}
10	7.61±0.03^{b}	7.64±0.00^{b}	7.64±0.00^{b}	21.47±7.83^{e}	42.49±0.66^{f}	45.14±0.21^{f}
15	7.57±0.02^{c}	7.58±0.01^{c}	7.55±0.01^{c}	19.73±4.37^{e}	28.12±4.26^{efg}	27.52±1.43^{efg}
20	7.40±0.02^{d}	7.41±0.02^{d}	7.39±0.02^{d}	15.97±2.77^{e}	17.28±0.95^{eg}	10.95±1.59^{eg}

The effect of Hb genotype on RBC sickling was studied in Atlantic cod captured in Scotland, to increase the chances of obtaining HbI 2/2 individuals with the HbI 2 allele, whose frequency increases northward along the North East Atlantic coastline. In the Atlantic cod captured in Scotland, sickling was found to occur in all observed haemoglobin genotypes, unlike in all mammalian species for which sickling has thus far been described (Bookchin *et al*., 1976; Butcher, 1979; Butcher and Hawkey, 1979. As with the cod from the Irish Sea, sickle cells were absent at high pH for all temperatures and rapidly increased in number as pH was decreased, such that 95 – 100% of cells sickled in all animals (Figure 4.5). Further, the same trend with increasing temperature was apparent for all genotypes, with the pK_a of sickling significantly decreasing as temperature was increased ($p < 0.005$, Two-way Repeated Measures ANOVA, with Holm-Sidak pairwise comparison) (Figure 4.5 and Table 4.2). The pK_a for sickling did not differ significantly between the three different genotypes at any of the

four temperatures tested between 5 and 20°C (Table 4.2). There was, however, a significant effect of Hb genotype on the n_HS.

n_HR of HbI 1/1 individuals was not significantly affected by temperature, yet, genotypes HbI 1/2 and HbI 2/2 showed significantly increased n_HR at 5 and 10°C compared to 20°C ($p < 0.02$, Two-way Repeated Measures ANOVA, with Holm-Sidak pairwise comparison). Also at 5 and 10°C n_HS was significantly higher for HbI 1/2 and HbI 2/2 genotypes when compared with HbI 1/1 ($p < 0.03$), this had the effect of reducing the amount of sickling at higher pH values (Figure 4.4 A and B).

4.5 Discussion

The present study describes the occurrence of RBC sickling and the Root effect in Atlantic cod at physiologically relevant pH and temperatures. Sickling in Atlantic cod RBCs was found to (1) occur in all HbI genotypes, (2) be strongly affected by acidosis, (3) decrease in incidence with increasing temperature and (4) have a potential link with Hb deoxygenation via the Root effect.

Sickling morphology and effect of HbI genotype

This study confirms evidence of *in vitro* sickle cells in juvenile Atlantic cod for all Hb genotypes. Similar morphological RBC alterations have previously been observed in Atlantic cod of varying age ranges but unspecified Hb genotype from Canada, Rhode Island, USA, and Aberdeen Bay, Scotland, and been shown to be due to Hb aggregation into large polymers, similar to RBC sickling in humans (Thomas, 1971; Harosi *et al*., 1998). The morphology of these sickle cells in Atlantic cod are comparable to those found in the close relative whiting, *Merlangius merlangus* (Koldkjaer and Berenbrink 2007). However, we found an additional morphology not reported in whiting, this is the 'spindle' variety (Figure 4.1F, centre), although the conditions these were viewed at were not consistent leading to the conclusion that it is merely a rare form and not induced by only a single set of conditions.

The occurrence of sickling in all cells of all HbI genotypes suggest that Met55Val or Lys62Ala polymorphisms cannot be attributed as the primary molecular mechanism which stimulates HbI polymerisation in cod. Indeed, the presence of sickling in the close cod relative, the whiting and other Gadidae suggest the mechanism is one likely shared throughout the family. Nevertheless, HbI genotype does appear to have some influence on sickling; in Scotland cod n_HS in HbI 2/2 individuals is more than double that of HbI 1/1 at the two lowest temperatures, an increase from 14.66 ± 6.43 in HbI 1/1 to 29.27 ± 0.02 in HbI 2/2 at 5.0°C and 21.47 ± 7.83 and 45.14 ± 0.21 at 10°C. Due to the polymorphism at position β62, from Lys (HbI 1/1) to Ala (HbI 2/2) interactions with water at the haem binding site is greater in HbI 1/1 individuals at low temperatures (Ooi and Oobatak, 1988; Andersen *et al*., 2009). This may inhibit the interaction with the ameliorating oxygen at low temperature thus inducing sickling at a slightly higher pH in the HbI 1/1 individuals (Koldkjaer and Berenbrink 2007). Further, interactions with water effect the R to T allosteric equilibrium (Haire and Hedlund, 1983) and can cause alterations in quarternary structure exposing or isolating proton binding sites, which could explain the differences in n_HS.

Effect of pH on sickling and deoxygenation

Both sickling and deoxygenation in HbI heterozygotes, were indeed triggered by decreasing pH as seen in previous studies (Root, 1931; Bridges *et al*, 1983; Brittain, 1987; Pelster and Weber 1991; Koldkjaer and Berenbrink 2007; Berenbrink *et al*., 2011; Koldkjaer *et al*,. 2013), with the onset of both sickling and the Root effect occurring at pH as high as 7.8 in Atlantic cod. While fish RBCs are regularly exposed to blood pH of this level and on occasion far below, both during short-term exposure in the *retia mirabilia* and for several hours during intense exercise and recovery (Steen, 1963; Holeton *et al*., 1983; Milligan and Wood, 1987; Kobayashi *et al*., 1990), the Hb is protected to a degree during exercise. Extreme exercise results in a release of adrenaline and noradrenaline (Berenbrink and Bridges, 1994; Perry *et al*., 1996), which triggers a β-adrenergically stimulated Na/H exchanger (βNHE) response thus increasing intracellular pH (Nikinmaa, 1982; Berenbrink *et al*., 2005) and reducing the

incidence of Hb polymerisation (Koldkjaer and Berenbrink, 2007). This effect will not be seen in our cod as catecholamines were removed during the overnight incubation. As such the occurrence of sickling within the animal may be slightly lessened than seen here. However, while the PO_2 in this study is not always comparable to values seen *in vivo*, the higher oxygen levels to which these experimental cells are exposed should have an ameliorating effect on sickling (Koldkjaer and Berenbrink 2007) thus reducing sickling levels, as such it is likely cod cells are regularly exposed to some degree of sickling.

Scotland cod had higher pK_a in comparison to Irish sea cod, this could be due to a number of factors, including: time of year the studies took place; age of the animals; geographical distribution and possible life history differences brought on by this. These factors may result in variations in relative quantities of the multiple Hb components found in the blood. HbI is the predominant Hb component found in Atlantic cod, however studies have shown a variety of Hb which can slightly increase or decrease in response to seasonal, diurnal and random temperature changes, they are also thought to vary with age (Brix *et al*, 2004). Unfortunately the individual properties of these Hb components are not well characterised and so present some difficulty to determine exact effects.

Effect of temperature

To our knowledge, this is the first time the effect of temperature on sickling and Root effect in fish has been reported. Increasing temperature decreased the occurrence of sickling and oxygen binding, contrary to what is found in mammalian vertebrates (Bookchin *et al*., 1976; Jain and Kono, 1977; Butcher, 1979; Butcher and Hawkey, 1979; Bunn, 1997). Given sickling in mammals is characterised as endothermic, the converse response to temperature suggests sickling in fish may be exothermic, and in conjunction so too is the Root effect. This trend in oxygenation is likely to be the effect of exothermic oxygenation of the haem groups (Wyman, 1948) and may result in insufficient oxygen uptake at increased temperatures.

The proposed exothermic nature of sickling may suggest it is involved in the reduction in thermal dependence of the oxygen transport system in Atlantic cod as suspected, although

further work is required. Working with RBCs, as opposed to Hb isolations makes it difficult to isolate the effects of individual allosteric modifiers or other factors, without interference by others. In isolated Hb solutions, use of inhibitors such as urea and tris can be used to prevent formation of hydrogen bonds and more accurate alterations can be made to pH and ionic strength, without protection from the RBC membrane (Freedman *et al*., 1973; Levine *et al.*, 1975; Hofrichter *et al*., 1974; Butcher, 1979; Butch and Hawkey, 1979). However, limitations with Hb isolates, such as reduced physiological significance, a need for extreme polymerisation conditions and the potential for dissociation of the Hb into dimers in solution (Kellett and Schachman, 1971; Fyhn and Sullivan, 1975) can also present problems.

Evidence of pK_aS and pK_aR changing in a non-parallel fashion to predicted *in vivo* pH (Ultsch and Jackson, 1996) changes suggest the exothermic nature of both the Root effect and sickling results in a reduction of these effects 'within' the animal. Thus, at increased temperatures a greater degree of pH change is required to initiate pH induced deoxygenation and sickling, this may be beneficial as Hb-O_2 affinity is believed to decrease at higher temperatures.

Sickling and the Root effect- a potential link and implications for a sickling mechanism

The similar response of RBC sickling and deoxygenation to both pH and temperature suggests a link between the two. Given the large Root effect of cod (more than 60% of Hb deoxygenation at low pH) and the apparently large fraction of Hb involved in sickling (light microscopy suggests that the entire cell cytoplasm may be filled with Hb polymer), we conclude that the major cod Hb isoform, HbI, is involved in producing this. A recent study by Wetten *et al.* (2010) suggests that β1 in Atlantic cod should not display a Root effect, as it lacks Asp β 99 and Asp β 101 (Yokoyama *et al*., 2004), which are reputed to be key to the structural basis of Root effect globins. However, given Andersen *et al*. and Borza *et al*. (2009) linked HbI to the β1 via the electrophoretic variations caused by the two linked polymorphisms, this suggests that β1 is part of the major Hb tetramer and thus it does indeed exhibit a Root effect.

Despite the fact that the mechanism for sickling in fish is still unconfirmed, the differences in thermal response suggest it involves a different type of polymerisation reaction than in humans. Converse to sickling in humans, where increasing temperature increases the occurrence of sickling (Bunn, 1997), in Atlantic cod sickling decreased at higher temperature, suggesting sickling in cod is exothermic in nature. As such, we can assume the cause is unlikely to be due to interactions between the hydrophobic sites, as is seen with valine which replaced the hydrophilic glutamic acid in human Hb (Odievre *et al*., 2011), as this binding mechanism tends to impart endothermy (Butcher, 1979; Butcher and Hawkey, 1979). Hb polymerisation in cod could be the result of hydrogen, electrostatic or ionic bonding, all of which can be exothermic in nature.

With the occurrence of sickling greatest at low pH and deoxygenation alone unable to induce Hb polymerisation (Koldkjaer and Berenbrink 2007), this suggests a significant role of protonable groups in the aggregation mechanism. The high n_HS values imply a large number of proton binding sites interact during sickling. Histidine substitutions, at β10 and β77 have been proposed to be involved in the sickling reaction in whiting (Koldkjaer and Berenbrink, 2007) and the ability of His to change surface charge could potentially be responsible for the pH-dependent polymerisation of β1. Interestingly, the β77 His is immediately preceded by a mutation from positively charged lysine-present in most teleost beta chains- to negatively charged aspartic acid (M.B., unpublished). Aspartic acid contains a carboxylic acid group (COO^-) which readily reacts with [H^+] to form a COOH side chain. This reduces potential of ionic interactions with other amino acids and removes its hydrophilic properties, potentially changing globin structure. Furthermore, although at different sites, the presence of spatially close Asp and His residues and two Asp residues both of which are found with the Lys to Asp mutation have been implicated in involvement of the Root effect in a number of fish Hbs (Brittain, 2005).

While we do not propose the genetic basis for the Root effect and occurrence of sickle cells is the same, in fact both seem to be influenced by multiple sites, we do suggest that they

share a proton binding site, which when protonated may trigger subsequent deoxygenation and sickling in the cells. The aspartic acid residues may be partially responsible for both Root effect and sickling mechanisms would account for the pH dependence, and exothermic nature of both phenomena and may benefit from further investigation.

Chapter 5. Red blood cell sickling in fish: evolution and potential marker

5.1 Abstract

Recent evidence has shown the occurrence of *in vivo* red blood cell (RBC) sickling in fish, however little is still known about this phenomenon. Understanding is primarily limited by a low number of studied species and in those for which there is data, observations have often been made under extremes of pH, PO_2 or cell dehydration, limiting physiological relevance. In this study we primarily use a reduction in extracellular pH to physiological possible levels and observe the occurrence of sickle cells in a range of species from different orders. Sickling was found to be prevalent throughout the Gadiformes, though no conclusive evidence was found in other tested species. Reconstruction of the evolution of fish RBC sickling on a composite phylogenetic tree of all studied species, including literature sources, suggests two possible maximum parsimony evolutionary pathways; 1) two independent gains of sickling in Gadiformes and Batrachoidiformes, or 2) a gain of sickling at the base of Acanthomorpha and subsequent loss in derived Percomorpha after their divergence from Batrachoidiformes. With a wider breadth of species and sequence data, two potential sickling genetic markers can be identified in the β chain. Given the pH dependence of sickling in fish the most likely of these is the presence of an Aspartic acid residue at position β76.

5.2 Introduction

With increasing evidence of the occurrence of red blood cell (RBC) sickling in fish under physiologically relevant conditions, including findings of sickle cells *in vivo* (Koldkjaer and Berenbrink, 2007; Koldjkaer *et al*., 2013), there is greater necessity to understand this phenomenon. While reports of sickling occurrence in fish RBCs are not uncommon, much of the observations have occurred under extremes of pH and oxygen tensions or on a limited number of species (Yoffey, 1929; Thomas, 1971; Harosi *et al*., 1998; Koldkjaer and Berenbrink, 2007). Furthermore, despite a recent resurgence in efforts to characterise sickling in fish, gaps in knowledge still remain. Indeed, it is still unknown if sickling incurs a penalty or advantage to the fish.

It has been suggested that sickling in fish may provide a defence against blood borne parasites (Koldjkaer *et al*., 2013), in a similar manner to the protective effects of human sickle cell trait against malaria. Humans homozygous for the sickling trait, haemoglobin (Hb) SS, suffer from increased red blood cell fragility, severe anaemia and obstruction of capillaries by sickled cells leading to severe pain, organ failure and death (Serjeant, 2001). These deleterious effects are greatly reduced in heterozygote carriers Hb AS and are compensated by the protection against *Plasmodium falciparum* infection (Bookchin *et al*., 1976). A number of parasite species are known to infect fish, in blood and RBCs (Davies and Johnston, 2000). Although the available evidence so far suggests that sickling in fish does not increase the fragility of RBCs, contrary to what is seen in humans, as no lysis of the cells is seen to occur (Koldkjaer and Berenbrink 2007; Koldkjaer *et al*, 2013). Interestingly, recent research has suggested Atlantic cod lack a number of immune response genes, specifically the major histocompatibility complex (MHC) II (Star *et al*., 2011), despite this, cod are not exceptionally susceptible to disease under natural conditions (Pilstrom *et al*., 2005). Expansion of the number of MHC I genes has been partially attributed to this, but their unusual immune system may be further facilitated by sickling. Our collaborators at the University of Oslo are currently working to observe how widespread this loss of MHC II is and those in the University of Stirling are seeking to observe if presence of sickled cells reduce the ability of blood borne parasites to infect RBCs. Here we seek to observe how widespread RBC sickling is in fish.

As in most vertebrate sickle cells, the polymers which distort the RBC shape in fish consist of deoxygenated haemoglobin (Hb) (Undritz *et al*., 1960; Holman and Dew, 1964; Hawkey and Jordan, 1967; Taylor and Easley, 1974; Whitten, 1967; Jain and Kono, 1977; Butcher and Hawkey, 1979; Harosi *et al*., 1998; Koldkjaer and Berenbrink, 2007). Recent research has suggested low pH is a necessity to induce sickling in fish (Koldkjaer and Berenbrink, 2007; Koldkjaer *et al*, 2013). Previous evidence in one Gadidae (whiting, *Merlangius merlangus*) and one Batrachoididae (gulf toadfish, *Opsanus beta*) showed physiologically possible acidosis levels induced cell sickling in fish RBCs (Koldkjaer and

Berenbrink 2007; Koldkjaer *et al*, 2013), this has been confirmed in Atlantic cod (*Gadus morhua*) (Chapter 4). In Atlantic cod, a close association was observed between acid induced RBC deoxygenation and sickling, in this study we find additional evidence to support this theory in the freshwater gadiform, Burbot (*Lota lota*).

In most vertebrates sickling is genetically based and often only occurs with a mutation in Hb. In humans, sickling occurs only in individuals with HbS, brought about by a single amino acid change from β6 Glu (in HbA) to Val (Ingram *et al*., 1957). Deoxygenation of HbS induces a change in conformation and quaternary structure, this exposes a hydrophobic site on the β chain of the mutant HbS, this then binds to a complementary hydrophobic site of another HbS molecule and polymers form. In sheep and deer, sickling is also dependent on Hb genotype and although the genotypic and molecular causes are not understood in these animals, their Hbs can be separated electrophoretically (Butcher and Hawkey, 1979; Taylor, 1983). We have previously shown that despite similar electrophoretic differences being found in cod as in sheep, the polymorphisms which cause genotypic variation in the main Hb component in Atlantic cod, HbI, did not cause sickling (Chapter 4). However, the occurrence in the close relative whiting (*Merlangius merlangus*) and other gadiformes suggest the genetic mechanism is likely to be shared between species and not restricted to certain genotypes within a species (Koldkjaer and Berenbrink, 2007). A number of potential molecular mechanisms have been suggested to explain sickling in fish. Gadiform globins are cysteine rich, the formation of disulphide bonds between Cys residues have been implicated in the in vitro formation of Hb polymers in some non-Gadidae teleosts (Borgese *et al*., 1988; Borgese *et al*., 1992; Fago *et al*., 1993; Riccio *et al*., 2011). However, this mechanism is not believed to be effected by pH, a strong influencing factor in the occurrence of sickling in both studied gadiformes, whiting and cod. An alternative mechanism which may explain this was proposed by Koldkjaer and Berenbrink (2007), who noted histidine residues at β10 and β77 in three sickling species, which were absent in non-sickling species. His residues can exhibit different charge at different pH, these changes, when found on the external surface of the protein, may

affect Hb surface charge, promoting the pH dependent Hb polymerisation. The His substitution at β77 is also proceeded by a change from a positively charged lysine at position β76 to negatively charged aspartic acid (M.B., unpublished), which has been suggested to facilitate proton binding by His β77. Unfortunately limits in number of species in which sickling has been observed limits a better understanding of such mechanisms.

There is further difficulty as fish haemoglobins have multiple α and β globin genes, the number of which vary between lineages. Atlantic cod have a total of nine α and β globin genes, all of which are transcriptionally active at some life stage, which has made identifying the components of the dominant Hb difficult to determine (Wetten *et al*., 2010). However, two independent studies have found that the dominant Hb component in adult Atlantic cod, HbI, contains the 'β1' subunit (Andersen *et al.*, 2009; Borza *et al*., 2009). A number of studies have proposed which of the multiple α genes comprises the subunit potentially present in Atlantic cod HbI, however these results are not conclusive (Andersen *et al.*, 2009; Riccio *et al*., 2011). As such until further data is provided by our collaborators we begin by looking at Atlantic cod β1 and its homologous counterparts in other species.

In this study we test for sickling in a wide range of species, using physiologically low pH to induce sickling, a necessity to induce Hb polymerisation in fish red blood cells. Using literature and our own data we attempt a basic reconstruction of sickling evolution and use sequence data available from National Centre for Biotechnology Information (NCBI) and provided by collaborators from University of Oslo to test if the molecular mechanisms previously proposed to induce sickling remain true in a wider variety of species.

5.3 Materials and Methods

Animals

All marine Gadiformes (see Table 5.1) and Atlantic mackerel (*Scomber scombrus*, $N = 10$) were captured by hook and line on a commercial fishing vessel in Lofoten, Norway in August 2014. Burbot (*Lota lota*, $N = 14$) were captured by hook and line under ice near Oslo, Norway

during January 2014. Atlantic salmon (*Salmo salar*, *N* = 12) blood samples were provided from University of Bergen, Norway. Pipefish (*Syngnathus typhle*, *N* = 7) were provided by GEOMAR Helmholtz Centre for Ocean Research Kiel. Lesser spotted dogfish (*Scyliorhinus canicula*, *N* = 6) were kept at the University of Liverpool for several weeks in a 1000 litre tank equipped with a biological filter and aerated, recirculated artificial seawater kept at a temperature range of 11-13°C. Rainbow trout (*Oncorhynchus mykiss*, *N* = 7) were similarly housed, but in a fresh water system. Nile tilapia (*Oreochromis niloticus*, *N* = 5), and North African catfish (*Clarius gariepinus*, *N* = 5) were kept individually in 80-200 litre tanks at approximately 28°C.

Blood sampling

All animals were killed by a British Home Office Schedule 1, concussion and destruction of the brain. Immediately following death, blood was removed by puncturing of the caudal vessels with a 1 ml syringe, whose dead space was filled with 50 mg ml^{-1} of heparin solution (180 units/mg, sodium salt from porcine intestinal mucosa, Sigma-Aldrich). Due to the small nature of pipefish, blood was obtained by removal of the tail and Samples were kept at 4°C until washing step could be performed this varied between groups. All samples were washed within 5 hours of sampling, with the exception of burbot samples and pipefish (24hrs). RBCs were isolated by centrifugation (3000 rcf, 4°C, 4 mins) and washed in an isotonic saline solution; (all concentrations given in mmol l^{-1}) NaCl (125.5), KCl (3), $MgCl_2$ (1.5), $CaCl_2$ (1.5), D-glucose (5) and Hepes (20) (as used by Koldkjaer and Berenbrink, 2007), adjusted to pH 7.97 at 15°C (using a Lazar Model FTPH-2S Micro Flow Through pH Measurement Electrode and Jenco 6230N combined pH/mV/temperature portable meter (Jenco Collaborative, California, USA). Washing was repeated a further two times before the cells were re-suspended at an approximate haematocrit (Hct) of 5-10 % and stored overnight at 4°C with a large air reservoir, placed on the side to maximise exchange surface area between saline and sedimented cells.

Prior to experiments the following day cells were washed again and resuspended at a Hct between 8-13%. This RBC suspension was then diluted 10 fold in 7.97 saline and a further 10 fold in saline of pH ranging from 6.5-8.05 (all adjusted at 15°C). These mixed solutions were incubated at temperatures 5.0°C under air, North African catfish and Nile tilapia samples were incubated at 27.5°C. pH of the saline solution mixtures without RBCs were measured at each temperature under air. RBC suspensions were equilibrated for at least 20 minutes before an aliquot fixed for later microscopy (see below).

Cell fixation, light microscopy and sickling analysis

A 2% gluteraldehyde fixation solution was made from a 25% stock (Grade I; Sigma-Aldrich Company Ltd, Gillingham, UK) using distilled water and saline to reach an osmolality of 300 mOsm. Equal quantities of sample and fixation solution, to reach a final 1% glutaraldehyde concentration and stored at 4°C until analysis on the microscope. The samples were counted on an Axiovert 135 TV microscope (Carl Zeiss Microlmaging GmbH, Göttingen, Germany; using a 100x oil immersion lens and direct interference contrast) and viewed with Scion Image software (Scion Corporation, Frederick, MD, USA).

Fractional oxygen saturation and sickle cells were plotted against measured pH values at each temperature. Mean apparent cooperativity constant for sickling-linked proton binding (n_H) and an apparent extracellular pH at half maximal sickling (pK_a) were determined using

$$f=-1*x^n/(x^n+(10^{-a})^n) \qquad \text{Equation 5.1}$$

where n is the slope of the curve and equates to n_H, a is pH when 50% of cells are sickled, x is the proton concentration (10^{-pH}) and f is the predicted dependent variable.

Phylogenetic tree construction

The evolution of fish RBC sickling was reconstructed on a composite phylogenetic tree of all species for which the presence or absence of sickling under physiologically relevant conditions of pH, temperature and PO_2 was documented in this study or could be reliably inferred from the literature. Phylogenetic relationships within the cod subfamily Gadidae

followed Owens (2015). Other relationships within the order Gadiformes were according to von der Heyden and Matthee (2008). Higher level relationships within the spiny-rayed fishes (Acantomorpha) and the more basal members of the class of ray-finned fishes (Actinoperygii), respectively, followed Near *et al*. (2013) and (2012). Interrelationships of the three elasmobranch species that were used as the outgroup were obtained from Naylor *et al*. (2012). MEGA version 6.06 was used to draw the composite phylogenetic tree and to reconstruct the evolution of RBC sickling by the parsimony criterion.

Haemoglobin β subunit alignment

Atlantic cod Hb β1 sequence was obtained from NCBI, Atlantic salmon, rainbow trout and tilapia were also found on NCBI, these were the only β subunit sequences available at time of collection (December, 2015). All other sequences were provided by collaborators from University of Oslo, these were obtained from full genome sequencing and aligned in BioEdit.

5.4 Results

Red blood cell morphology changes

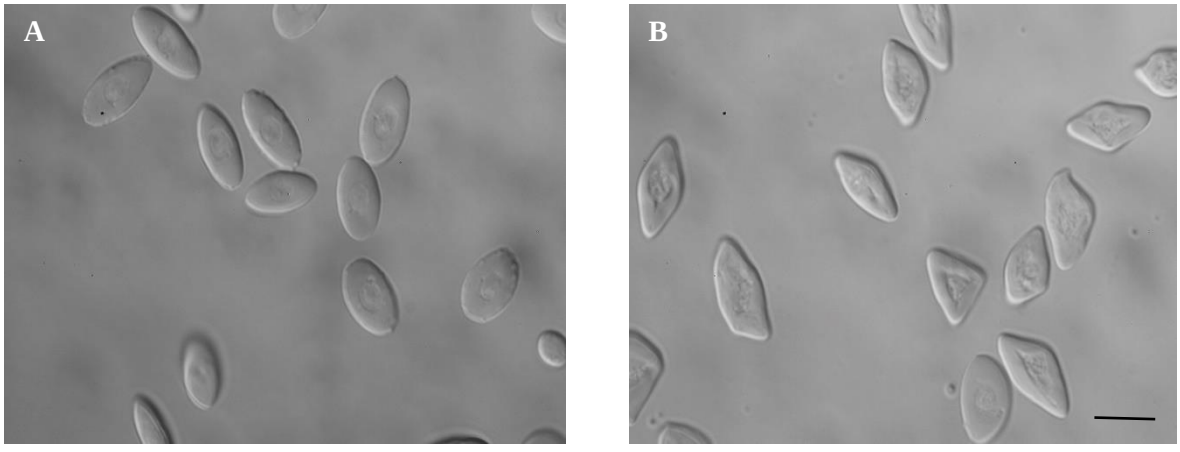


Figure 5.1: *Light microscopy images of representative normal (A) and sickled (B) gadiform RBCs fixed in 1% glutaraldehyde obtained using Zeiss Axiovert 135 TV microscope, x100 oil immersion objective and differential interference contrast. The scale bar applies to both panels and corresponds to 10 µm.*

'Normal' RBCs in all species were elliptical in shape and smooth in appearance with homogenous cytoplasms and nucleoplasms (Figure 5.1A). The occurrence of RBC sickling was found to be widespread at low pH in Gadiformes (Table 5.1). Sickle cells in these species were characterised by the presence of irregular, usually angular shaped RBCs with visible striations or granular texture in the nucleus and/or cytoplasm (Figure 5.1B).

With the exception of pipefish, no change in cell appearance was observed in any of the other tested species with changing pH. Pipefish showed evidence of morphological changes in a limited number of cells (about 1-3% of cells) also at low pH (Figure 5.2). However, the globular and crystal like structures present in the cytoplasm were not typical of sickle cells observed in other fish species, thus at this time sickling in pipefish remains uncertain.

Table 5.1: *Summary of fish species tested for sickling in this study and if sickling was present at any tested condition*

Common name	**Species name**	**Order**	***N***	**Sickle**
Atlantic cod	*Gadus morhua*	Gadiform	10	Yes
Whiting	*Merlangius merlangus*	Gadiform	10	Yes
Burbot	*Lota lota*	Gadiform	14	Yes
Haddock	*Melanogrammus aeglefinus*	Gadiform	5	Yes
Saithe	*Pollachius virens*	Gadiform	10	Yes
Cusk	*Brosme brosme*	Gadiform	1	Yes
Pollock	*Pollachius pollachius*	Gadiform	2	Yes
Ling	*Molva molva*	Gadiform	2	Yes
Hake	*Merluccius merluccius*	Gadiform	4	Yes
Norway pout	*Trisopterus esmarkii*	Gadiform	2	Yes
Poor cod	*Trisopterus minutus*	Gadiform	4	Yes
Pipefish	*Syngnathus typhle*	Syngnathiform	7	Unclear
Mackerel	*Scomber scombrus*	Scombriform	10	No
Lesser spotted dogfish	*Scyliorhinus canicula*	Carcharhiniform	6	No
North African catfish	*Clarius gariepinus*	Siluriform	5	No
Nile tilapia	*Oreochromis niloticus*	Perciform	5	No
Rainbow trout	*Oncorhynchus mykiss*	Salmoniform	7	No
Atlantic salmon	*Salmo salar*	Salmoniform	12	No

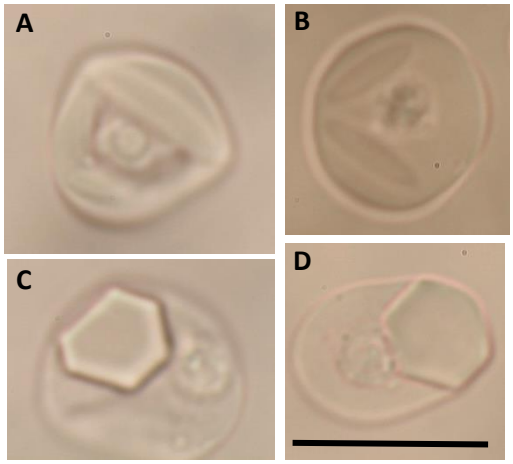


Figure 5.2: *Light microscopy images using differential interference contrast representative of the two types of unusual pipefish RBCs characterised by presence of globular (A-B) and crystal (C-D) structures in the cytoplasm. The scale bar applies to all panels and corresponds to 10 µm.*

Sickling in Gadiformes

Sickling curves for six gadiform species at 5°C can be seen in Figure 5.3. In all tested Gadiformes decreasing pH facilitates Hb polymerisation and production of sickled cells. The transition from no observable sickled RBCs at high pH to 100% sickled RBCs at lower pHs took place within a relatively small pH range ~0.2 pH units in most individuals (Figure 5.3). This was reflected by the high n_H (mean apparent cooperativity constant for sickling-linked proton binding) ranging between 10.7-15.5 for all individuals (where calculable using Eq. 5.1, Figure 5.3) and did not seem to consistently vary with species.

Limited variation was seen in sickling pK_a within and between four of the gadiforms (Figure 5.3), with no significant difference between haddock and cod (7.66 ± 0.04 and 7.70 ± 0.03, respectively; $p = 0.002$, Student's T-test) and ling (7.66 ± 0.00) and pollock (7.69 ± 0.01) pK_a values falling within the range of the former two species.

Sickling within individual saithe (*Pollachius virens*) and burbot RBCs varied quite considerably. The former has pK_a values ranging from 7.52 to 7.84, the highest of these,

means that a reduction of only 0.07 pH units from typical arterial blood pH may be required to induce 50% of cells to sickle (value predicted from predicted *in vivo* pH; Ultsch and Jackson, 1996).

Unlike saithe, where 100% sickling was observed in all individuals, not all burbot cells could be induced to sickle within the pH range used. Figure 5.3 shows sickling only reaches a maximum of 40% of cells even at the lowest pH in two burbot, while the remaining two animals reached 100% sickled cells. Interestingly this difference in pH induced sickling in burbot also correlated with a pH induced deoxygenation or Root effect, as first suggested in Atlantic cod (Chapter 4). Figure 5.4 indicates a reduced Root effect magnitude in the two burbot which displayed reduced sickle cell levels, appears to confirm that sickling is linked to the Root effect.

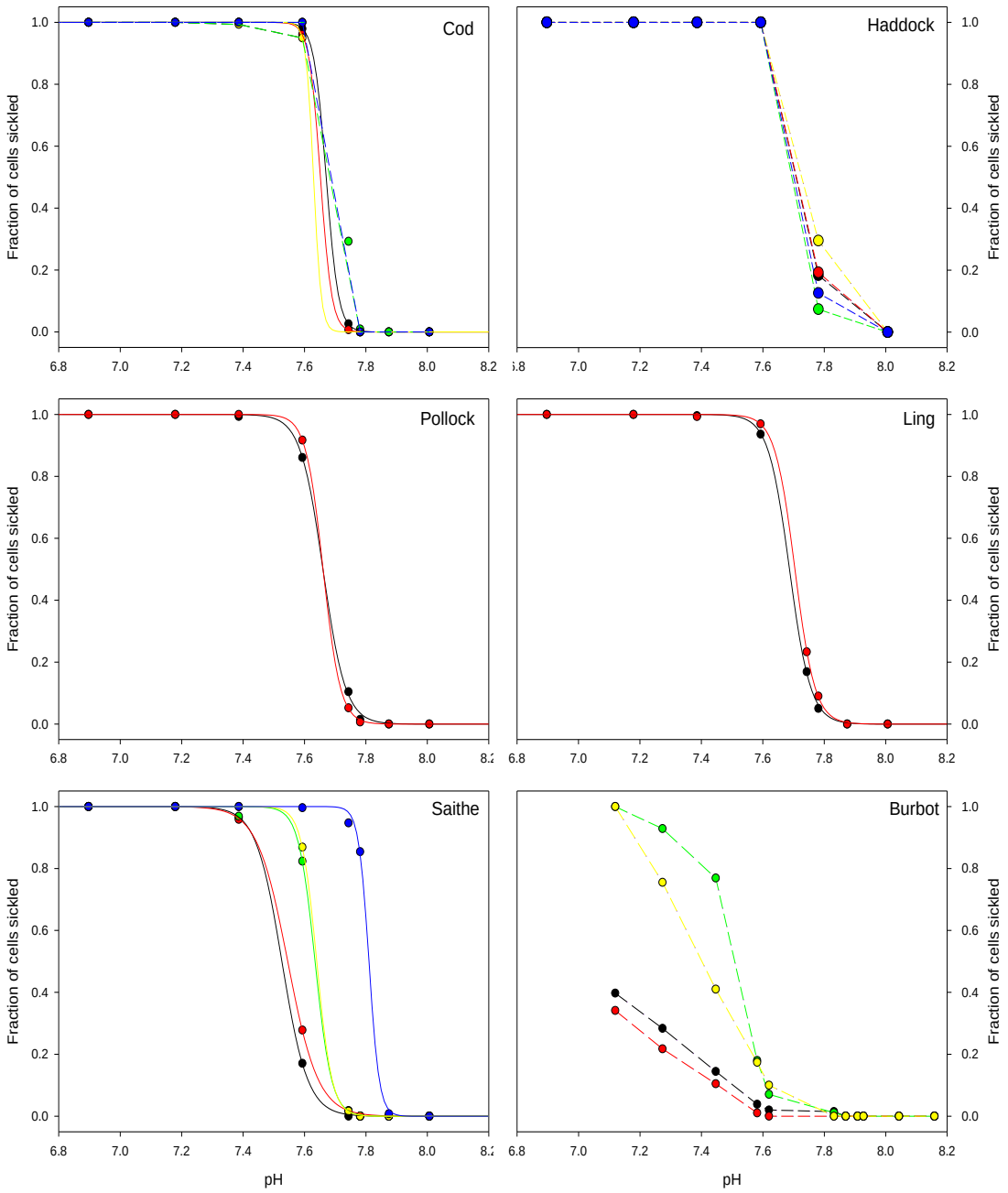


Figure 5.3: *Fraction of sickle cells against saline pH of six gadiform red blood cell suspensions equilibrated with air and at 5 °C. Colours indicate different individuals Atlantic cod (N=5), Haddock (5), Pollock (2), Ling (2) Saithe (5) Burbot (4); symbols represent measured values and curves are fitted using the* pK_a *and* n_H *for each individual (see Materials and Methods); if use of this equation was not possible measured points were joined by dashed lines.*

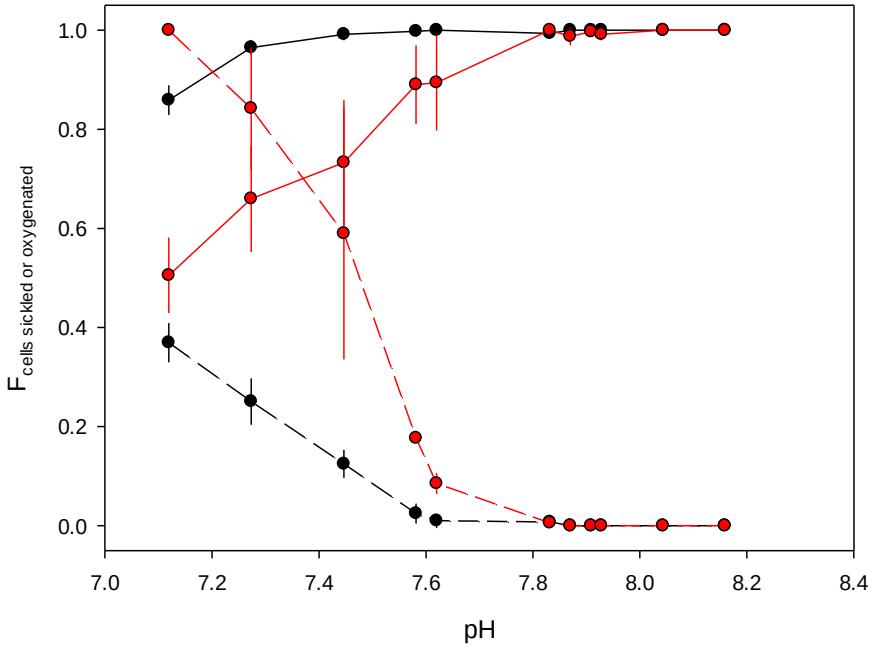
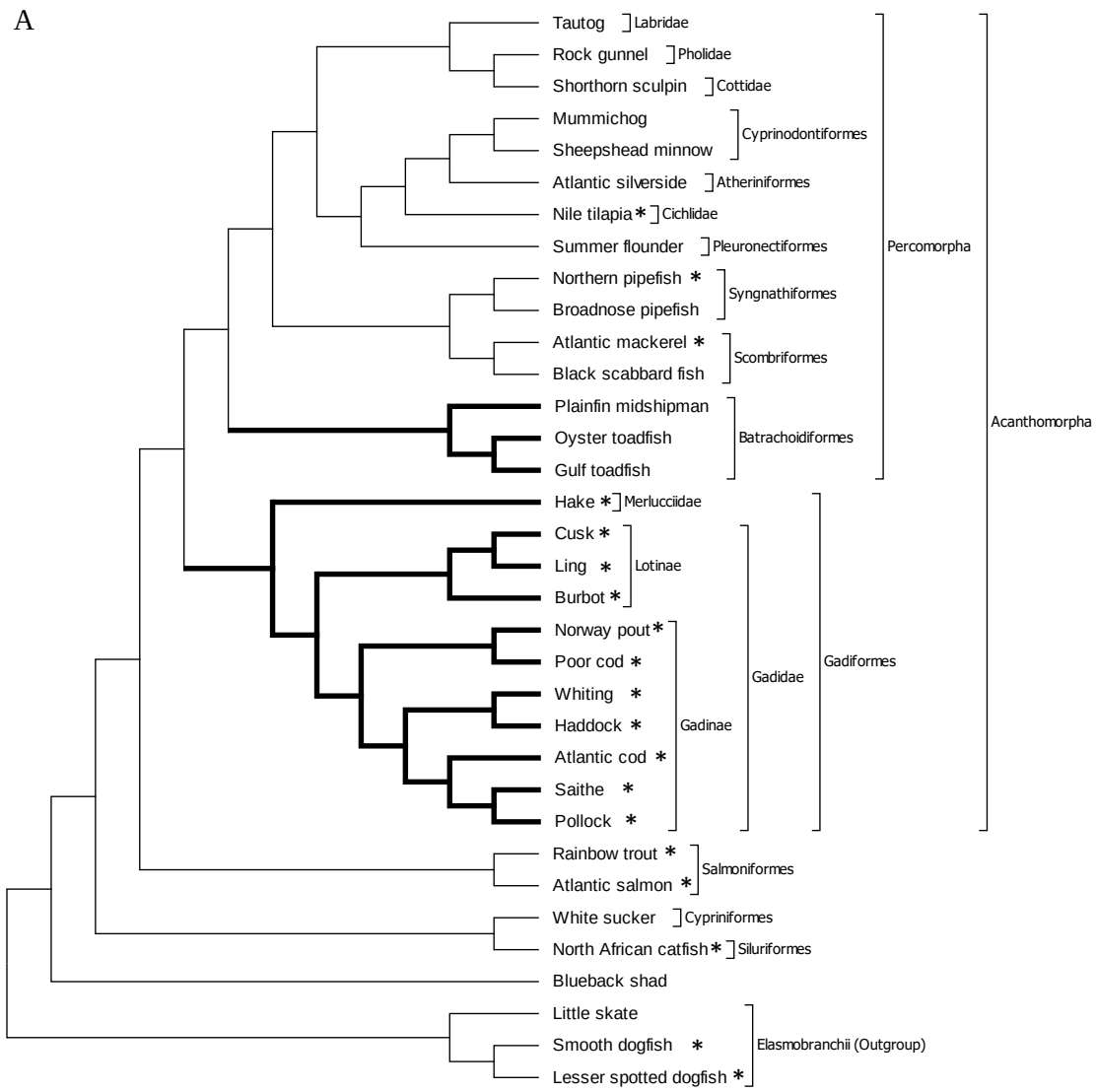


Figure 5.4: *Fraction of sickle cells (dashed lines) and fractional oxygen saturation (solid lines) in red blood cell suspensions of burbot as a function of saline pH at 5°C. Symbols represent mean values ± range for two burbot exhibiting high pH induced sickling and deoxygenation (red) and two burbot with low levels of pH induced sickling and deoxygenation (black).*

Evolution of sickling

Initial work suggests two possible equiparsimonious sickling reconstructions, with either two independent origins of sickling in Gadiformes (codfishes) and Batrachoidiformes (toadfishes) (Figure 5.5A), or a gain of sickling at the base of Acanthomorpha and subsequent loss in derived Percomorpha after their divergence from Batrachoidiformes (Figure 5.5B).

A


Tautog
Labridae
Rock gunnel
Pholidae
Shorthorn sculpin
Cottidae
Mummichog
Sheepshead minnow
Cyprinodontiformes
Atlantic silverside
Atheriniformes
Nile tilapia*
Cichlidae
Summer flounder
Pleuronectiformes
Percomorpha
Northern pipefish *
Broadnose pipefish
Syngnathiformes
Atlantic mackerel *
Black scabbard fish
Scombriformes
Plainfin midshipman
Oyster toadfish
Gulf toadfish
Batrachoidiformes
Acanthomorpha
Hake *
Merlucciidae
Cusk *
Ling *
Burbot *
Lotinae
Norway pout*
Poor cod *
Whiting *
Haddock *
Atlantic cod *
Saithe *
Pollock *
Gadinae
Gadidae
Gadiformes
Rainbow trout *
Atlantic salmon *
Salmoniformes
White sucker
Cypriniformes
North African catfish*
Siluriformes
Blueback shad
Little skate
Smooth dogfish *
Lesser spotted dogfish *
Elasmobranchii (Outgroup)

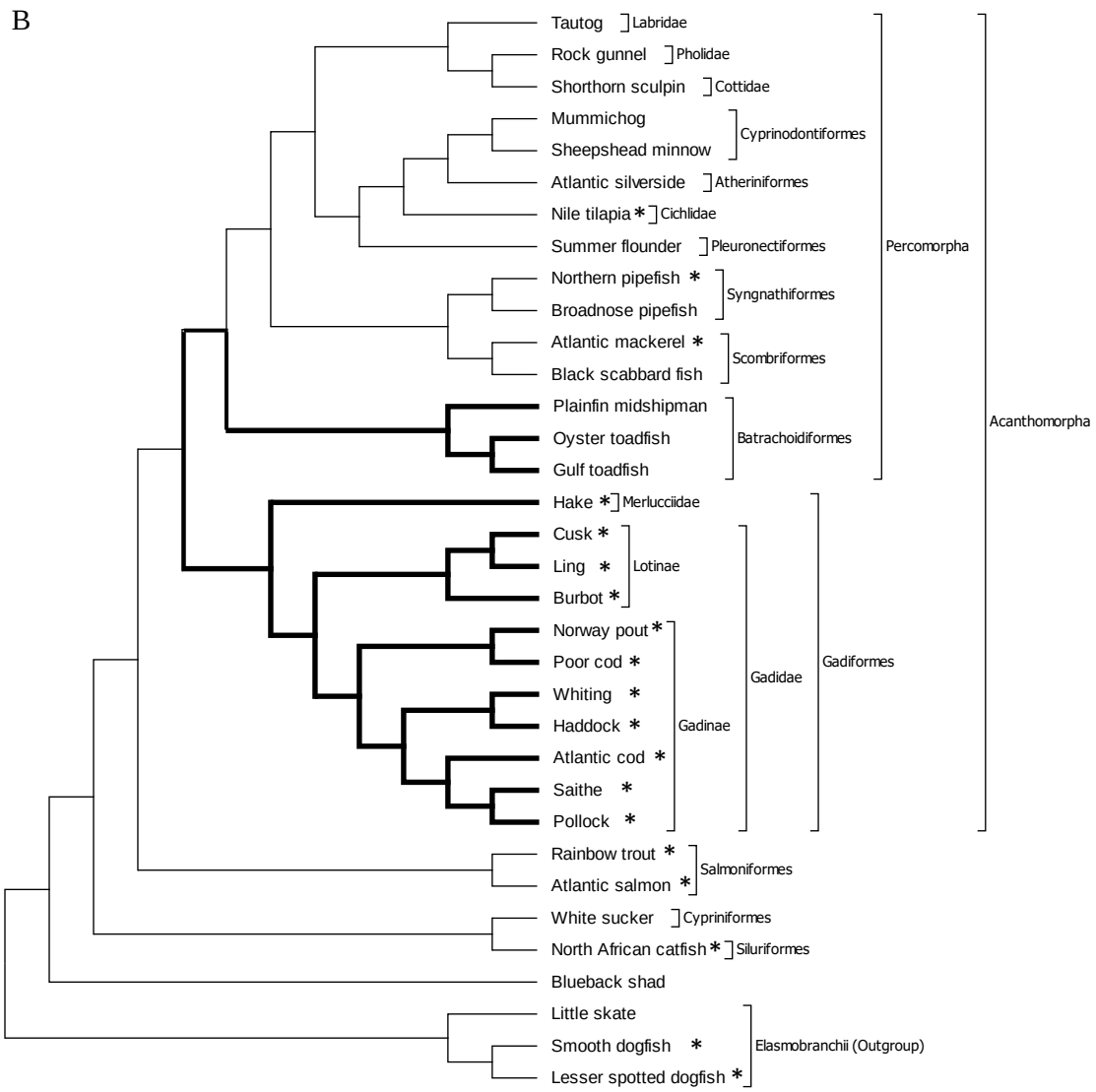


Figure 5.5: *Evolutionary reconstruction of fish red blood cell sickling on a composite phylogenetic tree (von der Heyden and Matthee, 2008; Naylor et al., 2012; Near et al., 2012 and 2013; Owens, 2015; constructed in Mega 6.0). Rooted with Elasmobranchs as outgroup. The presence and absence of sickling indicated by thick and thin branches, respectively. A and B show two equi-parsimonious reconstructions. Individuals tested in this study indicated by (*).*

Proposed sickling genetic marker

The adult Hb beta chain amino acid sequence is well conserved among Gadiformes, with many variations often present in all members within subfamilies (Figure 5.6). Alignments of the β sequences show two potential sites where amino acid variations is consistent and present in only sickling species. These are; aspartic acid residue at position 76 and cysteine residue at position 107. An additional site indicated is position 51 where most sickling species possess either the aspartic acid but the Merluciidae (hake) has a glutamic acid present.

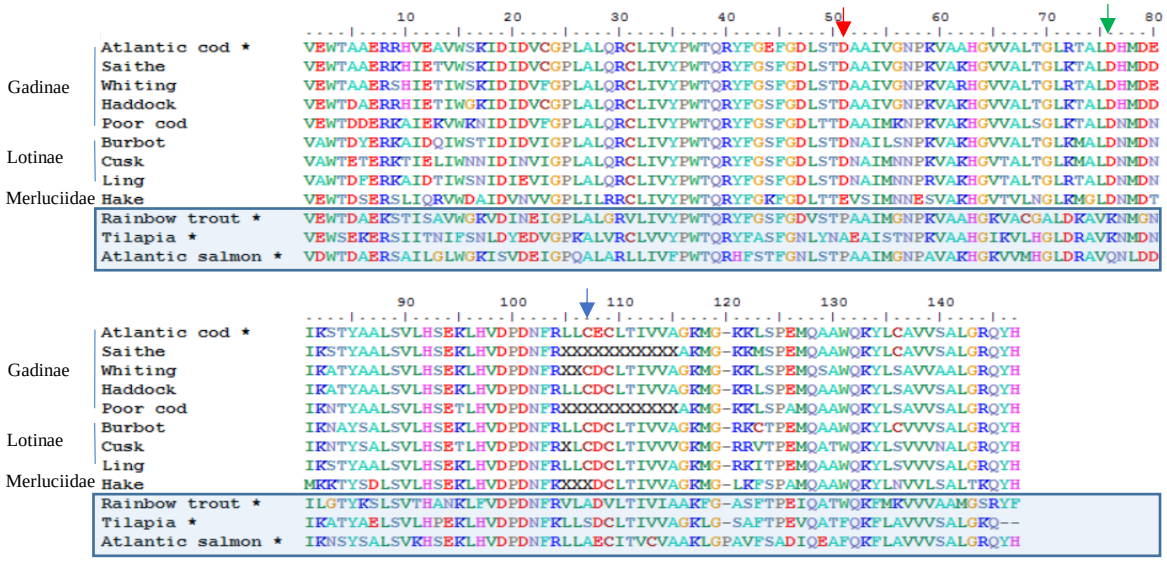


Figure 5.6*: Haemoglobin beta chain protein sequences from 12 teleost fishes which are known to have sickling absent (blue box) or present (no box) in red blood cells. Sequences taken from NCBI indicated by *, those without were provided by collaborators from University of Oslo. Proposed sites for sickling marker are indicated by arrows; cysteine (blue); aspartic acid (green); aspartic/glutamic acid (red).*

5.5 Discussion

This research forms part of an ongoing collaborative study to gain insight into the causes and consequences of Hb polymerisation and subsequent RBC sickling in fish. However, work in this field is often hindered due to limited number of species tested and the non- physiological conditions under which sickling is often observed. Thus the initial step towards understanding

sickling in fish is a more thorough investigation of a wider spectrum of species where it may occur under physiological like conditions.

Throughout the Gadiformes, sickle cells exhibited similar morphological changes as previously seen *in vitro* in Atlantic cod and haddock (Thomas, 1971; Harosi *et al*., 1998) and *in vivo* in whiting (Koldkjaer and Berenbrink 2007). The most commonly occurring are those which form triangular, diamond-shaped or polygonal shaped cells with evidence of long, aligned filament of Hb. Much less frequently observed were RBCs which retained their normal oval shape but acquire a granular appearance. These differences are believed to be the result of speed of Hb polymer formation, as in humans, where more rapid polymerisation causes short, disordered fibres (Eaton and Hofrichter, 1987; Bunn, 1997; Christoph *et al*., 2005; Koldkjaer and Berenbrink 2007).

Previous research by Mork (1985) which exposed cells to low pH and prolonged anoxia, found evidence of morphological changes in mackerel RBCs, which were classified as sickle cells. However, our observations show no evidence of any change in appearance of mackerel RBCs and so these cells do not sickle under our tested conditions. It is possible that after exposure to more extreme pH and low PO_2 sickle cells may be observed in mackerel, but due to equipment limitations in the field lab we were unable to attempt to replicate this finding at this time.

Presently this study is unable to clarify the occurrence of sickling in Northern pipefish. While there is evidence for changes in RBC morphology at low pH, they are not typical of those observed in codfishes or toadfishes (Thomas, 1971; Harosi *et al*., 1998; Koldkjaer and Berenbrink 2007; Koldkjaer *et al*, 2013). Dawson (1932) exposed Northern pipefish RBCs to air drying for 10 days and was able to induce cell changes which did resemble sickle cells in other species, suggesting Hb polymerisation is possible in this species, although possibly only under cell dehydration. Interestingly, the formations observed within pipefish RBCs resemble structures formed by human HbC. HbC is an abnormal haemoglobin in which substitution of a glutamic acid residue with a lysine residue has occurred at position 6 of the β-globin chain. In humans, HbC polymerises less readily than its more severe counterpart, HbS, but forms

elongated hexagonal crystals. However, more intensive studies of the structures formed in Northern pipefish RBCs are necessary to determine if they too consist of Hb polymers. Interestingly, as in Atlantic cod, pipefish is believed to have lost important immumological genes (unpublished report from collaborators), if sickling is confirmed in this group it will provide stronger support for sickling providing protection against blood borne parasites, as in humans (Bookchin *et al*., 1976).

This study aimed to simulate physiologically relevant conditions as such pH was only dropped as low as 6.8 and P_{O_2} of 157 mmHg was used. While such a high P_{O_2} will not be indicative of *in vivo* values at all times, they are possible. Indeed, Bohr (1894) showed that P_{O_2} in the Atlantic cod swimbladder may rise to 900 mmHg or above, while values of nearly 1200 mmHg have been measured in the choroid *rete mirabile* of the eye of this species (Wittenberg and Wittenberg, 1962). The extreme conditions RBCs are exposed to in the *rete mirable* continue with low pH, as values as low as 6.5–7.0 have been recorded in the swimbladder *rete mirabile* of the European eel (Steen, 1963; Kobayashi *et al*., 1990). Nevertheless, it is unknown if the period of exposure to these conditions will be sufficient to induce sickling in fish RBCs. In humans, Hb polymerisation is delayed for a sufficient length of time that passage through capillaries and the venous system at low P_{O_2} is rapid enough to prevent extensive in vivo RBC sickling (Mozzarelli *et al*., 1987; Christoph *et al*., 2005). Despite this, general blood acidosis as a result of intensive exercise can reduce pH levels to between 7.3 and 7.5 and require several hours to return to resting values (Steen, 1963; Holeton *et al*., 1983; Milligan and Wood, 1987; Kobayashi *et al*., 1990; Nelson *et al*., 1996; Perry *et al*., 1991). Under these conditions, a possible delay time before acid induced Hb polymerisation occurs, is unlikely to protect from sickling.

Gadiform sickling curves showed pH induced sickling occurring as high as pH 7.8 with 100% of cells sickled occurring by 7.4 in most individuals (Figure 5.3). This suggests there is a high possibility of the occurrence of sickle cells *in vivo*. However, the present study used an overnight incubation period for the cells, this process results in removal of catecholamines from the RBC suspensions. Within the animals during exercise a release of

adrenaline and noradrenaline (Berenbrink and Bridges, 1994a and b; Perry *et al*., 1996) triggers a β-stimulated response (βNHE), whereby catecholamines bind to β-receptors on RBC surface increasing the activity of Na^+ /H^+ exchangers in the cell membrane. This causes an increase in H^+ loss and Na^+ uptake to the cell and consequently will maintain or increase intracellular pH (pHi) and cell water content (Nikinmaa, 1982; Holk and Lykkeboe, 1995; Berenbrink *et al*., 2005) reducing the incidence of Hb polymerisation (Koldkjaer and Berenbrink, 2007).

The acidosis-induced sickling in Gadiformes confirms findings in previous studies, both *in vitro* and *in vivo* (Harosi *et al*., 1998; Koldkjaer and Berenbrink, 2007; Koldkjaer *et al*, 2013; Chapter 4) and contrasts with human sickling which is primarily induced by low PO_2 (Lange *et al*., 1951; Bookchin *et al*., 1976; Ueda and Bookchin, 1984). In whiting deoxygenation alone could not induce sickling (Koldkjaer and Berenbrink, 2007) this was also the case in Atlantic cod (unpublished data, Barlow 2015). However to ensure sickling was not promoted by low PO_2 in non-gadiform species, lesser spotted dogfish, Nile tilapia, North African catfish and rainbow trout RBCs were also tested under anoxia and a range of pHs, but this also did not induce sickling (data not shown).

Reconstruction of presence and absence of sickling under physiologically relevant conditions of pH, temperature and PO_2 determined from this study or from reliable literature data (Thomas, 1971; Mattisson, 1982; Harosi *et al.*, 1998; Koldkjær *et al*., 2013) on a composite phylogenetic tree (von der Heyden and Matthee, 2008; Naylor *et al*., 2012; Near *et al*., 2012 and 2013; Owens, 2015) showed two potential evolutionary pathways. The first suggests sickling in Gadiformes evolved independently from sickling in Batrachoidiformes (toadfishes); the second proposed the evolution of sickling occurs an Acanthomorpha ancestor and was lost in Percomorpha post divergence from Batrachoidiformes. In Gadiformes a reduction in PO_2 increases the occurrence of sickling (Koldkjaer and Berenbrink, 2007), however in Gulf toadfish (*Opsanus beta*) PO_2 has a biphasic effect on sickling (Koldkjær *et al*., 2013), this difference in cause of sickling may indicate a different molecular mechanism, supporting independent evolution.

The Cys residue observed at position 107, although not observable in all sequences, as found only in sickling animals. The globins of Gadiformes are rich in Cys and the formation of disulphide bonds between two Cys amino acids has been implicated in the *in vitro* formation of Hb polymers in some other teleosts (Borgese *et al*., 1988; Borgese *et al*., 1992; Fago *et al*., 1993; Riccio *et al*., 2011). However, this mechanism would not explain the high pH dependence observed in RBC sickling in codfishes. The negatively charged aspartic acid present at position 76 in all sickling fish species for which sequence data was available, is strongly influenced by pH. Reducing pH, increases $[H^+]$, these hydrogen ions react with carboxylic acid group (COO^-) of the aspartic acid, to form a COOH side chain. This reduces potential of ionic interactions with other amino acids and removes its hydrophilic properties, potentially changing globin structure. Another aspartic acid was present in most sickling fish at position 51 of the β chain, however Hake which also exhibits RBC sickling, had a glutamic acid. Glu has similar properties to Asp, both contain the acidic polar, negative COO- side chain and are likely to respond in a similar manner.

With additional species sequence and sickling data now available, the His residues at positions 10 and 77 which Koldkjaer and Berenbrink (2007) suggested to be responsible for the pH-dependent polymerisation, are evidently not necessary for Hb to polymerise. However they may still influence sickling, particularly as the variation in sickling curves between indiviuals of the same species (i.e. saithe and burbot) suggest the sickling mechanism may be more complex than a single amino acid mutation and may be influenced by many sites. Indeed, the steep pH dependence (high n_H) of sickling suggests that the molecular mechanism involves several interacting proton-binding sites. As in whiting and toadfish (Koldkjaer and Berenbrink 2007; Koldkjaer *et al*, 2013) presence of additional binding sites or alterations in protein surface charge may influence how readily Hb polymerises. Unfortunately, without sequence data of individual such variations are difficult to predict.

Future work

Understanding of both the evolution and molecular mechanism of sickling will be further enhanced with additional data on the presence or absence of sickling in a wider variety of

species. Particular interest could be shown to the Gadiform, to establish if sickling did evolve at the base of the order or at an early point during its evolutionary history. Unfortunately this is problematic, as the more basal families of the codfishes including Macrouridae (Grenadiers) and Euclichthyidae (Eucla cod) are deepwater fish, captured only rarely.

Using the data available, we were able to suggest a potential candidate involved in the mechanism of sickling in the primary structure of the Hb beta chain of Gadiformes. Nevertheless, additional functional sites may occur in the α subunits and it is likely the different globin isoforms within species exhibit different functional properties. These must be studied in isolation to establish this. Determination of whether sickling mechanisms between codfishes, toadfishes and potentially pipefishes are different require more thorough sequence data. Furthermore, to fully understand the consequences of primary structure variations detailed molecular structure–function analyses are required, ideally on otherwise similar, polymerising and non-polymerising Hbs.

Finally, work has begun to understand the consequences of sickling, particularly on the immune system. This involves reconstructing the evolutionary loss of immune genes, including MHC II, and tests of RBC vulnerability to parasite invasion in sickled and non-sickled cells.

Chapter 6. DNA extraction from fin tissue and sequencing of Hb β1 polymorphism in Atlantic cod

6.1 Abstract

A number of experimental studies have suggested the different Atlantic cod (*Gadus morhua*) haemoglobin (Hb) I alleles may influence the animal's response to temperature. A knowledge of wild thermal experience is key to understanding if HbI genotype may effect changing cod distribution in respect to climate change. A number of recent studies are using internal data storage tags (DSTs) to monitor direct thermal experience of individual cod. This study aimed to devise a method of genotyping HbI genotype from the archival fin clips taken from tagged cod. Here, we outline a method for reliable, cost effective genomic DNA (gDNA) extraction which yields high concentration and quality from small quantities of fin tissue. The isolated gDNA was used to test a simple method of polymerase chain reaction (PCR) amplification to determine HbI genotype. In this study we compare these methods between two tissue types, muscle and fin, and test the effect of age and storage conditions of the fin clips.

6.2 Introduction

Atlantic cod (*Gadus morhua*) is a commercially valuable species found throughout shelf seas in the North Atlantic. A combination of heavy exploitation by fisheries and warming seas as a consequence of climate change, have resulted in North Sea cod populations at a record low and some stocks are thought to be close to collapse (Hutchings and Myers, 1994; Pope and Macer, 1996; Cook *et al*., 1997; O'Brien *et al*., 2000; Christensen *et al*., 2003; Hutchings, 2004; Rose, 2004; Worm *et al*., 2006; Pitcher *et al*., 2009). The rate of cod stock decline has varied across the North Sea (Hedger *et al*., 2004; Horwood *et al*., 2006), suggesting spatially discrete populations which are believed to be largely reproductively isolated (Kritzer and Sale, 2004; Reiss *et al*., 2009; Ciannelli *et al*., 2013; Kritzer and Liu, 2013; Neat *et al*., 2014) and differentially adapted to physico-chemical environmental conditions (Nissling and Westin, 1997; Brix *et al*., 1998 and 2004). This is particularly problematic as spatially intensive fishing practices may result in a depletion of particular populations, potentially

reducing diversity and ability to cope with changing environments (Schindler *et al*., 2010). While the effects are still not fully understood, the haemoglobin (Hb) I genotype may be one such factor (Andersen, 2012; Ross *et al*., 2013).

The two main Hb components present in adult cod, HbI and HbII, can be separated by agar gel electrophoresis, of these the former is predominant in the adult animals (Sick, 1961). HbI was further shown to have two alleles, which result in three genotypic variants; HbI 1/1, HbI 1/2, HbI 2/2. The distribution of the two Hb alleles has led to suggestions these are strongly influenced by environmental temperature (Andersen, 2012; Ross *et al*., 2013), with the HbI 2 allele dominant in colder waters to the North, while HbI 1 predominantly occurs in warmer regions (Sick, 1965; Jamieson and Birley, 1989; Andersen *et al*., 2009; Ross *et al.* 2013). A similar, less defined cline is seen in the western North Atlantic (Sick, 1965).

Furthermore, Ross *et al.* (2013) collated HbI frequency data from 1961 to 2002, spatially covering most of Atlantic cod distribution range and seem to show HbI 1 allele frequency increased with increasing temperature in all regions. Indeed, a number of laboratory based studies suggest different water temperature preferences between two homozygotes, with HbI 2/2 favouring colder waters (Petersen and Steffensen, 2003; Behrens *et al*., 2012). However, these results are also not consistent. While Petersen and Steffensen (2003) showed wide variations in preferences between the genotypes from 8.2°C (HbI 2/2) and 15.4°C (HbI 1/1), Behrens *et al*. (2012) found a much smaller difference 8.9°C and 11.0°C, respectively and Gamperl *et al*. (2009) were unable to demonstrate a significant difference in upper thermal tolerance between the genotypes. These results suggest HbI 1/1 may have a selective advantage at warmer temperatures at least under some circumstances.

While discrepancies between these studies may be the result of differing methodology, it suggests experimental studies do not fully recognise the full range of physiological and ecological factors which may influence individual cod habitat. Determining

how HbI genotype may or may not affect thermal limitations of cod in a natural environment is important, in conjunction with experimental studies, to ensure spatially intensive fishing does not result in a loss of diversity which may help cod survive future warming.

Large scale electronic tagging programs are being used to record temperature experiences of wild cod (Righton *et al.*, 2007 and 2010; Neat *et al*., 2014) and are unique as they provide direct observation of the preferred thermal niche of individuals. A number of these expeditions have taken fin clips for genetic analysis, a resource which has been remarkably underutilised.

Fin clips are a form of non-destructive sampling, which implies the use of tissues (blood, skin, scales, muscle biopsy) without critical damages to the animals. Some success has been had with DNA extraction from such samples (Shiozawa *et al*., 1992; Taggart *et al*., 1992; Zhang *et al*. 1994; Nielsen *et al*., 1999; Adcock *et al*., 2000; Wasko *et al*., 2003; Lucentini *et al*., 2006). However, there are some difficulties due to the consistency and small size of these fin tissues which can lead to a low amount and poor quality of total recovered DNA.

The genetic and molecular basis of the Atlantic cod HbI polymorphism is now understood to be due to three non-synonymous mutations resulting in two amino acid changes in the β1 Hb chain (Andersen *et al*., 2009; Borza *et al*., 2009). Andersen *et al*. (2009) outline a method for genotyping HbI by single nucleotide polymorphism (SNP) analysis, unfortunately repeated attempts to utilise this method in the present study failed.

The present study aimed to: (1) devise and test a cheap and efficient method to extract high quality DNA from small quantities of fresh and archival Atlantic cod fin tissue and compare quality change with age, storage and with one of the most common sources of genetic data, muscle biopsy; (2) develop a method to PCR amplify and sequence HbI polymorphisms from genomic DNA (gDNA); and (3) look at preliminary data to observe a possible trend in wild cod thermal preference between HbI genotypes.

6.3 Materials and Methods

Sample origins and storage

24 Atlantic cod were line caught from the Mersey Estuary adjoining the Irish Sea near Liverpool, U.K., (53°25' North, 3.02°1' East). Animals were killed by a British Home Office approved Schedule 1 method, involving concussion and destruction of the brain. A portion of fin and muscle were removed, and stored at -20°C, with the fin clip also in ethanol (EtOH). gDNA was extracted from all muscle and fin clips within a month as outlined in *DNA extraction*, and repeated for 8 fin clips within 4 years. From 16 of these 24 cod, blood samples were available, which had been removed by puncturing of the caudal vessels with a 1 ml syringe, whose dead space was filled with 9.0 units ml^{-1} sodium heparin solution (from porcine intestinal mucosa, Sigma-Aldrich). Agarose gel electrophoresis (see below) was used to determine HbI genotype from blood samples and confirm conformity between genotyping methods.

A small number of Atlantic cod fin clips from Western Irish Sea (N = 4) and South Western English Channel (N = 17) were provided by the Centre for Environment, Fisheries and Aquaculture Science (CEFAS). These had been collected as part of a large scale tagging study of wild cod and were linked to tracking data (Righton *et al.*, 2007). Animals were captured using a modified commercial bottom trawl or by line in inshore areas where the seabed was too rough to trawl. Cod were anaesthetized in sea water containing MS222 or phenoxyethanol (Sigma-Aldrich Company Ltd, Gillingham, UK) and sterilized G5 data storage tags (DST) (Cefas Technologies Ltd, Lowestoft, UK) were inserted into the body cavity via a small incision in the belly along the mid-ventral line. A plastic filament (~7 cm long) from the DST remained external and secured with a rubber bead. The incision was then sutured with two stitches using braided nylon (Ethicon Ltd, Johnson & Johnson International). A second conventional fish tag with return details was sewn through the dorsal musculature. The tagged cod were allowed to recover from the procedure in a tank and, provided they

showed normal buoyancy control, were released overboard. A reward for return was offered to fishermen. In the United Kingdom, this research was carried out by Home Office licensed personnel under a number of Animals (Scientific Procedures) Act project licenses issued by the UK Home Office during the course and across the range of the study. The DSTs were programmed to record temperature every 10 mins (Righton *et al.*, 2007).

DNA Extraction

Approximately 10 mg of fin clip tissue was dried of ethanol and suspended in 0.5 mL of TNES lysis buffer (10 mM Tris-HCl, pH 7.5, 125 mM NaCl, 10 mM EDTA, 1% SDS) and 2.5 µL RNase A (Invitrogen, Life Technologies, Paisley, UK), after 1 hour incubation at 42ºC, 2.5 µL proteinase K (Invitrogen, Life Technologies, Paisley, UK) was added and a further incubation at 42ºC was performed for a minimum of 10 hours. DNA was isolated using 0.5 mL phenol:chloroform:isoamyl alcohol (25:24:1, Invitrogen, Life Technologies, Paisley, UK), inverted for 15 minutes and centrifuged for 15 minutes at 15000 rcf and 4ºC before the aqueous layer was isolated. DNA was precipitated with 50 µL 5 M NaCl and 2 volumes of 100% ice-cold ethanol and left for two hours at -20ºC. DNA was then washed with ethanol, resuspended in TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0) and stored at −20°C.

DNA quantity and purity were determined using spectrophotometric readings (at 260 and 280 nm) using a NanoDrop ND-1000 spectrophotometer (LabTech International, UK). DNA integrity was checked on 1 % agarose gel stained with ethidium bromide.

PCR amplification, product purification and DNA sequencing

PCR primers were designed using cod globin sequences available on NCBI. 1uL of gDNA or cDNA was PCR-amplified in a 10 µl reaction volume containing 5 µL 2x BioMix Red (Bioline, UK) and 10 pM (each) of the forward and reverse primers from either primer pair (Table 6.1, Eurofins MWG Operon, Germany). Cycling parameters were 95 °C for 10 mins, 30 three step cycles (30 s at 95 °C, 30 s at 63 °C, 30 s at 72 °C) and 72 °C for 10 mins.

Table 6.1: *Two pairs of forward and reverse primers used in PCR, numbers in brackets are positions are relative to the Atlantic cod Hb β1 gene (GenBank accession number FJ666972).*

	Primers	
	Forward	Reverse
Pair 1	(5'-GGACCACTCGCGTTGCAGAG-3') (120-139)	(5'-CCTGTGCGCGGTGGTTTCCG-3') (1142-1161)
Pair 2	(5'-GTAGGCCTAATCGACGATTTG-3') (283-303)	(5'-ATCAAGTCCACCTACGCTG-3') (557-575)

Unconsumed primers and nucleotides were removed from PCR products using an ExoSAP-IT digest. 10 µL reaction mix, containing 5 µL PCR product, 1 µL ExoSAP-IT (USB, UK) and 4 µL nuclease free water was incubated at 37°C then 80°C each for 15 minutes. The cycle sequencing reaction was set up using the BigDye® Terminator v3.1 Cycle Sequencing kit. One µl of ExoSAP-IT product was added to a 9 µl reaction mix containing 1.5 µL 5X sequencing buffer, 1.0 µl BigDye 3.1 (Applied Biosystems, Life Technologies, Paisley, UK) and 1.6 pM of forward or reverse primer. Cycling parameters were 30 three step cycles (96 °C for 10 sec, 50 °C for 5 sec, 60°C for 4 mins). The sequencing product was then precipitated using 3 M sodium acetate prior to resuspension in HiDi™ formamide (Applied Biosystems, Life Technologies, Paisley, UK), and then run on an ABI3130xl sequencer. Genotype was determined by viewing spectrograms of DNA sequences on BioEdit (Ibis Biosciences, Carlsbud, CA) and visually identifying polymorphic sites.

Agarose gel electrophoresis

Within 12 hours of capture and extraction red blood cells (RBCs) were separated from plasma and buffy coat by centrifugation (3000 rcf at 4°C for 4 mins). 20 µl of RBCs were lysed using 64 µl cold distilled water, cell debris was isolated by further centrifugation (15000 rcf at 4°C for 4 mins). The Hb isolates were then mixed with 40 % sucrose solution (4:1 ratio) and

separated by horizontal agarose gel electrophoresis, modified from Sick (1961). Smithies Buffer (45 mM tris, 25 mM boric acid and 1 mM EDTA) at pH 8.8 was used as an electrode buffer, and diluted 1:1 with distilled water for the gel buffer. A 1% agar gel was used and samples were run towards the cathode at 120 volts for 40 minutes in a cold room (set to 4°C). Process was repeated after 1 month with RBCs frozen at -80°C as a pellet.

6.4 Results and Discussion

The DNA extraction method employed in this study showed sufficient amounts of high molecular weight gDNA could be purified from fish fins. Comparison of extraction product from fresh (<1 month old) muscle and fin tissue from the same individuals (N = 24) actually showed a considerably larger yield of DNA from fin clips (372 ± 58 ng/µl) than muscle (177 ± 45 ng/µl) (Table 6.2). While the integrity of the isolated gDNA from both tissue types appeared satisfactory with limited evidence of DNA degradation, without RNase treatment there was evidence of RNAs present in the samples (Figure 6.1).

The extraction procedure on fin tissue after 4 years storage in EtOH at -20°C, yielded similar gDNA quantity and quality, both in terms of size and lack of degradation (Table 6.2, Figure 6.1). However, fin clip samples exposed to room temperature storage in EtOH for 6-8 years resulted in less than a third of the DNA isolated than in other fin clips (102 ± 52 ng/µl). A lack of DNA band and presence of smears on agarose gel suggest large amount of DNA degradation in these samples (Figure 6.1). This level of degradation led to a reduction in PCR amplification success (Table 6.2).

100% PCR amplification of the polymorphic HbI locus was achieved with DNA samples from muscle tissue and fin clips stored at -20°C with primer pair 1 (Table 6.1, 6.2). While high success was also seen with pair 2 for these samples, it could not be completed for all samples. Primer pair 2 was preferential for the lower quality DNA extracted from the 6/8 year old fin clips, increasing amplification success (AS) from 0 to 80 %, however amplification was still not possible for all samples (Table 6.1, 6.2).

Table 6.2: *Length of storage (Time) and storage conditions (Conditions), DNA yield ± S.E.M (ng/µl), DNA purity ± S.E.M achieved by extracting DNA from fin tissue of various ages and muscle tissue for comparison and number of samples (n) tested and PCR amplification success using the two primer pairs (%).*

Sample type	***N***	**Time**	**Conditions**	**DNA yield** *(ng/µl)*	**Purity**	**Amplification success** (%) Pair 1, Pair 2
Muscle	24	<1 month	-20°C	177 ± 45	1.89 ± 0.03	100, 92
Fin clip	24	<1 month	EtOH, -20°C	372 ± 58	1.90 ± 0.02	100, 96
Fin clip	8	<4 yrs	EtOH, -20°C	352 ± 56	1.91 ± 0.03	100, 88
Fin clip	21	6-8 yrs	EtOH, RT	102 ± 52	1.76 ± 0.03	0, 80

RT= Room temperature

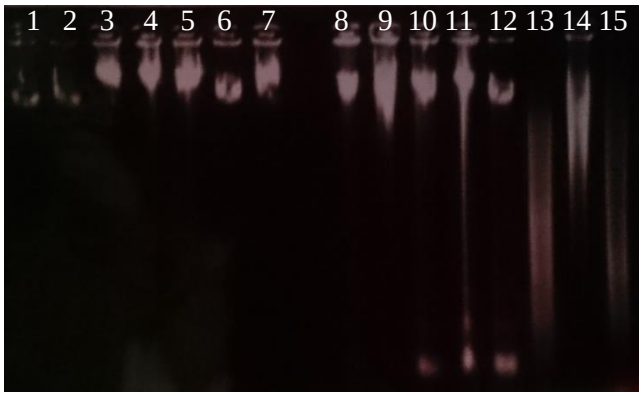


Figure 6.1: *1 % agarose gel of example DNA extraction products obtained from fish muscle and fin tissue of Atlantic cod. Lanes correspond to RNase treated gDNA obtained from: muscle <1 month old (1-3), fin <1 month old (4-6), <4 years old (7-9), and 6-8 years old (13-15). Lanes 10-12 show gDNA with no RNase treatment: fin <1 month old (10), <4 years old (11), muscle <1 month old (12).*

Agarose gel electrophoresis of red blood cell proteins highlighted HbI was indeed the dominant Hb component in these adult cod (Figure 6.2), with banding for HbI much brighter

than for HbII. Determination of HbI genotype by agarose gel electrophoresis conformed unambiguously with polymorphisms suggested by Andersen *et al*. (2009) and Borza *et al*. (2009), for the sixteen cod tested. Sequence chromatograms confirmed primers allowed amplification and easy identification of the polymorphic sites. An A/G mutation can be seen at the 1st position of the codon for position 55 of β1 globin; resulting in Met (ATG) in HbI 1/1 individuals and Val (GTG) in HbI 2/2. Heterozygotes display peaks for both A and G nucleotides at this site. Two mutations in the 1st and 2nd codon positions result in the amino acid changes at position 62; AAG in HbI 1/1 individuals codes for Lys, while GCG produces an Ala in HbI 2/2 cod (Figure 6.2). Good quality sequence data was obtained from all muscle and fin clips stored at -20°C (Figure 6.2). However, the fin clips exposed to room temperatures for a period of 6-8 years resulted in sequence failure of 18% of those successfully amplified and a further 11% were of very poor quality.

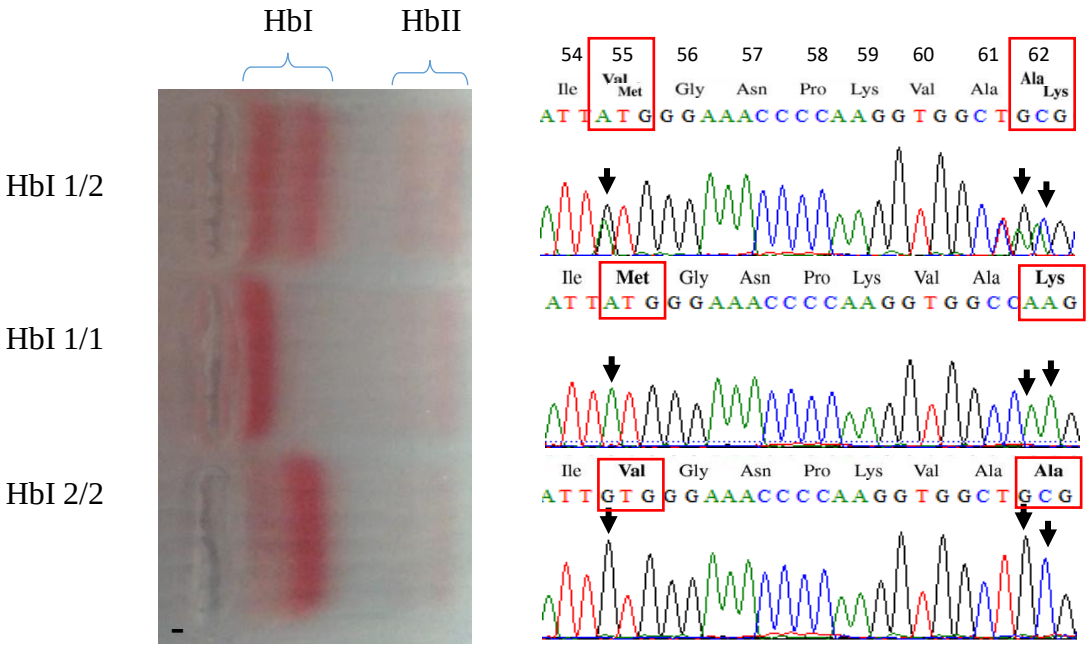


Figure 6.2: *HbI genotypes displayed as observed from agarose gel electrophoresis and chromatograms from sequencing the same individuals. Arrows indicate position of nucleotide polymorphisms and boxes highlight resulting amino acid changes. Numbers above ABI chromatogram images indicate amino acid position in β1 haemoglobin.*

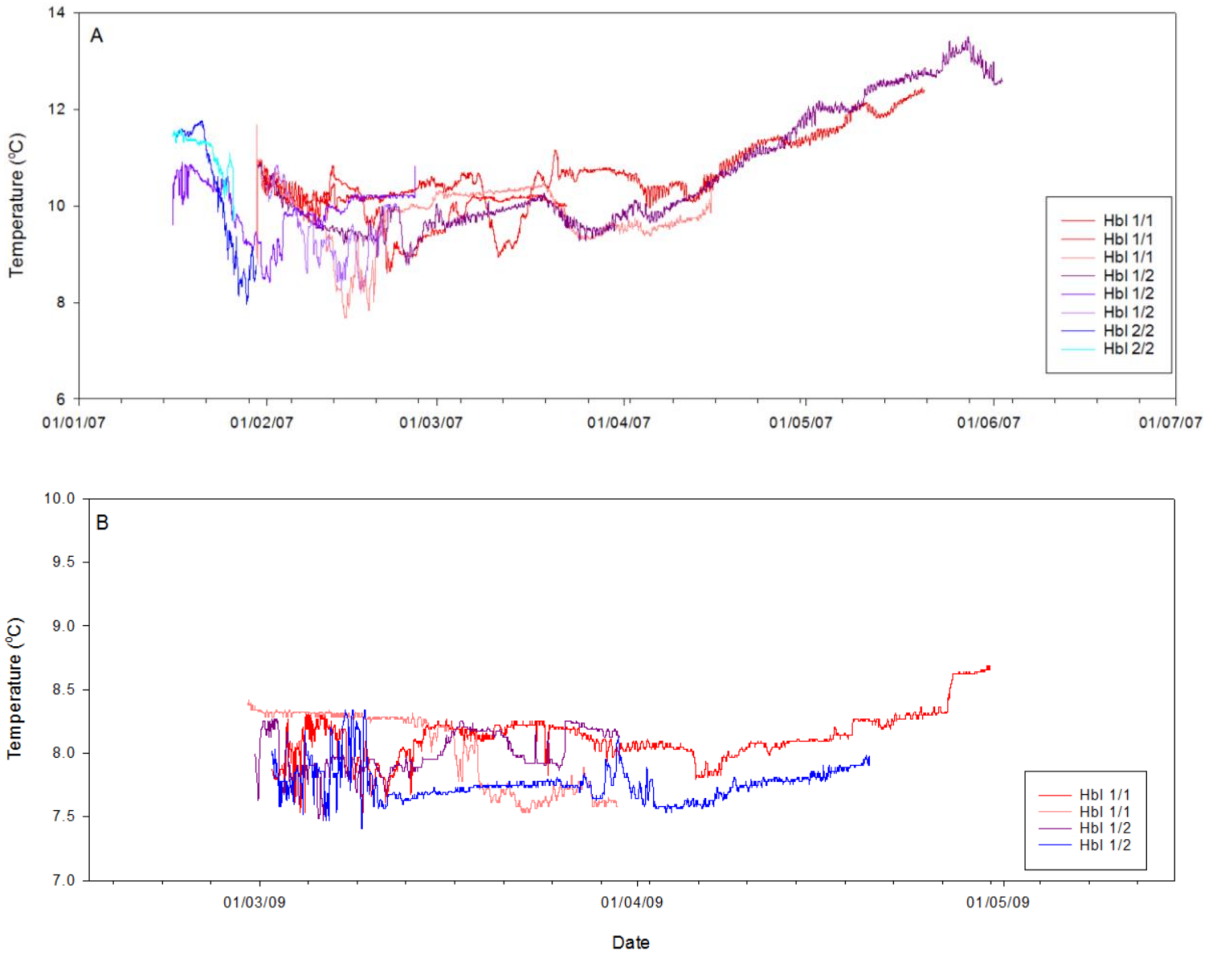


Figure 6.3: *Temperature experiences of individual tagged Atlantic cod indicting HbI genotype. Release region A) South Western English Channel and B) Western Irish Sea.*

Figure 6.3 displays the individual thermal experience of successfully sequenced individuals. Unfortunately, due to limitations in sample number and short length of time before recapture for a number of individuals, little can be inferred about the effect of HbI genotype from this data (Figure 6.3). However, during February- May there appears to be no defined differences between the genotypes thermal experience. Nevertheless, it is clear to see differences between the two populations. During March and April, the two months with data available for both regions, cod in the South Western English Channel (SWEC) had a higher average temperature (10.2 ± 0.09°C) compared to those in the Western Irish Sea (WIS) (8.0 ± 0.05°C). Furthermore, SWEC cod experience temperatures ranges twice as large as WIS, a

difference of 1.85°C and 0.95°C respectively. The largest temperature variation experienced by an individual was 4.7°C in SWEC.

6.5 Discussion

The isolation of high-quality DNA for genetic analysis is one of the major concerns for DNA-based techniques. Fin clips provide a non-destructive form of genetic sampling and have been used by recent Atlantic cod tracking studies as a source of genomic information. However this tissue type can present some difficulty during DNA extraction and methods can yield low quality and quantity of DNA. This study (1) presented a cheap method to extract high quality gDNA from small amounts of fin clip tissue, (2) utilised the DNA extracted to determine HbI genotype and (3) applied this method to archival fin clip samples linked to tagging data.

The proposed method was successful in extracting a large quantity of gDNA from 10 mg of fresh fin clip, indeed higher than that from muscle tissue, currently one of the more widely used sources of DNA. Unfortunately gDNA extracted from archival fin clips, while of reasonable quantity, had reduced purity when compared to fresh and all other samples and was poor in quality, being heavily degraded. PCR amplification of the HbI genotype using the isolated gDNA was largely successful, however in the case of the archival finclips it was largely hampered by the level of degradation and the length of the DNA template.

Studies have suggested full lysis of fin tissue is difficult to achieve without the addition of urea to the lysis buffer to assist in protein breakdown (Asahida *et al*., 1996; Wasko *et al*., 2003) or the use of liquid nitrogen maceration (Chen *et al*., 1995; Strassmann *et al*., 1996; Pinto *et al*., 2000). However, this study found full lysis could be achieved with concentrations of 0.1 mg/mL Proteinase K with a minimum incubation of time of 10 hr, without the necessity of a homogenisation step of any form including use of liquid nitrogen or urea supplementation and the risk of DNA degradation it can pose. Mukhopadhyay and Bhattacharjee (2014) had success with a higher Proteinase K concentrations of 0.3 mg/mL and as little incubation time as1 hr, while this approach appeared to facilitate sufficient tissue

breakdown, this study found this reduction in lysis time led to a decrease in purity with a reduction of 260/280, suggesting protein was still present in the sample.

The extraction quality of the DNA in this method was improved by the use of an RNase treatment step, which resulted in DNA samples with lower quantities of RNA (Figure 6.1). When tested this did not interfere with quality of sequence data for HbI genotype, it may interfere with other downstream procedures (Wasko *et al*., 2003) and its removal allowed for accurate quantification of DNA.

Comparison of the gDNA extraction method between Atlantic cod fin and muscle tissue show higher DNA yield in the former, Taggart *et al*. (1992) found this to be the case in salmonids also. However they suggested that storage of fin clips for greater than a year, even in EtOH at -20°C, resulted in partially degraded gDNA. Here, this was not found to be the case. After almost 4 years, Atlantic cod fin clips in EtOH at -20°C showed very little fragmentation. As such, the large reduction in integrity of gDNA seen in older fin clips, is most likely due to storage at room temperature, which is generally not recommended for long term storage. DNA preservation in fin samples at ambient temperature may be better facilitated by storage in ethanol/EDTA buffer, whereby presence of EDTA inhibits DNase activity (Wasko *et al*., 2003).

PCR amplification of the isolated gDNA provided a simple method, requiring minimal handling, of identifying HbI genotypes, allowing for processing of a large number of samples. This confirmed the Met/Lys and Val/Ala were confirmed to unambiguously correlate with the HbI 1/1 and 2/2 variants, respectively as observed through gel electrophoresis. The cause of the Hb separation seen on the agarose gel is the result of the amino acid change at position 62, whereby the possession of a neutral Ala as opposed to the positive Lys resulted in the HbI 2/2 variant travelling faster towards the positive anode.

Reduced PCR amplification success and reduced sequence quality in archival fin clips is likely to be due to fragmentation of the genome. The primers likely could not find a continuous regions of sufficient length to amplify. Use of the primer pair 2 positioned in the

introns and closer to the polymorphic regions, reduced the length of gDNA required and was more appropriate for sheared DNA.

The limitations in number and quality of the sequences from cod with available data tracks limited the opportunity to observe the effect of HbI genotype on thermal behaviour in the wild from these samples. However, this data is clearly an invaluable resource in understanding direct thermal experience of wild cod with the different HbI genotypes, potentially helping formulate an answer to the 50 year old question of the role of HbI genotypes. Futhermore, it may provide information to assess how the Hb variants may affect the future of wild cod in response to climate change and if protection is required for certain populations. This study suggested a method which allowed gDNA extraction from 81% of archival samples and had a HbI genotyping success rate of 66% from these samples. Also highlighted is the importance of correct storage of this resource, if better storage is observed this method resulted in 100% successful gDNA extraction and HbI genotyping.

Chapter 7. Conclusions and future perspectives

In this thesis we aimed to explore the effect of temperature on red blood cell (RBC) oxygen binding in Atlantic cod. Focusing particularly upon the effects of HbI genotype, temperature, pH, and Hb polymerisation or sickling.

7.1 HbI polymorphism in Atlantic cod

RBC oxygen affinity.

This work set out to better understand how the enigmatic HbI polymorphism in Atlantic cod affected RBC oxygen binding and its response to temperature (Sick, 1961; Karpov and Novikov, 1980; Andersen, 2012; Ross *et al*., 2013).

The study by Karpov and Novikov (1980) was the first to propose an explanation for the changes in HbI genotype frequency observed along both coasts of the Atlantic. Their work suggested the genotype (HbI 2/2) found most abundantly in the colder waters in the North resulted in temperature sensitive oxygen binding, whereby increasing temperature resulted in reduced oxygen affinity. This is likely to affect blood oxygen saturation at the gills. Conversely the HbI 1/1 genotype, found at more southerly latitudes, has oxygen binding properties which are thermally independent. A number of studies have found similar, if less pronounced, findings in Hb isolates (Brix *et al*. 1998 and 2004). However, the difficulties in fully reproducing the genotype effects found in RBCs by Karpov and Novikov (1980) in purified Hb by others (Brix *et al*. 1998; Colosimo *et al*., 2003; Brix *et al.*, 2004) suggest that the adaptive value of different Hb genotypes on O_2 supply rates in different environments may have been overemphasized, as discussed by Gamperl *et al.* (2009). As such we predicted that HbI genotype will have little effect upon the thermal sensitivity of RBC oxygen binding.

The present work assessed the thermal sensitivity of O_2 binding in Atlantic cod red blood cells with different HbI genotypes near their upper thermal distribution limit. Contrary to findings on more northerly cod populations (Karpov and Novikov, 1980; Brix *et al*. 1998 and 2004), wild caught Atlantic cod from the Irish Sea showed statistically indistinguishable

RBC O_2 binding between any of the three HbI genotypes. As such, we can accept the proposed hypothesis.

Differences in the results on HbI significance in cod in this study compared to others may be due to variations in methodology. The present study aimed to reduce and account for any confounding factors potentially overlooked in previous studies, hopefully improving the validity of the work. Removal of catecholamines with washing and overnight incubation ensured return of intracellular pH and nucleotide triphosphate (NTP) concentrations to levels comparable with *in vivo* (Leray, 1982; Berenbrink and Bridges, 1994a and b). Extreme dilution of RBCs in highly buffered salines allowed full control of extracellular pH, a highly influencing factor in Atlantic cod. Monitoring of pH changes with temperature, made it possible to remove the influence of fluctuating pH as a result of temperature. Full spectrophotometric assessment of RBC O_2 saturation between 500 and 700 nm in the present study also avoided having to assume full RBC O_2 saturation at some arbitrary high PO_2 which may have led to a systematic overestimation of O_2 saturation and affinity in some previous studies. These precautions make this present study the most comprehensive test yet for HbI genotype differences in RBC O_2 binding properties.

Alternatively, the difference may be due to effects of acclimation or life history between southerly populations used in this study, in contrast to the northern populations often used in other studies. Due to the defined time period in which the fish were captured, primarily during February, when water was well mixed and temperature was stable, , effects of different acclimatisation background were removed to a large degree (Neat *et al*. 2014; O'Boyle & Nolan, 2010). Different long term or even seasonal temperature variations may alter the effect of HbI genotype, which may explain the findings of thermally induced differences in HbI polymorphisms seen in northern cod populations. Use of consistent methods on cod along the European coast may provide some context as to whether the differences are the result of adaptation or merely variations in methodology.

The negative finding in this study gives rise to the question what other characteristic(s), if any, of the different HbI alleles is behind the documented differences in geographical distribution; growth rates, hypoxia tolerance, and preference temperature (reviewed by Andersen, 2012; Ross *et al*., 2013)?

Temperature preference of wild cod.

As yet the effect of HbI genotype on Atlantic cod thermal preference is also inconclusive (Petersen and Steffensen, 2003; Behrens *et al*., 2012). This work aimed to give a more accurate portrayal of the influence of HbI genotype on environmental temperature selection in wild cod. A method of Hb genotyping was successfully devised for low quality fin clip samples. Thermal experience data from tagged cod could be linked to this information, unfortunately the experience data from the few samples provided were of insufficient length and quantity to provide any information on the influence of HbI genotype on temperature preference of wild cod, at this time. However, it is hoped future collaboration with CEFAS may make it possible to isolate if HbI genotype effects thermal preference in wild populations by monitoring individual cod experience and genotyping from the proposed DNA extraction and sequencing method.

RBC sickling

In most vertebrates, the sickling mechanism is genetically based and often only occurs with a mutation in Hb (Ingram *et al*., 1957; Butcher and Hawkey, 1979; Taylor, 1983). In sheep and deer the Hb mutant which causes sickling can be separated from the wildtype electrophoretically (Butcher and Hawkey, 1979; Taylor, 1983). Similar electrophoretic properties occur between the two HbI polymorphisms in Atlantic cod. With the molecular mechanism causing sickling in fish remaining unclear, we explored the effect of HbI polymorphism influencing RBC sickling. The substitution at position 55, from a Met (HbI 1) to a smaller Val (HbI 2) amino acid side chain, is believed to destabilise the low oxygen affinity T(ense)- state of haemoglobin (Andersen *et al*., 2009). We proposed it may be

possible that animals with the HbI 2 variant haemoglobin may employ sickling as a mechanism to stabilise haemoglobin when deoxygenated.

The occurrence of sickling in all animals regardless of genotype suggests the genotypic variation in main Hb component in Atlantic cod, HbI, did not cause sickling (Chapter 4). As such, we reject our hypothesis pertaining to HbI genotype being the molecular mechanism causing sickling in Atlantic cod. Nevertheless, HbI genotype did appear to have a small influence on sickling. An increase in n_HS in HbI 2/2 individuals compared to HbI 1/1 at the two lowest temperatures may be due to the polymorphism at position β62. The change from Lys (HbI 1/1) to Ala (HbI 2/2) alter interactions with water at the haem binding site, this is greater in HbI 1/1 individuals at low temperatures (Ooi and Oobatak, 1988; Andersen *et al*., 2009). This may inhibit the interaction with the ameliorating oxygen at low temperature thus inducing sickling at a slightly higher pH in the HbI 1/1 individuals (Koldkjaer and Berenbrink 2007). Further, interactions with water effect the R to T allosteric equilibrium (Haire and Hedlund, 1983) and can cause alterations in quarternary structure exposing or isolating proton binding sites, which could explain the differences in n_HS.

With this largely negative result the molecular mechanism causing sickling remained unclear. However, the occurrence of sickling in the close relative whiting (*Merlangius merlangus*) and other gadiformes suggest the genetic mechanism is likely to be shared between species and not restricted to certain genotypes within a species (Koldkjaer and Berenbrink, 2007). A number of mechanisms had already been proposed (Borgese *et al*., 1988; Borgese *et al*., 1992; Fago *et al*., 1993; Riccio *et al*., 2011; Koldkjaer and Berenbrink, 2007) but limitations in number of species sampled make it difficult to pinpoint a genetic marker or when sickling evolved.

We determined the presence and absence of RBC sickling under physiologically relevant conditions in a large number of species. By comparing Hb genetic sequence (when available) and the occurrence of sickling we were able to eliminate some previously proposed markers, although it was not possible to conclusively identify the mechanism. We suggest

possible genetic markers include; position 76 and 51 of the β chain. All sickling species contained an aspartic acid or glutamic acid at these positions. Both these amino acids contain a carboxylic acid group (COO^-). By reducing pH, $[H^+]$ increases and these hydrogen ions can react with these sites to form COOH side chains. This reduces potential of ionic interactions with other amino acids and removes its hydrophilic properties, potentially changing globin structure. At this stage, limitations in sequence data restricted our search for a viable potential mechanism to a β primary structure of the Gadiform. Nevertheless, additional functional sites may occur in the α subunits and it is likely the different globin isoforms within species exhibit different functional properties. These must be studied in isolation to establish this. Determination of whether mechanisms for sickling between codfishes, toadfishes and potentially pipefishes are similar require more thorough sequence data. Additionally, detailed molecular structure–function analyses are required to fully understand the consequences of primary structure variations, ideally on otherwise similar, polymerising and non-polymerising Hbs.

Reconstructing the sickling data from this study and data from reliable sources (Thomas, 1971; Mattisson, 1982; Harosi *et al*., 1998; Koldkjær *et al*., 2013) on a composite phylogenetic tree (von der Heyden and Matthee, 2008; Naylor *et al*., 2012; Near *et al*., 2012 and 2013; Owens, 2015), we were also able to determine a potential evolutionary pathway. We suggest sickling evolved in an early gadiform ancestor. To identify when precisely, more species are required particularly in the more basal gadiformes, such as Macrouridae (Grenadiers) and Euclichthyidae *(*Eucla cod).

However, the evolution of sickling may have occurred in an earlier ancestor before separation of toadfishes and resulted in a secondary loss of sickling in other species. Nevertheless, we believe sickling in Gadiformes evolved independently from sickling in Batrachoidiformes (toadfishes), because of the slightly different responses to sickle stimulating factors i.e the mixed response in toadfish to PO_2, supports independent evolution.

As in humans, where RBC sickling is proposed to provide a level of protection against *Plasmodium falciparum* malaria, a similar theory has been suggested in fish (Koldkjær and

Berenbrink, 2007; Koldjkaer *et al*., 2013). The widespread occurrence of sickling in Gadidae gives credence to this theory. The recently discovered loss of an immunological gene complexes (MCH II; Star *et al*., 2011) may have been possible due to the evolution of sickling in the Gadiformes. Explorations into the links between these as well as other causes and consequences of sickling in fish are already underway.

Summary

The lack of effect of the HbI polymorphism on the traits we studied does not preclude its relevance. Indeed, natural selection may act on a different life history stage than the juveniles or adults that are most commonly studied, for example if the maternal HbI genotype in eggs affects their fertilisation success, then this may explain the significantly skewed HbI genotype ratios in offspring of heterozygote parents that was observed by Gamperl *et al*. (2009) and was later in life balanced by significantly higher growth rates of the underrepresented genotype. Thus, differing costs and benefits during different life history stages and/or in different micro environments may lead to balanced HbI polymorphisms that differ in HbI 1 frequencies across the distributional range.

Alternatively it is possible it is genetically linked to other traits that are under selection, such as the regulatory polymorphism of the HbI promoter in Atlantic cod (Star *et al*., 2011; Andersen, 2012) that may be responsible for HbI genotype-associated differences in Hct and Hb concentration observed in some studies (Mork and Sundnes, 1984). Clearly our work on this perplexing mutation is still not complete.

7.2 Effects of temperature and pH

RBC oxygen binding

Previous studies have suggested that limitations to circulatory O_2 transport may play a role in setting thermal tolerance in this species (Pörtner, 2001; Pörtner and Knust, 2007). The oxygenation of the haem is exothermic nature, this results in a reduction in oxygen affinity with increasing temperatures (Weber and Campbell, 2011). However, this exothermy has

been reduced in a number of heterothermic, such as tuna, billfish and some sharks, which have heated swimming muscles and/or warm eyes and brains (Carey *et al*., 1971; Carey and Gibson, 1977; Block and Carey, 1985; Block, 1986; Larsen *et al*., 2003) supported by the countercurrent heat-exchangers systems (Larsen *et al*., 2003). In these animals the typical decrease in Hb-O_2 affinity with increasing temperatures may cause abrupt O_2 unloading from blood perfusing the warm, red swimming muscles while unloading may be inhibited in cold organs such as the liver and gut (Clark *et al*., 2008; Weber and Campbell, 2011). As such they evolved reduced or reversed temperature sensitivity of oxygen binding, utilising the endothermic dissociation of allosteric effectors to counteract the release of heat during oxygenation of the haem (Cech *et al*., 1984; Jones *et al*., 1986; Brill and Bushnell, 1991; Lowe *et al*., 2000; Brill and Bushnell, 2006; Clark *et al*., 2008). This phenomenon had only previously been observed in one ectothermic teleost, the Pacific mackerel (Clark *et al*., 2008).

Excitingly, low or even reversed thermal sensitivity of oxygen binding was found in Atlantic cod. That this coincided with a very large Bohr effect suggested the influence of the allosteric effector H^+. At low pH, reduced thermal dependence of oxygen binding can likely be attributed to increased H^+ concentration, this allosteric effector preferentially binds to deoxy Hb and during oxygenation its endothermic dissociation can compensate for the heat released by exothermic haem oxygenation. This is the same mechanism observed in heterothermic fish (Larsen *et al*., 2003; Clark *et al*., 2008; Weber and Campbell, 2011). Comparisons between multiple species, as hypothesised, correlated the pH sensitivity with thermal sensitivity in RBC oxygen binding in six species and showed reduced/reversed temperature sensitivity to be much more widespread than previously believed. However, this study did not take into account the effect of other allosteric factors, such as organic phosphates, which may moderate or enhance these findings. As such, elucidation of the detailed molecular mechanism(s) behind reduced or even reversed thermal sensitivity of RBC O_2 affinity awaits detailed studies on purified Hbs under tightly controlled conditions of allosteric modifiers.

The finding of thermally independent and reversed thermal dependence of blood–O_2 binding exist in the ectothermic mackerel, suggests that reduced or reversed thermal effects on blood–O_2 binding may have evolved prior to regional heterothermy, not in response to it (Clark *et al*., 2008). However, this earlier evolution of reduced or reverse temperature dependence was still only predicted within the Scombroidei lineage. Its widespread occurence discovered in the present study may have implications for the evolution of mechanisms to successfully overcome thermal influence on oxygen binding. Its occurrence much earlier in the teleost lineage may have allowed enhanced gill oxygen uptake over a broad range of ambient temperatures and potentially facilitated thermal niche expansion. The presence of reduced thermal sensitivity of oxygen binding across such a wide range of physiological conditions in both gadiforms (Atlantic cod and burbot) may indicate more advance thermal tolerance in this lineage. Further phylogenetic analysis is required to better understand this potential.

The oxygen and capacity limited thermal tolerance model (OCLTT) suggests thermal tolerance in marine ectotherms is limited primarily by the capacity of the blood oxygen transport system (Pörtner and Knust, 2007; Pörtner, 2010). As temperature increases above the thermal optima for a species, metabolic rate and subsequently oxygen demand also increase. Aerobic capacity can be increased by increasing either cardiac output (heart rate or stroke volume), or the arterial-venous O_2 difference ($S_{a\text{-}v}$). This study modelled $S_{a\text{-}v}$ at physiological pH, temperature and arterial and venous O_2 partial pressures in Atlantic cod. This revealed a substantial capacity for increases in $S_{a\text{-}v}$ to meet rising tissue O_2 demands at 5.0 and 12.5°C, but not at 20°C. There was no evidence for an increase of maximal $S_{a\text{-}v}$ with temperature. This suggested that Atlantic cod at such high temperatures may solely depend on increases in cardiac output and blood O_2 capacity, or thermal acclimatisation of metabolic rate, for matching circulatory O_2 supply to tissue demand.

RBC sickling

Previous research has shown that Hb polymerisation and subsequent RBC sickling in fish occurs under decreased pH (Harosi *et al*., 1998; Koldjkaer and Berenbrink, 2007; Koldjkaer *et al*., 2013), but the temperature sensitivity of this phenomenon has, to our knowledge not previously been characterised. In all gadiforms tested in this study, reduced pH also increased the occurrence of RBC sickling. Sickle cells began to appear at pH as high as 7.8. With general blood acidosis reaching pH levels between 7.3 and 7.5 as a result of intensive exercise (Steen, 1963; Holeton *et al*., 1983; Milligan and Wood, 1987; Kobayashi *et al*., 1990; Nelson *et al*., 1996; Perry *et al*., 1991), *in vivo* sickling is likely to occur. Throughout the Gadiformes, sickle cells exhibited similar morphological changes as previously seen *in vitro* in Atlantic cod and haddock (Thomas, 1971; Harosi *et al*., 1998) and *in vivo* in whiting (Koldkjaer and Berenbrink 2007).

In most species of fish, decreasing pH also results in a reduction in maximum Hb oxygen binding, known as the Root effect (Root, 1931; Bridges *et al*., 1983, Berenbrink *et al*., 2005; Berenbrink *et al*., 2011). The Hb polymers formed during sickling only consist of deoxygenated Hb (Harosi *et al*., 1998). Given that both sickling and the Root effect are strongly effected by pH and given both their association with Hb deoxygenation, it is possible that both processes may influence each other. This work indeed found a strong link between acid induced deoxygenation and sickling in Atlantic cod and burbot. There was no significant difference between pH at 50% sickling and 50%change in oxygen saturation (pK_aS and pK_aR) and the onset of both phenomena occurred at similar pH levels. With the mechanisms for the Root effect and sickling still unclear, this link may provide some insight: while this shared mechanism may not be causational it may be a triggering one.

In mammalian vertebrates increasing temperature increased the occurrence of sickling (Bookchin *et al*., 1976; Jain and Kono, 1977; Butcher, 1979; Butcher and Hawkey, 1979; Bunn, 1997), the reverse was found here in fish. This study found increasing temperature decreased the occurrence of sickling and oxygen binding, suggesting both are exothermic.

This indicates that it is likely involved in the reduction in thermal dependence of the oxygen transport system in Atlantic cod as hypothesised. However, working with RBCs, as opposed to isolated Hb in solution, makes it difficult to isolate specific allosteric modifiers or other singular factors, without interference by others. Further work is required to understand the contributions of exothermic sickling to off-setting the effect of exothermic haem oxygen binding.

Evidence of pK_aS and pK_aR changing in a non-parallel fashion to predicted *in vivo* pH changes (Ultsch and Jackson, 1996) suggest the exothermic nature of both the Root effect and sickling results in a reduction of these effects 'within' the animal. Thus, at increased temperatures a greater degree of pH change is required to initiate pH induced deoxygenation and sickling, this may be beneficial as Hb-O_2 affinity is believed to decrease at higher temperatures, although as suggested by our work in Atlantic cod this may not be true in all species.

Concluding remarks

Clearly our understanding of molecular mechanisms enabling adaptation of marine ectotherms to environmental temperature change is just at the beginning and more studies linking the genetics, physiology, ecology and evolution of these organisms are required. An understanding of this is vital to predict the effects of climate change and warming seas and appropriately manage populations.

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