Abundance and Function of Multiple Haemoglobin Isomorphs from Rainbow Trout (*Oncorhynchus mykiss*)

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I have yet to see any problem, however complicated, which, when you looked at it the right way, did not become still more complicated.

-- Paul Alderson (1926-...) in "New Scientist", 25 September 1969, 638

Errors using inadequate data are much less than those using no data at all.

-- Charles Babbage (1792-1871)

ABSTRACT

Haemoglobins perform the vital physiological function of transporting oxygen from the external environment to the tissues. Poikilothermic rainbow trout (*Oncorhynchus mykiss* = *Salmo gairdneri* = *S. irideus*) produce multiple forms of haemoglobin that respond differently to varying environmental and physiological conditions. However, the timescale and physiology of these changes are not known. Changes in isomorph abundance may potentially originate from the production of new isomorphs in already circulating erythrocytes. Alternatively, new isomorphs may be produced through the reassembly of extant haemoglobin subunits. The final hypothesis is that changes in isomorph abundance occur through the production of new erythrocytes with red blood cells 'pre-programmed' to produce a particular set of haemoglobins. Changes originating from production of new erythrocytes would require longer periods of time before being detectable; weeks to months depending on the temperature regime.

To test this, paired groups of rainbow trout were subjected to either 10ºC or 20ºC for 5, 7, 14, 21 and 28 days. A total of 14 isomorphs were observed after the haemolysate was separated using cellulose acetate electrophoresis. However, no detectable differences in isomorph abundance were found between treatment groups. In a follow-up experiment, anaemia was induced in rainbow trout to stimulate the production of new erythrocytes. The trout were then held at either 10ºC or 20ºC for 21 days. This resulted in relative increases in the abundance of anodal haemoglobin isomorphs in the 20ºC acclimated group and a corresponding decrease in cathodal haemoglobin isomorphs. To further confirm that changes in abundance were occurring through the production of new erythrocytes, separation of erythrocytes into age classes was undertaken to compare the isomorphs present in mature erythrocytes with those from erythrocytes produced under amended temperature regimes. Using Percoll density gradients, red blood cells from anaemia-induced trout acclimated to either 10ºC or 20ºC were enriched into mature and young erythrocyte fractions. Further significant differences in abundance were found between both anodal and cathodal isomorphs when compared between treatment groups. From these results it was concluded that changes in haemoglobin isomorph abundance originated from the production of new erythrocytes.

Cellulose acetate gel electrophoresis was carried out on seven haemoglobin fractions that had undergone prior separation from whole haemolysate by Fast Protein Liquid

Chromatography. Each haemoglobin fraction was found to be composed of two isomorphs for a total of 14 haemoglobin isomorphs. The oxygen binding properties of each fraction was examined under varying conditions of temperature, pH, ATP and chloride concentrations. Cathodal functional groups HbI to HbIII were found to be insensitive to temperature, pH, chloride and the organic phosphate ATP. In contrast, the anodal fractions (HbIV to HbVII) all responded to pH and temperature changes, while HbVI and HbVII responded to ATP. However, no fraction responded to increased chloride concentrations. These results suggest that different varieties of rainbow trout may produce different forms of haemoglobin as part of an adaptive response to local environmental conditions, leading to variation in the functional properties of some of the less abundant functional groups such as HbIII.

Despite the theory that cathodal haemoglobins function as emergency back-up supplies of oxygen being proposed more than thirty years ago, no published information can be found for it being tested in the laboratory. Two groups of anaemia-induced rainbow trout were placed in a divided annular flume for 24 days. The high activity group was subjected to a forced swimming speed of 2.5 body lengths (B.L.) s^{-1} for 6 h d^{-1}. When not undergoing forced exercise the treatment group were maintained at the same speed as the low activity group of 0.5 B.L. s^{-1}. Significant differences in haemoglobin isomorph abundance were present between the initial samples taken at the time of anaemia induction and high and low activity groups. However, only the C4 isomorph demonstrated a significant difference between high and low activity groups. When total anodal and cathodal isomorphs were compared between initial state, high activity and low activity groups, no differences were present. These data suggest that the induction of anaemia had an effect on the composition of the isomorphs but no physiological effect on oxygen delivery. In addition, the swimming velocity of 2.5 B.L. s^{-1} employed for the high activity group may have been an insufficient stimulus to induce changes in isomorph abundance.

It is concluded that changes in haemoglobin isomorph abundance occur in response to chronic changes in the environment. Increases in the abundance of anodal isoforms in response to increasing temperature allows for increased delivery of oxygen to tissues undergoing increases in metabolic activity associated with higher temperatures. The multiple haemoglobin isomorphs of rainbow trout provide an increased efficiency in delivery of oxygen to tissues under varying metabolic conditions of pH, temperature and oxygen saturation. The cathodal and anodal haemoglobin functional groups of rainbow trout exhibit different oxygen affinities in response to temperature, pH and ATP concentration but not to physiologically realistic chloride concentrations. The oxygen binding properties of the

isomorphs within the cathodal and anodal functional groups are broadly similar. However, differences in responses by the anodal functional groups to NTPs may exist. An examination of the hypothesis that cathodal haemoglobins act as reserve oxygen delivery sources under prolonged activity produced no significant results. However, this hypothesis still remains viable and needs to be tested under different experimental conditions.

This work provides a basis for further research into the adaptive abilities of rainbow trout. The selection of rainbow trout which better adapt to wide ranges of environmental conditions would allow for targeted introduction by fisheries managers to aquatic systems previously not considered optimal for trout growth thereby expanding the fishery.

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All PhD projects have things go wrong, but I think I am justified in saying that I have had more than my fare share of disasters. For that reason I would like to thank the following PhDers for being there to cut me down from the rafters and share their war stories; so thanks

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CHAPTER ONE : GENERAL INTRODUCTION

Introduction to Haemoglobin

Vertebrate haemoglobin has evolved to transport oxygen via homotropic and heterotropic interactions from areas of high oxygen partial pressure to regions of the body with low partial pressures (Weber and Jensen, 1988). In ectothermic vertebrates, haemoglobin increases the oxygen carrying capacity of blood by about twenty-fold compared to physically dissolved oxygen (Weber and Jensen, 1988). In most vertebrates haemoglobin takes the form of a tetrameric molecule consisting of two α and two β polypeptide chains termed 'globins'. Embedded in each globin is a haem group which contains one iron atom that can reversibly bind one oxygen molecule; this temporarily oxidises the iron atom from Fe^{2+} to Fe^{3+} (Riggs, 1970).

The binding of oxygen to haemoglobin and the subsequent release of oxygen at specific tissue sites is primarily controlled by the ambient oxygen partial pressure (PO_2). Under normoxic conditions PO_2 at the respiratory sites is comparatively high, allowing the haemoglobin to become saturated; as the haemoglobin circulates the body PO_2 drops and the oxygen dissociates from the haemoglobin and is taken up by the tissues (Johansen, 1971; Weber and Jensen, 1988). The oxygen affinity of haemoglobin is also influenced by a phenomenon termed the Bohr effect. The Bohr effect (reviewed by Riggs, 1988) is characterised by the release of oxygen from haemoglobin at lower pH. The modulation of oxygen binding by pH occurs from the release of protons (H^+) at oxygenation of haemoglobin, and the uptake of protons at deoxygenation (Riggs, 1988). Reciprocally, changes in pH modulate oxygen affinity. The practical effect of this is that as tissues undergo aerobic or anaerobic metabolism, localised respiratory or metabolic acidosis occurs from the production of CO_2 or lactate, respectively, which through the Bohr effect causes a release of oxygen from the haemoglobin effectively delivering it to the respiring tissues.

The Root effect (reviewed by Brittain, 2005) is related to the Bohr effect but is only present in fish species, in particular those with multiple haemoglobins. The Root effect is a high sensitivity by some haemoglobin isomorphs to low pH, such that the haemoglobin cannot become oxygen saturated even at atmospheric partial pressures. The Root effect works in conjunction with counter current circulation allowing

haemoglobins to deliver oxygen to tissues against high oxygen gradients such as those that occur in the eyes and swimbladders (Pelster and Weber, 1990).

Haemoglobin oxygen affinity can also be affected by interactions with allosteric effectors such as organic phosphates (i.e. adenosine triphosphate (ATP) and guanosine triphosphate (GTP)) (Weber and Jensen, 1988), chloride ions (Riggs, 1988) and carbon dioxide (Weber and Lykkeboe, 1978). ATP and GTP (collectively these molecules and others are known as nucleoside triphosphates or NTPs) lower the oxygen affinity of haemoglobin by preferentially binding to deoxy-haemoglobin, decreasing its oxygen affinity and slowing the uptake of oxygen (Weber and Jensen, 1988). In functional terms, this means that oxygen partial pressure must be higher before 50% oxygenation of haemoglobin occurs (Figure 1.1).

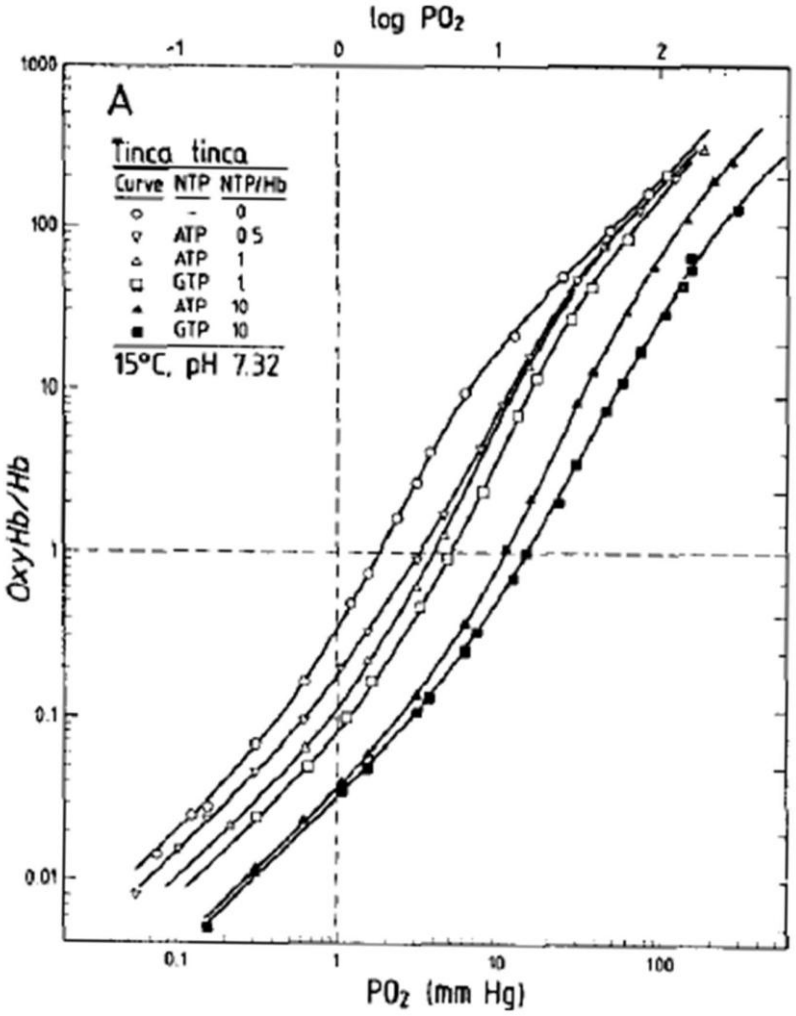


Figure 1.1: Oxygen equilibria of tench (*Tinca tinca*) haemoglobin at various NTP/Haemoglobin ratios. Horizontal dashed line at 1 oxyHb/Hb indicates 50% oxygenation. Plot taken from Weber and Jensen (1988).

In fish, hypoxia decreases erythrocyte NTP concentration which raises oxygen affinity directly via decreased allosteric interactions and indirectly via increases in erythrocyte pH (Wood and Johansen, 1972; Greaney and Powers, 1978; Weber and Lykkeboe, 1978; Soivio et al., 1980; Jensen and Weber, 1982). This response is graded to the ambient oxygen tension (Tetens and Lykkeboe, 1981) and serves to protect arterial oxygen loading and increase oxygen capacitance at low oxygen tensions. When hypoxia is encountered, the time required to physiologically adjust NTP levels can vary from hours to days (Weber and Lykkeboe, 1978; Soivio et al., 1980; Jensen and Weber, 1985; Tetens and Lykkeboe, 1985) depending on the species (e.g. 24 h in tench (*Tinca tinca*) (Jensen and Weber, 1985), 7 d in common carp (*Cyprinus carpio*) (Weber and Lykkeboe, 1978) and the severity of the hypoxia (i.e. PO_2 < 80 mm Hg) (Weber and Jensen, 1988).

It has been demonstrated that a diversity of fish species including catfish (*Silurus glanis*), European eel (*Anguilla anguilla*), rainbow trout (*Oncorhynchus mykiss = Salmo gairdneri = S. irideus*), northern pike (*Esox lucius*), goldfish (*Carassius auratus*), roach (*Rutilus rutilus*), perch (*Perca fluviatilis*), pumpkinseed (*Lepomis gibbosus*), dogfish (*Scyliorhinus caniculus*) and skate (*Raja clarata*) have variable ATP to GTP ratios (Leray, 1979). For example, ATP is the main NTP effector in species such as rainbow trout and dogfish sharks (*Carcharias taurus*), while GTP is the main modulator in species such as the American eel (*Anguilla rostrata*), common carp, tench and goldfish (Geoghegan and Poluhowich, 1974; Weber et al., 1975; Weber et al., 1976a; Weber and Lykkeboe, 1978; Leray, 1979; Jensen and Weber, 1982). The reason for high GTP levels in the red blood cells of so many fish species has not been explained. Interestingly, GTP levels are generally higher than ATP in fish species experiencing unstable oxygen conditions in their habitats (Val, 2000). Comparisons of the degree of effect equal concentrations of ATP and GTP have on haemoglobin oxygen affinity are not prevalent. However, GTP was reported to have a greater depressing effect on haemoglobin oxygen affinity than equal concentrations of ATP in both common carp and European eel (Weber et al., 1975; Weber and Lykkeboe, 1978)

Carbon dioxide is also an allosteric effector as it competes with NTPs to bind to β-globins (Weber and Lykkeboe, 1978). As with NTPs the effect slows the binding of oxygen to haemoglobin and shifts the oxygen equilibrium curve to the right.

However, in fish haemoglobins the effect is generally physiologically insignificant as carbon dioxide partial pressures are low in the blood of gill respirers. Also, acetylation of the α-amino groups of the α-globins limits carbon dioxide binding to the β-globins (Weber and Lykkeboe, 1978; Farmer, 1979).

Chloride concentrations in excess of 0.1 M are known to lower haemoglobin oxygen affinity and contribute to the alkaline Bohr effect in fish. This effect has been attributed to differential binding of chloride ions among oxy and deoxy haemoglobins (Brunori et al., 1975). Chloride binding to specific sites on the β-globin subunits of haemoglobin can lead to conformational changes, thus giving rise to significant changes in the oxygen affinity of the haem centre. In addition, the entry of chloride ions will widen the central cavity and reduce the excess positive charges in the cavity, thus reducing the free energy of the oxy-structure and therefore oxygen affinity (Sun et al., 2004).

Review articles by Riggs (1970) and Powers (1980) both state that in most fish species, an increase in temperature will cause a corresponding decrease in haemoglobin affinity for oxygen, as increased temperature weakens the bond between the oxygen and the haem unit. However, there are exceptions; crucian carp (*Carassius carassius*) are highly tolerant of wide temperature ranges and low oxygen levels. Initial experimental work has found that crucian carp haemoglobin increases its oxygen affinity in response to increased temperature, especially at pH levels below 7.0 (Kamshilov and Kamshilova, 2007). Alternatively, northern bluefin tuna (*Thunnus thynnus*) haemoglobin is insensitive to temperature at pH levels between 6.5 and 8.7. This allows them to move between waters of greatly changing temperature such as deep diving during hunting without changing the oxygen transport properties of the blood (Rossi-Fanelli and Antonini, 1960; Ikeda-Saito et al., 1983). In addition, tunas and other members of the Scombridae family such as chub mackerel (*Scomber japonicus*) display regional heterothermy. The high level of swimming activity undertaken by these fish produces large amounts of heat. This heat is preserved in the body core by counter-current vascular heat exchangers as blood flows from the body core to the extremities (Clark et al., 2010). The increased core body temperature allows them to maintain a higher metabolic rate and therefore a higher level of activity than would normally be expected for the ambient water temperature. For example, bluefin tuna have been reported to have muscle temperatures 15ºC above the surrounding water temperature (Carey and Gibson, 1983). However, regional

heterothermy creates a problem; the blood experiences abrupt temperature changes during its transit from the gills to those tissues served by heat exchangers. Therefore, the binding properties of the haemoglobin must be adapted to accommodate sufficient oxygen loading at the gills and delivery to the tissues while faced with extreme and abrupt changes in temperature. The haemoglobins of scombrid fish display reduced temperature dependence of blood–oxygen affinity or even a reversed temperature dependence where increasing temperature increases blood oxygen affinity to compensate (Clark et al., 2010).

Temperature acclimation plays an important role in mitigating the influence of temperature on haemoglobin oxygen affinity. Physiological responses to higher temperatures by fish may include increases in ventilation and heart rate (Prosser, 1973), increasing haemoglobin concentration and/or release of more erythrocytes from the spleen (DeWilde and Houston, 1967). Modification of erythrocyte NTP concentrations may also play a role in adapting to increased temperatures. For example, in brown bullhead catfish (*Amieurus nebulosus*) (Grigg, 1969) and common carp (Albers et al., 1983), erythrocyte ATP concentrations decreased after acclimation to 20ºC resulting in increased haemoglobin oxygen affinity. This effect may be construed as an adaptation to ensure oxygen loading at the respiratory surfaces at high temperature. In contrast, in the Australian blackfish (*Gadopsis marmoratus*), warm acclimation to 20ºC increased blood NTP concentration and decreased haemoglobin oxygen affinity (Dobson and Baldwin, 1982). This was suggested to be an adaptive increase in oxygen unloading as demand increased with temperature (Dobson and Baldwin, 1982). However, because oxygen availability and temperature are linked it is difficult to separate their influences on haemoglobin oxygen affinity. Both Vaccaro et al. (1975) and Weber et al. (1976) ruled out NTP as modifying factors of haemoglobin affinity after acclimation to increased temperatures by goldfish and rainbow trout, respectively, suggesting that haemoglobin concentration and abundance of individual haemoglobin isomorphs are greater modifiers of oxygen affinity.

Normal mammalian haemoglobins are composed of even numbers of α-globins (with A and B forms) and β-globins (also with A and B forms); there also exist haemoglobin isomorphs composed of even mixtures of the α and β-globins (i.e. $\alpha_2^A\beta_2^A$, $\alpha_2^B\beta_2^B$, $\alpha^A\alpha^B\beta_2^A$, $\alpha^A\alpha^B\beta^A\beta^B$) (Riggs, 1970). However, this appears not to be true for all fish haemoglobins. Both rainbow trout (Figure 1.2) and ohrid trout (*Salmo*

letnica) are known to possess haemoglobins with tetrameric conformations involving uneven numbers of α and β-globins (Cepreganova et al., 1992; Fago et al., 2002).

The presence of multiple haemoglobin isomorphs in fish, including rainbow trout, was first reported by Buhler and Shanks (1959). Since then, the number of haemoglobin isomorphs reported for rainbow trout has varied considerably, from the three reported by Buhler and Shanks (1959) to 16 reported by Tsuyuki and Gadd (1963). This variance can be attributed to the differing detection methods used and the varying conditions under which samples were run and stained. In general, early boundary electrophoresis methods produced less resolution (Buhler and Shanks, 1959; Buhler, 1963) than starch gel or cellulose acetate methods (Iuchi, 1973; Tun and Houston, 1986; Murad and Houston, 1991). Other methods such as isoelectric focusing and Fast Protein Liquid Chromatography (FPLC) tend to fall somewhere in the middle in terms of resolution with 8 to 12 bands (Weber et al., 1976b; Feuerlein and Weber, 1994; Fago et al., 2002). There have been no studies conducted comparing the resolution of rainbow trout haemoglobin isomorphs using different electrophoretic or chromatographic techniques. Currently, the true number of rainbow trout haemoglobin isomorphs remains elusive as some isomorphs may consist of less than 1% of total haemoglobin (Tun and Houston, 1986) and others appear to only be expressed under specific conditions such as developmental stage (Vanstone et al., 1964). However, a recent study by Fago et al. (2002) using FPLC found that rainbow trout had 9 different haemoglobin fractions composed of 9 different globin chains (5 α and 4 β globins).

The establishment of multiple haemoglobins in fish led Binotti et al. (1971) to the discovery that rainbow trout haemoglobin isomorphs had distinctive physico-chemical and physiological properties. Using column chromatography, the authors identified four distinct haemoglobin groups[*] and were able to successfully isolate two of the most abundant groups to examine their functional properties. The four groups were designated HbI to HbIV, with HbIV being the most abundant. Further work by Brunori et al. (1975), again using column chromatography, and Weber et al. (1976) using isoelectric focusing were able to further isolate HbII and HbIII with Weber et al. (1976) isolating a further two functional groups designated HbV and HbVI. The

[*] The term group is used here rather than isomorph as it is likely that each functional group was composed of multiple isomorphs.

collective results of Binotti et al. (1971), Brunori et al. (1975) and Weber et al. (1976) show a continuum of oxygen binding properties with the cathodal haemoglobins I-II being insensitive to temperature, pH and organic phosphates while the anodal haemoglobins IV-V are sensitive to pH, temperature and organic phosphates. HbIII demonstrates intermediate properties between the other groups (Table 1.1). While Weber et al. (1976) were able to isolate HbVI, an insufficient volume of protein was available to determine its functional properties.

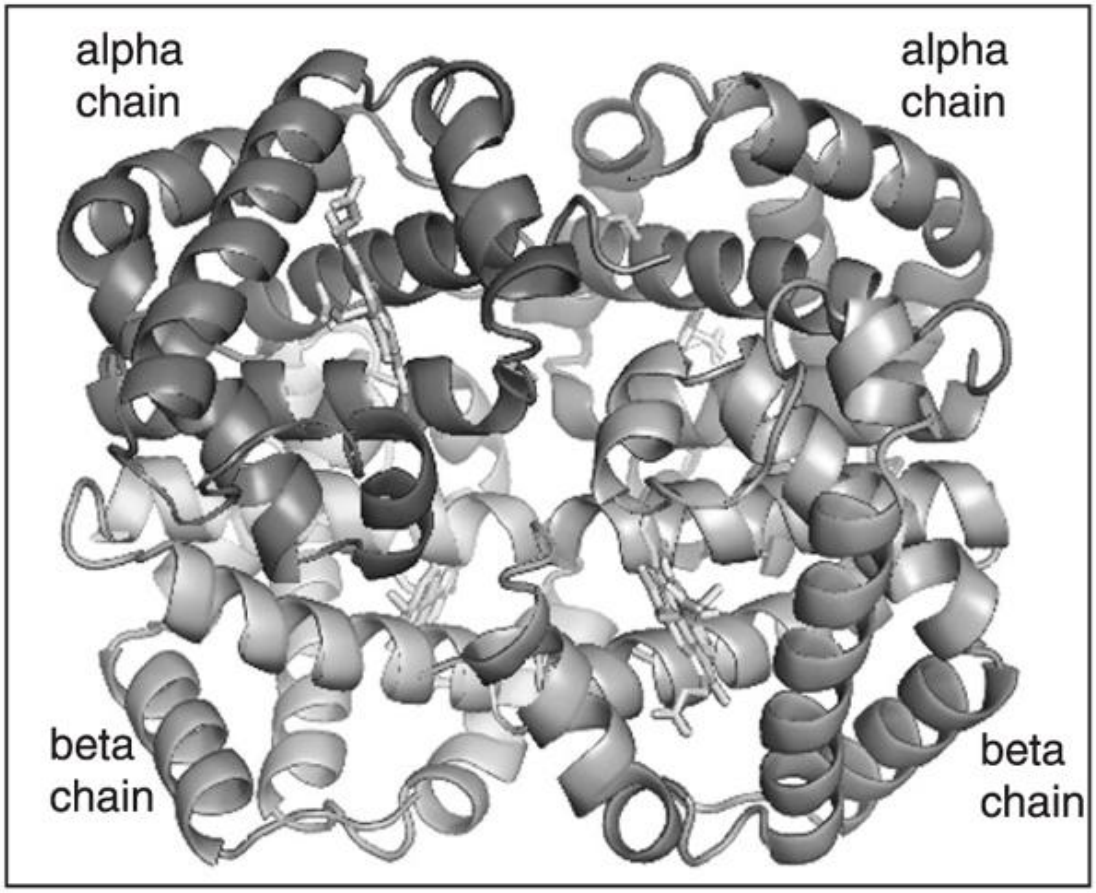


Figure 1.2: Schematic diagram of a typical rainbow trout (*Oncorhynchus mykiss*) tetrameric haemoglobin molecule with two alpha and two beta chains each containing a haem group. Reproduced from de Souza and Bonilla-Rodriguez (2007).

In fish such as trout, eels and some catfish (Powers, 1972; Weber et al., 1975), it is postulated that the cathodal haemoglobin components that have temperature and pH-insensitive oxygen affinities transport oxygen under conditions of internal hypoxia and respiratory acidosis as occur during extended periods of physical exertion. Alternatively, it is possible that cathodal haemoglobins have evolved to supply certain tissues with oxygen under persistent conditions of low PO_2. In fish, the

heart is located at the end of the circulatory system in terms of oxygen availability and therefore is subjected to a persistent low PO_2. Theoretically, blood entering the heart has already off-loaded a large proportion of its available O_2 to tissues throughout the body by the time it reaches the heart. Under conditions of exercise the oxygen demand from body tissues will further increase and more oxygen will be off-loaded from the anodal haemoglobins in response to the increased lactate and CO_2 levels. Although rainbow trout possess a coronary circulation this does not seem to adequately protect the heart from circulatory hypoxia. Steffensen and Farrell (1998) found that ligation of the coronary supply had no effect on swimming performance under hypoxic conditions. Because the cathodal haemoglobins in rainbow trout appear to only offload oxygen in response to low PO_2 they are suited to delivery of oxygen to low PO_2 areas such as the heart. As cathodal haemoglobins do not respond to changes in pH, this means that they are able to retain bound oxygen throughout body circulation. In contrast the low-affinity, pH-sensitive anodal haemoglobins appear functionally adapted to deliver oxygen to tissues performing increased metabolic function. Increased metabolic function produces either CO_2 or lactate causing a decrease in local pH. Because anodal haemoglobins respond to increased $[H^+]$ by reducing their affinity for oxygen, they appear to be better suited to delivery of oxygen to metabolically active tissues (Weber and Jensen, 1988).

Related groups of fishes generally have similar numbers of haemoglobin isomorphs. For example, *Liza ramada*, *Liza aurata*, *Liza saliens* and *Chelon labrosus* of the Mugilidae family have either five or six haemoglobin isomorphs when compared using isoelectric focusing (Basaglia, 2004). Multiple haemoglobins and polymorphic loci (i.e., isohaemoglobins and allohaemoglobins) may be adaptive responses to a variable environment (Powers, 1980).

Table 1.1: Properties of rainbow trout functional haemoglobin groups collated from the results of Binotti et al. (1971), Brunori et al. (1975) and Weber et al. (1976).

Functional Group	pH Sensitive	Temperature Sensitive	Organic Phosphate Sensitive	Chloride Sensitive
HbI	No	No	No	Yes
HbII	No	No	No	Yes
HbIII	Yes	No	Yes	Unknown
HbIV	Yes	Yes	Yes	No
HbV	Yes	Yes	Yes	Unknown
HbVI	Unknown	Unknown	Unknown	Unknown

Induced Haemoglobin Adaptation

In teleosts, haemoglobin isomorph changes commonly accompany temperature acclimation (Houston, 1980). In rainbow trout, the abundances of haemoglobin isomorphs also depend on day length and acclimation to hypoxic conditions (Tun and Houston, 1986). In the mudfish (*Labeo umbratus*), four weeks of hypoxia induced the synthesis of a cathodal haemoglobin, which had a significantly higher oxygen affinity than the anodal haemoglobins (Hattingh, 1976).

The ability of teleosts to change the relative abundance of haemoglobin isomorphs in response to environmental changes was established by Houston and Cyr (1974). Groups of goldfish were subjected to temperatures of 2ºC, 20ºC and 35ºC. After a period of at least 2 weeks goldfish kept at 2ºC exhibited two separate isomorphs while those kept at 20ºC and 35ºC produced three isomorphs. Groups of rainbow trout kept at 2ºC, 10ºC and 18ºC produced the same nine isomorphs at all temperatures, however, increases in abundances were seen in four of the isomorphs while one declined in abundance (Houston and Cyr, 1974).

Age and physiological status have also been found to play a role in structuring haemoglobin isomorph abundances. Coho salmon (*Oncorhynchus kisutch*), Atlantic salmon (*Salmo salar*) and sockeye salmon (*Oncorhynchus nerka*) were all found to undergo significant changes in isomorph abundance at the onset of smolting and seaward migration. Chinook salmon (*Oncorhynchus tshawytscha*) were also found to change isomorph abundances with increasing maturity. The changes were found to continue occurring well after the transition from freshwater to saltwater. The authors proposed that this meant they do not play an adaptive role in the transition from freshwater to saltwater life and further study was needed to explain the changes in isomorph abundance (Fyhn et al., 1991).

Changes in the abundance of rainbow trout haemoglobin functional groups have been induced by removal of 15% of the animals' blood volume. Functional group HbI increased in abundance while HbIV decreased in abundance. When bleeding was coupled with prior starvation for 30 days, functional groups HbI and HbIV again responded as well as increases in abundance from HbII and HbIII. In contrast, 30 day starvation by itself did not induce any change in functional group abundance (Lane, 1980). The reason for this is that starvation is an erythropoietic depressant and that all functional groups were uniformly reduced (Lane, 1980).

The induction of changes in fish haemoglobin isomorph abundances has been investigated for a number of physiological and environmental factors such as temperature, light, oxygen availability and maturity. However, it is of interest that other potential stimuli have not been explored, such as aestivation and sustained exercise. Both of these place large metabolic demands on the body along with the potential need to adapt to changing respiratory demands.

The Role of Multiple Haemoglobins in Rainbow Trout

Rainbow trout appear to fall at the top end of the scale in terms of number of haemoglobin isomorphs with > 10 isomorphs typically detected (Riggs, 1970). Why rainbow trout, along with most salmonid species, have so many haemoglobin isomorphs is potentially related to the environmental conditions typically encountered. For example, most salmonid species are facultatively anadromous, encountering waters of variable pH, temperature and salinity; all of which have an effect on the oxygen binding ability of haemoglobin (Riggs, 1970; Brunori, 1975). By differentially expressing haemoglobins with differing functional properties rainbow trout may be able to optimally adapt their haemoglobins to new environmental conditions.

It is theorised that the functional properties of HbI allow for the delivery of oxygen under physiological emergency conditions in cases of instantaneous and critical oxygen demand. The cooperative character of the ligand dissociation curve, together with the complete lack of oxygen-linked effects of protons, ATP (and possibly CO_2), mean that HbI can deliver oxygen under conditions of violent exercise because oxygen offloading is driven by falling PO_2. It is known that in hyperactive fish the production of lactic acid may be so great as to create problems of oxygen supply to the tissues, and the animal may even die of internal asphyxia (Black, 1958). Under these conditions the role of HbI would be that of providing a normal oxygen supply during emergency (Brunori, 1975).

The role of pH sensitive anodal haemoglobins such as HbIV in the function of the swimbladder is fundamental insofar as haemoglobin is the carrier of oxygen, which is the gas primarily secreted into the swimbladder. In fact it is reported that fish having a gas-filled swimbladder possess haemoglobin isomorphs characterised by the Root effect (Fange, 1966; Riggs, 1970). Thus the role of HbIV may be outlined as

follows. When the fish changes depth, the release of gas into the swimbladder is triggered by a neuro- controlled mechanism (Fange, 1966). Glucose is rapidly converted into lactic acid; and the ensuing drop in pH is transmitted from the blood leaving the gas gland to the blood arriving in the gas gland through the counter-current diffusion system of the *rete mirabile*. Carbon dioxide is likewise liberated, and it is of importance that the gas newly secreted in the swimbladder is very rich in CO_2 (Pelster and Scheid, 1992). The pH drop affects the haemoglobin isomorphs possessing the Root effect and oxygen dissociation follows. The molecular events occurring in the erythrocyte at the level of haemoglobin i.e., binding of protons, CO_2 and other ions (e.g. ATP), conformational changes, dissociation of the ligand, are all very fast events, and therefore never rate-limiting at a physiological level. Thus, HbIV provides oxygen to the secretion mechanism of the swimbladder in large quantities, and even against the very high back pressures that may be present inside the swimbladder (Brunori, 1975).

Specific theories as to the role of HbII and HbIII have not been put forward. Given the similarity in functional properties between HbI and HbII it would not be unreasonable to conclude that they both play the same physiological role (Brunori, 1975). In contrast HbIII appears to share functional properties of both HbI and HbIV. In rainbow trout, the relative abundance of HbIII is considerably lower than HbI or HbIV. This suggests that it plays only a minor physiological role and only responds in a limited manner to environmental challenges such as hypoxia and temperature (Weber et al., 1976b).

Additional Physiological Changes to Temperature and Hypoxia

Changes in haemoglobin isomorph abundance are just one of a number of physiological adjustments that fish undergo to adapt to changing environmental conditions. Rainbow trout frequently surface-breathe at the beginning of summer, suggesting that they have thermoacclimatory respiratory responses. In addition, both ventilation and heart rates of rainbow trout increase with declining PO_2 and rising temperature (by about 34% between 15ºC and 24ºC), but this response is insufficient to completely compensate for a nearly threefold increase in oxygen consumption observed (Heath and Hughes, 1973).

Several studies (Dewilde and Houston, 1967; Houston and DeWilde, 1968; Houston and DeWilde, 1969; Cameron, 1970; Houston and Cyr, 1974) report increases in total blood haemoglobin content in trout and other fish during adaptation to increased ambient temperatures. Although the associated augmentation in blood oxygen capacity may contribute to a seasonal adjustment in oxygen transport, this is far less than the amount of change in respiratory demand (Cameron, 1970). In trout, moreover, seasonal changes in haemoglobin content have also been observed at constant temperature (Denton and Yousef, 1975), indicating that this response may not be temperature-induced (Weber et al., 1976b). However, adaptation to environmental changes by haematological adjustments is not quite so clear-cut. The results from Houston and Cyr (1974) support the conclusion reached by several authors (Spoor, 1951; Bondar, 1957; DeWilde and Houston, 1967; Houston and DeWilde 1968, 1969; Cameron 1970) that acclimation to increased environmental temperatures is associated with haematological alterations which tend to enhance the oxygen carrying capacity of the blood. However, this is in contrast with studies in which insignificant responses or changes that are inconsistent with this interpretation have been encountered (Anthony, 1960; Falkner and Houston, 1966; Grigg 1969; Eddy 1973). Weber et al. (1976b) found that alterations in the respiratory properties of the blood of rainbow trout were not implicated in the acclimatory responses to environmental temperature even after acclimation periods of up to six months. This accords with the absence of temperature-induced changes in NTP or NTP/haemoglobin ratio. Research also shows that dilution of common carp haemoglobin increases its oxygen affinity, even when the phosphate/haemoglobin ratio is kept constant (Weber and Lykkeboe, 1978). The increase in haematocrit but not in NTP/haemoglobin of trout acclimated to 22ºC (Weber et al., 1976b) predicts a higher oxygen affinity in this fish than in cold acclimated ones, i.e. a response similar to those seen in brown bullhead catfish (Grigg, 1969). The findings of Weber et al. (1976b) and Cameron (1971) indicate the absence of a thermoacclimatory response in oxygen affinity of rainbow trout blood after both shorter (three weeks) and longer (six months) acclimation periods. Also, Black et al. (1966) found that the P_{50} and Bohr effect were the same in winter- and summer- acclimated brook trout (*Salvelinus frontinalis*), suggesting that as far as the respiratory function of blood is concerned, salmonids must rely almost completely on alternative mechanisms for seasonal

temperature adaptations, such as changes in the degree of utilisation of haemoglobin bound oxygen, and its delivery rate to the tissues.

Erythrocyte swelling is a common response in fish to stressors such as hypoxia, exercise and capture (Jensen and Weber, 1982; Nikinmaa et al., 1984; Ling and Wells, 1985; Primmett et al., 1986). Swelling occurs as part of a β-stimulated response, whereby catecholamines bind to β-receptors on the erythrocyte 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 an increase in intracellular pH (pHi) and cell water content (Holk and Lykkeboe, 1995). Erythrocytes of fish subjected to environmental hypoxia swell rapidly and typically remain swollen throughout long-term severe hypoxia (Holeton and Randall, 1967; Jensen and Weber, 1982, 1985) and revert to their original volume when normoxia returns (Jensen and Weber, 1985). Although swelling does not alter the NTP/haemoglobin ratio, it decreases the cellular haemoglobin and NTP concentrations and haemoglobin-NTP complexing, all of which promote an increase in blood oxygen affinity (Weber et al., 1976b; Weber and Lykkeboe, 1978). The increase in pHi associated with erythrocyte swelling also increases oxygen affinity by counteracting the Bohr effect (Holk, 1996).

Project Rationale and Aims

Teleost species inhabiting temperate freshwater systems have evolved to be able to adapt to fluctuating temperatures and dissolved oxygen levels. In addition, anthropogenic effects on temperate freshwater environments have increased markedly in the past hundred years with both thermal pollution from industrial waste water and eutrophication affecting oxygen availability. By studying the ability of fish to cope with environmental change we can better predict the effects of human activities on fish species.

Rainbow trout have been extensively distributed around the world from their native habitat range of the north-eastern Pacific. They often form the basis of recreational fisheries in many countries including New Zealand and as such are extensively stocked from hatchery sources (McDowall, 1990). Rainbow trout are moderately stenothermal compared to species such as the eurythermal common carp or some of the extremely stenothermal Antarctic marine species such as *Pagothenia*

borchgrevinki. Rainbow trout also produce numerous haemoglobins with a wide range of oxygen binding characteristics (Houston, 1980). These characteristics may partially explain the success of rainbow trout in adapting to a comparatively wide range of new environments.

The question 'what advantage does possession of multiple haemoglobin isomorphs offer rainbow trout?' has yet to be adequately addressed despite multiple studies being conducted on the function and changes in relative abundance of haemoglobin isomorphs. In the case of rainbow trout, a link with adaptation to habitat and changes in life-style is possible. But it may also be possible that multiple α and β genes provide no selective advantages and are simply surviving selectively neutral gene duplication, with no obvious correlation with environmental conditions. Fago et al. (2002) have shown that at least nine haemoglobins can be derived from rainbow trout blood using FPLC. In contrast, 10 to 14 isomorphs have been reported by authors using cellulose acetate electrophoresis under varying environmental conditions (Tun and Houston, 1986; Marinsky et al., 1990; Murad and Houston, 1991; Houston et al., 1996). Unlike other methods, FPLC separation of rainbow trout haemoglobin provides enough eluted volume for individual fractions to be run on cellulose acetate and to investigate the functional oxygen binding properties of each fraction. While extensive work has been conducted on the functional properties of trout HbI and HbIV (Binotti et al., 1971; Brunori et al., 1975; Weber et al., 1976b) HbII and HbIII have been only partially examined due to low isolation volumes; the functional properties of HbV, HbVI and HbVII have not been fully investigated as they have only recently been isolated. Electrophoresis of isolated fractions will also reveal the relationship between chromatography separated and electrophoretically separated haemoglobins. By establishing the functional properties of rainbow trout haemoglobin isomorphs, a hypothesis can be formed as to why changes in abundance occur and predictions made as to the conditions in which changes in haemoglobin isomorph abundance are likely to occur.

Despite the numerous studies published on changes in haemoglobin isomorph abundance (Houston and Cyr, 1974; Giles and Vanstone, 1976; Houston et al., 1976; Weber et al., 1976b; Tun and Houston, 1986; Fyhn et al., 1991; Murad and Houston, 1991) in response to environmental challenges, there is still doubt over the time-scale in which these changes occur. Recent work has shown that the nucleated erythrocytes of rainbow trout are able to synthesise new proteins while circulating in the blood;

although this ability declines with increasing erythrocyte age (Speckner et al., 1989; Lund et al., 2000; Phillips et al., 2000). This implies that when subjected to environmental change, circulating erythrocytes could quickly produce a new range of haemoglobin isomorphs, thereby adapting to the new environment. Alternatively, new isomorph abundances may only arise through the production of new erythrocytes containing the variant. Therefore, changes in the abundance of haemoglobin isomorphs would only occur over moderate to seasonal periods of time as the life-span of erythrocytes in fish generally ranges between 100 to 300 days (Soldatov, 2005).

The time-span for detectable physiological alteration of haemoglobin isomorphs in response to environmental challenges will be examined. Rainbow trout will be subjected to environmental challenges ranging from approximately 1-4 weeks and 6-months. Sampling over seasonal periods of time will also allow for the examination of changes in isomorph abundance with regard to age. Such changes have previously been detected in coho salmon, Atlantic salmon and sockeye salmon but similar work has not been conducted on rainbow trout.

Fractionation of fish erythrocytes into age classes has been successfully attempted by a number of researchers using a variety of techniques such as discontinuous density gradient centrifugation, velocity sedimentation and fixed-angle centrifugation (Houston et al., 1991; Hofer et al., 2000; Phillips et al., 2000). However, these techniques are comparatively crude, leading to enrichment of cells based on size and density rather than true separation by age (Lund et al., 2000). Flow cytometry potentially provides a superior quantitative method for separation of erythrocytes into age classes. Successful separation of human erythrocytes into various mature and reticulocyte age classes using flow cytometry coupled with RNA fluorescence was achieved by Tanke et al. (1980). Accordingly separation of erythrocytes into age classes would allow for the determination of whether changes in haemoglobin isomorph abundance occur in mature circulating erythrocytes or if the changes originate from newly produced erythrocytes. As fish erythrocytes mature the amount of RNA and protein synthesis declines (Lund et al., 2000). By using a RNA-binding fluorescent dye, flow cytometry may be able to separate young and immature erythrocytes from mature cells more efficiently than conventional techniques. Haemoglobin from separated erythrocytes could then be extracted and isomorph abundances quantified.

Exercise as a stimulus for changes in haemoglobin isomorph abundance has been proposed by both Brunorri (1975) and Powers (1980). However, the potential changes have never been examined under controlled laboratory conditions. It is theorised that increases in cathodal isomorphs would occur in response to sustained exercise as cathodal haemoglobins are thought to act as providers of oxygen to tissues under conditions of physiological stress.

Summary

Haemoglobin is the primary oxygen transportation molecule present in vertebrate species. Over time it has evolved a number of functional properties that allow organisms to inhabit fluctuating environments. Teleost haemoglobin is typically composed of four globin subunits arranged into a tetrameric structure. The number of haemoglobin isomorphs produced by any given fish species is highly variable and only loosely correlated to their environment. Functional properties such as the Bohr and Root effects, along with allosteric effectors, allow the delivery of oxygen to respiring tissues. In the case of the Root effect, oxygen delivery to tissues with high oxygen partial pressures, such as the swimbladder, can be achieved. To achieve this, species such as rainbow trout have multiple haemoglobin isomorphs which vary in abundance in response to environmental stressors such as temperature and hypoxia.

In order to better understand how and why these changes occur, a number of experiments will be conducted on rainbow trout. The hypotheses to be investigated herein include; (i) that changes in rainbow trout haemoglobin isomorph abundance originate from the production of new erythrocytes rather than *de novo* production of haemoglobin from circulating mature erythrocytes; (ii) that rainbow trout have more than five haemoglobin functional groups; (iii) changes in the abundance of the isomorphs, and therefore the functional groups, will be related to the physiological environment encountered by the fish. In addition, the relationships between the functional groups derived from FPLC and the haemoglobin isomorphs separated during electrophoresis on cellulose acetate gels will be ascertained; (iv) that exercise will induce an increase in cathodal isomorph abundance in rainbow trout undergoing periodic sustained exercise.

This research will provide a greater understanding of the physiological adaptations rainbow trout undergo when adapting to fluctuating environmental

conditions. Elucidation of the time scale at which isomorph abundance changes occur and the identification of variants that possess the ability to adapt to marginal conditions will help in the protection and management of rainbow trout fish stocks.

References

Albers C, Goetz KH, Hughes GM, 1983. Effect of acclimation temperature on intraerythrocytic acid-base balance and nucleoside triphosphates in the carp, *Cyprinus carpio*. Respiration Physiology 54:145-159.

Basaglia F, 2004. Comparative study of electrophoretic and isoelectrophoretic characteristics of osteichthyan and amphibian hemoglobin. Italian Journal of Zoology 71:287-295.

Binotti I, Giovenco S, Giardina B, Antonini E, Brunori M, Wyman J, 1971. Studies on the functional properties of fish hemoglobins : II. The oxygen equilibrium of the isolated hemoglobin components from trout blood. Archives of Biochemistry and Biophysics 142:274-280.

Black EC, 1958. Hyperactivity as a lethal factor in fish. Journal of the Fisheries Research Board of Canada 15:573-586.

Brittain T, 2005. Root effect hemoglobins. Journal of Inorganic Biochemistry 99:120-129.

Brunori M, 1975. Molecular adaptation to physiological requirements: the hemoglobin system of trout. In: Current Topics in Cellular Regulation (Horecker BL, Stadtman ER, eds). Academic Press Inc. New York. p 1-39.

Brunori M, Falcioni G, Fortuna G, Giardina B, 1975. Effect of anions on the oxygen binding properties of the hemoglobin components from trout (*Salmo irideus*). Archives of Biochemistry and Biophysics 168:512-519.

Buhler DR, 1963. Studies on fish hemoglobins. Chinook salmon and rainbow trout Journal of Biological Chemistry 238:1665-1674.

Buhler DR, Shanks WE, 1959. Multiple hemoglobins in fishes. Science 129:899-900.

Cameron JN, 1970. The influence of environmental variables on the hematology of pinfish (*Lagodon rhomboides*) and striped mullet (*Mugil cephalus*). Comparative Biochemistry and Physiology 32:175-192.

Carey FG, Gibson QH, 1983. Heat and oxygen exchange in the rete mirabile of the bluefin tuna, *Thunnus thynnus*. Comparative Biochemistry and Physiology Part A 74:333-342.

Cepreganova B, Wilson JB, Webber BB, Kjovkareska B, Efremov GD, Huisman THJ, 1992. Heterogeneity of the hemoglobin of the Ohrid trout (*Salmo* L *typicus*.). Biochemical Genetics 30:385-399.

Clark TD, Rummer JL, Sepulveda CA, Farrell AP, Brauner CJ, 2010. Reduced and reversed temperature dependence of blood oxygenation in an ectothermic scombrid fish: implications for the evolution of regional heterothermy? Journal of Comparative Physiology B 180:73-82.

Denton JE, Yousef MK, 1975. Seasonal changes in hematology of rainbow trout, *Salmo gairdneri*. Comparative Biochemistry and Physiology A 51:151-153.

de Souza PC, Bonilla-Rodriguez GO, 2007. Fish hemoglobins. Brazilian Journal of Medical and Biological Research 40:769-778.

Dewilde MA, Houston AH, 1967. Hematological aspects of thermoacclimatory process in rainbow trout *Salmo gairdneri*. Journal of the Fisheries Research Board of Canada 24:2267-2281.

Dobson GP, Baldwin J, 1982. Regulation of blood oxygen affinity in the Australian blackfish *Gadopsis marmoratus*: II. Thermal acclimation. Journal of Experimental Biology 99:245-254.

Fago A, Forest E, Weber RE, 2002. Hemoglobin and subunit multiplicity in the rainbow trout (*Oncorhynchus mykiss*) hemoglobin system. Fish Physiology and Biochemistry 24:335-342.

Fange R, 1966. Physiology of the swimbladder. Physiological Reviews 46:299-322.

Farmer M, 1979. The transition from water to air breathing: Effects of CO_2 on hemoglobin function. Comparative Biochemistry and Physiology Part A 62:109-114.

Feuerlein R, Weber R, 1994. Rapid and simultaneous measurement of anodic and cathodic haemoglobins and ATP and GTP concentrations in minute quantities of fish blood. Journal of Experimental Biology 189:273-277.

Fyhn UEH, Clarke WC, Withler RE, 1991. Hemoglobins in smoltifying chinook salmon, *Oncorhynchus tshawytscha*, subjected to photoperiod control. Aquaculture 95:359-372.

Geoghegan WD, Poluhowich JJ, 1974. The major erythrocytic organic phosphates of the American eel, *Anguilla rostrata*. Comparative Biochemistry and Physiology Part B 49:281-290.

Giles MA, Vanstone WE, 1976. Ontogenetic variation in multiple hemoglobins of coho salmon (*Oncorhynchus kisutch*) and effect of environmental factors on their expression. Journal of the Fisheries Research Board of Canada 33:1144-1149.

Greaney GS, Powers DA, 1978. Allosteric modifiers of fish hemoglobins: In vitro and in vivo studies of the effect of ambient oxygen and pH on erythrocyte ATP concentrations. Journal of Experimental Zoology 203:339-349.

Grigg GC, 1969. Temperature-induced changes in the oxygen equilibrium curve of the blood of the brown bullhead, *Ictalurus nebulosus*. Comparative Biochemistry and Physiology 28:1203-1223.

Hattingh J, 1976. Hemoglobins in *Labeo umbratus* - influence of temperature and oxygen. South African Journal of Science 72:27-28.

Heath AG, Hughes GM, 1973. Cardiovascular and respiratory changes during heat stress in rainbow trout (*Salmo gairdneri*). Journal of Experimental Biology 59:323-338.

Hofer R, Stoll M, Romani N, Koch F, Sordyl H, 2000. Seasonal changes in blood cells of Arctic char (*Salvelinus alpinus* L.) from a high mountain lake. Aquatic Sciences - Research Across Boundaries 62:308-319.

Holeton GF, Randall DJ, 1967. The effect of hypoxia upon the partial pressure of gases in the blood and water afferent and efferent to the gills of rainbow trout. Journal of Experimental Biology 46:317-327.

Holk K, 1996. Effects of isotonic swelling on the intracellular Bohr factor and the oxygen affinity of trout and carp blood. Fish Physiology and Biochemistry 15:371-375.

Holk K, Lykkeboe G, 1995. Catecholamine induced changes in oxygen affinity of carp and trout blood. Respiration Physiology 100:55-62.

Houston A, Freeman G, Plint A, Korcock D, 1991. Erythrocyte fractionation by velocity sedimentation and discontinuous density gradient centrifugation. Fish Physiology and Biochemistry 9:279-289.

Houston AH, 1980. Components of the hematological response of fishes to environmental change: A review. In: Environmental Physiology of Fishes (Ali, MA, ed.) Plenum Press. New York. p 241-298.

Houston AH, Cyr D, 1974. Thermoacclimatory variation in hemoglobin systems of goldfish (*Carassius auratus*) and rainbow trout (*Salmo gairdneri*). Journal of Experimental Biology 61:455-461.

Houston AH, Dewilde MA, 1968. Thermoacclimatory variations in haematology of common carp *Cyprinus carpio*. Journal of Experimental Biology 49:71-81.

Houston AH, DeWilde AM, 1969. Environmental temperature and the body fluid system of the fresh-water teleost--III. Hematology and blood volume of thermally acclimated brook trout, *Salvelinus fontinalis*. Comparative Biochemistry and Physiology 28:877-885.

Houston AH, Dobric N, Kahurananga R, 1996. The nature of hematological response in fish - Studies on rainbow trout *Oncorhynchus mykiss* exposed to simulated winter, spring and summer conditions. Fish Physiology and Biochemistry 15:339-347.

Houston AH, Mearow KM, Smeda JS, 1976. Further observations upon the hemoglobin systems of thermally-acclimated freshwater teleosts: Pumpkinseed (*Lepomis gibbosus*), white sucker (*Catostomus commersoni*), carp (*Cyprinus carpio*), goldfish (*Carassius auratus*) and carp-goldfish hybrids. Comparative Biochemistry and Physiology Part A 54:267-273.

Ikeda-Saito M, Yonetani T, Gibson QH, Gilbert GA, 1983. Oxygen equilibrium studies on hemoglobin from the bluefin tuna (*Thunnus thynnus*). Journal of Molecular Biology 168: 673-686.

Iuchi I, 1973. Chemical and physiological properties of larval and adult Hemoglobins in rainbow trout, *Salmo gairdnerii irideus*. Comparative Biochemistry and Physiology 44:1087-1094.

Jensen FB, Weber RE, 1982. Respiratory properties of tench blood and hemoglobin adaptation to hypoxic hypercapnic water. Molecular Physiology 2:235-250.

Jensen FB, Weber RE, 1985. Kinetics of the acclimational responses of tench to combined hypoxia and hypercapnia .1. respiratory responses. Journal of Comparative Physiology B 156:197-203.

Johansen K, 1971. Comparative physiology: gas exchange and circulation in fishes. Annual Review of Physiology 33:569-612

Johansen K, Lykkeboe G, Weber RE, Maloiy GMO, 1976. Respiratory properties of blood in awake and estivating lungfish, *Protopterus amphibius*. Respiration Physiology 27:335-345.

Kamshilov I, Kamshilova T, 2007. Effect of temperature on functional properties of hemoglobin of crucian carp (*Carassius carassius*). Journal of Ichthyology 47:469-472.

Lane HC, 1980. The response of the haemoglobin system of fed and starved rainbow trout, Salmo gairdneri Richardson, to bleeding. Journal of Fish Biology 16:405-411.

Leray C, 1979. Patterns of purine nucleotides in fish erythrocytes. Comparative Biochemistry and Physiology Part B 64:77-82.

Ling N, Wells RMG, 1985. Plasma catecholamines and erythrocyte swelling following capture stress in a marine teleost fish. Comparative Biochemistry and Physiology C 82:231-234.

Lund SG, Phillips MCL, Moyes CD, Tufts BL, 2000. The effects of cell ageing on protein synthesis in rainbow trout (*Oncorhynchus mykiss*) red blood cells. Journal of Experimental Biology 203:2219-2228.

Marinsky CA, Houston AH, Murad A, 1990. Effect of hypoxia on hemoglobin isomorph abundances in rainbow trout, *Salmo gairdneri*. Canadian Journal of Zoology 68:884-888.

McDowall RM, 1990. New Zealand Freshwater Fishes: A Natural History and Guide. Heinemann Reed. Auckland.

Murad A, Houston A, 1991. Haemoglobin isomorph abundances in splenectomized rainbow trout, *Oncorhynchus mykiss* (Walbaum). Journal of Fish Biology 38:641-651.

Nikinmaa M, Cech JJ, McEnroe M, 1984. Blood-oxygen transport in stressed striped bass (*Morone saxatilis*) role of beta-adrenergic responses. Journal of Comparative Physiology 154:365-369.

Pelster B, Scheid P, 1992. Countercurrent concentration and gas secretion in the fish swim bladder. Physiological Zoology 65:1-16.

Pelster B, Weber RE, 1990. Influence of organic phosphates on the Root effect of multiple fish Journal of Experimental Biology 149:425-437.

Phillips MC, Moyes CD, Tufts BL, 2000. The effects of cell ageing on metabolism in rainbow trout (*Oncorhynchus mykiss*) red blood cells. Journal of Experimental Biology 203:1039-1045.

Powers DA, 1972. Hemoglobin adaptation for fast and slow water habitats in sympatric catostomid fishes. Science 177:360-362.

Powers DA, 1980. Molecular ecology of teleost fish hemoglobins - strategies for adapting to changing environments. American Zoologist 20:139-162.

Primmett DR, Randall DJ, Mazeaud M, Boutilier RG, 1986. The role of catecholamines in erythrocyte pH regulation and oxygen transport in rainbow

trout (*Salmo gairdneri*) during exercise. Journal of Experimental Biology 122:139-148.

Riggs AF, 1970. Properties of fish hemoglobins. In: Fish Physiology (Hoar WS, Randall DJ, eds). Academic Press. New York. p 209-252.

Riggs AF, 1988. The Bohr effect. Annual Review of Physiology 50:181-204.

Rossi-Fanelli A, Antonini E, 1960. Oxygen equilibrium of haemoglobin from *Thunnus thynnus*. Nature 186:895-896.

Soivio A, Nikinmaa M, Westman K, 1980. The blood-oxygen binding properties of hypoxic *Salmo gairdneri*. Journal of Comparative Physiology 136:83-87.

Soldatov AA, 2005. Peculiarities of organization and functioning of the fish red blood system. Journal of Evolutionary Biochemistry and Physiology 41:272-281.

Speckner W, Schindler JF, Albers C, 1989. Age-dependent changes in volume and haemoglobin content of erythrocytes in the carp (*Cyprinus carpio* L.). Journal of Experimental Biology 141:133-149.

Steffensen JF, Farrell AP, 1998. Swimming performance, venous oxygen tension and cardiac performance of coronary-ligated rainbow trout, *Oncorhynchus mykiss*, exposed to progressive hypoxia. Comparative Biochemistry and Physiology 119A:585-592.

Sun Y, Liu X, Fan C, Zhang W, Li G, 2004. Electrochemical investigation of the chloride effect on haemoglobin. Bioelectrochemistry 64:23-27.

Tetens V, Lykkeboe G, 1981. Blood respiratory properties of rainbow trout, *Salmo gairdneri* - responses to hypoxia acclimation and anoxic incubation of blood invitro. Journal of Comparative Physiology 145:117-125.

Tetens V, Lykkeboe G, 1985. Acute exposure of rainbow trout to mild and deep hypoxia: O_2 affinity and O_2 capacitance of arterial blood. Respiration Physiology 61:221-235.

Tsuyuki H, Gadd REA, 1963. The multiple hemoglobins of some members of the Salmonidae family. Biochimica et Biophysica Acta 71:219-221.

Tun N, Houston AH, 1986. Temperature, oxygen, photoperiod, and the hemoglobin system of the rainbow trout, *Salmo gairdneri*. Canadian Journal of Zoology 64:1883-1888.

Vaccaro AM, Raschetti R, Ricciardi G, Morpurgo G, 1975. Temperature adaptation at the hemoglobin level in *Carassius auratus*. Comparative Biochemistry and Physiology Part A 52:627-634.

Val AL, 2000. Organic phosphates in the red blood cells of fish. Comparative Biochemistry and Physiology Part A 125:417-435.

Vanstone WE, Tsuyuki H, Roberts E, 1964. Changes in multiple hemoglobin patterns of some pacific salmon genus *Oncorhynchus* during parr-smolt transformation. Canadian Journal of Physiology and Pharmacology 42:697-703.

Weber RE, Jensen FB, 1988. Functional adaptations in hemoglobins from ectothermic vertebrates. Annual Review of Physiology 50:161-179.

Weber RE, Lykkeboe G, 1978. Respiratory adaptations in carp blood influences of hypoxia, red cell organic phosphates, divalent cations and CO_2 on hemoglobin-oxygen affinity. Journal of Comparative Physiology 128:127-137.

Weber RE, Lykkeboe G, Johansen K, 1975. Biochemical aspects of the adaptation of hemoglobin-oxygen affinity of eels to hypoxia. Life Sciences 17:1345-1349.

Weber RE, Lykkeboe G, Johansen K, 1976a. Physiological properties of eel haemoglobin: hypoxic acclimation, phosphate effects and multiplicity. Journal of Experimental Biology 64:75-88.

Weber RE, Wood SC, Davis BJ, 1979. Acclimation to hypoxic water in facultative air-breathing fish: blood-oxygen affinity and allosteric effectors. Comparative Biochemistry and Physiology A 62:125-129.

Weber RE, Wood SC, Lomholt JP, 1976b. Temperature-acclimation and oxygen-binding properties of blood and multiple hemoglobins of rainbow-trout. Journal of Experimental Biology 65:333-345.

Wood SC, Johansen K, 1972. Adaptation to hypoxia by increased hbO_2 affinity and decreased red-cell ATP concentration. Nature-New Biology 237:278-279.

CHAPTER TWO : HAEMATOPOIETIC CHANGES IN RAINBOW TROUT HAEMOGLOBIN ISOMORPH ABUNDANCES IN RESPONSE TO TEMPERATURE

Introduction

Multiple haemoglobin isomorphs have been detected in many fish species, including rainbow trout (*Oncorhynchus mykiss*) (Buhler and Shanks, 1959; Tsuyuki and Gadd, 1963; Tsuyuki et al., 1965; Wilkins, 1968; Sharp, 1973; Vaccaro et al., 1975; Weber and Jensen, 1988). Rainbow trout have variously been reported as producing between 3 (Buhler, 1963) and 16 haemoglobin isomorphs (Tsuyuki and Gadd, 1963), with 8 to 14 isomorphs most commonly detected (Iuchi, 1973; Houston and Cyr, 1974; Weber et al., 1976; Tun and Houston, 1986; Murad and Houston, 1991; Houston et al., 1996). The number of haemoglobins reported depends on the resolution of the methods used. For example, starch gel electrophoresis typically has less resolution than isoelectric focusing; while cellulose acetate electrophoresis is generally the most sensitive (Iuchi, 1973; Weber et al., 1976; Murad and Houston, 1991).

Haemoglobin isomorphs of rainbow trout are known to vary in abundance in response to environmental variables. For example, changes in isomorph abundance in response to temperature (Houston and Cyr, 1974), hypoxia (Marinsky et al., 1990) and light (Tun and Houston, 1986) have been reported. In conjunction with allosteric effectors these changes in abundance are believed to modify blood oxygen affinity demonstrating an adaptive response by the oxygen transport system to variability in the external environment.

While the effect of temperature on haemoglobin isomorph abundance in eels (*Anguilla* spp.), common carp (*Cyprinus carpio*), goldfish (*Carassius auratus*) and rainbow trout (Poluhowich, 1972; Houston and Cyr, 1974; Vaccaro et al., 1975; Houston et al., 1976; Tun and Houston, 1986; Houston et al., 1996) has been widely examined, the timescale and derivation of these changes have not been fully determined. Shifts in isomorph abundance in response to temperature have been observed after more than two weeks; however these changes were comparatively small, not exceeding a 6% shift in individual isomorph abundance (Houston and Cyr,

1974). Larger shifts of up to 50% individual isomorph abundance were observed by Tun and Houston (1986), after 4 weeks acclimation to various conditions of photoperiod, temperature and hypoxia. Investigations of shifts in rainbow trout isomorph abundance have not been conducted over periods shorter than 2 weeks or longer than 4 months. However, changes in the abundance of haemoglobin isomorphs over seasonal periods of time have been observed in yellowfish (*Barbus holubi*), common carp and mudfish (*Labeo umbratus* and *Labeo capensis*) (Fourie and van Vuren, 1976; van Vuren and Hattingh, 1978).

Changes in isomorph abundances could originate from three potential pathways. Teleost erythrocytes remain nucleated throughout their life-span and young erythrocytes are able to synthesise RNA and proteins while circulating in the blood stream (Lund et al., 2000; Phillips et al., 2000). It has also been demonstrated that both young and mature erythrocytes are able to respond to environmental stressors by way of heat shock protein production (Lund et al., 2000). Therefore, changes in isomorph abundance could occur over timescales of less than 7 days through the synthesis of new haemoglobin proteins. Similarly, changes in abundance may result from recombination of pre-existing haemoglobin subunits (Weber et al., 1976) which would again be observable over shorter periods of time. However, if changes in isomorph abundance primarily occur through the production of new erythrocytes then the acclimatory process is likely to occur over a timescale of weeks to months, as fish erythrocyte life-spans can range from 80 to 500 days, with a maturation period of 17–23 days (Cavas and Konen, 2007).

Modes of adaptation to changing environments by living organisms are an important field of investigation given current concern over anthropogenic environmental disturbance. In this study, groups of rainbow trout were subjected to two different environmental temperatures and sampled after varying time intervals to determine the timescale in which changes in haemoglobin isomorph abundance occurred. In addition, anaemia was induced in selected groups of rainbow trout to induce erythropoesis before being subjected to altered temperature conditions. Induction of erythropoesis was designed to increase the chance of detecting changes in isomorph abundance should adaptation to new environmental conditions occur through new erythrocyte production. Density gradient enrichment of young or mature erythrocytes was also conducted in order to test for changes in haemoglobin isomorph abundance originating from the production of new erythrocytes. Lastly, a group of

rainbow trout were kept under naturally varying conditions and were sampled every 6-months for 2-years to determine the extent of isomorph variation over extended periods of time.

Methods

Origin and maintenance of experimental animals

Rainbow trout parr were sourced from the Ngongotaha Hatchery (Rotorua, New Zealand) and housed in a 5000 L fibreglass tank with flow-through dechlorinated tap water. Water temperature varied with season, ranging from 10ºC to 20ºC. Fish were fed to satiation each day with a commercial pellet (Reliance Stock Foods, Dunedin, New Zealand), with faecal and other debris removed following cessation of feeding. Supplementary aeration ensured that dissolved oxygen content consistently exceeded 90% saturation. Overhead fluorescent lighting was set to provide a 12L:12D photoperiod initiated at 0700 h. Animals were monitored daily for feeding behaviour and overt disease symptoms.

Experimental conditions

Trout were housed in 90 L glass aquaria located in temperature controlled rooms under a 12L:12D photoperiod. Oxygenated water was recycled through individual activated carbon filtration units at 2 L min^{-1} with 25% of the total water volume replaced each day. Fish were fed to satiation each day on a commercial pellet with excess food and waste removed 1 h after feeding. In the first experiment, groups of 5 fish (12-25 cm fork length) were subjected to either 10ºC or 20ºC for 5, 7, 14, 21 or 28 days. For logistical reasons temperature acclimation periods were split and run on three separate occasions. Acclimation periods of 5 and 7 days were run in May, separately from the 21 and 28 day periods run in January; while the 14 day period was run individually in March. In the second experiment, 4 groups of 5 fish had approximately 25% of their blood volume removed on 2 consecutive days to induce erythropoesis. Each fish was first weighed and the volume (ml) of blood to be removed (25%) was calculated as 1% of the fish's body weight (g), which was then removed by caudal venipuncture. The fish were then held at either 10ºC or 20ºC for 24 days. The second experiment was run twice, the second time the resulting blood

samples underwent density gradient centrifugation in order to enrich the cell age classes in the samples. In the third experiment, fish were sampled every 6-months from the same cohort held outside in a 400 L fibreglass tank supplied with continuous flow-through dechlorinated tap water. Both photoperiod and temperature were allowed to vary with season; however tank water dissolved oxygen was maintained at a minimum of 90% saturation. Fish were fed to satiation every second day with a commercial pellet (Reliance Stock Food, Dunedin, New Zealand).

Sampling and analysis

At sampling, trout were anesthetised in a 1:10000 solution of buffered tricane methane sulphonate (MS222, Sigma, New Zealand). Fork length was measured to the nearest mm and blood was then drawn into a heparinised (sodium heparin, Sigma, New Zealand) syringe from the caudal vein. Within 30 min of collection, blood samples were analysed for haematocrit (Hct), total haemoglobin (Hb), red blood cell count (RBCC), mean erythrocyte haemoglobin concentration (MEHC), mean cell haemoglobin (MCH), and mean cell volume (MCV) according to standard methods (Dacie and Lewis, 1991). Haemoglobin for isomorph analysis was prepared by washing the cells twice in 0.9% saline and then cells were lysed by addition of an equal volume of 10 mM Tris, pH 8. The samples were then centrifuged at 10 000 rcf for 10 min at 4ºC and the supernatant frozen at -80ºC. The composite haemoglobins of the haemolysates were separated on a cellulose acetate medium (Helena Laboratories, United States) in conjunction with a Tris-glycine buffer (pH 8.1-8.4). Duplicate separations were carried out at 200 V for 1 h at 4ºC using the haemoglobin of an individual rainbow trout as a mobility standard. Haemoglobins were identified by staining with 4 ml of 0.025% benzidine (Sigma, New Zealand) for 5 min, followed by immersion in 5% hydrogen peroxide for 2 min and then rinsing under tap water. Relative abundances of haemoglobin isomorphs were quantified by photographing the gels and then using Image Pro plus version 4.5.1.22 for Windows (Media Cybernetics, United States) to quantify the differences in staining intensity as a measure of isomorph abundance.

Erythrocyte enrichment and staining

Successful differentiation and separation of human erythrocytes into mature and reticulocyte fractions using the fluorescent RNA binding dye pyronin Y and flow cytometry was achieved by Tanke et al. (1981). Following the methods of Tanke et al. (1981) separation of rainbow trout erythrocytes into mature and young cell fractions was attempted using flow cytometry. Staining of formalin preserved cells produced poor staining results with little differentiation between cells. However, better results were achieved with fresh erythrocytes. Newly drawn blood cells were washed twice in 0.9% saline before staining with chloroform purified pyronin Y (Sigma, New Zealand) (0.1%) and sodium chloride (0.9%) solution for 30 min. Stained cell samples were viewed by fluorescence microscopy (excitation filters SP 560 and LP 515, chromatic beam splitter at 580 nm, barrier filter LP 580) with differential staining of dull ovoid mature erythrocytes from brighter rounder young erythrocytes observed. This difference in staining intensity is due to the staining of ribosomal bodies in the cell cytoplasm which are larger and more numerous in young erythrocytes and reticulocytes (Tanke et al. 1981; Tavares-Dias 2006). Flow cytometry was conducted using a BD FACSVantage™ SE flow cytometer (BD Biosciences, USA), equipped with the BD FACSDiVa™ digital data processing electronics and software option. The flow cytometer was fitted with a Coherent INNOVA® Enterprise™ II ion laser regulated at 300 mW, providing 488 nm excitation, and a Spectra-Physics Model 127 helium neon laser, providing 633 nm excitation. The instrument sheath fluid was phosphate-buffered saline (PBS; Gibco, USA), adjusted to pH 7.2 and delivered through a 70 μm nozzle at 131 kPa. However, cell differentiation and therefore separation was not achieved by flow cytometry. There was insufficient dissimilarity in forward scatter (cell volume) and side scatter (cell complexity) signals from the erythrocyte population for cells to be categorised into separate groups based on stage of maturity.

In order to test for differences in haemoglobin isomorph abundance between young and mature erythrocytes a less efficient method of separation was employed; discontinuous gradient centrifugation. This method utilised 100 μl of whole blood taken from two groups of ten fish in which anaemia had been induced and then subsequently held at either 10ºC or 20ºC for 24 days. The whole blood was layered on top of an isotonic Percoll (Sigma, New Zealand) discontinuous density gradient. This

method is based on the assumption that cell density increases with age, as haemoglobin accumulates and the composition of other cell components change (Clark, 1988). It is important to note that this process does not fully separate erythrocytes into age classes, but rather enriches the proportion of an age class in a given fraction. Using pasteur pipettes, 1 ml of four different Percoll solutions with decreasing densities of 1.105, 1.095, 1.085 and 1.075 g/ml respectively, were carefully layered into 15 ml centrifuge tubes. Finally, 100 µl of whole blood was placed on top of the gradient and the tube transferred to a swing-out bucket centrifuge. Samples were centrifuged at 500 rcf for 90 min, the top and bottom fractions were removed and diluted in 3 volumes of 0.9% saline. After centrifuging at 3000 rcf for 5 min the supernatant was discarded and the cells washed in 0.9% saline before being diluted in 25 µl of 0.9% saline. From each top and bottom fraction as well as the corresponding whole blood sample, 5 µl aliquots were taken and stained for 30 min with 5 µl of a 0.8% NaCl / 1% Brilliant Cresyl Blue (Gurr) solution (Tavares-Dias, 2006). Blood smears were then prepared on microscope slides and allowed to air dry before being methanol fixed. They were then stained with Leishman-Giemsa stains to distinguish mature and immature erythrocytes. Erythrocyte age classes were designated according to the criteria used by Tavares-Dias (2006); with erythroblasts and small round erythrocytes classified as young erythrocytes, larger more oval erythrocytes were classified as mature erythrocytes. Young and immature erythrocytes appear to have much more blue stained basophilic ribonucleoprotein present in the cytoplasm compared to mature erythrocytes. Mature erythrocytes have a much larger proportion of the cell cytoplasm taken up with haemoglobin compared to young and immature erythrocytes. Counts of 500 cells were made and the percentage of leucocytes, mature erythrocytes and young erythrocytes calculated to determine the degree of enrichment of young and mature erythrocytes compared to whole blood (Hofer et al., 2000).

Statistical analysis

Significant differences between treatments were tested for using Student's *t*-test or one-way Analysis of Variance (ANOVA) coupled with Tukey's post-hoc tests using Statistica v.9.0 (Statsoft, USA).

Results

There were no significant differences in haematocrit (Hct), total haemoglobin (Hb), red blood cell count (RBCC), mean erythrocyte haemoglobin concentration (MEHC), mean cell haemoglobin (MCH), and mean cell volume (MCV) between 10ºC and 20ºC paired groups at either 5, 7, 14, 21 or 28 days (Table 2.1).

When whole blood haemolysate was electrophoresed on a cellulose acetate medium, fourteen distinct bands were observed and designated A (anodal) 1 to 8 and C (cathodal) 1 to 6 based on direction of movement and order of increasing mobility (Figure 2.1). No evidence was seen of polymerization or auto-oxidation. The bands exhibited the same degree of mobility and were observable under all treatment conditions in all subsequent experiments. There were no statistically significant differences in isomorph abundance between 10ºC and 20ºC treatment groups at any of the sampling periods (Student's *t*-test, $P > 0.05$) (Table 2.2). Comparisons between sampling periods for the same isomorphs cannot be made due to the different acclimation periods being conducted at different times of the year. In all acclimation periods, isomorph A3 was the most abundant comprising ~17% of the total haemoglobin. A4 and A2 were also both comparatively abundant, constituting ~12% and ~12.5% respectively, of total haemoglobin content. The most abundant cathodal isomorph was C5 which made up ~12% of the total haemoglobin. Other than fraction A5 no other individual fraction comprised more than 10% of total haemoglobin abundance.

Differences in isomorph abundance were observed between anaemia-induced fish after 24 days of being held at either 10ºC or 20ºC (Figure 2.2). Haemoglobin isomorphs C5, C6, A3 and A4 were significantly different between treatments (Student's *t*-test; d.f. = 8, $P < 0.05$). Total anodal and total cathodal isomorph abundance were also significantly different (Student's *t*-test; d.f. = 8, $P < 0.05$) with anodal isomorphs being more abundant at 20ºC compared to 10ºC, while cathodal isomorphs were more abundant at 10ºC compared to 20ºC. The 20ºC group had a higher RBCC (mean 1.36×10^{12} cells $L^{-1} \pm 8.6 \times 10^{10}$ SEM) in comparison to the 10ºC group (mean 1.07×10^{12} cells $L^{-1} \pm 4.3 \times 10^{10}$ SEM) (Student's *t*-test; d.f. =18, $n = 10$, $P = 0.009$). MEHC was also significantly higher in the 20ºC group (mean 133 g $L^{-1} \pm 3$ SEM) compared to the 10ºC group (mean 101 g $L^{-1} \pm 2$ SEM) (Student's *t*-test; d.f. =18, $n = 10$, $P = 0.007$). All other haematological variables were not significantly different.

Table 2.1: Means (± SEM) of length and blood parameters from paired groups of rainbow trout (*Oncorhynchus mykiss*) kept at 10ºC or 20ºC for 5, 7, 14, 21 and 28 days. Total haemoglobin (Hb), haematocrit (Hct), red blood cell count (RBCC), mean erythrocyte haemoglobin concentration (MEHC), mean cell haemoglobin (MCH), and mean cell volume (MCV).

	n	Fork Length (mm)	Hb ($g\ L^{-1}$)	Hct	RBCC ($x\ 10^{12}$ cells L^{-1})	MEHC ($g\ L^{-1}$)	MCH (pg)	MCV (fl)
Day 5, 10º C	5	128 ± 8	77.7 ± 6.1	0.39 ± 0.03	1.17 ± 0.12	204 ± 24	70.0 ± 11.2	339 ± 21
Day 5, 20ºC	5	153 ± 10	84.4 ± 3.6	0.41 ± 0.01	1.22 ± 0.05	208 ± 11	70.0 ± 4.6	337 ± 14
Day 7, 10ºC	5	137 ± 7	87.3 ± 4.0	0.34 ± 0.02	1.24 ± 0.9	259 ± 10	71.5 ± 5.1	277 ± 18
Day 7, 20ºC	5	145 ± 10	78.9 ± 5.0	0.38 ± 0.03	1.38 ± 0.10	209 ± 16	58.3 ± 4.5	279 ± 15
Day 14, 10ºC	5	157 ± 6	73.8 ± 1.1	0.32 ± 0.01	1.26 ± 0.6	233 ± 10	59.3 ± 3.5	256 ± 17
Day 14, 20ºC	5	142 ± 10	82.6 ± 4.0	0.39 ± 0.02	1.47 ± 0.11	215 ± 15	56.6 ± 2.6	271 ± 27
Day 21, 10ºC	5	162 ± 7	69.9 ± 6.2	0.43 ± 0.04	1.07 ± 0.11	165 ± 8	66.6 ± 4.6	403 ± 14
Day 21, 20ºC	5	155 ± 6	65.9 ± 1.5	0.47 ± 0.01	1.16 ± 0.09	142 ± 6	58.1 ± 4.9	407 ± 22
Day 28, 10ºC	5	156 ± 6	77.7 ± 1.4	0.43 ± 0.01	1.18 ± 0.06	183 ± 4	66.3 ± 2.5	362 ± 12
Day 28, 20ºC	5	134 ± 3	83.7 ± 2.5	0.43 ± 0.02	1.30 ± 0.10	195 ± 4	65.4 ± 3.3	335 ± 14

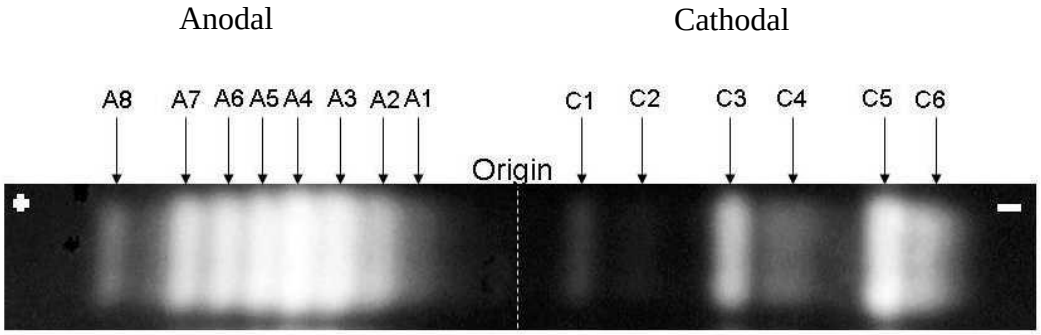


Figure 2.1: Haemoglobin isomorphs separated from rainbow trout (*Oncorhynchus mykiss*) haemolysate. Haemolysate was electrophoresed on a cellulose acetate medium using a Tris-glycine buffer (pH 8.1-8.4) for 1 hour at 4ºC. Isomorphs were designated A (anodal) 1 to 8 and C (cathodal) 1 to 6 based on direction of movement and order of increasing mobility.

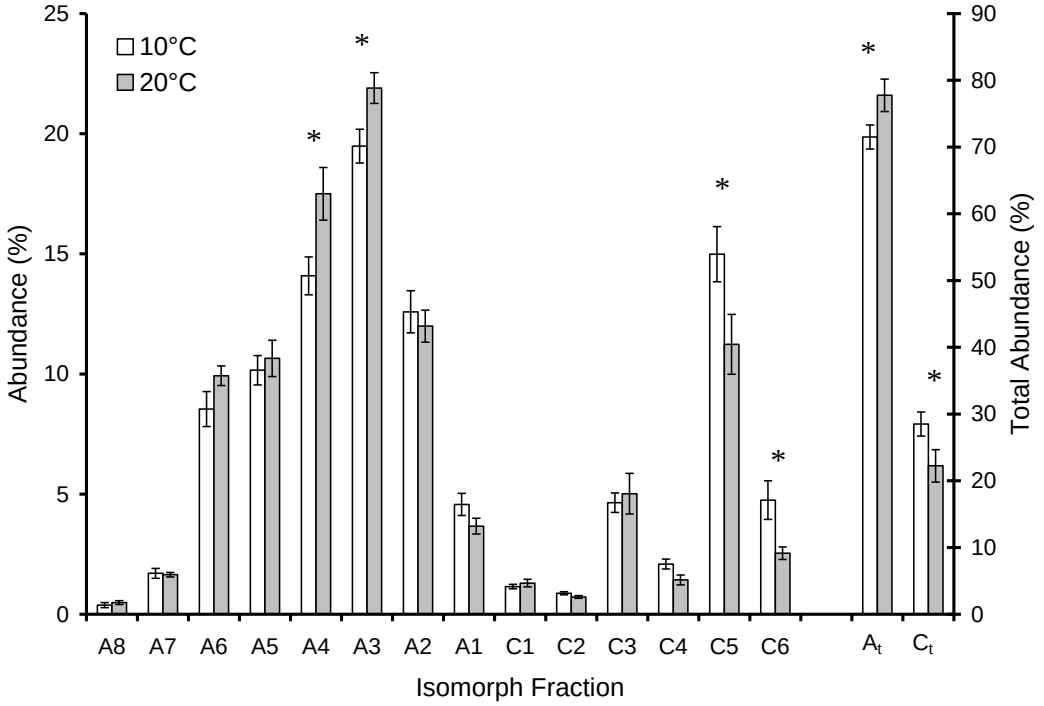


Figure 2.2: Mean (± SEM) percentage haemoglobin isomorph abundances of anaemia-induced rainbow trout (*Oncorhynchus mykiss*) after 25 days kept at either 10ºC or 20ºC. Total anodal (A_t) and total cathodal (C_t) isomorphs for both temperatures are also presented. ∗ indicates significant differences in isomorph abundance ($P < 0.05$).

Table 2.2: Mean (± SEM) percentage haemoglobin isomorph abundances from rainbow trout (*Oncorhynchus mykiss*) kept at 10ºC or 20ºC for 5, 7, 14, 21 and 28 days. Use of differently sourced animals and differing pre-experimental conditions precludes comparisons between sampling periods for the same isomorph.

		Time (days)				
		5	**7**	**14**	**21**	**28**
A8	10ºC	1.89 ± 0.29	1.73 ± 0.11	1.55 ± 0.12	1.67 ± 0.12	1.50 ± 0.10
	20ºC	1.33 ± 0.37	1.82 ± 0.18	1.67 ± 0.12	2.05 ± 0.15	1.49 ± 0.08
A7	10ºC	1.90 ± 0.61	1.89 ± 0.61	1.82 ± 0.45	1.62 ± 0.13	1.86 ± 0.45
	20ºC	2.09 ± 1.06	2.04 ± 0.21	1.71 ± 0.27	1.96 ± 0.09	2.70 ± 0.88
A6	10ºC	9.04 ± 1.23	8.03 ± 0.74	8.85 ± 0.40	7.84 ± 0.31	8.15 ± 0.58
	20ºC	8.01 ± 0.71	8.56 ± 0.35	9.92 ± 0.46	7.96 ± 0.42	8.43 ± 0.71
A5	10ºC	9.83 ± 0.63	10.88 ± 0.34	9.91 ± 1.12	9.98 ± 0.28	9.92 ± 0.69
	20ºC	11.08 ± 0.71	11.03 ± 0.21	10.85 ± 0.66	10.70 ± 0.16	10.46 ± 0.89
A4	10ºC	9.97 ± 1.07	12.84 ± 1.10	12.74 ± 0.83	11.34 ± 0.32	12.36 ± 1.56
	20ºC	11.93 ± 2.06	12.89 ± 1.53	11.41 ± 1.08	11.96 ± 0.61	13.73 ± 1.60
A3	10ºC	16.41 ± 1.17	17.72 ± 0.44	16.57 ± 0.69	17.19 ± 0.40	17.28 ± 1.26
	20ºC	17.03 ± 1.72	17.01 ± 0.29	17.37 ± 0.47	18.65 ± 0.54	18.78 ± 0.60
A2	10ºC	13.61 ± 1.06	13.30 ± 0.35	12.22 ± 0.89	13.90 ± 0.24	11.88 ± 0.38
	20ºC	12.49 ± 1.22	11.97 ± 1.46	12.94 ±1.62	13.89 ± 0.37	12.21 ± 1.59
A1	10ºC	6.02 ± 0.41	4.11 ± 0.13	4.38 ± 0.44	4.52 ± 0.36	4.07 ± 0.37
	20ºC	5.10 ± 0.42	4.13 ± 0.14	4.72 ± 0.22	4.37 ± 0.41	4.15 ± 0.97
C1	10ºC	1.91 ± 0.13	1.37 ± 0.27	1.89 ± 0.18	1.76 ± 0.32	2.32 ± 0.34
	20ºC	2.02 ± 0.20	1.60 ± 0.19	1.34 ± 0.28	1.36 ± 0.15	1.42 ± 0.37
C2	10ºC	1.30 ± 0.30	1.51 ± 0.15	0.92 ± 0.46	1.09 ± 0.16	0.88 ± 0.27
	20ºC	1.07 ± 0.20	1.50 ± 0.06	1.37 ± 0.12	1.18 ± 0.17	0.68 ± 0.12
C3	10ºC	8.28 ± 0.45	8.72 ± 0.67	8.21 ± 0.36	8.91 ± 0.64	8.70 ± 1.12
	20ºC	8.23 ± 0.80	9.16 ± 0.19	8.18 ± 0.68	9.76 ± 0.44	7.91 ± 0.92
C4	10ºC	3.53 ± 0.31	2.80 ± 0.25	2.53 ± 0.66	2.20 ± 0.12	2.15 ± 0.36
	20ºC	3.89 ± 0.54	2.93 ± 0.67	2.84 ± 0.51	2.48 ± 0.21	2.74 ± 0.19
C5	10ºC	11.42 ± 0.83	11.05 ± 0.43	11.38 ± 1.22	12.99 ± 0.59	12.72 ± 1.33
	20ºC	11.44 ± 1.17	10.01 ± 0.32	10.28 ± 1.88	11.11 ± 0.48	11.78 ± 1.30
C6	10ºC	4.86 ± 0.97	5.24 ± 0.68	3.93 ± 0.65	4.01 ± 0.24	4.79 ± 0.11
	20ºC	4.28 ± 0.97	5.55 ± 0.31	3.99 ± 0.44	3.57 ± 0.27	4.74 ± 0.65
Total Anodal	10ºC	68.67	70.75	68.04	68.06	67.02
	20ºC	69.06	69.45	70.59	71.54	71.95
Total Cathodal	10ºC	31.3	30.69	34.24	30.96	31.56
	20ºC	30.93	30.75	32.72	29.46	29.27

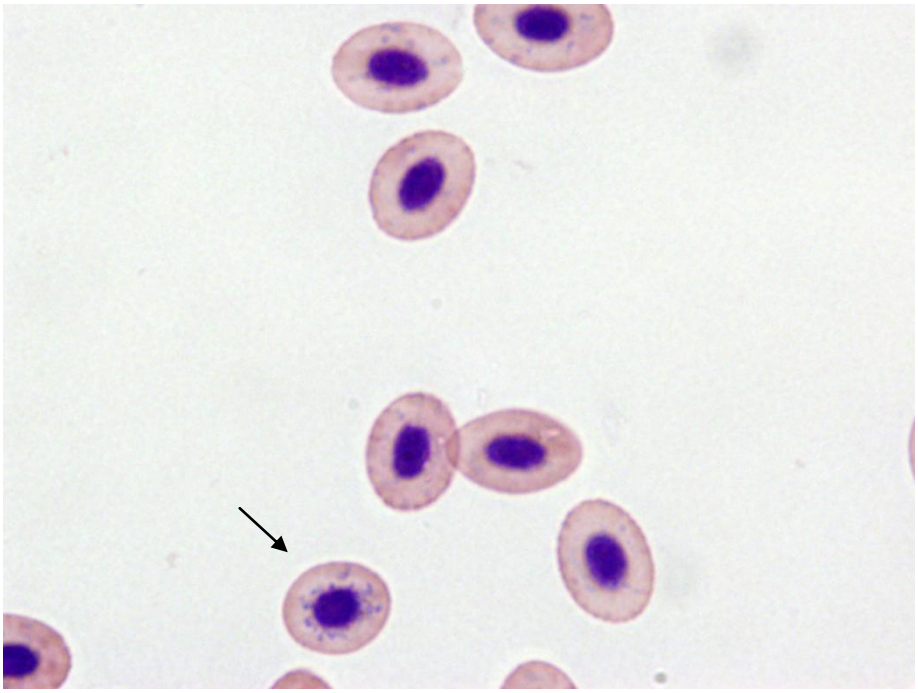
Upon conducting the anaemia-induced experiment a second time, significant differences (Student's *t*-test; d.f. =18, $n = 10$, $P < 0.05$) were present between total haemoglobin, Hct, RBCC and MEHC (Table 2.3). Enrichment of mature and young erythrocytes (Figure 2.3) by Percoll discontinuous density gradient centrifugation resulted in an increase in the percentage of young erythrocytes in the low density (top) layer (150.4% and 337.9% for the 10ºC and 20ºC treatment groups, respectively) over

whole blood fractions. In the high density (bottom) fraction the percentage of young erythrocytes was decreased by 93% and 80.3% compared to the whole blood fractions in the 10ºC and 20ºC treatment groups respectively (Table 2.4). The mean percentage of mature erythrocytes in the whole blood was significantly higher in the 20ºC treatment group compared to the 10ºC treatment group (Student's *t*-test, $P < 0.05$).

It was observed that additional staining with 1% Brilliant Cresyl Blue prior to Leishmann-Giemsa staining was superior in comparison to staining with only May-Grünwald-Giemsa stain or Leishmann-Giemsa stain. Brilliant Cresyl Blue staining provided enhanced identification of erythroblasts and proerythrocytes through staining of basophilic ribonucleoprotein material in the cytoplasm.

When haemoglobin from the density separated erythrocytes was electrophoresed on cellulose acetate, significant differences in abundance were present between a number of isomorphs (Figure 2.4A & B). In the high density bottom fraction, significant differences were observed in isomorph fractions A8, A6, C1, C2 and C6 (ANOVA; d.f. = 8, $n = 10$, $P < 0.05$). Isomorph fractions A8 and A6 were more abundant in the 20ºC treatment group; in comparison, fractions C1, C2 and C6 were more abundant in the 10ºC treatment group (Figure 2.4A). In the young erythrocyte enriched fraction significant differences were observed in most isomorph fractions (ANOVA; d.f. = 8, $n = 10$, $P < 0.05$), except for fractions A1, A2, C2 and C3 ($P > 0.05$. Total anodal isomorphs were more abundant (79.7%) (Student's *t*-test; d.f. = 8, $n = 5$, $P < 0.05$) in the 20ºC treatment groups, compared to the 10ºC treatment groups (66.3%). Concordantly cathodal isomorphs were more abundant ($P < 0.05$) in the 10ºC treatment groups for both mature enriched and young erythrocyte enriched fractions (Figure 2.4A & B).

A



B

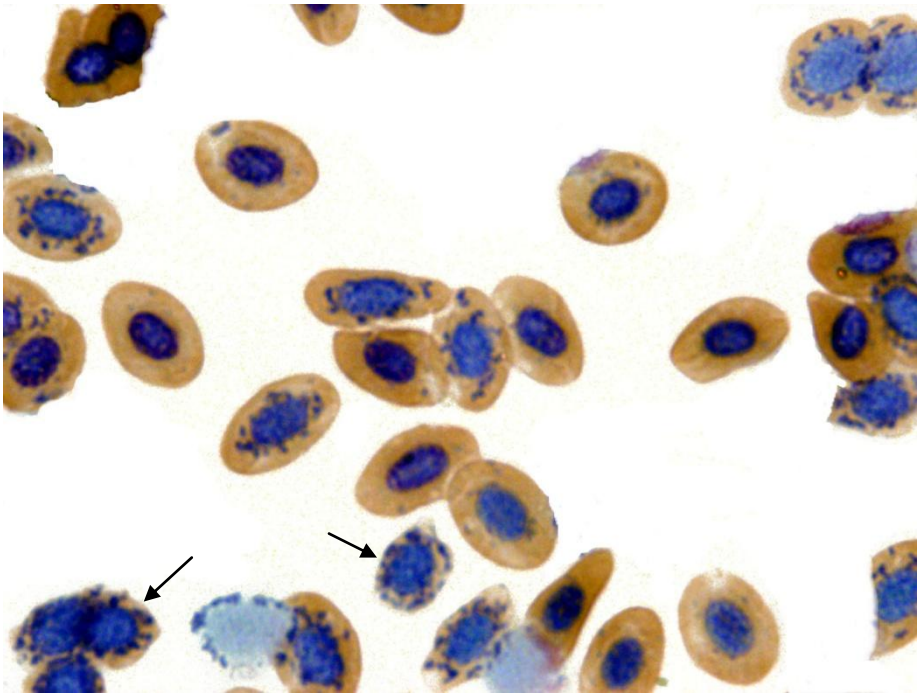


Figure 2.3: Rainbow trout (*Oncorhynchus mykiss*) erythrocytes stained with Brilliant Cresyl Blue and Leishman-Giemsa stains. Figure 2.3A is an image from a mature erythrocyte enriched fraction; the young erythrocyte (indicated by the arrow) in the image has a more spherical appearance with more blue stained basophilic ribonucleoprotein present in the cytoplasm compared to the other mature erythrocytes in the image. Figure 2.3B is an image taken from a young erythrocyte enriched fraction with erythroblasts indicated by the arrows.

Table 2.3: Means (± SEM) of body weight and blood parameters from anaemia-induced rainbow trout (*Oncorhynchus mykiss*) after 25 days held at either 10ºC or 20ºC. * indicates significant differences ($P < 0.05$).

	n	Weight (g)	Hb (g L^{-1})*	Hct (%)*	RBCC (x 10^{12} cells L^{-1})*	MEHC (g L^{-1})*	MCH (pg)	MCV (fl)
10ºC	10	40.2 ± 4.3	42.5 ± 1.8	31.3 ± 0.5	0.76 ± 0.05	136 ± 5	59.3 ± 4.1	436 ± 26
20ºC	10	45.2 ± 4.5	70.6 ± 1.2	40.2 ± 1.0	1.08 ± 0.03	176 ± 5	66.1 ± 3.3	375 ± 13

Table 2.4: Mean (± SEM) percentage of mature and young erythrocytes present in the whole blood and then the top and bottom fractions after density gradient centrifugation from anaemia induced rainbow trout (*Oncorhynchus mykiss*) after 24 days held at either 10ºC or 20ºC.

	10ºC Treatment Group			20ºC Treatment Group		
Erythrocytes	Whole Blood	Top Fraction	Bottom Fraction	Whole Blood	Top Fraction	Bottom Fraction
Mature	75.6% ± 3.90	63.6% ± 5.41	98.3% ± 0.48	93.4% ± 1.39	77.7% ± 7.33	97.8% ± 0.40
Young	24.2% ± 3.81	36.4% ± 4.40	1.7% ± 0.24	6.6% ± 1.05	22.3% ± 3.85	1.3% ± 0.40

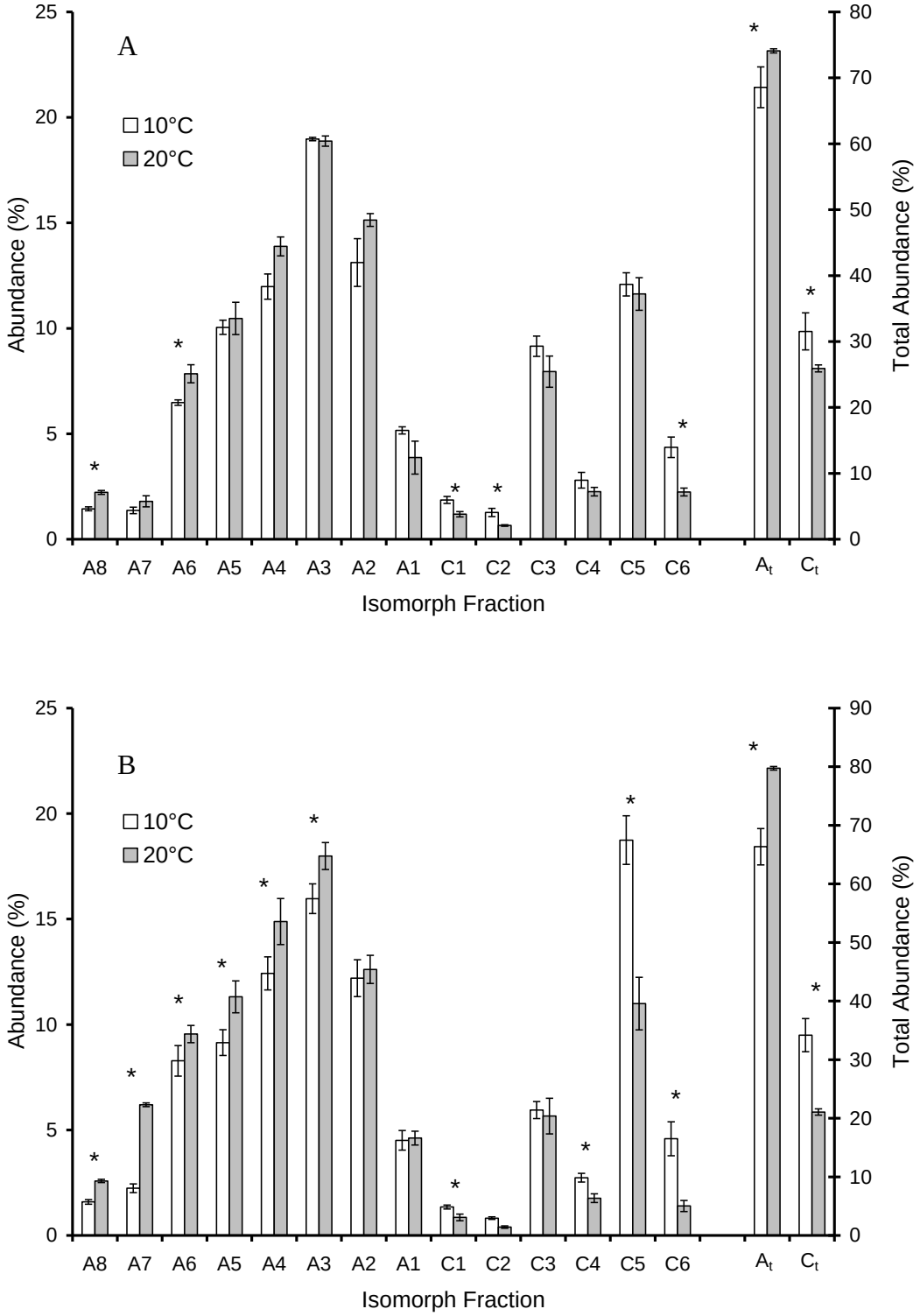


Figure 2.4: Mean (± SEM) haemoglobin isomorph fraction abundance from rainbow trout (*Oncorhynchus mykiss*) kept at either 10ºC or 20ºC for 24 days. Blood samples were separated by Percoll discontinuous density gradient centrifugation into fractions of either numerically enriched mature erythrocytes (A) or young erythrocytes (B). Total anodal (A_t) and total cathodal (C_t) isomorphs for both temperatures are also presented. ∗ indicates significant differences in isomorph abundance ($P < 0.05$).

Rainbow trout were seasonally sampled in mid-January and mid-July for two years; monthly mean water temperature and hours of daylight are presented in Figure 2.5 and means (± SEM) for haematological variables in Table 2.5. Single fish from the second winter and second summer sampling periods were excluded from the analysis due to anaemia. Total haemoglobin levels were higher (ANOVA, $P < 0.05$) in the summer than in the winter sampling periods, however there were no significant differences in MEHC between the sampling periods. Red blood cell counts for fish in the first winter sampling period were significantly lower (ANOVA, $P < 0.05$) compared to the other sampling periods; while mean haematocrit values were not reduced, this resulted in significantly higher MCV values.

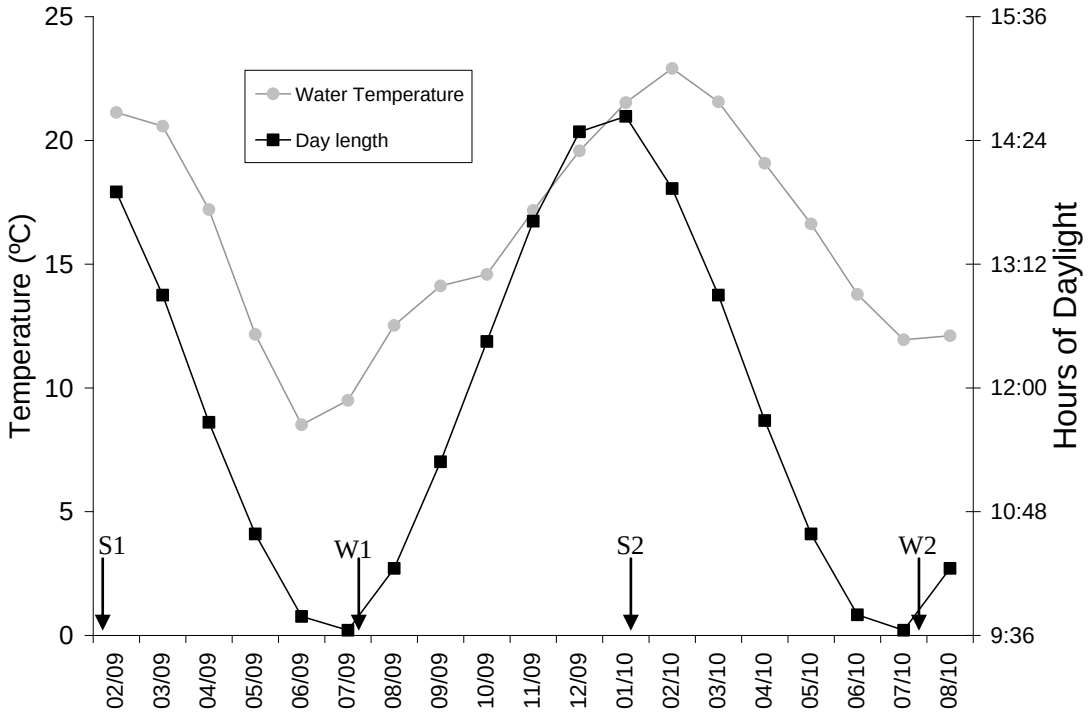


Figure 2.5: Mean monthly water temperature and hours of daylight experienced by rainbow trout (*Oncorhynchus mykiss*) housed outside in a 400 L flow-through fibreglass tank for 18 months. Arrows indicate sampling occasions at first summer (S1), first winter (W1), second summer (S2) and second winter (W2).

Table 2.5: Means (± SEM) of body weight and blood parameters from captive seasonally sampled rainbow trout (*Oncorhynchus mykiss*). S1 (1st Summer), W1 (1st Winter), S2 (2nd Summer) W2 (2nd Winter) indicate significant differences (ANOVA, $P < 0.05$) between groups.

	n	Weight (g)	Hb (g L^{-1})	Hct (%)	RBCC (x 10^{12} cells L^{-1})	MEHC (g L^{-1})	MCH (pg)	MCV (fl)
1st Summer	10	39.2 ± 4.3^{S2,W2}	56.9 ± 2.5	37.6 ± 0.7^{S2}	1.18 ± 0.08 ^{W1}	151 ± 7	49.4 ± 2.0	334 ± 23
1st Winter	10	56.7 ± 3.6^{W2}	49.4 ± 1.2^{S2}	38.4 ± 0.8^{S2}	0.86 ± 0.03 ^{S1,S2,W2}	129 ± 4	58.2 ± 3.0^{S2}	451 ± 15 ^{S1,S2W2}
2nd Summer	9	101.7 ± 16.9^{S1,W2}	59.2 ± 1.0^{W1}	42.9 ± 0.5^{S1,W1,W2}	1.35 ± 0.03 ^{W1}	140 ± 3	44.4 ± 1.3^{W1}	317 ± 6
2nd Winter	9	234.5 ± 28.2^{S1,W1,S2}	54.2 ± 3.2	36.4 ± 1.3^{S2}	1.23 ± 0.04^{W1}	149 ± 9	44.9 ± 4.0	299 ± 16

There was statistically significant variation (ANOVA, $P < 0.05$) in the mean abundance of all individual isomorph fractions between the first and second years (Figure 2.6). However, there was no concordance in isomorph abundance between the first and second summer and winter sampling periods. In addition, the maximum range in individual isomorph mean abundance between sampling periods was 5.7% in isomorph C3. When total anodal and cathodal abundances were compared between the first and second years, there was a 7.5% increase in total cathodal isomorph abundance and a corresponding decrease in anodal isomorph abundance.

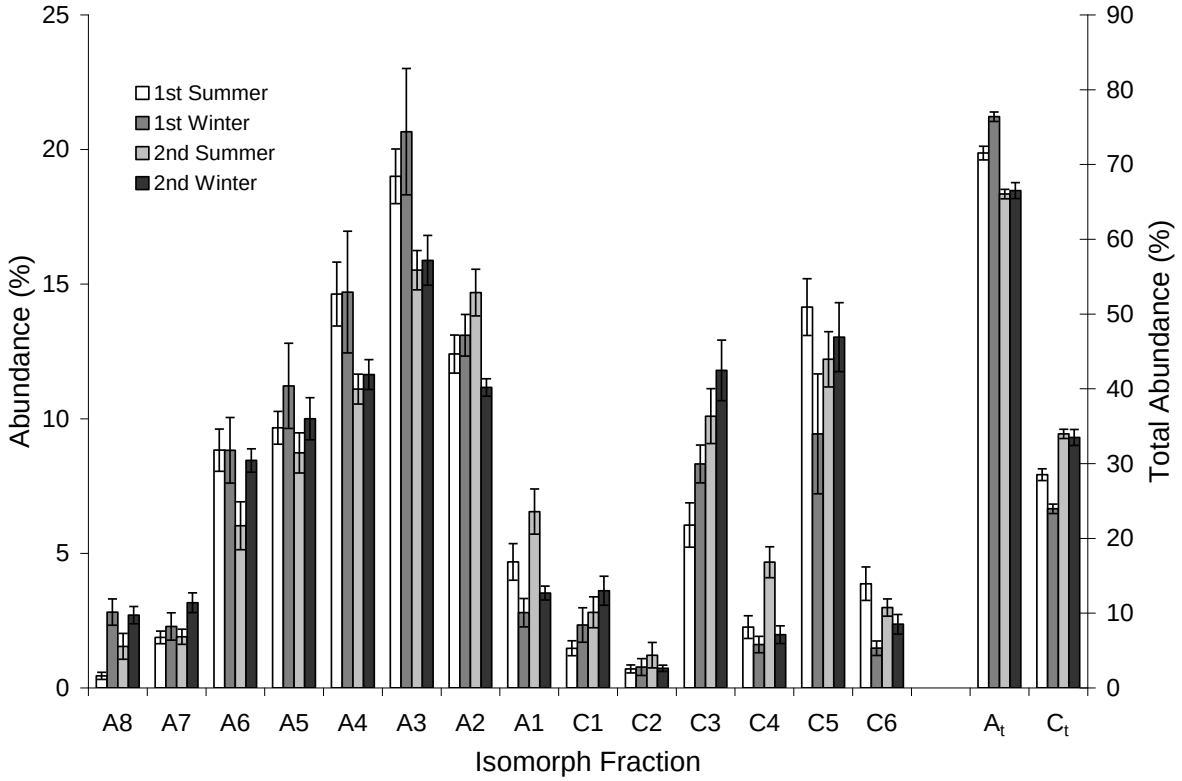


Figure 2.6: Mean (± SEM) haemoglobin isomorph fraction abundance from captive reared rainbow trout (*Oncorhynchus mykiss*) sampled on a 6-monthly consecutive basis. Total anodal (A_t) and total cathodal (C_t) isomorphs for both temperatures are also presented. Significant differences have not been indicated for clarity.

Discussion

No differences in haemoglobin isomorph abundance could be detected between rainbow trout kept at either 10ºC or 20ºC for up to 28 days. This is in contrast to the findings of Houston and Cyr (1974) who reported small changes (max. 6%) in the abundance of all nine identified isomorphs when rainbow trout were kept at 2ºC, 10ºC

or 18ºC for "not less than two weeks" (Houston and Cyr, 1974. p 456). The greater temperature range employed by Houston and Cyr is unlikely to explain the observed differences in abundance, as erythropoesis is reduced at lower temperatures in ectothermic vertebrates (Cline and Waldmann, 1962; Chudzik and Houston, 1983). This is reflected in the fact that while there were some significant differences in isomorph abundance present between 2ºC and 18ºC treated fish, there was more similarity in individual isomorph abundance levels between 2ºC and 18ºC treated fish than between 10ºC and 2ºC or 18ºC treated fish. A more plausible explanation for the failure to detect differences is that the lower sample numbers (5, compared to 12 used by Houston and Cyr) used in this experiment had insufficient statistical power to detect the small changes in isomorph abundance. Due to the use of acrylamide gel electrophoresis and the omission to report direction of movement during electrophoresis by individual isomorphs, no comparison can be made between total anodal and total cathodal abundance of the treatment groups reported by Houston and Cyr.

In contrast, studies where rainbow trout have been exposed to differing environmental temperatures for periods of 1 to 4 months (Weber et al., 1976; Tun and Houston, 1986; Houston et al., 1996) have reported much greater changes in individual isomorph abundance, in some cases in excess of 50% of the individual isomorph abundance (8% of total haemoglobin abundance) (Houston et al., 1996). Changes of a similar magnitude were observed in this study of seasonal isomorph changes, with isomorph C3 increasing in total haemoglobin abundance by 5.7% (> 80% individual abundance) between the first summer and second winter sampling periods. The findings of increased total anodal and decreased total cathodal isomorph abundance in response to increased temperature are in agreement with the findings of Weber et al. (1976).

While significant changes in isomorph abundance were observed in seasonally sampled fish in this study, there was no consistent pattern between seasons. One explanation for this is that water temperatures were not as low in the second winter as in the first winter; however, this does not explain why there was no consistent pattern of abundance between summer seasonally sampled fish that had much more comparable temperature regimes. Changes in isomorph abundance have been reported during different stages of development in rainbow trout (Iuchi and Yamagami, 1969), herring (*Clupea harengus*), sprat (*Sprattus sprattus*) (Wilkins and Iles, 1966) and

chinook salmon (*Oncorhynchus tshawytscha*) (Fyhn and Withler, 1991). It is therefore not unexpected that differing patterns of isomorph abundance were present over the two year period that fish were sampled.

Anodal haemoglobin isomorphs exhibit decreasing oxygen affinity with increasing temperature (Weber et al., 1976). Increased abundance of anodal isomorphs, as seen in the anaemia-induced 20ºC acclimation groups, would therefore increase delivery of oxygen to tissues, in part compensating for the increase in tissue metabolic activity associated with higher environmental temperatures. However, there is likely to be a physiological limit to the extent that anodal isomorph abundance can be increased. Many freshwater environments become hypoxic with increasing temperature (Horne and Goldman, 1994), the lower oxygen partial pressure coupled with the lower oxygen affinity of the anodal isomorphs would restrict oxygen uptake at the gills. Further increases in anodal isomorph abundance would then become detrimental to the animal as insufficient oxygen would be supplied to the tissues with continued temperature increases.

The induction of anaemia prior to exposure to differing water temperatures was expected to induce erythropoesis (Lecklin and Nikinmaa, 1988). Seasonal changes in temperature are known to induce erythropoiesis in fish as a mechanism to compensate for increased oxygen demand (Chudzik and Houston, 1983). In addition, higher temperatures (> 15°C) are known to increase the maturation rate of erythrocytes (Houston and Murad, 1992). Therefore, production of new erythrocytes and therefore new haemoglobins appears to be part of a seasonal adaptive response. If changes in haemoglobin isomorph abundance were originating from the production of new red blood cells then large-scale release of young erythrocytes into the circulation would produce a level of change that would be more readily detectible using the described methods. This was confirmed with the detection of changes in abundance of four haemoglobin isomorphs. However, the extent of the changes was small, amounting to no more than 4% of individual isomorph abundance. The divergence in total cathodal and anodal isomorph abundance was also comparatively small. This may again be attributed to the reduced rate of erythropoesis at lower temperatures. The lower rate of recovery from anaemia in the 10ºC treated group is evident from the lower RBCC and MEHC. This lower rate of haemoglobin production translates to smaller apparent changes in haemoglobin isomorph abundance.

To address this issue, the experiment was repeated and the enrichment of young erythrocytes was attempted in order to increase the quantity of newly synthesised haemoglobin in the sample and thereby enhance the detection limits of isomorph differences. This was achieved by density gradient centrifugation using Percoll. This method enriches the abundance of cell fractions based on their density rather than truly separating them into age classes. It has previously been successfully used by Houston et al. (1991) and a slightly modified method by Hofer et al. (2000). The successful enrichment of age fractionated cells was confirmed by staining and counting the relative abundance of red blood cell age classes. In comparison to previous studies where only May-Grünwald-Giemsa stain (Hofer et al., 2000) or Leishmann-Giemsa stain (Houston et al., 1991) was used, it was found that using the method of Tavares-Dias (2006), involving additional staining with 1% Brilliant Cresyl Blue prior to Leishmann-Giemsa staining, helped to identify erythroblasts and proerythrocytes through identification of basophilic ribonucleoprotein material in the cytoplasm.

The differences in the profusion of young erythrocytes in the whole blood of 10ºC (24.2%) and 20ºC (6.6%) treated fish can be ascribed to differential rates of erythrocyte development that occurs at high and low environmental temperatures (Chudzik and Houston, 1983). At 20ºC, newly produced erythrocytes mature faster than at 10ºC and so would have reached a more developed stage after 3 weeks. This is supported by the fact that total haemoglobin, haematocrit, red blood cell count and mean erythrocyte haemoglobin content were all significantly higher in the 20ºC treatment group compared to the 10ºC group.

The increased abundance of young erythrocytes in the low density fractions produced the greatest observed difference in individual isomorph abundances, with 10 of 14 isomorph fractions exhibiting statistically significant differences in abundance. Differences in isomorph abundance were also observed in fractions enriched in mature erythrocytes. This can be explained by the fact that as the separation method only enriches fractions rather than completely separating cell age classes, some differences would still be expected to be observed. This is confirmed by the fact that the pattern of abundance was the same between the young and mature erythrocyte fractions but the magnitude of differences was reduced. In addition, the faster rate of maturation brought about by the higher temperatures in the 20ºC treatment group

would have caused some of the new erythrocytes to be sorted into the high density fraction, resulting in mixtures of old and younger erythrocytes.

The results presented here are comparable to some of the largest changes in individual mean isomorph abundance for rainbow trout under any experimental conditions (Houston and Cyr, 1974; Weber et al., 1976; Lane, 1980; Tun and Houston, 1986; Marinsky et al., 1990; Houston et al., 1996). The evidence presented here also indicates that changes in haemoglobin isomorph abundance occur through the production of new erythrocytes and the subsequent production of new haemoglobins. However, the magnitude of the change in abundance of any given individual isomorph is still so small as to bring into question the physiological benefit of altering its abundance.

Explanations regarding the physiological benefit of multiple haemoglobins in rainbow trout have been inadequate. The cathodal haemoglobins of rainbow trout are reported to be strongly insensitive to both allosteric effectors, such as ATP, and temperature (Binotti et al., 1971; Weber et al., 1976), while anodal haemoglobins are highly sensitive to temperature and allosteric effectors (Binotti et al., 1971). It has been proposed that anodal haemoglobins perform an important role in swimbladder function; their sensitivity to proton concentrations allowing them to supply oxygen to the swimbladder against high oxygen partial pressures (Brunori, 1975). In addition, the sensitivity of anodal haemoglobins to temperature means that oxygen is more readily delivered to tissues under increased temperature conditions when metabolic rates are raised (Powers, 1980). This theory is supported by the findings in this study; anodal haemoglobins were found to be more abundant in fish acclimated to 20ºC (79.7%) compared to fish acclimated to 10ºC (66.3%). Similar findings of increased anodal isomorph abundance in response to increased temperature (anodal abundances of 70.7% and 67.4% to 20ºC and 5ºC respectively) have also been reported by Tun and Houston (1986).

In contrast, Brunori (1975) has advocated that the insensitivity of cathodal haemoglobins to allosteric effectors means they are suited to act as an emergency supply of oxygen under conditions of extensive exercise (Brunori, 1975). However, this theory has never been tested and alternative explanations for the evolution of cathodal haemoglobins are generally cursory at best.

If haemoglobin isomorphs are viewed collectively as functional groups with similar oxygen binding properties rather than as individual isomorphs, changes in the

abundance of these 'functional groups' can reach a magnitude where they have a physiological effect on the animal. When coupled with other physiological changes associated with oxygen transport such as regulation of allosteric effectors, the resulting adaptations in the oxygen transport system provide the increased efficiency required for oxygen delivery to the tissues. For example, there was approximately a 27% (~ 1 kPa) difference in mean P_{50} (the PO_2 at which half the haemoglobin is saturated) between the whole haemoglobin rainbow trout acclimated to 5ºC or 22ºC when measured at either of the acclimation temperatures. This is associated with a 15% decrease in cathodal isomorph abundance between 22ºC and 5ºC (Weber et al., 1976).

Conclusion

The results presented in this study suggest that changes in rainbow trout haemoglobin isomorph abundance originate from erythropoesis as opposed to *de novo* synthesis of new haemoglobin isomorphs in mature circulating erythrocytes or re-aggregation of haemoglobin subunits. This adaptation of the oxygen transport system appears to be a chronic response to environmental change requiring weeks to seasonal periods of time to adjust. Viewed in isolation, the magnitude of variation in abundance of any given individual haemoglobin isomorph would appear to be insufficient to provide a physiological gain against changing environmental conditions. However, when haemoglobin isomorphs are collectively viewed as functional groups the magnitude of the changes is of a sufficient size to have a physiological effect. This will be further explored in Chapter Three where the oxygen binding properties of individual haemoglobin isomorphs will be studied. In addition, Brunori's (1975) theory that cathodal haemoglobins act as an emergency supply of oxygen under conditions of exercise will be tested in Chapter Four.

References

Binotti I, Giovenco S, Giardina B, Antonini E, Brunori M, Wyman J, 1971. Studies on the functional properties of fish hemoglobins : II. The oxygen equilibrium of the isolated hemoglobin components from trout blood. Archives of Biochemistry and Biophysics 142:274-280.

Brunori M, 1975. Molecular adaptation to physiological requirements: the hemoglobin system of trout. In: Current Topics in Cellular Regulation (Horecker BL, Stadtman ER, eds). Academic Press Inc. New York. p 1-39.

Brunori M, Bonaventura J, Bonaventura C, Giardina B, Bossa F, Antonini E, 1973. Hemoglobins from trout: Structural and functional properties Molecular and Cellular Biochemistry 1:189-196.

Buhler DR, 1963. Studies on fish hemoglobins. Chinook salmon and rainbow trout Journal of Biological Chemistry 238:1665-1674.

Buhler DR, Shanks WE, 1959. Multiple hemoglobins in fishes. Science 129:899-900.

Cavas T, Konen S, 2007. Detection of cytogenetic and DNA damage in peripheral erythrocytes of goldfish (*Carassius auratus*) exposed to a glyphosate formulation using the micronucleus test and the comet assay. Mutagenesis 22:263-268.

Chudzik J, Houston AH, 1983. Temperature and erythropoiesis in goldfish. Canadian Journal of Zoology 61:1322–1325.

Clark MR, 1988. Senescence of red blood cells: progress and problems. Physiological Reviews 68:503-554.

Cline MJ, Waldmann TA, 1962. Effect of temperature on erythropoiesis and red cell survival in the frog. American Journal of Physiology 203:401-403.

Dacie JV, Lewis SM, 1991. Practical Haematology. 7th edition. Churchill Livingstone. New York.

Fourie FR, van Vuren JHJ, 1976. A seasonal study on the hemoglobins of carp (*Cyprinus carpio*) and yellowfish (*Barbus holubi*) in South Africa. Comparative Biochemistry and Physiology Part B 55:523-525.

Fyhn UEH, Withler RE, 1991. Ontogeny of hemoglobins in chinook salmon, *Oncorhynchus tshawytscha*. Comparative Biochemistry and Physiology Part B 98:201-208.

Giovenco S, Binotti I, Brunori M, Antonini E, 1970. Studies on the functional properties of fish haemoglobins, I. The O_2 equilibrium of trout haemoglobin. International Journal of Biochemistry 1:57-61.

Hofer R, Stoll M, Romani N, Koch F, Sordyl H, 2000. Seasonal changes in blood cells of Arctic char (*Salvelinus alpinus* L.) from a high mountain lake. Aquatic Sciences - Research Across Boundaries 62:308-319.

Horne AJ, Goldman CR, 1994. Limnology. 2nd edition. McGraw-Hill Inc. New York.

Houston AH, Cyr D, 1974. Thermoacclimatory variation in hemoglobin systems of goldfish (*Carassius auratus*) and rainbow trout (*Salmo gairdneri*). Journal of Experimental Biology 61:455-461.

Houston AH, Mearow KM, Smeda JS, 1976. Further observations upon the hemoglobin systems of thermally-acclimated freshwater teleosts: Pumpkinseed (*Lepomis gibbosus*), white sucker (*Catostomus commersoni*), carp (*Cyprinus carpio*), goldfish (*Carassius auratus*) and carp-goldfish hybrids. Comparative Biochemistry and Physiology Part A 54:267-273.

Houston A, Freeman G, Plint A, Korcock D, 1991. Erythrocyte fractionation by velocity sedimentation and discontinuous density gradient centrifugation. Fish Physiology and Biochemistry 9:279-289.

Houston AH, Murad A, 1992. Erythrodynamics in goldfish, *Carassius auratus* L.: temperature effects. Physiological Zoology 65:55–76.

Houston AH, Dobric N, Kahurananga R, 1996. The nature of hematological response in fish - Studies on rainbow trout *Oncorhynchus mykiss* exposed to simulated winter, spring and summer conditions. Fish Physiology and Biochemistry 15:339-347.

Iuchi I, 1973. Chemical and physiological properties of larval and adult Hemoglobins in rainbow trout, *Salmo gairdnerii irideus*. Comparative Biochemistry and Physiology 44:1087-1094.

Iuchi I, Yamagami K, 1969. Electrophoretic pattern of larval haemoglobins of the salmonid fish, *Salmo gairdnerii irideus*. Comparative Biochemistry and Physiology 28:977-978.

Lane HC, 1980. The response of the haemoglobin system of fed and starved rainbow trout, *Salmo gairdneri* Richardson, to bleeding. Journal of Fish Biology 16:405-411.

Lecklin T, Nikinmaa M, 1998. Erythropoiesis in Arctic charr is not stimulated by anaemia. Journal of Fish Biology 53:1169-1177.

Lund SG, Phillips MCL, Moyes CD, Tufts BL, 2000. The effects of cell ageing on protein synthesis in rainbow trout (*Oncorhynchus mykiss*) red blood cells. Journal of Experimental Biology 203:2219-2228.

Marinsky CA, Houston AH, Murad A, 1990. Effect of hypoxia on hemoglobin isomorph abundances in rainbow trout, *Salmo gairdneri*. Canadian Journal of Zoology 68:884-888.

Murad A, Houston A, 1991. Haemoglobin isomorph abundances in splenectomized rainbow trout, *Oncorhynchus mykiss* (Walbaum). Journal of Fish Biology 38:641-651.

Phillips MC, Moyes CD, Tufts BL, 2000. The effects of cell ageing on metabolism in rainbow trout (*Oncorhynchus mykiss*) red blood cells. Journal of Experimental Biology 203:1039-1045.

Powers DA, 1980. Molecular ecology of teleost fish hemoglobins - strategies for adapting to changing environments. American Zoologist 20:139-162.

Poluhowich JJ, 1972. Adaptive significance of eel multiple hemoglobins. Physiological Zoology 45:215-222.

Sharp GD, 1973. An electrophoretic study of hemoglobins of some scombroid fishes and related forms. Comparative Biochemistry and Physiology Part B 44:381-384.

Tanke HJ, Nieuwenhuis IAB, Koper GJM, Slats JCM, Ploem JS, 1981. Flow cytometry of human reticulocytes based on RNA fluorescence. Cytometry 1: 313-320.

Tavares-Dias M, 2006. A morphological and cytochemical study of erythrocytes, thrombocytes and leukocytes in four freshwater teleosts. Journal of Fish Biology 68:1822-1833.

Tsuyuki H, Gadd REA, 1963. The multiple hemoglobins of some members of the Salmonidae family. Biochimica et Biophysica Acta 71:219-221.

Tsuyuki H, Roberts E, Vanstone WE, 1965. Comparative zone electropherograms of muscle myogens and blood hemoglobins of marine and freshwater vertebrates and their application to biochemical systematics. Journal of the Fisheries Research Board of Canada 22:203-213.

Tun N, Houston AH, 1986. Temperature, oxygen, photoperiod, and the hemoglobin system of the rainbow trout, *Salmo gairdneri*. Canadian Journal of Zoology 64:1883-1888.

Vaccaro AM, Raschetti R, Ricciardi G, Morpurgo G, 1975. Temperature adaptation at the hemoglobin level in *Carassius auratus*. Comparative Biochemistry and Physiology Part A 52:627-634.

van Vuren JHJ, Hattingh J, 1978. Seasonal changes in the haemoglobins of freshwater fish in their natural environment. Comparative Biochemistry and Physiology Part A 60:265-268.

Weber RE, Jensen FB, 1988. Functional adaptations in hemoglobins from ectothermic vertebrates. Annual Review of Physiology 50:161-179.

Weber RE, Wood SC, Lomholt JP, 1976. Temperature-acclimation and oxygen-binding properties of blood and multiple hemoglobins of rainbow-trout. Journal of Experimental Biology 65:333-345.

Wilkins NP, 1968. Multiple haemoglobins of atlantic salmon (*Salmo salar*). Journal of the Fisheries Research Board of Canada 25:2651-2663.

Wilkins NP, Iles TD, 1966. Haemoglobin polymorphism and its ontogeny in herring (*Clupea harengus*) and sprat (*Sprattus sprattus*). Comparative Biochemistry and Physiology 17:1141-1152.

CHAPTER THREE : OXYGEN BINDING PROPERTIES OF RAINBOW TROUT HAEMOGLOBIN ISOMORPHS SEPARATED BY ANION-EXCHANGE CHROMATOGRAPHY

Introduction

The individual haemoglobins of rainbow trout (*Oncorhynchus mykiss*) are typically classified into functional groups based on their elution or electrophoretic mobility and oxygen binding properties. Initially, four functional groups were isolated and designated HbI to HbIV by Binotti et al. (1971) based on the order of decreasing anodic mobility when separated by starch gel electrophoresis. Brunori (1975) summarised the oxygen binding properties of haemoglobin functional groups HbI, HbII and HbIV; but due to its low abundance, HbIII could not be isolated in sufficient quantities for analysis of its oxygen binding properties. Using isoelectric focusing, Weber et al. (1976) were able to isolate sufficient quantities of HbIII to be able to determine its oxygen binding properties. They were also able to isolate a further two functional groups which they designated HbV and HbVI, although, again, sufficient quantities of HbVI were not available for analysis. A further two haemoglobin groups were isolated by Fago et al. (2002) using Fast Protein Liquid Chromatography (FPLC); they were also able to further separate HbI into two subgroups. However, the purpose of that study was to investigate the structure of the α and β-globin subunits present, rather than the oxygen binding properties of the haemoglobin groups.

In contrast to chromatography and isoelectric focusing studies, investigations using starch gel electrophoresis or cellulose acetate electrophoresis are generally able to provide greater resolution of individual haemoglobin isomorphs with 9-16 components (Tsuyuki and Gadd, 1963; Iuchi, 1973; Tun and Houston, 1986; Murad and Houston, 1991; Houston et al., 1996) being reported depending on electrophoretic buffer conditions and the variety of rainbow trout sampled (di Prisco and Tamburrini, 1992). Apart from the initial work undertaken by Binotti et al. (1971), no study on the functional properties of these haemoglobins, separated using these methods has been published. Typically, isomorphs separated by these methods have been simply designated as either cathodal or anodal depending on their direction of mobility.

Several of the haemoglobin functional groups of rainbow trout are known to respond differently to temperature and various ligands such as organic phosphates, carbon dioxide and chloride ions (Binotti et al., 1971; di Prisco and Tamburrini, 1992). The oxygen binding curves of HbI, HbII and HbIII are temperature independent, while HbIV and HbV exhibit increased P_{50} values in response to increased temperature (Weber et al., 1976).

The oxygen affinities of the HbI and HbII fractions were found to be insensitive to pH, whereas HbIV and HbV exhibited large Bohr effects (Binotti et al., 1971, Weber et al., 1976). Interestingly, in addition to the alkaline Bohr effect, HbIII also had an acid Bohr effect, suggesting that HbIII remains immobilised in the low-affinity, deoxy conformation at low pH (Lau et al., 1975; di Prisco and Tamburrini, 1992).

The oxygen affinity of HbI and HbII is also insensitive to the presence of organic phosphates, whereas adenosine-5'-triphosphate (ATP), and in particular guanosine-5'-triphosphate (GTP) in rainbow trout (Weber et al., 1976; Pelster and Weber, 1990), shifts the oxygen dissociation curves of HbIII and HbIV to the right thereby providing a regulating mechanism for oxygen delivery to the tissues (Brunori et al., 1975). The response of HbV to organic phosphates is currently unknown.

Brunori et al. (1975) investigated the effect of increased chloride concentration on the oxygen binding properties of HbI, HbII and HbIV. HbI and HbII decreased in oxygen affinity, while HbIV did not respond to increased chloride concentrations (Brunori et al., 1975). However, the maximal responses reported were conducted under chloride concentrations 5 – 10 times those of normal rainbow trout blood (Eddy et al., 1977). The effects of chloride on HbIII and HbIV have not been studied.

The number of haemoglobin isomorphs known to be produced by rainbow trout has increased over time as analysis procedures and techniques have developed. Techniques for the separation of haemoglobin functional groups have also advanced allowing the functional properties of new groups to be determined that previously could not be isolated in sufficient quantities. The purpose of this study is to determine how the 14 isomorphs previously detected in Chapter Two relate to the functional groups isolated in other studies using anion-exchange chromatography. The basic functional oxygen binding properties of these isomorphs will then be determined. This will also provide data on some of the more difficult to isolate functional groups such as HbIII and HbVI.

Methods

Origin and maintenance of experimental animals

Rainbow trout parr were sourced from the National Institute of Water and Atmospheric Research (Hamilton, New Zealand) and held outside in a 400 L fibreglass tank supplied with continuous flow-through dechlorinated tap water. Both photoperiod and temperature were allowed to vary with season; however, dissolved oxygen was maintained above a minimum of 90% saturation. Fish were fed to satiation every second day with a commercial pellet (Reliance Stock Food, Dunedin, New Zealand).

Sampling and haemoglobin separation

Six adult trout (> 350 mm FL) were anesthetised in a 1:10000 solution of buffered tricane methane sulphonate (MS222, Sigma, New Zealand). Approximately 10 ml of blood was then drawn into a heparinised (sodium heparin, Sigma, New Zealand) syringe from the caudal vein and placed on ice. The haemolysate was prepared by washing the cells twice in two volumes of 0.9% saline and then lysing by addition of an equal volume of 10 mM Tris, pH 8. The samples were then centrifuged at 10 000 rcf for 10 min at 4ºC and the supernatant recovered. The samples were then saturated with carbon monoxide and stored at -80ºC for several days before being run on FPLC. Samples were analysed for methaemoglobin formation by scanning spectrophotometry (Shimadzu Model 1601, Shimadzu Corporation, Japan) between 450 – 650 nM at a dilution factor of 1:200 haemoglobin to 0.01 M HEPES buffer pH 7.66. No oxidation was evident from the absorption spectrum.

Three samples were randomly selected for separation of the haemoglobin components. This was achieved following the procedures of Fago et al. (2002) using anion-exchange chromatography on a Mono Q HR 10/10 (Pharmacia) column using a Akta Explorer 100 Fast Protein Liquid Chromatography System, (Pharmacia Biotec). Following thawing, 5 ml of ~30 mg ml^{-1} haemoglobin was applied to the column and a linear gradient of 0.0 – 0.1 M NaCl in 0.01 M HEPES, pH 7.66, was applied at a flow rate of 1 ml min^{-1}. The separation run was followed by flushing the column with two volumes of 0.2 M NaCl to ensure all haemoglobin was removed from the column. No visible haemoglobin could be detected during this flushing process. Elution

volumes were collected in 1 ml aliquots and kept on ice before being concentrated using Pierce Concentrators (20K MWCO, Thermo Scientific, Rockford USA). Haemoglobin lysate and individual fraction concentrations were determined by spectrophotometry (Shimadzu Model 1601, Shimadzu Corporation, Japan) at 800 nm wavelength according to the methods of Kim et al. (2005).

Subsamples of the haemoglobin fractions and whole haemolysate samples were then run on a cellulose acetate medium (Helena Laboratories, United States) in conjunction with a Tris-glycine buffer (pH 8.1-8.4). Duplicate separations were carried out at 200 V for 1 h at 4ºC with rainbow trout haemoglobin as an electrophoretic standard. Haemoglobins were identified by staining with 4 ml of 0.025% benzidine (Sigma, New Zealand) for 5 min, followed by immersion in 5% hydrogen peroxide for 2 min and then rinsing under tap water.

Haemoglobin oxygen binding analysis

Oxygen binding characteristics were analysed using a Hemox analyzer (TCS Scientific, USA). This instrument uses dual spectrophotometric measurement and continuous monitoring of oxygen partial pressure by way of a Clark oxygen electrode to determine haemoglobin oxygen saturation. Oxygen dissociation curves and the P_{50} values (the PO_2 where 50% of the oxygen is saturated) were automatically calculated using Hemox Analytical Software (TCS Scientific, USA). Temperature was regulated by a water bath connected to the Hemox analyzer water jacket via insulated hoses and kept within a tolerance of ± 0.2ºC. Approximately 0.5 mg of haemoglobin was added to 2.5 ml of Hemox buffer solution (17.5 mM NaCl, 145 mM KCl, 1.4 mM $MgSO_4$, 10 mM HEPES, pH 7.5 or 7.2 at 20ºC) and 10 µl of antifoam solution (Antifoam A, Fluka, Sigma, New Zealand). Each sample was flushed with oxygen-free nitrogen to a PO_2 of less than 1% saturation to remove any bound carbon monoxide prior to analysis and then fully equilibrated with air (dry air; BOC Gases, New Zealand) before the data acquisition run was begun. The air-saturated PO_2 value was determined as follows:

PO_2 = (Patm – Pwv) x 0.209 where Patm is the ambient barometric pressure corrected for temperature and Pwv is the water vapour pressure at the respective test temperature. Ambient barometric pressure was determined using a mercury barometer

(Kew Barometer, Casella London Ltd, United Kingdom) with appropriate corrections for altitude and temperature.

The sample was deoxygenated by bubbling oxygen-free N_2 gas (zero-grade N_2; BOC Gases, New Zealand) through it. The dissociation curve was recorded from 100% to 0% saturation using the TCS Scientific data acquisition software. Oxygen binding characteristics of the blood were examined at temperatures of 15°C and 20°C (± 0.2°C) and pH values of 7.2 and 7.5 (± 0.02 pH unit). In addition, the effect of ATP on oxygen binding was examined by adding 10× the molar haemoglobin concentration of ATP to the sample and running under conditions of pH 7.5, 20ºC. The effect of chloride was also examined by increasing the sample chloride concentration by 100 mM and running under conditions of pH 7.5, 20ºC. All samples were run in duplicate, with the mean of the two samples used in the analysis.

Statistical analysis

Significant differences between fraction oxygen affinities were tested for using Student's *t*-test or one-way Analysis of Variance (ANOVA) coupled with Tukey's post-hoc tests using Statistica v.9.0 (Statsoft, USA).

Results

Anion-exchange chromatography resolved the trout haemolysate into seven different fractions (Figure 3.1). Similar results were obtained for haemolysates from two other fish sampled (data not shown). As with the published results of Fago et al. (2002), HbI passed unretarded through the anion-exchange column.

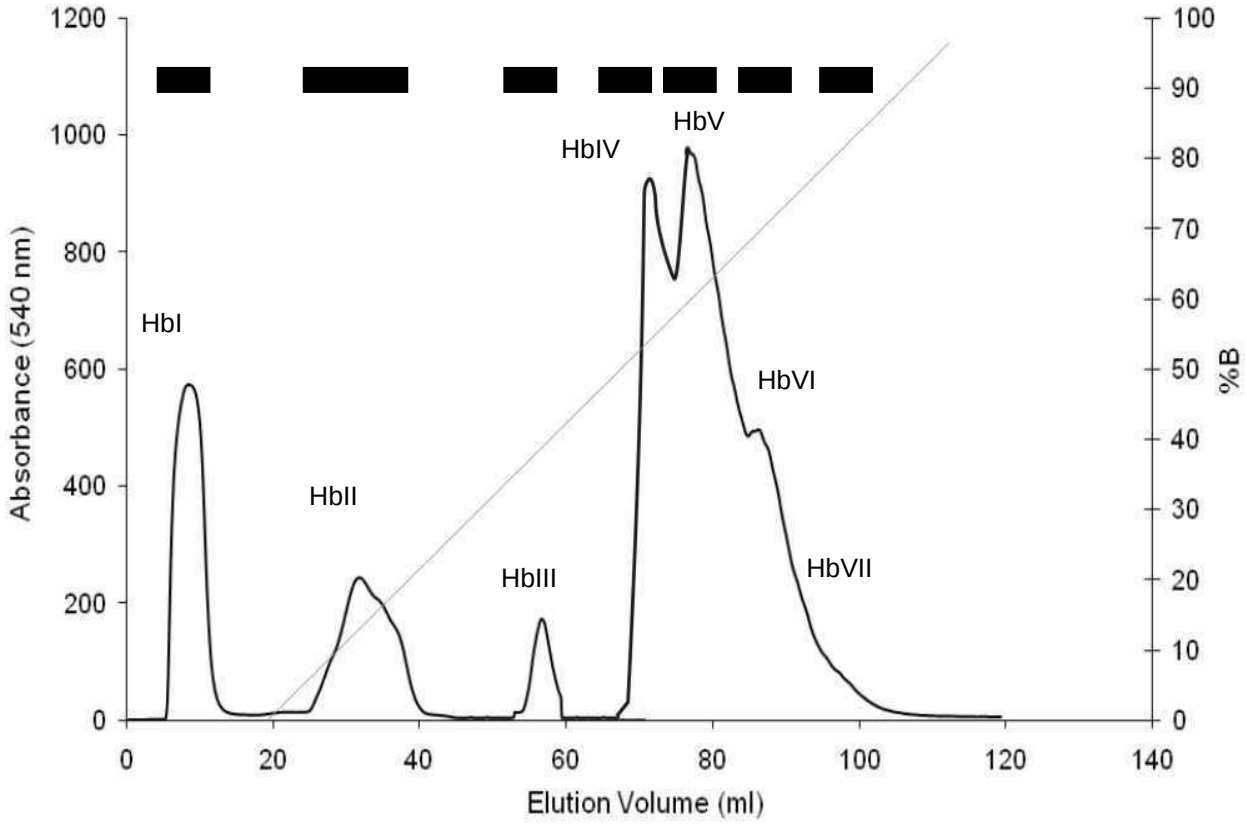


Figure 3.1: FPLC separation of trout haemoglobins on a Mono Q column. The black horizontal bars indicate the fractions that were collected for analyses. Diagonal grey line represents the percentage of salt buffer (%B) to HEPES buffer injected onto the column.

Sub-samples taken from the seven fractions were subsequently run on duplicate cellulose acetate gels and representative results are presented in Figure 3.2. Each fraction was composed of two bands for a total of 14 isomorphs. Fractions HbI, HbII and HbIII were cathodal while fractions HbIV – HbVII were anodal.

Each of the seven fractions was assessed for its oxygen binding properties. Unlike previous studies, sufficient volumes of HbIII, HbVI, and a new fraction HbVII, were available for analysis. A comparison between fractions of mean P_{50}

values under conditions of 20ºC at pH 7.5 revealed significant differences (ANOVA; d.f. = 16, $n = 8$, $P < 0.05$) between the anodal fraction HbIV and the cathodal fractions HbI and HbIII. A significant difference was also present between HbV and HbIII (Figure 3.3). It was found that decreasing the buffer pH from 7.5 to 7.2 caused an increase in mean P_{50} values for all the anodal fractions HbIV to HbVII and the whole blood haemolysate. Analysis of the Hemox data for fractions HbIV, to HbVII and the whole blood lysate (Students's *t*-test; d.f. = 4, $n = 3$, $P < 0.05$) demonstrated they were pH sensitive (Figure 3.4).

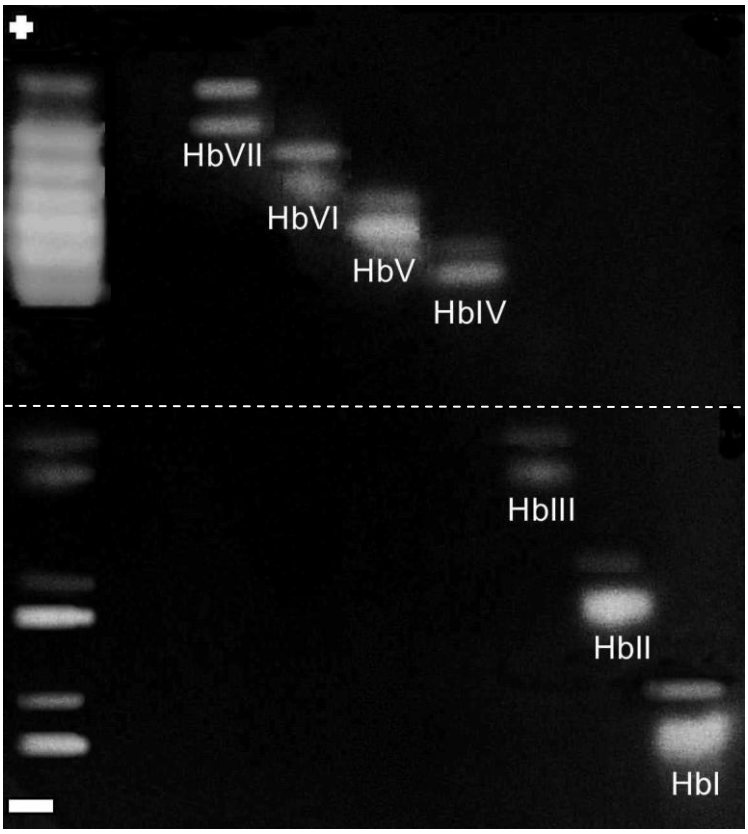


Figure 3.2: Cellulose acetate gel of selected fractions from rainbow trout haemolysate separated by anion exchange FPLC chromatography, with comparison to a rainbow trout whole blood haemolysate (left hand column). Dashed line indicates the electrophoretic origin.

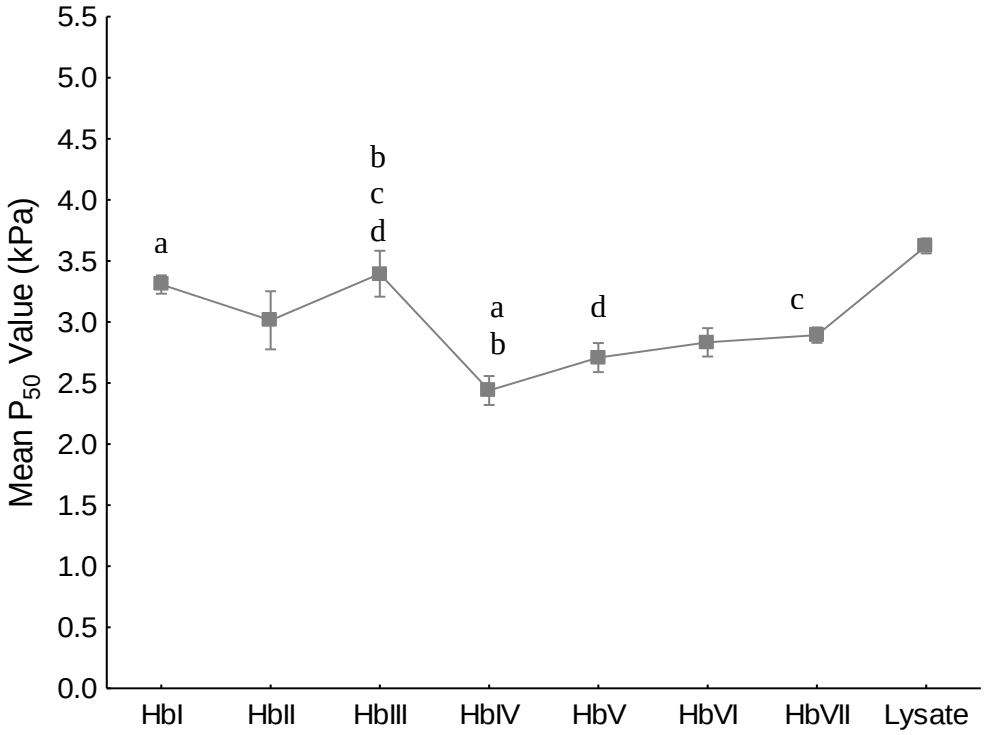


Figure 3.3: Mean (± SEM) P_{50} values (n=3) at pH 7.5, 20ºC for each of the separated haemoglobin fractions. a, b, c Indicates significant differences (Student's t-test, $P < 0.05$) between means with the same letters.

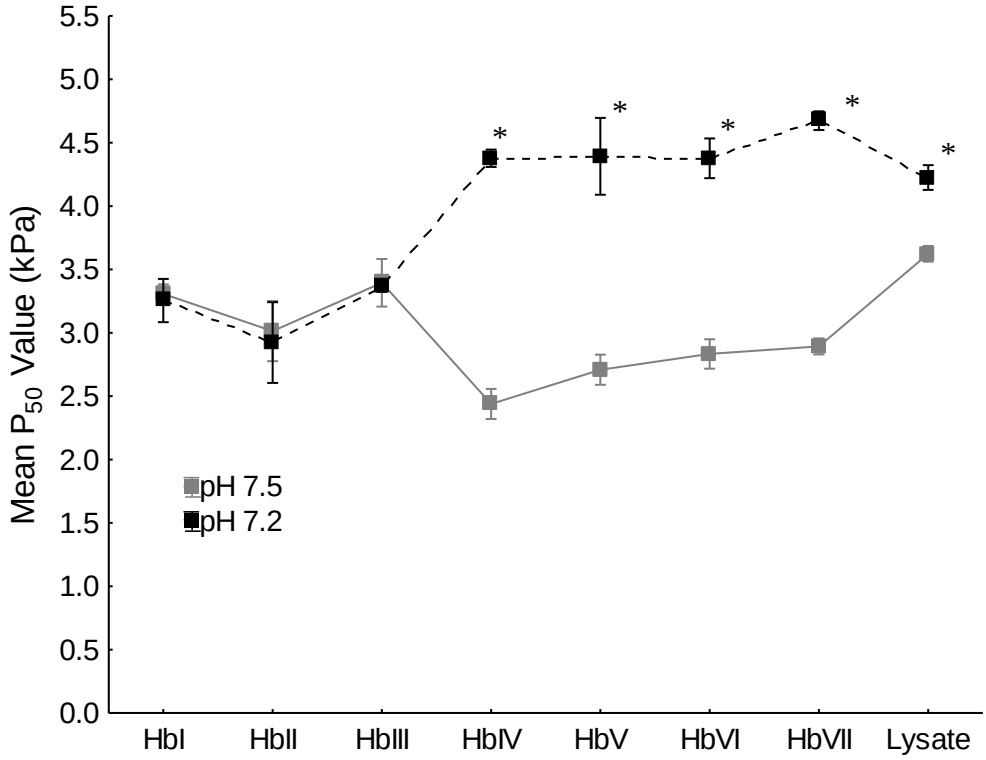


Figure 3.4: Comparison of mean (± SEM) P_{50} values (n=3) at pH 7.2 and pH 7.5, 20ºC for each of the FPLC separated rainbow trout haemoglobin fractions and whole blood haemolysate. * Indicates significant differences (Student's t-test, $P < 0.05$).

Fractions HbI to HbIII were insensitive to temperature (Figure 3.5). However, the anodal fractions HbIV to HbVII and the whole blood haemolysate were sensitive to temperature changes (Students *t*-test; d.f. = 4, $n = 3$, $P < 0.05$).

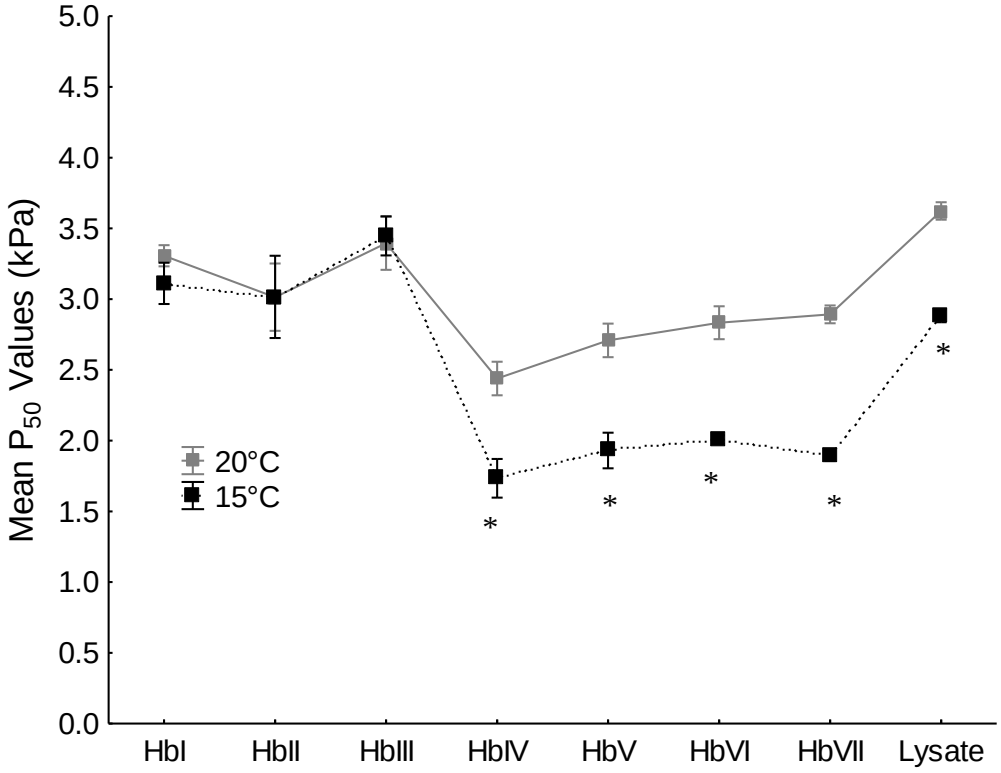


Figure 3.5: Comparison of mean (± SEM) P_{50} values (n=3) at 20ºC and 15ºC, pH 7.5 for each of the FPLC separated rainbow trout haemoglobin fractions and whole blood haemolysate. * Indicates significant differences (Student's *t*-test, $P < 0.05$).

Haemoglobin fraction responses to ATP tended to increase with increasing anodal electrophoretic mobility although only HbVI and HbVII were statistically different (Student's *t*-test; d.f. = 4, $n = 3$, $P < 0.05$) (Figure 3.6). Chloride caused small but non-significant decreases in mean P_{50} values for the cathodal fractions (HbI – HbIII) (Student's *t*-test; d.f. = 4, $n = 3$, $P > 0.05$) (Figure 3.7). No differences were observed between any of the anodal fractions ($P > 0.05$)

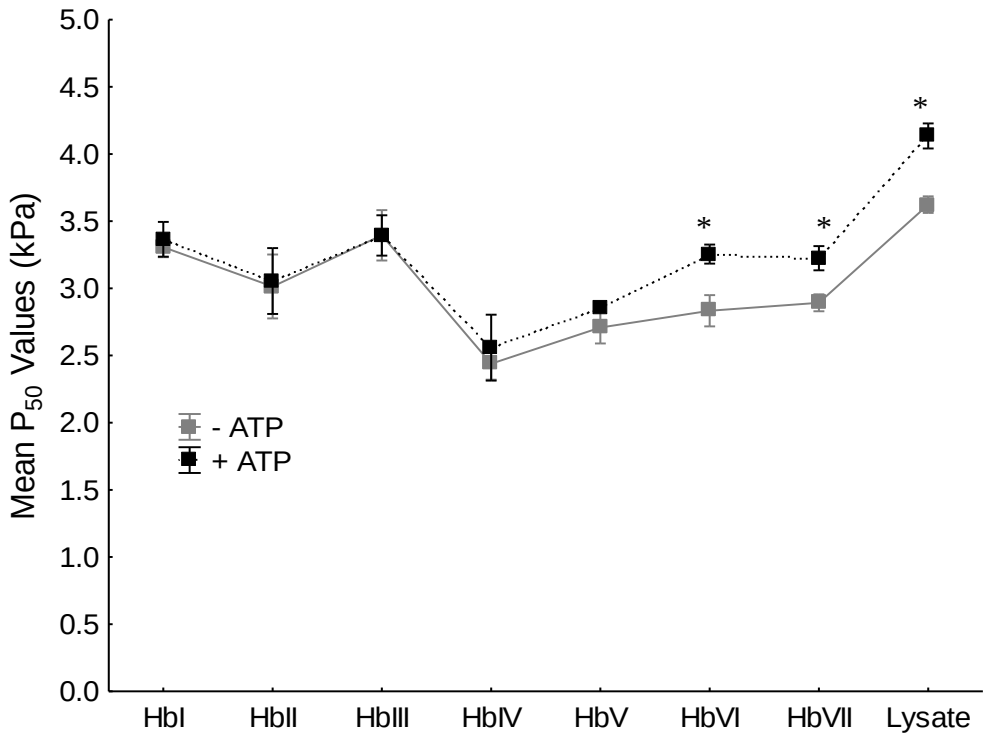


Figure 3.6: Comparison of mean (± SEM) P_{50} values (n=3) under stripped and increased ATP conditions at pH 7.5, 20ºC for each of the FPLC separated rainbow trout haemoglobin fractions and whole blood haemolysate. * Indicates significant differences (Student's t-test, $P < 0.05$).

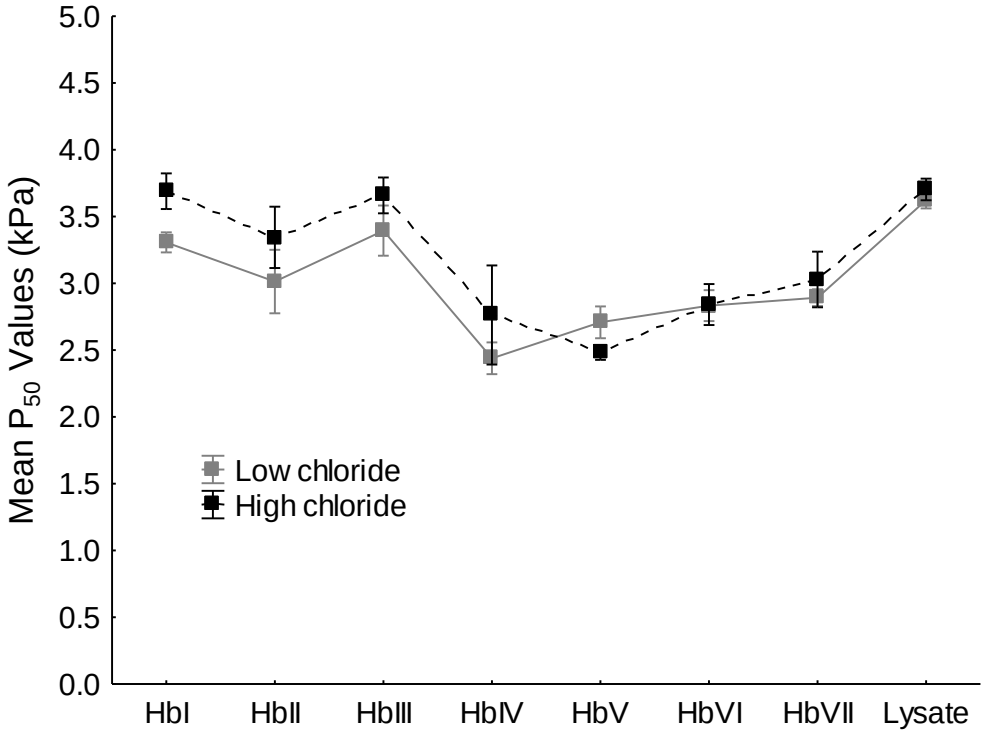


Figure 3.7: Comparison of mean (± SEM) P_{50} values (n=3) under normal and increased chloride (+100 mM) at pH 7.5, 20ºC buffer conditions for each of the FPLC separated rainbow trout haemoglobin fractions and whole blood haemolysate.

Discussion

Detection of haemoglobin isomorphs can vary significantly with the method used. Variation in the abundance of haemoglobin isomorphs from rainbow trout and other fish species can also arise in response to temperature, hypoxia, light and stage of development (Iuchi and Yamagami, 1969; Houston and Cyr, 1974; Tun and Houston, 1986; Marinsky et al., 1990; Fyhn and Withler, 1991; Murad and Houston, 1991; Houston et al., 1996). In addition, *in vitro* artefacts such as polymerization, dissociation and autooxidation (Powers, 1980) can have a distorting effect on the reporting of haemoglobin isomorphs. Therefore, it is not unexpected that discrepancies in the number of haemoglobin fractions should be present in the literature.

In this study anion-exchange chromatography resolved the haemolysate of rainbow trout into seven identifiable fractions. This is in contrast to the eight fractions reported by Fago et al. (2002) and the four components obtained by Binotti et al. (1971) using anion-exchange chromatography. Both Fago et al. and Binotti et al. detected the same three cathodal fractions reported in this study. However, Fago et al. reported one additional anodal fraction while Binotti et al. reported only one anodal fraction. Weber et al. (1976) detected six to eight fractions using isoelectric focusing with variation in the abundance of a number of fractions depending on whether they were from 5ºC or 15ºC acclimated fish. With this variation in abundance comes the possibility that some of the less abundant fractions may be expressed below the limit of detection or simply not expressed in response to ambient environmental conditions. In addition, the diversity of genotypes within *Oncorhynchus mykiss* (Overturf et al., 2003; Taylor et al., 2011) may mean that different varieties which are known to have different environmental tolerances could differentially express haemoglobin isomorphs as part of their environmental adaptation (Weber et al., 1976; di Prisco and Tamburrini, 1992). For example, three separate stocks of Arctic charr (*Salvelinus alpinus*) were shown to express different numbers of haemoglobin isomorphs and this was associated with differences in oxygen affinity and swimming performance (Giles, 1991).

Fago et al. (2002) were able to determine the amino-acid structure of nine major globin chains, with isomorphs consisting of symmetric tetramers (i.e. $\alpha_2\beta_2$) and asymmetric tetramers i.e. those with two different α and/or β chains. Coupled with the fact that a subunit can be present in more than one functional group (i.e. the same α-

globin was found to be present in both the HbI and HbIV fractions (Fago et al., 2002)) determining the individual isomorphs that compose a functional group or even the scope of the possible isomorphs becomes increasingly difficult.

Cellulose acetate gel electrophoresis of the concentrated fractions from the FPLC separation revealed that each fraction was comprised of an electrophoretic doublet. This agrees to some extent with the two different components found to comprise HbI when further separated using FPLC by Fago et al. (2002). However, given the potential for heteromorphy among isomorphs that have the same functional properties it is possible that more isomorphs were present but were below the detection limits of the methods applied in this study. For example, cellulose acetate based electrophoretic studies of torrent fish (*Cheimarrichthys fosteri*) haemoglobin have detected variable numbers of haemoglobin isomorphs in relation to staining intensity. Torrent fish were initially assayed as having 12 isomorphs composed of 6 doublets, but after increasing the staining intensity, 18 isomorphs were detected composed of 6 triplets (Ling pers. com.).

Cathodal groups HbI to HbIII showed no response in P_{50} values to changes in temperature, pH, ATP or chloride concentrations. Anodal groups HbIV to HbVII all responded to temperature and pH; groups HbVI and HbVII also responded to increased ATP concentrations. No anodal fraction responded to increased chloride concentrations. These results partly conform to those reported by Binotti et al. (1971) and Weber et al. (1976). However, for HbIII neither the Bohr effect at alkaline pH reported by Lau et al. (1975) and Weber et al. (1976), nor the ATP effect was observed. It could be argued that the mixed functional properties previously observed for HbIII may be the result of incomplete separation of cathodal and anodal fractions. As can be seen in Figure 3.2, HbIII displays only a marginal anodal shift and coupled with its low abundance may be difficult to fully discriminate from HbIV. However, if this were the case then a shift in the P_{50} value of HbIII would have been observed in response to temperature (Weber et al., 1976). Alternatively, as has been previously established, the haemoglobin functional groups observed are composed of heterogeneous isomorphs (Fago et al., 2002), indicating that the functional properties of HbIII change in response to environmental or physiological stimuli as the group varies in haemoglobin isomorph composition. However, it is unlikely that temperature would be a driving factor because the Bohr effect from HbIII has been observed from animals housed at either 5ºC or 15ºC for 4 months (Weber et al., 1976). This again

leads to the possibility that different varieties of *O. mykiss* may differentially express haemoglobin isoforms as part of their environmental adaptation (Weber et al., 1976).

In fish such as rainbow trout and common carp (*Cyprinus carpio*) ATP and other organic phosphates are known to be important regulators of oxygen uptake in response to environmental stimuli such as temperature and hypoxia (Smit and Hattingh, 1981; Albers et al., 1983; Pelster and Weber, 1990). In this study, only two of the anodal haemoglobins (HbVI and HbVII) were found to have a statistically significant response in oxygen binding to the addition of ATP. The response to ATP by anodal haemoglobins (HbIV and HbV) reported by Weber et al. (1976) were at ATP levels four times greater than those used in this study. This fact, coupled with the apparent increase in response to ATP by the anodal functional groups with increasing anodal migration seen in Figure 3.5, suggest that there may be an increasing response to ATP concentration the more electronegative the isomorph. This theory is partially supported by previously published work by Pelster and Weber (1990). Haemolysates that were separated into either cathodal or anodal haemoglobins from European eel (*Anguilla anguilla*) and rainbow trout were subjected to a range of ATP concentrations; the cathodal haemoglobins did not respond to any of the ATP concentrations while anodal haemoglobins exhibited strong responses that increased with increasing ATP/Haemoglobin ratios (Pelster and Weber, 1990). This would mean that regulation of the abundance of haemoglobin isomorphs in functional groups HbVI and HbVII would have a larger effect on oxygen uptake when coupled with ATP regulation than modulation of isomorphs that were less sensitive to ATP concentrations. Further research is needed to determine if this is a real phenomenon or an artefact of the small sample size used.

Chloride ions are also recognized as having an influence on the oxygen affinity of haemoglobin (Powers, 1980). Brunori et al. (1975) reported that the oxygen affinity of rainbow trout HbI and HbII decreased as chloride concentration is increased to 0.7 M. In contrast, at chloride concentrations above 1 M the effect is reversed and an increase in oxygen affinity is observed. This effect appears to be independent of pH and temperature (Brunori et al., 1975). The problem with these observations is that the chloride concentrations used were in excess of those likely to be physiologically relevant to the animal under normal conditions (i.e. 100 mM $Cl^- L^{-1}$) (Hille, 1982). In the current study, exposure of FPLC separated fractional groups to a more relevant increased chloride concentration found no change in oxygen affinity

by any of the fractions. This suggests that rather than being a regulatory component for oxygen binding as with organic phosphates, high concentrations of chloride ions inhibit normal oxygen affinity requiring the animal to regulate its physiology to compensate. An example of this was demonstrated for white sucker (*Catostomus commersoni*) which were exposed to an environmental salinity of 300 mosmol kg^{-1} (0.9%) NaCl, thereby increasing extracellular and intracellular chloride concentrations. However, there was no change in the oxygen affinity of the whole blood between fish that had been exposed to high salinity and a low salinity exposed control group. There was a corresponding decrease in erythrocyte NTP concentrations in the saline exposed fish which would appear to compensate for the reduced oxygen affinity caused by the chloride-haemoglobin binding (Walker et al., 1989).

The Bohr effect describes the reduction in oxygen affinity by haemoglobin in response to a decrease in pH (Riggs, 1970; Riggs, 1988). It results in increased oxygen unloading at tissue sites experiencing acidosis from carbon dioxide and/or lactic acid increases, which are the result of increased metabolic activity (Weber and Jensen, 1988). This preferential delivery of oxygen to tissues with high metabolic demands has obvious physiological advantages and from the research carried out here it is known that all the anodal haemoglobins of rainbow trout (HbIV – HbVII) exhibit an alkaline Bohr effect. The anodal haemoglobin isomorphs account for approximately 65% of the total haemoglobin forms (Chapter Two). While not investigated here, rainbow trout anodal haemoglobins also exhibit an extreme form of Bohr effect known as the Root effect (Weber et al., 1976). The Root effect is the failure of the haemoglobin to reach full oxygen saturation when equilibrated in air at acid pH (Brittain, 2005). The explanation for this phenomenon is that it allows oxygen to be delivered to tissues that require high oxygen partial pressure such as the swimbladder and/or choroid rete (Brunori, 1975; Pelster and Weber, 1990; Brittain, 2005). The anodal haemoglobins of rainbow trout also respond to changes in temperature, with haemoglobin-oxygen affinity increasing as temperature decreases. This fits physiologically with the fact that at lower temperatures metabolic activity is reduced and concomitantly oxygen demand (Powers, 1980).

Explanations for the presence of the rainbow trout cathodal haemoglobin functional groups of HbI to HbIII which do not respond to temperature, pH and organic phosphates have not been as extensively developed. Haemoglobins with functional properties similar to those of rainbow trout HbI and HbII are more likely to

be found in species living in fast-flowing waters requiring a high degree of sustained activity (Brunori, 1975). It has been proposed that these isomorphs may function as an emergency back-up system during lactic acid build up following intensive exertion (Brunori, 1975; Powers, 1980). Hyperactive species such as chinook salmon (*Onchorynchus tshawytscha*), sockeye salmon (*Onchorynchus nerka*) and tench (*Tinca tinca*) are known to produce lactic acid in sufficient quantities to induce a disruption of oxygen supply to the tissues leading to death of the animal (Black, 1958). Under these conditions the role of HbI and HbII would be to provide a normal oxygen supply (Brunori, 1975). An example in support of this idea comes from a study of two sympatric catostomid species. Members of the subgenus *Catostomus* live in fast flowing waters, whereas members of the *Pantostomus* subgenus prefer slow moving or still waters. It was found that only members of the *Catostomus* subgenus possess cathodal haemoglobins with similar functional properties to rainbow trout HbI and HbII (Powers, 1972).

The insensitivity to ATP of the oxygen binding properties of HbI and HbII may also be related to their functioning as emergency oxygen suppliers. The binding of ATP to HbIV is much stronger in its deoxygenated form (Brunori, 1975). If HbI and HbII were sensitive to ATP, under conditions of increased lactic acid this would cause HbIV to sequester the cellular pool of ATP and strip ATP from HbI and HbII. This would cause an increase in the oxygen affinity of HbI and HbII under conditions where they should function as emergency oxygen suppliers (Brunori, 1975).

Tunas (family Scombridae) are able to maintain a core body temperature up to 14ºC above ambient water temperature through possession of vascular heat exchangers which allow heat retention in specific regions of the body and permit the animal to maintain a metabolic rate well above what would normally be expected from a poikilotherm (Riggs, 1970; Clark et al., 2010). As blood flows from the cool extremities of the tuna it warms. If tuna haemoglobins were temperature sensitive, oxygen affinity would be reduced as it warmed and oxygen would be lost to the warm tissues and an adequate supply of oxygen to tissues positioned efferent to the heat exchangers would not be possible (Clark et al., 2010). However, no evidence can be found for regional heterothermy in rainbow trout, therefore an adequate explanation for the temperature insensitivity of rainbow trout functional groups HbI to HbIII is still to be ascertained and further research is needed.

The different oxygen binding properties observed between cathodal and anodal fractions observed in this study signify that rather than interpreting changes in individual isomorphs, changes in abundance should be compared between total anodal and cathodal fractions. Increases in the abundance of individual anodal isomorphs in response to higher temperature seen in the paired temperature experiments in Chapter Two appear to be of insufficient magnitude to have a physiological effect on the fish. However, if anodal isomorphs are taken as a whole, with the same oxygen binding properties, then the magnitude of the change is sufficient to provide a physiological benefit to the fish. While the temperatures of 10ºC and 20ºC used for the experiments in Chapter Two were well within the known environmental tolerances for rainbow trout (Tun and Houston, 1986), an increase in total anodal haemoglobins at 20ºC would provide a greater oxygen supply to tissues with increased metabolic requirements due to the increased temperature. An increase in the abundance of anodal haemoglobin isomorphs would deliver more oxygen to the tissues due to the Bohr effect and the pH differential between the gills and the tissues (Brauner et al., 2000). As blood passes through the gills, protons are excreted, causing an increase in blood pH and resulting in increased oxygen affinity by the anodal isomorphs. As the blood passes through metabolically active tissues the pH drops, decreasing haemoglobin oxygen affinity and releasing oxygen (Riggs, 1988; Brauner et al., 2000). An increase in abundance of anodal haemoglobins would necessitate a decrease in cathodal haemoglobin abundance. As the trout were not under extreme physiological duress, a reduction in haemoglobins providing an ‘emergency backup’ oxygen supply was not critical to the animal.

Conclusion

Haemolysate stripped of organic phosphates from rainbow trout was separated into seven fractions by Fast Protein Liquid Chromatography. Subsequently the fractions were run on duplicate cellulose acetate gels. Three of the fractions moved cathodally while four of the fractions moved anodally. Each fraction was composed of doublets for a total of 14 isomorphs, matching the number observed in the experiments described in Chapter Two. Analysis of the oxygen binding properties of each of the fractions found that the cathodal fractions designated HbI and HbII and the anodal fractions HbIV and HbV had similar properties to those previously described. Anodal

fractions HbVI and HbVII were separated in sufficient volumes to investigate their oxygen binding properties for the first time. It was observed that they exhibited similar oxygen binding properties to HbIV and HbV under varying conditions of temperature and pH. However, there is the potential that there is a graded response to ATP across the anodal fractions which has not been previously observed and further research is needed. In addition, the Bohr effect and response to ATP by HbIII observed in other studies was not observed in this study; further research is needed to determine if the haemoglobin isomorphs that comprise this fraction are variably expressed and thereby the oxygen binding properties of the fraction change in response to environmental and/or physiological stimuli.

The results from this study support the hypothesis that studies on the changes in haemoglobin isomorph abundance would have more relevance when interpreted as changes in the abundance of functional groups instead of changes in individual isomorph abundance. In Chapter Four, rainbow trout are subjected to physical exercise in an attempt to induce the need for an increase in the cathodal haemoglobins which have been theorised to function as backup oxygen supplies in times of physiological stress.

References

Albers C, Goetz KH, Hughes GM, 1983. Effect of acclimation temperature on intraerythrocytic acid-base balance and nucleoside triphosphates in the carp, *Cyprinus carpio*. Respiration Physiology 54:145-159.

Binotti I, Giovenco S, Giardina B, Antonini E, Brunori M, Wyman J, 1971. Studies on the functional properties of fish hemoglobins : II. The oxygen equilibrium of the isolated hemoglobin components from trout blood. Archives of Biochemistry and Biophysics 142:274-280.

Black EC, 1958. Hyperactivity as a lethal factor in fish. Journal of the Fisheries Research Board of Canada 15: 573-586.

Brauner CJ, Thorarensen H, Gallaugher P, Farrell AP, Randall DJ 2000. The interaction between O_2 and CO_2 exchange in rainbow trout during graded sustained exercise. Respiration Physiology 119:83-96.

Brunori M, 1975. Molecular adaptation to physiological requirements: the hemoglobin system of trout. In: Current Topics in Cellular Regulation (Horecker BL, Stadtman ER, eds). Academic Press Inc. New York. p 1-39.

Brunori M, Falcioni G, Fortuna G, Giardina B, 1975. Effects of anions on the oxygen binding properties of the hemoglobin components from trout (*Salmo irideus*). Archives of Biochemistry and Biophysics 168:512-519.

Brittain T, 2005. Root effect hemoglobins. Journal of Inorganic Biochemistry 99:120-129.

Clark TD, Rummer JL, Sepulveda CA, Farrell AP, Brauner CJ, 2010. Reduced and reversed temperature dependence of blood oxygenation in an ectothermic scombrid fish: implications for the evolution of regional heterothermy? Journal of Comparative Physiology B 180:73-82.

di Prisco G, Tamburrini M, 1992. The hemoglobins of marine and freshwater fish: the search for correlations with physiological adaptation. Comparative Biochemistry and Physiology 102B:661-671.

Eddy FB, Lomholt JP, Weber R, Johansen K, 1977. Blood respiratory properties of rainbow trout (*Salmo gairdneri*) kept in water of high CO_2 tension. Journal of Experimental Biology 67:37-47.

Fago A, Forest E, Weber RE, 2002. Hemoglobin and subunit multiplicity in the rainbow trout (*Oncorhynchus mykiss*) hemoglobin system. Fish Physiology and Biochemistry 24:335-342.

Fyhn UEH, Withler RE, 1991. Ontogeny of hemoglobins in Chinook salmon *Oncorhynchus tshawytscha*. Comparative biochemistry and Physiology 98B:201-208.

Giles MA, 1991. Strain differences in haemoglobin polymorphism, oxygen consumption, and blood oxygen equilibria in three hatchery broodstocks of Arctic charr (*Salvelinus alpinus*). Fish Physiology and Biochemistry 9:291-301.

Hille S, 1982. A literature review of the blood chemistry of rainbow trout, *Salmo gairdneri* Rich. Journal of Fish Biology 20:535-569.

Houston AH, Cyr D, 1974. Thermoacclimatory variation in hemoglobin systems of goldfish (*Carassius auratus*) and rainbow trout (*Salmo gairdneri*). Journal of Experimental Biology 61:455-461.

Houston AH, Dobric N, Kahurananga R, 1996. The nature of hematological response in fish - Studies on rainbow trout *Oncorhynchus mykiss* exposed to simulated winter, spring and summer conditions. Fish Physiology and Biochemistry 15:339-347.

Iuchi I, 1973. Chemical and physiological properties of larval and adult hemoglobins in rainbow trout, *Salmo gairdnerii irideus*. Comparative Biochemistry and Physiology 44:1087-1094.

Iuchi I, Yamagami K, 1969. Electrophoretic pattern of larval haemoglobins of the salmonis fish, *Salmo gairdneri irideus*. Comparative Biochemistry and Physiology 28:977-979.

Kim JG, Xia M, Liu H, 2005. Extinction coefficients of hemoglobin for near-infrared spectroscopy of tissue. Engineering in Medicine and Biology Magazine, IEEE 24: 118-121.

Lau HKF, Wallach DE, Pennelly RR, Noble RW, 1975. Ligand binding properties of haemoglobin 3 of the trout, *Salmo gaidneri*. The occurrence of an acid Bohr effect in the absence of heme-heme interaction. Journal of biological Chemistry 250:1400-1404.

Marinsky CA, Houston AH, Murad A, 1990. Effect of hypoxia on hemoglobin isomorph abundances in rainbow trout, *Salmo gairdneri*. Canadian Journal of Zoology 68:884-888.

Murad A, Houston A, 1991. Haemoglobin isomorph abundances in splenectomized rainbow trout, *Oncorhynchus mykiss* (Walbaum). Journal of Fish Biology 38:641-651.

Overturf K, Casten MT, LaPatra SL, Rexroad C, Hardy RW, 2003. Comparison of growth performance, immunological response and genetic diversity of five strains of rainbow trout (*Oncorhynchus mykiss*). Aquaculture 217:93-106.

Pelster B, Weber RE, 1990. Influence of organic phosphates on the Root effect of multiple fish. Journal of Experimental Biology 149:425-437.

Powers DA, 1980. Molecular ecology of teleost fish hemoglobins - strategies for adapting to changing environments. American Zoologist 20:139-162.

Powers DA, 1972. Hemoglobin adaptation for fast and slow water habitats in sympatric catostomid fishes. Science 177:360-362.

Riggs AF, 1970. Properties of fish hemoglobins. In: Fish Physiology (Hoar WS, Randall DJ, eds). Academic Press. New York. p 209-252.

Riggs AF, 1988. The Bohr effect. Annual Review of Physiology 50:181-204.

Smit GL, Hattingh J, 1981. The effect of hypoxia on haemoglobins and ATP levels in three freshwater fish species. Comparative Biochemistry and Physiology Part A 68:519-521.

Taylor EB, Tamkee P, Keeley ER, Parkinson EA, 2011. Conservation prioritization in widespread species: the use of genetic and morphological data to assess population distinctiveness in rainbow trout (*Oncorhynchus mykiss*) from British Columbia, Canada. Evolutionary Applications 4:100-115.

Tsuyuki H, Gadd REA, 1963. The multiple hemoglobins of some members of the Salmonidae family. Biochimica et Biophysica Acta 71:219-221.

Tun N, Houston AH, 1986. Temperature, oxygen, photoperiod, and the hemoglobin system of the rainbow trout, *Salmo gairdneri*. Canadian Journal of Zoology 64:1883-1888.

Walker RL, Wilkes PRH, Wood CM, 1989. The effects of hypersaline exposure on oxygen-affinity of the blood of the freshwater teleost *Catostomus commersoni*. Journal of Experimental Biology 142:125-142.

Weber RE, Jensen FB, 1988. Functional adaptations in hemoglobins from ectothermic vertebrates. Annual Review of Physiology 50:161-179.

Weber RE, Wood SC, Lomholt JP, 1976. Temperature-acclimation and oxygen-binding properties of blood and multiple hemoglobins of rainbow-trout. Journal of Experimental Biology 65:333-345.

CHAPTER FOUR : SUSTAINED SWIMMING AS A STIMULUS FOR CHANGES IN HAEMOGLOBIN ISOMORPH ABUNDANCE IN RAINBOW TROUT

Introduction

Salmonids such as rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), brook trout (*Salvelinus fontinalis*) and brown trout (*Salmo trutta*) are able to sustain swimming velocities of up to 3 body lengths per second for indefinite periods of time (Nahhas et al., 1982; Peake et al., 1997). The derived energy for this level of sustained activity almost exclusively comes from the aerobic activity of red muscle (McKenzie et al., 2004). Delivery of oxygen to areas of red muscle is therefore critical to their function. The aerobic function of red muscle produces CO_2, which under resting conditions is stoichiometrically exchanged at the gills for oxygen (Brauner et al., 2000). Under conditions of exercise it has been reported that excretion of CO_2 does not linearly increase with oxygen uptake resulting in decreased extracellular and intracellular pH and therefore changes in the binding properties of some haemoglobin isomorphs (Brauner et al., 2000).

As established in the previous chapter, the anodal haemoglobins of rainbow trout exhibit changes in their oxygen affinity in response to variation in pH (Bohr effect) and temperature. The Bohr effect provides preferential delivery of oxygen to tissues experiencing respiratory and metabolic acidosis (Riggs, 1988). However, while respiratory and metabolic acidosis decreases the oxygen affinity of the anodal haemoglobins resulting in increased oxygen delivery it also reduces oxygen uptake at the gills, reducing overall oxygen delivery to the tissues, especially at low environmental oxygen saturation levels (Riggs, 1988).

In contrast to the anodal haemoglobins, the cathodal haemoglobins display no evidence of pH or temperature sensitivity. Both Brunori (1975) and Powers (1980) have suggested that cathodal haemoglobins function as an emergency backup oxygen delivery system in response to hyperactivity and excess proton production. This is because their oxygen affinity is unaffected by changes in pH and therefore oxygen uptake at the gills remains constant (Powers, 1980).

Despite this theory first being proposed more than thirty years ago no published information can be found for it being tested in the laboratory. An extensive body of literature can be found regarding changes in the physiology of captive fish species including changes in oxygen consumption rate by Arctic charr (*Salvelinus alpinus*) (Giles, 1991), increased cardiovascular function in chinook salmon (*Oncorhynchus tshawytscha*) (Gallaugher et al., 2004) and lactate increases in rainbow trout (Bannon, 2006) in response to forced swimming. Differences in isomorph abundance have also been observed between closely related subspecies of desert sucker (*Catostomus clarkii*) that live in either still or fast flowing water (Powers, 1972). Desert sucker subspecies living in fast flowing waters had more abundant pH insensitive haemoglobins than those living in pools and sluggish waters (Powers, 1972). This indicates that an adaptive response to different environmental conditions is possible by the circulatory system. However, no information is available on whether increases in the abundance of cathodal or anodal haemoglobin isomorphs can be induced through forced swimming.

The aim of this study was to determine if changes in the haemoglobin isomorph abundances of rainbow trout could be induced in response to forced periodic sustained swimming over a period of approximately 3 weeks.

Methods

Origin and maintenance of experimental animals

Rainbow trout parr were sourced from the National Institute of Water and Atmospheric Research (Hamilton, New Zealand) and reared outside in a 400 L fibreglass tank supplied with continuous flow-through dechlorinated tap water. Both photoperiod and temperature were allowed to vary with season; however tank water dissolved oxygen was maintained at a minimum of 90% saturation by provision of supplementary aeration. Fish were fed to satiation every second day with a commercial pellet (Reliance Stock Food, Dunedin, New Zealand).

Flume design

The experiment was conducted in a rectangular acrylic channel that was 7.2 m long, 0.5 m wide and 0.5 m deep (Figure 4.1). A 0.4 m diameter return pipe ran beneath the

flume and an impeller in the descending arm of the return pipe regulated flow speed via a variable-speed AC motor, which produced velocities up to 0.65 m s^{-1}. A 3 m-long by 0.5 m-wide acrylic sheet was used to divide the channel. Plastic grills (0.5 m x 0.6 m, mesh size 1 cm^2) were installed at either end of the divider to straighten the flow and contain the fish. Plastic mesh sections (6 mm mesh) were placed over the grill on the side containing the low activity group of fish to retard the flow speed (Figure 4.1). Flow velocity was measured using a mechanical flowmeter (General Oceanics, Model 2030R, Miami, USA). The stability of flow profiles were previously assessed by Bannon (2006) with no difference in flow speed and height from the bottom at either of the flow velocities used.

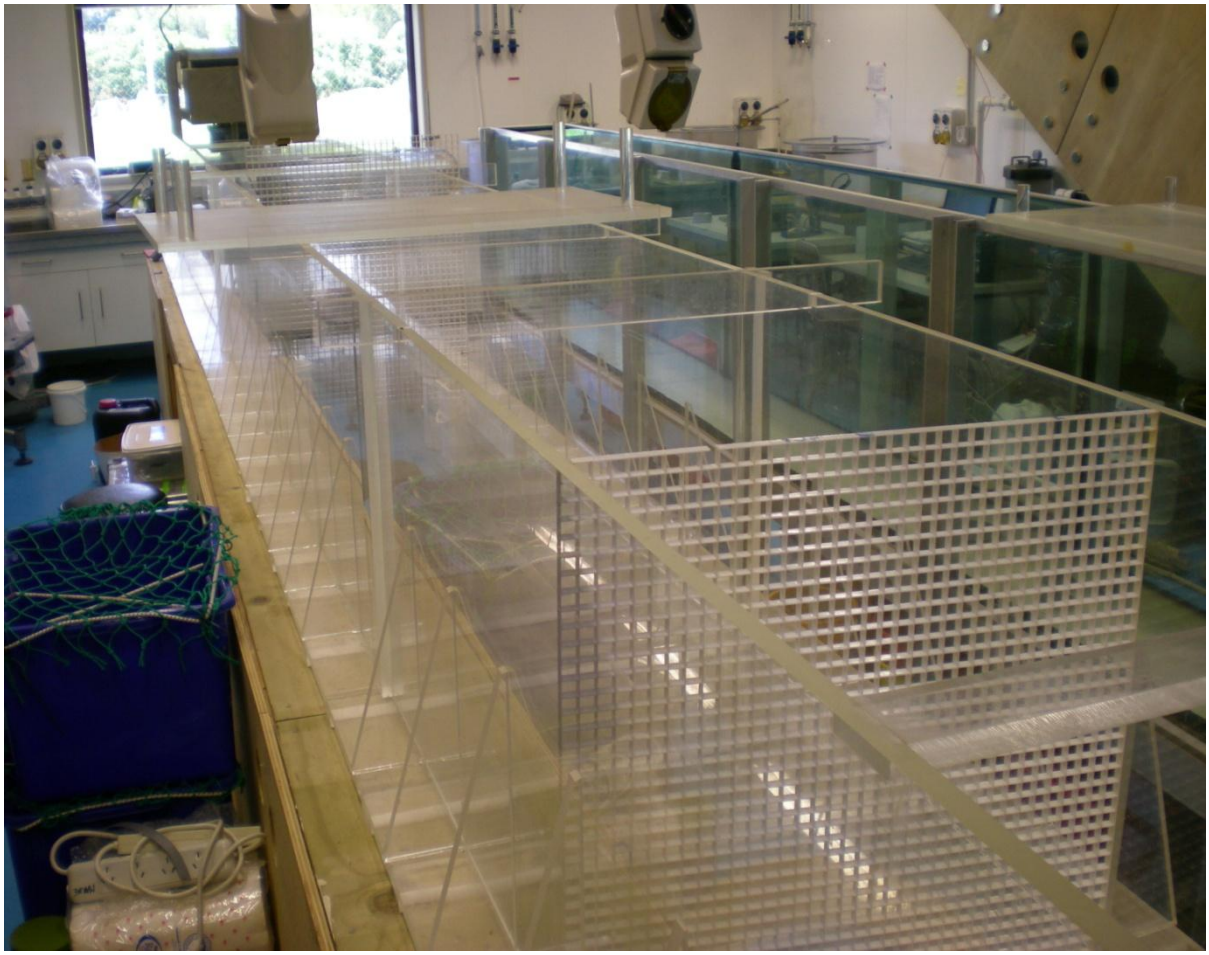


Figure 4.1: Flume used in experiment. Plastic mesh was placed at either end of the central acrylic divider to contain the fish and straighten the flow. A stretched netting cover was placed over the top to prevent fish from escaping.

Experimental design

Twelve rainbow trout (mean fork length 222 mm) were randomly divided into two groups of six fish and placed on either side of the divided flume. Black plastic sheeting was taped along the length on the flume to reduce disturbance to the fish. They were then allowed to acclimatise for two days before commencement of the experiment with the flow kept at a constant 0.5 body lengths (B.L.) s^{-1}. After the acclimatisation period both groups had approximately 25% of their blood volume removed on two consecutive days to induce anaemia; during this period flow velocity was maintained at 0.5 B.L. s^{-1}. To determine the volume of blood to be removed, each fish was first weighed and the volume (ml) then calculated as 1% of the fish's body weight (g). Anaemia was induced in order to stimulate the production of erythrocytes as it was previously observed (Chapter Two) that changes in the abundance of haemoglobin isomorphs originate from the production of new erythrocytes. Blood from the first blood sample was used to determine the abundance of haemoglobin isomorphs from the fish under initial conditions. Following bleeding on the second day the fish were allowed to recover for 1 h before the flume speed was increased to 2.5 B.L. s^{-1}. This flow velocity was selected based on the work of Bannon (2006) who determined the maximum swimming (U_{crit}) velocity of anaemic juvenile rainbow trout (140 mm FL) to be approximately 4 B.L. s^{-1} at 20ºC. Given that water temperature could not be controlled in the flume and rainbow trout were larger than those sampled by Bannon (2006) (as fish size increases, relative speed decreases) a conservative high activity rate of 2.5 B.L. s^{-1} was selected. Although prolonged swimming employs both aerobic and anaerobic muscle, studies of salmonids indicate that white muscle is only recruited at velocities above 70% of U_{crit} (MacNutt et al., 2006). Therefore, the selected high activity rate of 2.5 B.L. s^{-1} (~65% of U_{crit}) would be unlikely to recruit significant white muscle mass during exercise periods, resulting in sustained swimming being entirely supported by aerobic muscle function. Plastic mesh was placed over the grill on the low activity side to reduce the flow to 0.5 B.L. s^{-1}, this was subsequently removed after 6 h. The increased flow was maintained for 6 h and then reduced to 0.5 B.L. s^{-1}. This was repeated every day for a further 23 days. Flow was completely stopped for 1 h each day while the fish were fed and maintenance and cleaning was performed on the flume. Oxygenation was provided throughout the experiment, with dissolved oxygen levels never less than 95% saturation. Water

temperature was allowed to fluctuate naturally and was monitored every 30 min using a Hobo TidbiT® v2 temperature logger (Onset Computer Corporation, USA); daily mean water temperature varied between 16.9ºC and 20.3ºC with a mean of 18.8ºC. Overhead fluorescent lighting was set to provide a 12L:12D photoperiod initiated at 0700 h. Approximately one-third of the water was removed daily and replaced with dechlorinated tap water. Animals were fed daily with a commercial pellet (Reliance Stock Food, Dunedin, New Zealand) and monitored for feeding behaviour and overt disease symptoms such as white spot. On day three a low activity fish was found dead and was removed from the experiment. On day seven a high activity fish showed signs of poor health by being unable to maintain station in the flow and was subsequently removed from the experiment and euthanized.

Sampling and analysis

At sampling, trout were anesthetised in a 1:10000 solution of buffered tricane methane sulphonate (MS222, Sigma, New Zealand). Fork length was measured to the nearest mm and weight to the nearest 0.1 g. Blood was then drawn into a heparinised (sodium heparin, Sigma, New Zealand), syringe from the caudal vein. Within 30 min of collection, blood samples were analysed for haematocrit (Hct), total haemoglobin (Hb), red blood cell count (RBCC), mean erythrocyte haemoglobin concentration (MEHC), mean cell haemoglobin (MCH), and mean cell volume (MCV) according to standard methods (Dacie and Lewis, 1991).

Haemoglobin for isomorph analysis was prepared by washing the cells twice in 0.9% saline and then cells were lysed by addition of an equal volume of 10 mM Tris, pH 8. The samples were then centrifuged at 10 000 rcf for 10 min at 4ºC and the supernatant frozen at -80ºC. The composite haemoglobins of the haemolysates were separated by running on a cellulose acetate medium (Helena Laboratories, United States) in conjunction with a Tris-glycine buffer (pH 8.1-8.4). Duplicate separations were carried out at 200 V for 1 h at 4ºC using the haemoglobin of an individual rainbow trout as a mobility standard. Haemoglobins were identified by staining with 4 ml of 0.025% benzidine (Sigma, New Zealand) for 5 min, followed by immersion in 5% hydrogen peroxide for 2 min and then rinsing under tap water. Relative abundances of haemoglobin isomorphs were quantified by photographing the separations and then using the image analysis software Image Pro plus version

4.5.1.22 for Windows (Media Cybernetics, United States) to quantify the differences in staining intensity as a measure of isomorph abundance.

Statistical analysis

Significant differences between treatments were tested for using Student's *t*-test or one-way Analysis of Variance (ANOVA) coupled with Tukey's post-hoc tests using Statistica v.9.0 (Statsoft, USA).

Results

Other than the two fish removed from the experiment, none of the fish appeared to have difficulty with maintaining station within the flow. The fish removed from the treatment group was the smallest of the group and appeared to have suffered damage to its caudal fin due to aggression from other members of its cohort. It was assumed that the death of the fish from the control group was due to infection subsequent to bleeding as no signs of external physical injury could be observed.

After 24 d all haematological parameters were lower in the low activity group compared to the high activity group indicating faster recovery of red cell mass in the active group. However, presumably due to low sample size, significant differences (Student's *t*-test; d.f. = 8, $n = 5$, $P < 0.05$) in mean values were only observed for total haemoglobin (Hb) and mean cell haemoglobin (MCH). No significant differences (Student's *t*-test; d.f. = 8, $n = 5$, $P > 0.05$) were observed between haematocrit, (Hct), red blood cell counts (RBCC), mean erythrocyte haemoglobin concentrations (MEHC) and mean cell volumes (MCV) (Table 4.1).

Table 4.1: Means (± SEM) of blood parameters from anaemia-induced rainbow trout (*Oncorhynchus mykiss*) after 24 days of periodic sustained activity (2.5 B.L. s^{-1} for 6 h d^{-1}) compared to low level activity (0.5 B.L. s^{-1} continuously). * indicates parameters with significant differences (Student's *t*-test, $P < 0.05$).

	n	Hb ($g\ L^{-1}$)*	Hct (%)	RBCC ($\times 10^{12}$ cells L^{-1})	MEHC ($g\ L^{-1}$)	MCH (pg)*	MCV (fl)
Low activity	5	39.4 ± 5.5	23.0 ± 3.5	0.835 ± 0.087	171 ± 5	46.7 ± 3.2	274 ± 15
High activity	5	53.1 ± 3.9	29.0 ± 1.9	0.947 ± 0.068	185 ± 5	56.1 ± 1.3	305 ± 13

As with the results presented in Chapter Two, 14 haemoglobin isomorphs were detected when the haemolysate was separated by cellulose acetate electrophoresis. Isomorphs were designated A (anodal) 1 to 8 and C (cathodal) 1 to 6 based on direction of movement and order of increasing mobility. No evidence was seen of polymerization or auto-oxidation. The bands exhibited the same degree of mobility and were observable under both treatment conditions. Haemoglobin isomorph abundances were compared between high activity fish, low activity fish and the isomorph abundances from all fish at the start of the experiment (Figure 4.2).

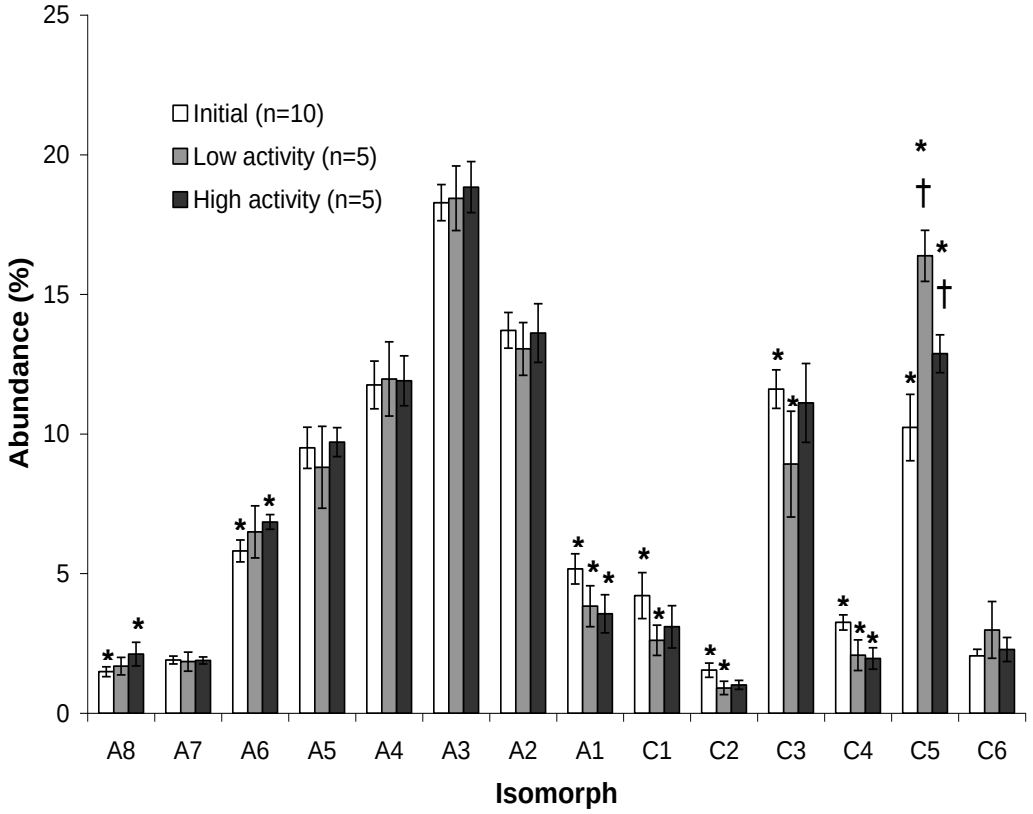


Figure 4.2: Mean (± 95% C.I.) haemoglobin isomorph abundances from anaemia-induced rainbow trout (*Oncorhynchus mykiss*) at initial conditions and after 24 d of low activity (0.5 B.L. s^{-1} continuously) and high activity (2.5 B.L. s^{-1} for 6 h d^{-1} then maintained at 0.5 B.L. s^{-1}). Isomorphs marked * indicate significant differences (ANOVA; $P < 0.05$) between initial conditions and low and/or high activity groups. Isomorphs with the † symbol indicate significant differences (ANOVA, $P < 0.05$) between the isomorph abundances of the high and low activity groups.

Significant differences (ANOVA, $P < 0.05$) between haemoglobin isomorph abundances were primarily observed between the initial samples taken during the induction of anaemia and the low activity and high activity groups. Anodal isomorphs

A6 and A8 were more abundant in the high activity group compared to the initial samples. In comparison, the A1, C1, C2, C3, and C4 isomorphs from the initial samples were more abundant than those from the low activity group. The cathodal C5 isomorph was the only isomorph to have a significant difference (ANOVA, $P < 0.05$) between the high activity and low activity groups (Figure 4.2).

When total anodal isomorph abundance and total cathodal isomorph abundance was compared between the initial, high and low activity groups (Figure 4.3) there were no significant differences between any of the groups (ANOVA, $P > 0.05$).

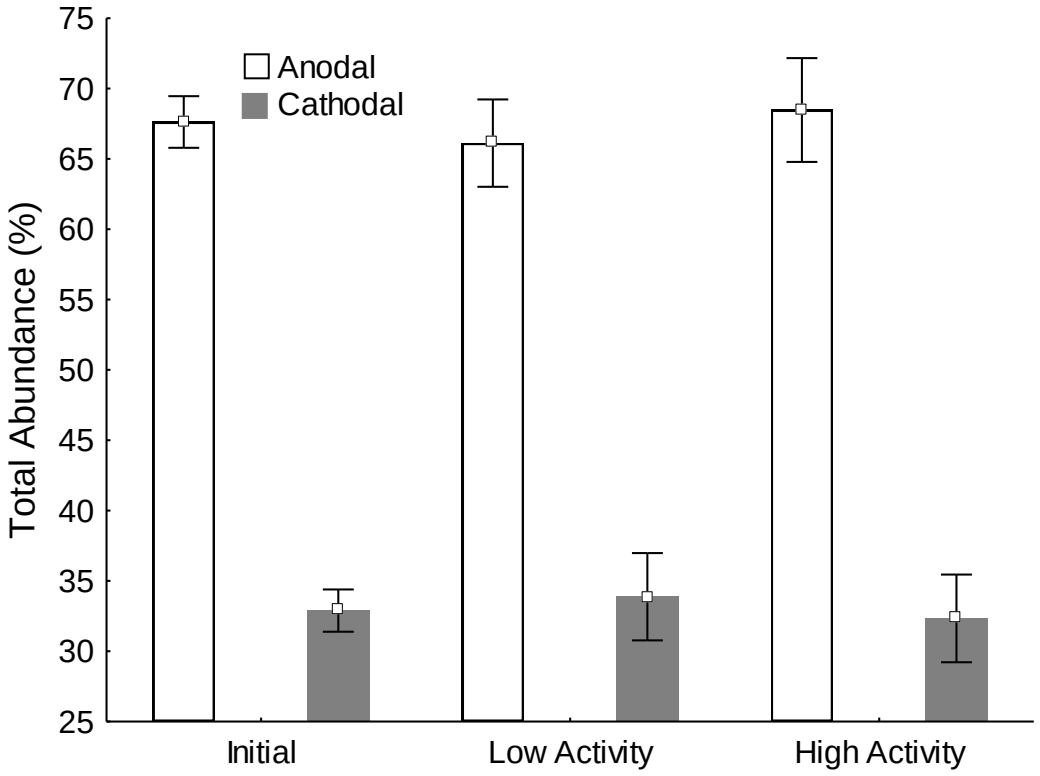


Figure 4.3: Mean (± 95% C.I) of total anodal and cathodal haemoglobin isomorph abundance from anaemia-induced rainbow trout (*Oncorhynchus mykiss*) at initial conditions and after 24 d of low activity (0.5 B.L. s^{-1} continuously) and high activity (2.5 B.L. s^{-1} for 6 h d^{-1} then maintained at 0.5 B.L. s^{-1}).

Discussion

The finding of increased total haemoglobin and mean cell haemoglobin in the high activity group is in contrast to that reported by Davie et al. (1986) where no such change was observed in rainbow trout. It was reported that a level of 1 B.L. s^{-1}

sustained aerobic activity induced an increase in the number of capillaries per red muscle fibre but did not affect blood lactate, haemoglobin concentration, haematocrit or whole blood oxygen affinity (Davie et al., 1986). However, experimental conditions may be dissimilar enough to account for this finding. For example, the rainbow trout of Davie et al. (1996) were subjected to continuous 1 B.L. s^{-1} exercise, compared with the 2.5 B.L. s^{-1} for 6 h d^{-1} in this study. In addition, bleeding the trout in the current study prior to subjecting them to exercise is likely to have initially decreased the physiological scope for physical activity by decreasing the amount of oxygen able to be transported to the tissues. A previous study has found that at 20ºC the critical swimming speed (U_{crit}) for anaemic rainbow trout parr (140 mm F.L.) was reduced from approximately 5.5 B.L. s^{-1} to 4 B.L. s^{-1} (Bannon, 2006). The requirement for increased oxygen transport to the tissues could explain the increase in cellular haemoglobin content of the high activity fish. Similar increases in haemoglobin concentration in response to increased sustained activity have been reported for other fish species (Soldatov, 2005) such as chinook salmon (Gallaugher et al., 2004).

The only observed change in haemoglobin isomorph abundance between the low activity and high activity fish was that of the C5 isomorph becoming slightly more abundant in the low activity group. Given that this difference accounted for only a 4% difference in total isomorph composition, it is unlikely that it would have had any detectable effect on the oxygen transport characteristics of the blood. Changes in isomorph abundance were observed between initial and low activity as well as high activity fish. However, no discernible pattern of change was evident with both increases and decreases in abundance of cathodal isomorphs between initial state and low activity fish. While changes in isomorph abundance between the initial state and high activity fish were not as common, the same phenomenon of both increased and decreased abundances were present. These results suggest that the induction of anaemia prior to induced swimming activity had a larger effect on isomorph abundance than the forced activity levels. This is possibly due to internal hypoxia generated by the combination of anaemia and forced exercise (Cook et al., 2011). Further research is needed to determine the physiological drivers of isomorph abundance change in anaemia-induced fish.

Contrary to expectations, no changes were observed in the abundance of the combined anodal or cathodal isomorphs between either the initial state, high activity

or low activity groups. However, the hypothesis that cathodal isomorphs act as supplementary oxygen suppliers under conditions of increased activity still remains viable. It is possible that the swimming speed of 2.5 B.L. s^{-1} and duration (6 h d^{-1}) used as an arbitrary level of high activity was too conservative to induce changes in isomorph abundance. The inducement of anaemia in order to stimulate erythropoesis also added a complicating factor to the selection of activity levels and there are few studies examining the effect of anaemia on swimming (Bannon, 2006). Therefore, selection of a sustainable swimming speed that balances animal welfare with potentially stressful exercise was difficult to achieve. Furthermore, factors such as temperature, hypoxia and level of physical activity (i.e. burst swimming versus prolonged swimming) are known to have synergistic effects on swimming performance (Bannon, 2006).

Given that changes in haemoglobin isomorph abundance originate from the production of new erythrocytes, the alternative to inducement of anaemia would have been to conduct the experiment for a considerably longer period of time in order to allow for the natural replacement of erythrocytes. The life span of erythrocytes is somewhat dependent on temperature (Soldatov, 2005), but the reported life span of erythrocytes for rainbow trout is on average 105 ± 17 days (Soldatov, 2005). This timeframe would require continuously running the flume for a minimum of 3 months.

Conclusion

The attempt to induce changes in haemoglobin isomorph abundance by subjecting rainbow trout to sustained exercise was not successful. However, the theory that cathodal haemoglobins act as reserve sources of oxygen under conditions of respiratory and metabolic acidosis due to their insensitivity to changes in pH cannot be discounted. These data suggest that while the induction of anaemia had an effect on the composition of isomorph abundance, there was no physiological effect on oxygen transportation. The induction of anaemia coupled with forced exercise may have resulted in internal hypoxia. This resulted in unexpected significant differences in isomorph abundance between initial conditions and treatment groups, masking differences between treatment groups. Also, it is possible that the intermittent level of forced activity used was insufficient to induce changes in isomorph abundance and a continuous low level (1 B.L. s^{-1}) of activity may have produced better results. In

related experiments investigating changes in isomorph abundance in rainbow trout, the stimuli of temperature and hypoxia used were close to the known tolerance limits of the fish (Houston and Cyr, 1974; Marinsky et al., 1990; Houston et al., 1996). Therefore, changes in haemoglobin isomorph abundances may be a mechanism of adaptation in response to living in environmental extremes. Other physiological modifications such as variations in total haemoglobin concentration, NTP concentration, cardiac and gill ventilation frequency are likely to be sufficient to allow the animal to adapt to lesser fluctuations in the environment.

References

Bannon HJ, 2006. Effects of water quality parameters on prolonged swimming ability of freshwater fishes. PhD Thesis. University of Waikato, Hamilton. p 102.

Brauner CJ, Thorarensen H, Gallaugher P, Farrell AP, Randall DJ, 2000. The interaction between O_2 and CO_2 exchange in rainbow trout during graded sustained exercise. Respiration Physiology 119:83-96.

Brunori M, 1975. Molecular adaptation to physiological requirements: the hemoglobin system of trout. In: Current Topics in Cellular Regulation (Horecker BL, Stadtman ER, eds): Academic Press Inc. New York. p 1-39.

Cook DG,Wells RMG, Herbert NA 2011. Anaemia adjusts the aerobic physiology of snapper (*Pagrus auratus*) and modulates hypoxia avoidance behaviour during oxygen choice presentations. Journal of Experimental Biology 214: 2927-2934.

Davie PS, Wells RMG, Tetens V, 1986. Effects of sustained swimming on rainbow trout muscle structure, blood oxygen transport, and lactate dehydrogenase isozymes: Evidence for increased aerobic capacity of white muscle. Journal of Experimental Zoology 237:159-171.

Dacie JV, Lewis SM, 1991. Practical Haematology, 7th edition. Churchill Livingstone. New York.

Gallaugher PE, Thorarensen H, Kiessling A, Farrell AP, 2004. Effects of high intensity training on cardiovascular function, oxygen uptake, internal oxygen transport and osmotic balance in Chinook salmon (*Oncorhynchus tshawytscha*) during critical speed swimming. Journal of Experimental Biology 204:2861-2872.

Giles MA, 1991. Strain differences in haemoglobin polymorphism, oxygen consumption, and blood oxygen equilibria in three hatchery broodstocks of Arctic charr (*Salvelinus alpinus*). Fish Physiology and Biochemistry 9:291-301.

Houston AH, Cyr D, 1974. Thermoacclimatory variation in hemoglobin systems of goldfish (*Carassius auratus*) and rainbow trout (*Salmo gairdneri*). Journal of Experimental Biology 61:455-461.

Houston AH, Dobric N, Kahurananga R, 1996. The nature of hematological response in fish - Studies on rainbow trout *Oncorhynchus mykiss* exposed to simulated winter, spring and summer conditions. Fish Physiology and Biochemistry 15:339-347.

MacNutt MJ, Hinch SG, Lee CG, Phibbs JR, Lotto AG, Healey MC, Farrell AP, 2006. Temperature effects on swimming performance, energetics, and aerobic capacities of mature adult pink salmon (*Oncorhynchus gorbuscha*) compared with those of sockeye salmon (*Oncorhynchus nerka*). Canadian Journal of Zoology 84:88-97.

Marinsky CA, Houston AH, Murad A, 1990. Effect of hypoxia on hemoglobin isomorph abundances in rainbow trout, *Salmo gairdneri*. Canadian Journal of Zoology 68:884-888.

McKenzie DJ, Wong S, Randall DJ, Egginton S, Taylor EW, Farrell AP, 2004. The effects of sustained exercise and hypoxia upon oxygen tensions in the red muscle of rainbow trout. Journal of Experimental Biology 207:3629-3637.

Nahhas R, Jones NV, Goldspink G, 1982. Some aspects of sustained training of rainbow trout, *Salmo gairdneri* Richardson. Journal of Fish Biology 20:351-358.

Peake S, McKinley RS, Scruton DA, 1997. Swimming performance of various freshwater Newfoundland salmonids relative to habitat selection and fish-way design. Journal of Fish Biology 51:710-723.

Powers DA, 1980. Molecular ecology of teleost fish hemoglobins - strategies for adapting to changing environments. American Zoologist 20:139-162.

Powers DA, 1972. Hemoglobin adaptation for fast and slow water habitats in sympatric catostomid fishes. Science 177:360-362.

Riggs AF, 1988. The Bohr effect. Annual Review of Physiology 50:181-204

Soldatov AA, 2005. Peculiarities of organization and functioning of the fish red blood system. Journal of Evolutionary Biochemistry and Physiology 41:272-281.

CHAPTER FIVE : CONCLUSIONS

Research Summary

Research into the oxygen transport system of fish has shown that the vast majority of fish species possess multiple haemoglobins (di Prisco and Tamburrini, 1992). However, the exact number of haemoglobins possessed by any given species is difficult to determine due to the resolution provided by the different detection methods employed. For example, rainbow trout have been reported as having between 4 to 16 isomorphs depending on the detection methods used. Boundary electrophoresis methods produce less resolution (Buhler and Shanks, 1959; Buhler, 1963) than starch gel or cellulose acetate methods (Iuchi, 1973; Tun and Houston, 1986; Murad and Houston, 1991). Other methods such as isoelectric focusing and anion exchange chromatography tend to fall somewhere in the middle in terms of resolution with 8 to 12 bands (Weber et al., 1976b; Feuerlein and Weber, 1994; Fago et al., 2002). There have been no studies conducted comparing the resolution of rainbow trout haemoglobin isomorphs using different electrophoretic or chromatographic techniques.

The selective advantage that possession of multiple haemoglobin isomorphs provides is still a matter of on-going research and a robust theory linking fish ecology and life-style with multiplicity and function of haemoglobins has yet to be developed. It has been demonstrated that in species such as eels (*Anguilla* spp.), common carp (*Cyprinus carpio*), goldfish (*Carassius auratus*) and rainbow trout (*Oncorhynchus mykiss*) haemoglobin isomorph abundance varies in response to temperature and hypoxia (Houston and Cyr, 1974; Weber et al., 1976a; van Vuren and Hattingh, 1978, Tun and Houston, 1986). The reason for this is related to the fact that individual isomorphs display different oxygen binding properties (Weber et al. 1976b). In rainbow trout, the cathodal isomorphs are insensitive to temperature, pH and organic phosphates, while the oxygen binding properties of the anodal isomorphs vary in response to temperature, pH and organic phosphates (Binotti et al., 1971; Weber et al., 1976b).

While it has been demonstrated that changes in haemoglobin isomorph abundance occur in response to environmental stimuli, there is still doubt over the

time-scale in which these changes occur. In addition, it has yet to be identified whether changes in isomorph abundances originate from the production of new erythrocytes or if *de novo* synthesis of new haemoglobin isomorphs occurs in already circulating erythrocytes. This was addressed in Chapter Two where three separate experiments were undertaken. In the first, paired groups of rainbow trout were acclimated to 10ºC or 20ºC for either 5, 7, 14, 21 or 28 days. In the second experiment, paired groups of rainbow trout were rendered anaemic to induce erythropoesis and then acclimated at either 10ºC or 20ºC for 24 days. Numerical enrichment of young and mature erythrocytes by density gradient centrifugation from the blood of these anaemic acclimated fish was also undertaken. In the third experiment, a captive population of rainbow trout was kept under conditions of seasonally variable light and temperature and sampled every six months for two years. Assessment for changes in haemoglobin isomorph abundance was under-taken using cellulose acetate electrophoresis.

Rainbow trout haemoglobin isomorphs have traditionally been grouped into four functional groups even though only two functional groups, the cathodal HbI and the anodal HbIV, have been extensively investigated. In contrast, recent work published by Fago et al. (2002) using anion exchange chromatography has revealed the presence of eight distinct haemoglobin fractions composed of 5 α-globin and 4 β-globin chains. However, the functional properties of these haemoglobin fractions are not currently known; nor is the relationship of haemoglobin fractions separated by anion exchange chromatography to isomorphs separated from whole haemolysate using cellulose acetate electrophoresis. In Chapter Three rainbow trout haemolysate was separated into seven fractions using the methods of Fago et al. (2002); subsamples of the haemoglobin fractions were then electrophoresed on cellulose acetate to determine their isomorph composition. In addition, the oxygen binding properties of the separated fractions were examined under varying temperature, chloride ion concentration, ATP concentration and pH conditions.

It has been proposed that the cathodal haemoglobins of rainbow trout may act as a reserve oxygen delivery system to muscles under-going sustained exercise, but this theory has not been tested (Brunori, 1975; Powers, 1980). Furthermore, while the effects of temperature and hypoxia on isomorph abundance have been examined in rainbow trout, the effects of swimming activity on isomorph abundance have not been researched. These questions were examined in Chapter Four. In a paired experiment,

two groups of five anaemia induced rainbow trout were placed in a divided 7 m annular flume for 24 days. The high activity group was subjected to a forced swimming speed of 2.5 body lengths per second (B.L. s^{-1}), for 6 hours a day. When not undergoing forced exercise the treatment group were maintained at the same speed as the low activity group of 0.5 B.L. s^{-1}. Assessment for changes in haemoglobin isomorph abundance was under-taken using cellulose acetate electrophoresis.

Haemoglobin Isomorph Abundance in Response to Temperature

A total of 14 isomorphs were observed when whole blood haemolysate was separated using cellulose acetate electrophoresis, however, no detectable differences in isomorph abundance were found between 10ºC and 20ºC treatment groups of fish acclimated for either 5, 7, 14, 21 or 28 days. In contrast, significant differences in the abundance of individual haemoglobin isomorphs as well as total anodal and cathodal isomorphs were observed in anaemia-induced fish. These differences were enhanced in samples enriched in young erythrocytes. Differences in individual isomorph abundances were also observed between trout sampled every 6 months for 2 years.

The results presented in Chapter Two support the theory that changes in the abundance of haemoglobin isomorphs transpires through the production of new erythrocytes rather than the production of new isomorphs in already circulating red blood cells. Data on the life-span of erythrocytes in fish is limited, but it is reported that a life-span of ~100 days is typical for rainbow trout erythrocytes (Soldatov, 2005). This indicates that changes in haemoglobin isomorph abundance of a sufficient magnitude to exert a physiological effect are unlikely to be a response to acute environmental challenges. It is more probable that changes in isomorph abundance are an adaptive response to seasonal or regional environmental variability. For example, Atlantic cod (*Gadus morhua*) were shown to have distinct haemoglobin isomorph abundance patterns with regard to area of habitation within the North Atlantic (Karpov and Novikov, 1980). When Atlantic cod from two different geographic areas were acclimated to temperatures of 8, 12 and 15ºC for more than 10 months an optimal oxygen binding temperature of 12ºC was indentified for both populations. However, one population was able to perform comparatively well at 8ºC and the other population at 15ºC. This was attributed to the differential expression of anodal and

cathodal haemoglobin isomorphs. The population expressing more of the anodal forms performed better at 15ºC while the other population was able to perform better at 8ºC and had more abundant cathodal isomorphs (Brix et al., 2004). Under raised temperature conditions, increases in anodal haemoglobin isomorph abundance facilitate increased delivery of oxygen to the tissues due to the Bohr effect and the pH differential between the gills and the tissues (Brauner et al., 2000). As blood passes through the gills protons are excreted causing an increase in blood pH and resulting in increased oxygen affinity by the anodal isomorphs. As the blood passes metabolically active tissues the pH drops, decreasing haemoglobin oxygen affinity and releasing oxygen to the tissues (Riggs, 1988; Brauner et al., 2000).

Oxygen Binding Properties of Rainbow Trout Haemoglobins

Anion-exchange chromatography was performed on rainbow trout haemolysate separating it into seven distinct functional groups (designated HbI to HbVII). These were further separated by cellulose acetate electrophoresis producing eight anodal and six cathodal isomorphs with each group represented by an electrophoretic doublet. These separation patterns matched those seen from the temperature acclimation experiments performed in Chapter Two. Each functional group was composed of two haemoglobin isomorphs and confirms the findings of Fago et al. (2002) that each functional group is composed of mixtures of haemoglobin isomorphs. Haemoglobin-oxygen binding analysis of the seven fractions found that the cathodal fractions (HbI-HbIII) were insensitive to temperature, chloride, ATP and pH while all anodal fractions (HbIV-HbVII) were sensitive to temperature and pH, but only HbVI and HbVII were sensitive to ATP.

The discovery of multiple forms of haemoglobin raises the question of what is the advantage of such high multiplicity. As has already been discussed in Chapters Three and Four, cathodal and anodal forms allow for the optimal delivery of oxygen to tissues under normal conditions and metabolic acidosis as well as variable environmental conditions of temperature and oxygen saturation. The existence of multiple haemoglobin isomorphs with varying isoelectric points may expand buffering of intracellular pH to a wider range or allow a higher intracellular total haemoglobin concentration by increasing the solubility of the individual components (Weber, 1990, Fago et al., 2002). Changes in the abundance of different haemoglobins

will also modify the oxygen binding characteristics of the blood allowing adaptation to a broader range of environmental conditions and expanding the range of the animal.

Examination of the oxygen binding characteristics of the haemoglobin fractions HbI, HbII and HbIV found similar results to those of Binotti et al. (1971) and Weber et al. (1976). The HbIII fraction exhibited no response to an alkaline pH shift which did not match the results published by Lau et al. (1975) and Weber et al. (1976b). This may be due to the fact that the HbIII fraction typically has a very low abundance and complete separation of a sufficient volume to perform oxygen binding analysis has proved difficult in the past. Alternatively, it may mean that the oxygen binding properties of this fraction are variable depending on environmental conditions and that changes in the composition of the isomorphs that constitute this fraction are possible.

The oxygen binding properties of HbVI and HbVII have not previously been ascertained. They both exhibited similar responses to pH and temperature as those observed for HbIV and HbV. However, in contrast to the study by Weber et al. (1976), HbIV and HbV did not exhibit any statistically significant decreases in oxygen affinity in response to increased ATP concentrations while HbVI and HbVII did show statistically significant decreases. The ATP concentration used in Chapter Four was a quarter of that employed by Weber et al. (1976b). This, combined with the trend to increasing effect of ATP with increasing anodal displacement of isomorphs suggests that there may be a graded response to ATP concentration among the anodal isomorphs. In addition, guanosine triphosphate (GTP) is known to have a larger effect on oxygen affinity than ATP in rainbow trout even though ATP is more prevalent than GTP (Jensen, 1993). While a response to ATP was not observed in the HbIV and HbV functional groups in the current study, it is possible that a response to GTP may still occur and coupled with the high abundance of the HbIV and HbV fraction would explain the larger reduction in oxygen affinity induced by GTP compared to ATP.

Chloride concentration is known to have a direct effect on the oxygen affinity of rainbow trout haemoglobins (Binotti et al., 1971; Weber et al., 1976). However, the chloride concentrations used in these studies were up to 25 times more than those realistically expected to occur in rainbow trout. The use of chloride concentrations within an expected normal range for rainbow trout found no direct effect of chloride on oxygen affinity for any of the haemoglobin functional groups. It is more likely that normal metabolic concentrations of chloride act in an indirect manner to affect

haemoglobin oxygen affinity. For example, when 0.1 M NaCl was added to the cathodal haemoglobin fractions of *Catostomus clarkii*, the effect of ATP on haemoglobin oxygen affinity was negated (Powers et al., 1980).

The generally analogous oxygen binding properties of haemoglobin isomorphs within the cathodal and anodal functional groups suggests that researchers interested in adaptive responses by rainbow trout to environmental stimuli should look for changes in total cathodal and anodal isomorph abundance. From the observations presented here and studies by Houston and Cyr (1974), Tun and Houston (1986) Marinsky et al. (1990) and Houston et al. (1996), changes in individual haemoglobin isomorph abundance for rainbow trout rarely exceed 6% of the total isomorph abundance.

Effect of Exercise on Haemoglobin Isomorph Abundance

The hypothesis that cathodal haemoglobins act as supplementary supplies of oxygen to tissues under sustained metabolic activity was tested in Chapter Four. Alternatively, it is possible that cathodal haemoglobins have evolved to supply certain tissues with oxygen under persistent conditions of low PO_2. The fish heart is located at the end of the circulatory system in terms of oxygen availability and therefore is subjected to a persistent low PO_2. Theoretically, blood entering the heart has already off-loaded a large proportion of its available O_2 to tissues throughout the body by the time it reaches the heart. Under conditions of exercise the oxygen demand from body tissues will increase further and more oxygen will be off-loaded from the anodal haemoglobins in response to the increased lactate and CO_2 levels. Because cathodal haemoglobins appear to only off-load oxygen in response to low PO_2 they appear suited to delivery oxygen to low PO_2 areas such as the heart. Because cathodal haemoglobins do not respond to changes in pH, this means that they are able to retain bound O_2 throughout the circulation while O_2 is preferentially supplied by anodal haemoglobins. Total haemoglobin and mean cell haemoglobin was found to be higher in the high activity fish compared to the low activity fish. However, while significant differences in haemoglobin isomorph abundance were present between the initial samples taken at the time of anaemia induction and high and low activity groups; only the C4 isomorph demonstrated a significant difference between high and low activity groups. When combined total anodal isomorphs and total cathodal isomorphs

abundances were compared between initial state, high activity and low activity groups, no differences were observed.

It was anticipated that by placing rainbow trout under extended periods of moderate intensity exercise over a period of weeks, more cathodal haemoglobins would be produced to allow the trout to deliver oxygen to tissues despite decreased metabolic pH levels. However, no changes in total cathodal or total anodal isomorph abundance were observed. It is possible that the observed results were due to the trout not being placed under enough physiological stress to induce shifts in anodal and cathodal isomorph abundance. Alternatively, the induction of anaemia prior to the experiment to stimulate erythropoesis may have had a confounding effect on the physiology of the fish. The effect of anaemia induction in fish is not well understood and studies by Murad and Houston (1991) and Bryne and Houston (1988) both had unexpected transient changes in isomorph abundance that could not be explained when anaemia was induced by phenylhydrazine HCl.

Study Outcome

This study provides a basis for further research to identify rainbow trout varieties that exhibit haemoglobin isomorph abundance traits that allow them to adapt to different environmental conditions. For example, varieties that have more abundant anodal functional groups would be suited to warmer environmental conditions. Conversely, varieties producing more cathodal forms would be suited to colder temperatures. Concordantly the same set of characteristics has been observed in Atlantic cod and is associated with increased growth (Brix et al., 2004). By determining which variety of rainbow trout is suited to particular environmental conditions, fisheries managers can preferentially stock areas with trout varieties that can optimally adapt to that environment and produce larger catches for anglers. Also, identification of traits that facilitate adaptation to marginal environmental conditions would be of benefit in managing teleost species under future climate change conditions.

The use of more sophisticated separation techniques such as FPLC has allowed the discovery of more functional haemoglobin groups than the four originally reported by Binotti et al. (1971) or six by Weber et al. (1976b). In addition, these techniques have provided superior quantities of haemoglobin, allowing a more comprehensive range of oxygen-binding assessments to be performed. This study has

also demonstrated the relationship between individual haemoglobin isomorphs separated by cellulose acetate electrophoresis and haemoglobin functional groups separated by anion-exchange chromatography.

The information presented here and by Fago et al. (2002) provides evidence that some of the early detection methods used in studies of fish haemoglobins may have resulted in under-reporting of the number of haemoglobin isomorphs in a range of fish species.

Recommendations for Further Research

Despite being studied for more than forty years, the extent and composition of the haemoglobin isomorphs produced by rainbow trout are still not completely understood. While sequencing of the haemoglobin protein subunits has progressed (e.g. Fago et al., 2002), sequencing of the haemoglobin alleles has lagged behind, with only a handful of α and β-subunits currently published (Yoshizaki et al., 1996; Yoshizaki et al., 1997). Elucidation of the genetic sequences would allow for the use of methods such as quantitative PCR in determining the stimuli for up-regulation of individual isomorphs.

A definitive theory explaining the reason for haemoglobin multiplicity and function has yet to be developed. It could be speculated that comparative studies of haemoglobin function in diverse fish lineages may provide greater insights into the evolutionary significance of multiple haemoglobins. In the case of some species such as rainbow trout and tuna, a link with adaptation to habitat becomes possible. But in the majority of cases it is not clear whether multiple haemoglobin genes provide selective advantages or whether they are surviving selectively neutral gene duplications, with no obvious correlation with environmental conditions (di Prisco and Tamburrini, 1992).

The multiple haemoglobin forms found in this study and by other authors such as Tun and Houston (1986) and Marinsky and Houston (1990) appear to form functional groups of isomorphs with similar oxygen binding properties. Further separation of these functional groups is needed to determine the number of haemoglobin isomorphs that compose each fraction and the oxygen binding properties of those isomorphs. In particular, the functional group HbIII requires more research to determine why its oxygen binding properties appear to be pH sensitive in some cases

and not in others. Further research is also required to examine the interaction of effects between chloride concentrations, NTP concentrations and whether there is a graded response in oxygen affinity between NTP concentrations and the anodal isomorph fractions. A graded response would suggest that the multiple haemoglobins of rainbow trout originate from adaptive mutations rather than duplications or neutral or nearly neutral substitutions caused by random drift. Finally, a modified investigation is needed to test the hypothesis that cathodal haemoglobins act as supplementary suppliers of oxygen to tissues during periods of increased high proton production by metabolic activity.

References

Binotti I, Giovenco S, Giardina B, Antonini E, Brunori M, Wyman J, 1971. Studies on the functional properties of fish hemoglobins : II. The oxygen equilibrium of the isolated hemoglobin components from trout blood. Archives of Biochemistry and Biophysics 142:274-280.

Brauner CJ, Thorarensen H, Gallaugher P, Farrell AP, Randall DJ, 2000. The interaction between O_2 and CO_2 exchange in rainbow trout during graded sustained exercise. Respiration Physiology 119:83-96.

Brix O, Thorkildsen S, Colosimo A, 2004. Temperature acclimation modulates the oxygen binding properties of the Atlantic cod (*Gadus morhua* L.) genotypes--HbI*1/1, HbI*1/2, and HbI*2/2--by changing the concentrations of their major hemoglobin components (results from growth studies at different temperatures). Comparative Biochemistry and Physiology Part A 138:241-251.

Bryne AP, Houston AH, 1988. Use of phenylhydrazine in the detection of responsive changes in haemoglobin isomorph abundances. Canadian Journal of Zoology 66:758-762.

Brunori M, 1975. Molecular adaptation to physiological requirements: the hemoglobin system of trout. In: Current Topics in Cellular Regulation (Horecker BL, Stadtman ER, eds). Academic Press Inc. New York. p 1-39.

Buhler DR, 1963. Studies on fish hemoglobins. Chinook salmon and rainbow trout Journal of Biological Chemistry 238:1665-1674.

Buhler DR, Shanks WE, 1959. Multiple hemoglobins in fishes. Science 129:899-900.

di Prisco G, Tamburrini M, 1992. The hemoglobins of marine and freshwater fish: the search for correlations with physiological adaptation. Comparative Biochemistry and Physiology 102B:661-671.

Fago A, Forest E, Weber RE, 2002. Hemoglobin and subunit multiplicity in the rainbow trout (*Oncorhynchus mykiss*) hemoglobin system. Fish Physiology and Biochemistry 24:335-342.

Feuerlein R, Weber R, 1994. Rapid and simultaneous measurement of anodic and cathodic haemoglobins and ATP and GTP concentrations in minute quantities of fish blood. Journal of Experimental Biology 189:273-277.

Houston AH, Cyr D, 1974. Thermoacclimatory variation in hemoglobin systems of goldfish (*Carassius auratus*) and rainbow trout (*Salmo gairdneri*). Journal of Experimental Biology 61:455-461.

Houston AH, Dobric N, Kahurananga R, 1996. The nature of hematological response in fish - Studies on rainbow trout *Oncorhynchus mykiss* exposed to simulated winter, spring and summer conditions. Fish Physiology and Biochemistry 15:339-347.

Iuchi I, 1973. Chemical and physiological properties of larval and adult hemoglobins in rainbow trout, *Salmo gairdnerii irideus*. Comparative Biochemistry and Physiology 44:1087-1094.

Jensen F, 1993. Influence of nucleoside triphosphates, inorganic salts, NADH, catecholamines, and oxygen saturation on nitrite-induced oxidation of rainbow trout haemoglobin. Fish Physiology and Biochemistry 12:111-117.

Karpov AK, Novikov GG, 1980. Hemoglobin alloforms in cod *Gadus morhua* (Gadiformes, Gadidae), their functional characteristics and occurrence in populations. Journal of Ichthyology 6:45–49.

Lau HKF, Wallach DE, Pennelly RR, Noble RW, 1975. Ligand binding properties of haemoglobin 3 of the trout, *Salmo gaidneri*. The occurrence of an acid Bohr effect in the absence of heme-heme interaction. Journal of biological Chemistry 250:1400-1404.

Marinsky CA, Houston AH, Murad A, 1990. Effect of hypoxia on hemoglobin isomorph abundances in rainbow trout, *Salmo gairdneri*. Canadian Journal of Zoology-Revue Canadienne De Zoologie 68:884-888.

Murad A, Houston A, 1991. Haemoglobin isomorph abundances in splenectomized rainbow trout, *Oncorhynchus mykiss* (Walbaum). Journal of Fish Biology 38:641-651.

Powers DA, 1980. Molecular ecology of teleost fish hemoglobins - strategies for adapting to changing environments. American Zoologist 20:139-162.

Riggs AF, 1988. The Bohr effect. Annual Review of Physiology 50:181-204

Soldatov AA, 2005. Peculiarities of organization and functioning of the fish red blood system. Journal of Evolutionary Biochemistry and Physiology 41:272-281.

Tun N, Houston AH, 1986. Temperature, oxygen, photoperiod, and the hemoglobin system of the rainbow trout, *Salmo gairdneri*. Canadian Journal of Zoology-Revue Canadienne De Zoologie 64:1883-1888.

van Vuren JHJ, Hattingh J, 1978. Seasonal changes in the haemoglobins of freshwater fish in their natural environment. Comparative Biochemistry and Physiology Part A 60:265-268.

Weber RE, 1990. Functional significance and structural basis of multiple hemoglobins with special reference to ectothermic vertebrates. In: Animal Nutrition and Transport Processes. 2. Transport, Respiration and Excretion: Comparative

and Environmental Aspects. Truchot JP and Lahlou B (eds). Karger. Basel. pp. 58–75.

Weber RE, Lykkeboe G, Johansen K, 1976a. Biochemical aspects of the adaptation of hemoglobin-oxygen affinity of eels to hypoxia. Life Sciences 17:1345-1349.

Weber RE, Wood SC, Lomholt JP, 1976b. Temperature-acclimation and oxygen-binding properties of blood and multiple hemoglobins of rainbow-trout. Journal of Experimental Biology 65:333-345.

Yoshizaki G, Kang JH, Sakuma K, Aoki T, Takashima F, 1996. Cloning and sequencing of rainbow trout beta-globin cDNA. Fisheries Science 62:723-726.

Yoshizaki G, Takano A, Aoki T, Takashima F, 1997. Rapid communication: Nucleotide sequence of rainbow trout alpha-globin I and IV cDNA. Journal of Animal Science 75:1426.